Hemostasis and Thrombosis

Basic Principles and Clinical Practice

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Victor J. Marder
William C. Aird
Joel S. Bennett
Sam Schulman
Gilbert C. White, II

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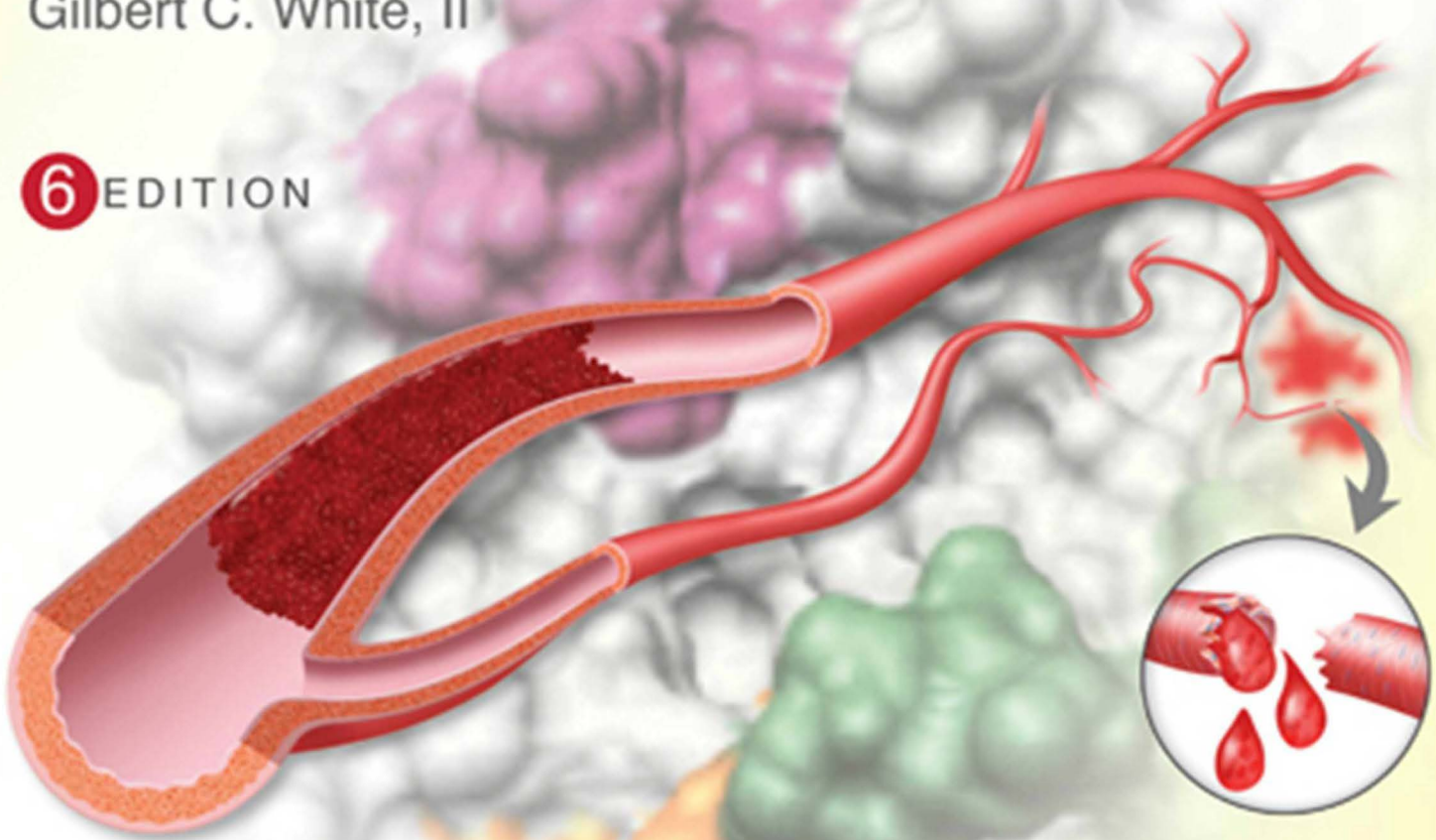

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Front Cover Note

The front cover symbolizes the basic (background) and clinical aspects (foreground) of hemostasis and thrombosis considered in this volume. The background is a surface representation of thrombin in standard orientation (see Figure 9.16B) indicating the location of the 60-loop (magenta) and the g-loop (green) that border the substrate-binding cleft. The foreground is a schematized artery (modified from Figure 112.3) containing a thrombus and a downstream (remote) bleeding site.

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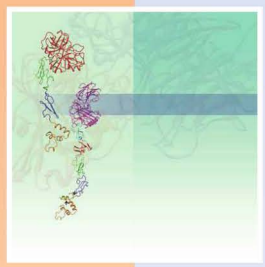
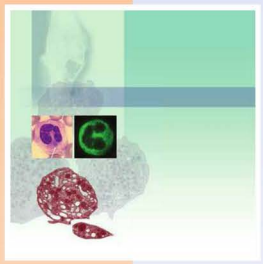
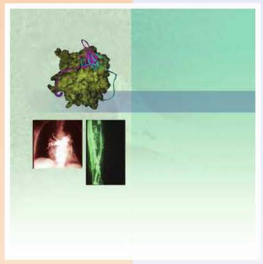

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EDITORS

Victor J. Marder, MD
Professor of Medicine
David Geffen School of Medicine
at University of California, Los Angeles
Los Angeles, California

William C. Aird, MD
Professor of Medicine
Harvard Medical School
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Joel S. Bennett, MD
Professor of Medicine
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Sam Schulman, MD
Professor of Medicine
McMaster University
Hamilton, Ontario, Canada

Gilbert C. White, II, MD
Executive Vice President for Research
BloodCenter of Wisconsin
Milwaukee, Wisconsin

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Senior Executive Editor: Jonathan W. Pine, Jr.
Senior Product Manager: Emilie Moyer
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Senior Manufacturing Manager: Benjamin Rivera
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Senior Designer: Stephen Druding
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CONTRIBUTING AUTHORS

Charles S. Abrams, MD
Professor
Department of Medicine
University of Pennsylvania
Associate Chief
Divison of Hematology-Oncology
University of Pennsylvania Hospital
Philadelphia, Pennsylvania

Anna Ågren, MD, PhD
Assistant Professor
Coagulation Unit
Division of Hematology
Karolinska University Hospital
Department of Clinical Sciences
Karolinska Institutet
Stockholm, Sweden

Anat Aharon, PhD
Department of Hematology and Bone Marrow Transplantation
Rambam Health Care Campus
Bruce Rappaport Faculty of Medicine
Technion
Israel Institute of Technology
Haifa, Israel

Martine Aiach, PhD
Dean
Faculté de pharmacie
Université Paris Descartes
Paris, France

William C. Aird, MD
Professor of Medicine
Harvard Medical School
Beth Israel Deaconess Medical Center
Boston, Massachusetts

John H. Alexander, MD
Associate Professor of Medicine
Cardiovascular Medicine
Duke Clinical Research Institute
Cardiologist
Cardiovascular Medicine
Duke University Medical Center
Durham, North Carolina

Sonia Anand, MD, PhD
Professor
Department of Medicine and Epidemiology
McMaster Unviersity
Hamilton, Ontario, Canada

Robert K. Andrews, PhD
Associate Professor
Australian Centre for Blood Diseases
Monash University
Melbourne, Australia

Robert A.S. Ariëns, PhD
Reader in Molecular Vascular Biology
Division of Cardiovascular and Diabetes Research
University of Leeds
Leeds, United Kingdom

Richard H. Aster, MD
Professor of Medicine
Medical College of Wisconsin
Senior Investigator
Blood Research Institute
BloodCenter of Wisconsin
Milwaukee, Wisconsin

Kenneth I. Ataga, MBSS
Associate Professor
Director, UNC Comprehensive Sickle Cell Program
Division of Hematology/Oncology
University of North Carolina
Chapel Hill, North Carolina

S. Paul Bajaj, PhD
Professor
Protein Chemistry Laboratory
UCLA/Orthopaedic Hospital
Department of Orthopaedic Surgery
David Geffen School of Medicine
University of California, Los Angeles
Los Angeles, California

David L. Bark, Jr., BS, MS, PhD
Research Fellow
Australian Centre for Blood Diseases
Monash University
Melbourne, Victoria, Australia

Kenneth A. Bauer, MD
Professor
Department of Medicine
Harvard Medical School
Director, Thrombosis Clinical Research
Department of Medicine
Beth Israel Deaconess Medical Center
Boston, Masachusetts

Joel S. Bennett, MD
Professor of Medicine
Hematology-Oncology Division
Department of Medicine
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Michael C. Berndt, PhD
Director
Biomedical Diagnostics Institute
Dublin City University
Dublin, Ireland

Leslie Roy Berry, MD, PhD
Assistant Professor
Department of Pediatrics
McMaster University
Hamilton, Ontario, Canada

Kamila Bledzka, PhD
Postdoctoral Fellow
Department of Molecular Cardiology
Lerner Research Institute
The Cleveland Clinic
Cleveland, Ohio

Susan C. Bock, PhD
Professor
Departments of Medicine and Bioengineering
University of Utah
Salt Lake City, Utah

Françoise Boehlen, MD
Haemostasis Unit
University Hospital Geneva
Geneva, Switzerland

Nuala A. Booth, PhD
Personal Chair
School of Medical Sciences
University of Aberdeen
Aberdeen, United Kingdom

Henri Bounameaux, MD
Professor of Medicine
Department of Medicine
Faculty of Medicine
Director
Division of Angiology and Hemostasis
University Hospital of Geneva
Geneva, Switzerland

Lawrence F. Brass, MD, PhD
Professor
Department of Medicine
University of Pennsylvania
Division of Hematology Oncology
University of Pennsylvania Hospital
Philadelphia, Pennsylvania

Benjamin Brenner, MD
Professor
Caster Chief in Leukemia Research
Department of Hematology
Technion Israel Institute of Technology
Director
Department of Thrombosis and Hematology
Rambam Health Care Campus
Haifa, Isreal

George J. Broze, Jr., MD
Professor
Department of Medicine
Washington University
Attending Physician
Dpeartment of Internal Medicine/Hematology
Barnes-Jewish Hospital
St. Louis, Missouri

Keith R. Brunt, PhD
Division of Cardiovascular Surgery
Faculty of Medicine
Toronto General Hospital and University of Toronto
University Health Network
Toronto, Ontario, Canada

James B. Bussel, MD
Professor of Pediatrics
Departments of Pediatrics in Obstetrics and Gynecology and Medicine
Department of Pediatric Hematology Oncology
Weill-Cornell Medical College
Attending Physician
Department of Pediatrics
New York Presbyterian Hospital
New York, New York

Jagdish Butany, MBBS
Laboratory Medicine and Pathobiology
Faculty of Medicine
Toronto General Hospital and University of Toronto
University Health Network
Toronto, Ontario, Canada

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Stephen H. Caldwell, MD
Professor
Director of Hepatology
Department of GI/Hepatology
University of Virginia
Charlottesville, Virginia

Rodney M. Camire, PhD
Associate Professor
Department of Pediatrics
The University of Pennsylvania
The Children's Hospital of Philadelphia
Philadelphia, Pennsylvania

Peter Carmeliet, MD, PhD
Professor, Director
Versalius Research Center
Katholieke Universiteit Leuven
Leuven, Belgium

Elizabeth A. Chalmers, MB ChB
Department of Haematology
Royal Hospital for Sick Children
Yorkhill, Glasgow, United Kingdom

Anthony K. Chan, MBBS, FRCPC, FRCPath
Professor
Department of Pediatrics
Division Chief, Pediatric Hematology/Oncology
McMaster University
Hamilton, Ontario, Canada

Jing Chen, PhD
Department of Ophthalmology
Harvard Medical School
Children's Hospital Boston
Boston, Massachusetts

Dominic W. Chung, PhD
Professor
Department of Biochemistry
University of Washington
Seattle, Washington

Douglas B. Cines, MD
Professor
Pathology and Laboratory Medicine and Medicine
University of Pennsylvania
Director, Coagulation Laboratory
Vice-Chair, Academic Programs
Pathology and Laboratory Medicine and Medicine
Hospital of the University of Pennsylvania
Philadelphia, Pennsylvania

Jennifer Cohn, MD, MPH
Assistant Professor
Division of Infectious Diseases
Co-Director
Global Health Equities Residency Track, Department of Medicine
University of Pennsylvania
Doctors Without Borders/Médecins Sans Frontières
Access Campaign, East Africa

Peter W. Collins, MBBS, MD, FRCP, FRCPath
Reader
Department of Haematology
School of Medicine Cardiff University
Consultant
Department of Haematology
University Hospital of Wales
Cardiff, United Kingdom

Robert W. Colman, MD
Professor of Medicine
Temple University Health Sciences Center
Hematology-Oncology Section
Temple University School of Medicine
Philadelphia, Pennsylvania

Edward M. Conway, MD, PhD
Professor of Medicine
Director of Centre for Blood Research
Department of Medicine
University of British Columbia
Staff Hematologist
Division of Hematology
Department of Medicine
Vancouver General Hospital
Vancouver, British Columbia, Canada

Michiel Coppens, MD, PhD
Fellow
Department of Vascular Medicine
University of Amsterdam
Thrombosis and Hemostasis Consultant
Department of Vascular Medicine
Academic Medical Center
Amsterdam, The Netherlands

Elisabeth Cramer Bordé, MD, PhD
Professor
Faculté de médecine
Université de Versailles St-Quentin-En-Yvelines
Head
Service d'hématologie et immunologie
Hôpital Ambroise Paré
Boulogne, France

Kevin Croce, MD, PhD
Instructor in Medicine
Cardiovascular Medicine, Brigham and Womens Hospital
Boston, Massachusetts

Mark A. Crowther, MD
Professor
Department of Medicine
McMaster University
Chief
Department of Laboratory Medicine
St. Joseph's Healthcare and Hamilton Health Sciences
Hamilton, Ontario, Canada

Earl W. Davie, PhD
Professor
Department of Biochemistry
University of Washington
Seattle, Washington

Peter F. Davies, PhD
Robinette Foundation Professor of Cardiovascular Medicine
Pathology and Laboratory Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Hervé Decousus, MD
Coordinator
Centre d'Investigation Clinique-Epidémiology
Chief
Department of Medicine and Therapeutics
Saint-Etienne Hospital
Saint-Etienne, France

Gregory J. del Zoppo, MD, MS, FAHA
Professor of Medicine (in Hematology)
Adjunct Professor of Neurology
Department of Medicine (in Hematology)
University of Washington School of Medicine
Harborview Medical Center
Seattle, Washington

Philippe de Moerloose, MD
Professor
Department of Internal Medicine
Faculty of Medicine
Head
Haemostasis Unit
University Hospital Geneva
Geneva, Switzerland

Carl-Erik H. Dempfle, Prof Dr Med
Associate Professor
Medical Faculty Mannheim
University of Heidelberg
Institut fürMarke und Design Mannheim
Mannheim, Germany

Anne de Paepe, MD, PhD
Professor
Medical Genetics
Ghent University
Head, Department of Medical Genetics
Ghent University Hospital
Ghent, Belgium

Karl C. Desch, MD
Assistant Professor
Department of Pediatrics and Communicable Diseases
University of Michigan
Attending Physician
Divison of Neonatal and Perinatal Medicine
C.S. Mott Children's Hospital
Ann Arbor, Michigan

James D. Douketis, MD
Professor
Department of Medicine
McMaster University
Staff Physician
St. Joseph's Healthcare Hamilton
Hamilton, Ontario, Canada

Ludovic Drouet, MD, PhD
CREATIF (Centre de Référence des Antithrombotiques Ile de France)
Service d'Hématologie Biologie Hôpital
Paris, France

Hillary A. Dunlevy, MD
Department of Internal Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Cédric Duval, PhD
The Leeds Institute of Genetics, Health and Therapeutics
Division of Cardiovascular and Diabetes Research
The LIGHT Laboratories
Leeds, United Kingdom

Charles S. Eby, MD
Associate Professor
Department of Pathology and Immunology
Washington University
Medical Director
Hematology Laboratory
Barnes-Jewish Hospital
St. Louis, Missouri

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L. Henry Edmunds, Jr., MD
Julian Johnson Professor of Cardiothoracic Surgery
Department of Surgery
University of Pennsylvania
Editor, The Annals of Thoracic Surgery
Philadelphia, Pennsylvania

Joseph Edmunds, Jr., MD, PhD
Professor of Vascular Medicine
INSERM U765
University Paris Descartes
Hôpital Européen Georges Pompidou
Paris, France

Suzanne G. Eskin, PhD
Principal Research Scientist
The Wallace H. Coulter Department of Biomedical Engineering
Georgia Institute of Technology
Atlanta, Georgia

Lesley Everett, MPhil
Fellow, Medical Scientist Training Program
Department of Human Genetics
University of Michigan
Ann Arbor, Michigan

Anna Falanga, MD
Chief Division Immunohematology
Department of Oncology-Hematolgoy
Ospedela Riuniti DiBergamo
Bergamo, Italy

Jawed Fareed, PhD
Professor
Pathology and Pharmacology
Stritch School of Medicine
Loyola University Medical Center
Maywood, Illinois

Richard W. Farndale, PhD
Professor of Matrix Biochemistry
Department of Biochemistry
University of Cambridge
Cambridge, United Kingdom

Philip J. Fay, PhD
Professor
Department of Biochemistry and Biophysics
University of Rochester School of Medicine
Rochester, New York

Garret A. FitzGerald, MD
Chair
Department of Pharmacology
Director
Institute for Transitional Medicine and Therapeutics
University of Pennsylvania
Philadelphia, Pennsylvania

Massimo Franchini, MD
Director
Department of Pathology and Laboratory Medicine
Immunohematology and Transfusion Center
University Hospital of Parma
Parma, Italy

Charles W. Francis, MD
Professor Emeritus
Department of Medicine
University of Rochester
Attending Physician
Department of Medicine
Strong Memorial Hospital
Rochester, New York

David Gailani, MD
Professor of Pathology and Medicine
Department of Pathology
Vanderbilt University
Medical Director
Clinical Coagulation Laboratory
Department of Pathology
Vanderbilt University Medical Center
Nashville, Tennessee

Dennis K. Galanakis, MD
Associate Professor of Pathology and Medicine
School of Medicine, State University of New York at Stony Brook
Stony Brook, New York

Miriam Galbusera, Biol Sci D
Mario Negri Institute for Pharmacological Research
Centro Anna Maria Astori
Science and Technology Park
Kilometro Rosso
Bergamo, Italy

James N. George, MD
George Lynn Cross Professor
Biostatics and Epidemiology/Medicine
University of Oklahoma Health Scineces Center
Oklahoma City, Oklahoma

Jeffrey Ginsberg, MD
Professor of Medicine
Department of Medicine
McMaster University
Clinician
Department of Hematology/Thrombosis
Hamilton Health Sciences, McMaster Site
Hamilton, Ontario, Canada

Mark H. Ginsberg, MD
Distinguished Professor
Department of Medicine
University of California at San Diego
La Jolla, California

David Ginsburg, MD
James V. Neel Distinguished University Professor
Departments of Internal Medicine, Human Genetics, and Pediatrics and Communicable Diseases
Howard Hughes Medical Institute
University of Michigan Medical School
Ann Arbor, Michigan

Lawrence Tim Goodnough, MD
Professor
Department of Pathology and Medicine
Stanford University
Director of Transfusion Service
Director, Transfusion Medicine Fellowship Program
Associate Medical Director, Stanford Blood Center
Stanford Hospital and Clinics
Stanford, California

Maud B. Gorbet, PhD
Assistant Professor
Systems Design Engineering
School of Optometry, Cross Appointed
University of Waterloo
Waterloo, Ontario, Canada

Marianne A. Grant, PhD
Instructor in Medicine
Department of Medicine
Harvard Medical School
Instructor in Medicine
Center for Vascular Biology Research
Beth Israel Deaconess Medical Center
Boston, Massachusetts

Andreas Greinacher, MD
Professor
Immunology and Transfusion Medicine
Ernst-Moritz-Arndt University
Head of Institute
Immunology and Transfusion Medicine
University Hospital
Greifswald, Germany

John H. Griffin, PhD
Professor
Department of Molecular and Experimental Medicine
The Scripps Research Institute
La Jolla, California

Jean-Christophe Gris, MD
Department of Haematology
University Hospital
Nîmes, France
Laboratory of Haematology
Faculty of Pharmacy and Biological Sciences Montpellier 1 University
Montpellier, France

Peter L. Gross, MD
Associate Professor
Department of Medicine
McMaster University
Thrombosis Service
Department of Medicine
Juravinski Hospital, Hamilton Health Sciences
Hamilton, Ontario, Canada

Tilo Grosser, MD
Institute for Translational Medicine and Therapeutics
Translational Research Center
Perelman School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania

Sandra L. Haberichter, PhD
Director of Hemostasis
Associate Investigator
Blood Research Institute
BloodCenter of Wisconsin
Investigator
Children's Research Institute
Children's Hospital of Wisconsin
Milwaukee, Wisconsin

Jonathan L. Halperin, MD
Robert and Harriet Heilbrunn Professor of Medicine (Cardiology)
Director, Cardiology Clinical Services
The Zena and Michael A. Wiener Cardiovascular Institute and Marie-Josée and Henry R. Kravis Center for Cardiovascular Health
Mount Sinai School of Medicine
Mount Sinai Medial Center
New York, New York

Paul Harrison, PhD
Clinical Scientist
Oxford Haemophilia and Thrombosis Centre
Churchill Hospital
Oxford, United Kingdom

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John H. Hartwig, PhD
Professor
Department of Medicine
Harvard Medical School
Senior Biologist
Translational Medicine Division
Brigham & Women's Hospital
Boston, Massachusetts

Charles R.M. Hay, MD
Honorary Senior Lecturer
Director
Manchester Haemophilia Comprehensive Care Centre, Consultant Haematologist
Manchester University Department of Haematology
The University of Manchester
Manchester Royal Infirmary
Manchester, United Kingdom

Robert P. Hebbel, MD
Regents Professor and Clark Professor
Deparment of Medicine
University of Minnesota Medical School
Minneapolis, Minnesota

John A. Heit, MD
Professor of Medicine
Division of Cardiovascular Diseases
College of Medicine
Director, Coagulation Laboratories
Division of Hematopathology
Mayo Clinic
Rochester, Minnesota

Ian Hitchcock, PhD
Assistant Professor
Department of Medicine
Stony Brook University
Stony Brook, New York

John B. Holcomb, MD
Professor
Department of Surgery
University of Texas Health Science Center
Chief, Trauma Services
Memorial Hermann Hospital
Houston, Texas

Sonya Hui, MSc
Department of Physiology
Faculty of Medicine
Toronto General Hospital and University of Toronto
University Health Network
Toronto, Ontario, Canada

Menno V. Huisman, MD, PhD
Associate Professor
Chair of Vascular Medicine
Department of Thrombosis and Hemostasis
Department of Endocrinology
Leiden University Medical Center
Leiden, The Netherlands

Mansoor Husain, MD, FRCPC
Associate Professor
Department of Medicine
University of Toronto
Senior Scientist
Toronto General Research Insitute
University Health Network
Toronto, Ontario, Canada

Vera Ignaoatovic, PhD
Senior Research Fellow
Department of Pediatrics
The University of Melbourne
Royal Children's Hospital
Senior Reseach Fellow
Haematology Research
Murdoch Children's Research Institute
Parville, Victoria, Australia

Helen A. Ireland, PhD
Principal Research Fellow
Division of Medicine
University College London
London, United Kingdom

Joseph E. Italiano, Jr., PhD
Assistant Professor
Department of Medicine
Harvard Medical School
Associate Biologist
Translational Medicine/Hematology
Brigham & Women's Hospital
Boston, Massachusetts

Shaun Jackson, MBBS, BMedSci, PhD
Principle Research Fellow
Australian Centre for Blood Diseases
Monash University
Melbourne, Victoria, Australia

Juan M. Jiménez, PhD
Post-doctoral Fellow
Institute for Medicine and Engineering
University of Pennsylvania
Philadelphia, Pennsylvania

Hylton V. Joffe, MD
Director
Division of Reproductive and Urologic Products
U.S. Food and Drug Administration
Silver Spring, Maryland
Assistant Professor
Department of Medicine
The Johns Hopkins Hospital
Baltimore, Maryland

Jill M. Johnsen, MD
Assistant Professor
Department of Medicine
University of Washington
Assistant Member
Puget Sound Blood Center
Seattle, Washington

Phillip Joseph, MD
Fellow
Population Health Research Insitute
McMaster University
Hamilton, Ontario, Canada

Stephanie M. Jung, PhD
Senior Research Associate
Department of Biochemistry
University of Cambridge
Cambridge, United Kingdom

Susan R. Kahn, MD, MSc
Professor
Department of Medicine
McGill University
Director
Thrombosis Program
Jewish General Hospital
Montreal, Quebec, Canada

Mark L. Kahn, MD
Professor
Department of Medicine
University of Pennsylvania
Translational Research Center
Philadelphia, Pennsylvania

Ajay Kakkar, BSc MB, PhD, FRCS
Professor of Surgery
University College London
Director, Thrombosis Research Institute
Consultant Surgeon
University College Hospital London National Health Service Foundation Trust
London, United Kingdom

Evi Kalodik, MD BA DIC PhD FRCS
Honorary Senior Research Fellow
Faculty of Medicine
Imperial College
Senior Vascular Research Fellow
Department of Vascular Surgery
Ealing Hospital
London, United Kingdom

Philip A. Kalra, MD
Honorary Professor
University of Manchester
Manchester, United Kingdom
Consultant Nephrologist
Department of Renal Medicine
Salford Royal Foundation Trust
Salford, United Kingdom

Saurabh Kalra, II, BHSc
Undergraduate Student
Faculty of Health Sciences
McMaster University
Hamilton, Ontario, Canada

Sangita Kapur, MD
Department of Radiology
University of Cincinnati College of Medicine
Cincinnati, Ohio

Ana Kasirer-Friede, PhD
Associate Project Scientist
Department of Medicine
University of California, San Diego
La Jolla, California

Randal J. Kaufman, PhD
Professor and Director
Degenerative Disease Research Program
Del E. Webb Neuroscience, Aging, and Stem Cell Research Center
Sanford-Burnham Medical Research Institute
La Jolla, California

Kenneth Kaushansky, MD
Dean, School of Medicine
Department of Medicine
Stony Brook University
Senior Vice President, Health Sciences
Stony Brook Health Sciences Center
Stony Brook, New York

Thomas L. Kawano, MD
Fellow in Cardiology
The Cardiovascular Institute
Mount Sinai School of Medicine
Mount Sinai Medical Center
New York, New York

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Nigel S. Key, MB, ChB, FRCP
Harold R. Roberts Distinguished Professor
Department of Medicine
University of North Carolina
Chief, Section of Hematology
Division of Hematology/Oncology
University of North Carolina Hospitals
Chapel Hill, North Carolina

Thomas S. Kickler, MD
Professor of Medicine, Oncology, and Pathology
Department of Medicine, Oncology, and Pathology
The Johns Hopkins University School of Medicine
Director, Hemostasis Coagulation Laboratory
The Johns Hopkins Hospital
Baltimore, Maryland

R. Manjunatha Kini, PhD
Professor
Biological Science
National University of Singapore
Singapore

Barbara A. Konkle, MD
Professor
Department of Medicine
University of Washington
Director, Translational Research
Puget Sound Blood Center
Seattle, Washington

Sriram Krishnaswamy, PhD
Professor
Department of Pediatrics
University of Pennsylvania
Stokes Investigator
Children's Hospital of Philadelphia
Philadelphia, Pennsylvania

Michael J. Krisinger, PhD
Postdoctoral Research Fellow
Centre for Blood Research
University of British Columbia
Vancouber, British Columbia, Canada

Taco W. Kuijpers, MD, PhD
Professor
Pediatric Immunologist & Head
Division of Pediatric Hematology, Immunology, and Infectious Diseases
Emma Children's Hospital Academic Medical Centre
Amsterdam, The Netherlands

Thomas J. Kunicki, PhD
Senior Scientist II
Children's Hospital of Orange County
Hematology Research
Orange, California

Shiyang Kwong, PhD
Protein Science Laboratory
Department of Biological Sciences
Faculty of Science
National University of Singapore
Singapore

Agnes Y.Y. Lee, MD
Associate Professor
Department of Medicine
University of British Columbia
Director, Thrombosis Program
Department of Medicine
Vancouver General Hospital
Vancouver, British Columbia, Canada

Kerry L. Lee, PhD
Professor of Biostatistics
Department of Biostatistics and Bioinformatics
Duke University School of Medicine
Faculty Director
Biostatistics
Duke Clinical Research Institute
Durham, North Carolina

Michelle Letarte, PhD
Professor
Department of Immunology
University of Toronto
Senior Scientist
Molecular Structure and Function Program
Hospital for Sick Children
Toronto, Ontario, Canada

Marcel Levi, MD, PhD
Professor of Medicine
Department of Medicine
University of Amsterdam
Chairman
Department of Medicine
Academic Medical Center
Amsterdam, The Netherlands

Klaus Ley, MD, PhD
Head
Inflammation Biology
La Jolla Institute for Allergy and Immunology
La Jolla, California

Peter Libby, MD
Chief, Cardiovascular Medicine, Brigham and Women's Hospital
Mallinckrodt Professor of Medicine, Harvard Medical School
Cardiovascular Medicine
Boston, Massachusetts

Andrew H. Lichtman, MD, PhD
Professor
Department of Pathology
Harvard Medical School
Staff Pathologist
Department of Pathology
Brigham and Women's Hospital
Boston, Massachusetts

Howard A. Liebman, MD
Professor
Departments of Medicine and Pathology
University of Southern California
Attending Physician
Division of Hematology, Department of Medicine
Norris Comprehensive Cancer Center
Los Angeles, California

David Lillicrap, MD
Professor
Pathology and Molecular Medicine
Queen's University
Kingston, Ontario, Canada

Gregory Y.H. Lip, MD
Professor of Cardiovascular Medicine
University of Birmingham
Visiting Professor of Haemostasis Thrombosis & Vascular Sciences
University of Aston in Birmingham
Centre for Cardiovascular Sciences
City Hospital
Birmingham, England, United Kingdom

José A. López, MD
Professor of Medicine and Biochemistry
University of Washington
Executive Vice-President for Research
Research Division
Puget Sound Blood Center
Seattle, Washington

Rommel P. Lu, MD
Clinical Assistant Professor
Department of Pathology & Laboratory Medicine
Department of Medicine
University of North Carolina at Chapel Hill
Associate Director, Transfusion Medicine Service
Department of Pathology & Laboratory Medicine
University of North Carolina Hospitals
Chapel Hill, North Carolina

Aernout Luttun, PhD
Associate Professor
Center for Molecular and Vascular Biology
Katholike Universiteit Leuven
Leuven, Belgium

Isabelle Mahe, MD, PhD
Professor
Universite Paris
Chief
Department of Internal Medicine
Hopital Louis Mourier
Colombes, France

Fransiska Malfait, MD, PhD
Post-doctoral Fellow
Centre for Medical Genetics
Ghent University
Ghent University Hospital
Ghent, Belgium

Pier Mannuccio Manucci, MD
Professor of Medicine
Department of Internal Medicine
University of Milan
Director
Department of Medicine and Medical Specialties
Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Ca Granda Maggiore Hospital
Milan, Italy

Douglas A. Marchuk, PhD
Professor and Vice-Chair
Department of Molecular Genetics and Microbiology
Duke University School of Medicine
Durham, North Carolina

Victor J. Marder, MD
Professor of Medicine
Division of Hematology/Medical Oncology
Department of Medicine
David Geffen School of Medicine at University of California, Los Angeles
Los Angeles, California

Paris Margaritis, DPhil
Research Assistant Professor
Department of Pediatrics
The University of Pennsylvania
The Children's Hosptial of Philadelphia
Philadelphia, Pennsylvania

Bradley A. Maron, MD
Fellow in Cardiovascular Medicine
Department of Internal Medicine
Harvard Medical School
Brigham and Women's Health
Boston, Massachusetts

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M. Patricia Massicotte, MSc, MD, FRCPC, MHSc
Peter Olley Chair
Pediatric Thrombosis
Professor Pediatrics
Director KIDClot Program
University of Alberta
Stollery Children's Hospital
Edmonton, Alberta, Canada

Emer R. McGrath, MB
Neurology Research Fellow
Department of Medicine
National University of Ireland, Galway
Galway, Ireland

Larry V. McIntire, PhD
Professor
Biomedical Engineering
Georgia Institute of Technology
Atlanta, Georgia

Nicolas Meneveau, MD, PhD
Author
Physiologie Cardiovasulaire et Prévention
Université de Franche-Comté
Professor
Cardiology Department
University Hospital Jean Minjoz
Besançon, France

Thomas Michel, MD, PhD
Professor of Medicine and Biochemistry
Department of Medicine
Harvard Medical School
Senior Physician in Cardiovascular Medicine
Department of Medicine
Brigham and Women's Hospital
Boston, Massachusetts

Alan D. Michelson, MD
Professor of Pediatrics
Harvard Medical School
Director, Center for Platelet Research Studies
Director, Thrombosis Program and Anticoagulation Service
Division of Hematology/Oncology
Children's Hospital Boston
Dana-Farber Cancer Institute
Boston, Massacusetts

Saskia Middeldorp, MD, PhD
Professor
Department of Vascular Medicine
University of Amsterdam
Co-chair
Department of Vascular Medicine
Academic Medical Center
Amsterdam, The Netherlands

Patrick Mismetti, MD, PhD
Author
Gourpe de Recherche sur la Thrombose
Université de St-Etienne Jean Monnet
Professor
Department of Internal Medicine
University Hospital Saint-Etienne
Saint-Etienne, France

Paul Monagle, MD
Stevenson's Chair and Head
Department of Pediatrics
University of Melbourne
Director
Department of Haematology
Royal Children's Hospital
Melbourne, Australia

Robert R. Montgomery, MD
Professor of Pediatric Hematology
Blood Research Institute
Milwaukee, Wisconsin

Micah J. Mooberry, MD
Clinical Instructor
Department of Medicine
University of North Carolina
Clinical Instructor
Hemostasis and Thrombosis Program
University of North Carolina Hospital
Chapel Hill, North Carolina

Pierre-Emmanuel Morange, MD, PhD
Professor
Department of Hematology
Aly-Marseille University
Chief
Department of Hematology
Timane Hospital
Marseille, France

Masaaki Moroi, PhD
Visiting Professor
Department of Biochemistry
University of Cambridge
Cambridge, United Kingdom

James H. Morrissey, PhD
Professor
Department of Biochemistry
University of Illinois at Urbana-Champaign
Urbana, Illinois

Laurent O. Mosnier, PhD
Assistant Professor
Department of Molecular and Experimental Medicine
The Scripps Research Institute
La Jolla, California

Jon G. Moss, MBChB, FRCS, FRCR
Professor
Department of Interventional Radiology
North Glasgow University Hospitals
Glasgow, United Kingdom

Swaminathan Murugappan, MD, PhD
Hematology Oncology Fellow
University of Washington School of Medicine
Seattle, Washington

Nikki J. Mutch, PhD
Lecturer
School of Medicine and Dentistry
University of Aberdeen
Aberdeen, United Kingdom

Yona Nadir, MD, PhD
Lecturer
Department of Hematology
Technion Institute of Technology
Senior Hematologist
Thrombosis and Hemostatis Unit
Rambam Health Care Center
Haifa, Israel

Warwick S. Nesbitt, BSc, PhD
Senior Research Fellow
The Australian Centre for Blood Diseases
Monash University
Melbourne, Australia

Mathilde Nijkeuter, PhD
Postdoctorate Fellow
Department of Vascular Medicine
Academic Medical Center, University of Amsterdam
Amsterdam, The Netherlands

Diane J. Nugent, MD
Director of Hematology
Children's Hospital of Orange County Blood and Donor Center
Children's Hospital of Orange County
Orange, California
Principal Investigator Region IX Maternal and Child Health Bureau
Centers for Disease Control Hemophilia Grants
Chair of the National Coordinating Committee
Centers for Disease Control Joint Outcome and Surveillance Project
Bethesda, Maryland
Professor
University of California, Los Angeles Mattel Children's Hospital
Los Angeles, California

Alan T. Nurden, PhD
Emeritus Director
Technology and Innovation Platform
University Victor Segalen
Bordeaux, France
Scientific Advisor
French Network for Inherited Platelet Disorders
Hôpital Xavier Arnozan
Pessac, France

Paquita Nurden, MD, PhD
National Coordinator
French Network for Inherited Platelet Disorders
University Victor Segalen
Hôpital Xavier Arnozan
Pessac, France

Martin J. O'Donnell, PhD
Associate Professor, Translational Medicine
Department of Medicine
National University of Ireland, Galway
Galway, Ireland

Patrizia Ondei, MD
Department of Immunology and Transplantation
Azienda Pspedallera Ospedall Rlunjitl di Bergamo
Bergamo, Italy

Thomas L. Ortel, MD, PhD
Professor
Departments of Medicine and Pathology
Duke University Medical Center
Durham, North Carolina

Sharif Mohammed Pasha, MD
Medical Doctor
Department of Thrombosis and Haemostasis
Leiden University
Leiden, The Netherlands

Carlo Patrono, MD
Professor and Chair of Pharmacology
Department of Pharmacology
Catholic University School of Medicine
Rome, Italy

Vittorio Pengo, MD
Professor
Cardiac Thoracic and Vascular Sciences
University of Padova
Padova, Italy

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Arnaud Perrier, MD
Professor of Medicine
Department of Medicine
Faculty of Medicine
Director
Division of General Internal Medicine
University Hospital of Geneva
Geneva, Switzerland

Flora Peyvandi, MD
Professor of Medicine
Department of Internal Medicine
University of Milan
Medical Doctor
Department of Medicine and Medical Specialities
Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Ca Grand Maggiore Hospital
Milan, Italy

Helen Philippou, PhD
The Leeds Institute of Genetics, Health and Therapeutics
Division of Cardiovascular and Diabetes Research
The LIGHT Laboratories
Leeds, United Kingdom

Edward F. Plow, PhD
Department Chairman
Department of Molecular Cardiology
Lerner Research Institute
The Cleveland Clinic
Cleveland, Ohio

Laura Popolo, PhD
Associate Professor of Molecular Biology
Dipartimento di Scienze Biomolecolarie Biotecnologie
Universita degli Studi di Milano
Milano, Italy

Jun Qin, PhD
Professor
Department of Molecular Medicine
Cleveland Clinic Lerner College of Medicine
Staff
Department of Molecular Cardiology
Lerner Research Institute, Cleveland Clinic
Cleveland, Ohio

Jacob H. Rand, MD
Professor
Department of Pathology & Medicine
Albert Einstein College of Medicine of Yeshiva University
Director
Hematology Laboratories
Montefiore Medical Center
Bronx, New York

A. Koneti Rao, MBBS
Sol Sherry Professor of Medicine
Professor of Thrombosis Research
Professor of Pharmacology
Chief, Hematology Division
Co-Director, Sol Sherry Thrombosis Research Center
Temple University School of Medicine
Philadelphia, Pennsylvania

Giuseppe Remuzzi, MD
Director
Marlo Negri Institute for Pharmacological Research
Centro Anna Maria Asiori
Science and Technology Park
Kilometro Rosso
Director
Department of Immunology and Transplantation
Azienda Ospedaliera Ospedali Riunili di Bergamo
Bergamo, Italy

Thomas Renné, MD, PhD
Clinical Chemistry
Department of Molecular Medicine and Surgery and Center of Molecular Medicine
Karolinska Institutet
Stockholm, Sweden

Frederick R. Rickles, MD
Professor
Departments of Medicine
Pediatrics and Pharmacology and Physiology
The George Washington University
Attending Physician
Department of Medicine
The George Washington University School of Medicine and Health Sciences and the Veterans Administration Medical Center
Washington, District of Columbia

Paul M. Ridker, MD, MPH
Eugene Braunwald Professor of Medicine
Cardiovascular Medicine, Brigham and Women's Hospital
Division of Preventive Medicine
Boston, Massachusetts

Timolaos Rizos, MD
Neurologist
Department of Neurology
University of Heidelberg
Im Neuenheimer Feld
Heidelberg, Germany

Harold R. Roberts, MD
Sarah Graham Kenan Professor of Medicine
Department of Medicine
University of North Carolina
Attending Emeritus
Department of Medicine
University of North Carolina Hospitals
Chapel Hill, North Carolina

Matthew T. Roe, MD, MHS
Associate Professor
Division of Cardiovascular Medicine
Duke Clinical Research Institute
Physician/Cardiologist
Department of Medicine
Duke University Medical Center
Durham, North Carolina

Wolfram Ruf, MD
Professor
Department of Immunology and Microbial Science
The Scripps Research Institute
La Jolla, California

Zaverio M. Ruggeri, MD
Professor
Molecular and Experimental Medicine
The Scripps Research Institute
La Jolla, California

J. Evan Sadler, MD, PhD
Professor and Director
Division of Hematology
Department of Medicine
Washington University
St. Louis, Missouri

Michael Meyer Samama, MD, PharmD
Emeritus Professor
Department of Hematology
Hotel Dieu University Hospital
Paris, France

Carlos E. Sanchez, MD
Division of Cardiovascular Diseases
University of Cincinnati College of Medicine
Cincinnati, Ohio

Brian Savage, PhD
Staff Scientist
Molecular and Experimental Medicine
The Scripps Research Institute
La Jolla, California

Jeffrey L. Saver, MD, FAHA, FAAN
Professor of Neurology
David Geffen School of Medicine at University of California, Los Angeles
Director, UCLA Stroke Center
Los Angeles, California

Amy E. Schmidt, PhD
Professor
Department of Orthopedic Surgery and Molecular Biology Institute
University of California, Los Angeles
Los Angeles, California

Sam Schulman, MD
Professor of Medicine
McMaster University
Hamilton Health Sciences - General Hospital
Hamilton, Ontario, Canada

Michael V. Sefton, ScD
University Professor
Institute of Biomaterials and Biomedical Engineering
University of Toronto
Toronto, Ontario, Canada

Nirmish Shah, MD
Division of Pediatric Hematology/Oncology, Department of Pediatrics
Division of Hematology
Department of Medicine
Duke University Medical Center
Durham, North Carolina

Eduard Shantsila, PhD
Postdoctoral Research Fellow
Centre for Cardiovascular Sciences, City Hospital
Birmingham, England, United Kingdom

Sanford J. Shattil, MD
Professor
Department of Medicine
University of California, San Diego
La Jolla, California

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Claire L. Shovlin, PhD
Senior Lecturer
National Heart & Lung Institute Cardiovascular Sciences
Imperial College London
Imperial Centre for Translational and Experimental Medicine
Hammersmith Campus
Honorary Consultant
Respiratory Medicine and HHTIC London
Imperial College Healthcare National Health Service Trust
Hammersmith Hospital
London, United Kingdom

Lois E. H. Smith, MD, PhD
Professor of Ophthamology
Department of Ophthalmology
Harvard Medical School
Associate in Ophthalmology
Department of Ophthalmology
Children's Hospital Boston
Boston, Massachusetts

Andreas Stahl, MD
Department of Ophthalmology
Harvard Medical School and Children's Hospital Boston
Boston, Massachusetts

Thorsten Steiner, MME
Vice Director
Department of Neurology
Heidelberg University
Heidelberg, Germany

Daphne B. Stewart, MD, MS
Assistant Professor
Department of Medicine
David Geffen School of Medicine at University of California, Los Angeles
Los Angeles, California
Physician Specialist
Department of Medicine
Olive View - University of California, Los Angeles Medical Center
Sylmar, California

John H. Stone, MD, MPH
Associate Professor
Department of Medicine
Harvard Medical School
Director, Clinical Rheumatology
Department of Medicine
Massachusetts General Hospital
Boston, Massacusetts

James R. Stone, MD, PhD
Assistant Professor
Department of Pathology
Harvard Medical School
Head of Cardiovascular Pathology
Pathology Service
Massachusetts General Hospital
Boston, Massachusetts

Angelina Wei Ling Tan
National University of Singapore Graduate School for Integrative Sciences and Engineering
Protein Science Laboratory
Department of Biological Sciences
Faculty of Science
National University of Singapore
Singapore

Ayalew Tefferi, MD
Professor of Medicine
Department of Hematology
Mayo Clinic
Rochester, Minnesota

L. Gayani Tillekeratne, MD
Infectious Diseases Fellow
Department of Medicine
Division of Infectious Diseases
University of Pennsylvania
Philadelphia, Pennsylvania

Douglas M. Tollefsen, MD, PhD
Professor
Departments of Medicine and Pathology & Immunology
Washington University
Attending Physician
Department of Medicine
Barnes-Jewish Hospital
St. Louis, Missouri

Edward G.D. Tuddenham, MD
Emeritus Professor of Haemophilia
Department of Haematology
University College London Medical School
Honorary Consultant Haematologist
Department of Haematology
Royal Free Hospital
National Health Service Trust
London, United Kingdom

Kanagasabai Vadivel, PhD
Postdoctoral Fellow
Protein Chemistry Laboratory
University of California, Los Angeles
Los Angeles, California/Orthopaedic Hospital
Department of Orthopaedic Surgery
David Geffen School of Medicine
University of California, Los Angeles
Los Angeles, California

William Vainchenker, MD, PhD
Director of Research
INSERM
Vaillant, France

Edith van de Vijver
PhD Candidate
Department of Blood Cell Research
Sanquin Blood Supply
Amsterdam, The Netherlands

John Paul Vavalle, MD
Cardiovascular Disease Fellow
Department of Medicine and Cardiology
Duke Clinical Research Institute
Duke University Medical Center
Durham, North Carolina

Elliott P. Vichinsky, MD
Adjunct Professor
Department of Hematology and Oncology
University of California at San Francisco
San Francisco, California
Medical Director
Department of Hematology and Oncology
Children's Hospital Oakland
Oakland, California

Jeanine M. Walenga, PhD
Professor
Department of Thoracic and Cardiovascular Surgery
Stritch School of Medicine
Director, Clinical Coagulation Laboratory
Department of Pathology
Loyola University Medical Center
Maywood, Illinois

Peter N. Walsh, MD, PhD
Professor
Department of Medicine, Biochemistry, and Thrombosis Research
Temple University School of Medicine
Philadelphia, Pennsylvania

Theodore E. Warkentin, MD
Professor
Department of Pathology and Molecular Medicine
Department of Medicine
McMaster University
Hematologist and Regional Director Transfusion Medicine
Hamilton Regional Laboratory Medicine Program
Hamilton Health Sciences - General Hospital
Hamilton, Ontario, Canada

Stephen P. Watson, PhD
British Heart Foundation Professor
Centre for Cardiovascular Sciences
University of Birmingham
Birmingham, United Kingdom

Neal L. Weintraub, MD
Director
Division of Cardiovascular Diseases
Internal Medicine
University of Cincinnati
Cincinnati, Ohio

John W. Weisel, PhD
Professor
Department of Cell and Developmental Biology
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

Jeffrey I. Weitz, MD
Professor
Medicine & Biochemistry & Biomedical Sciences
McMaster University
Executive Director
Thrombosis & Atherosclerosis Research Institute
Hamilton Health Sciences - General Hospital
Hamilton, Ontario, Canada

Gilbert C. White, II, MD
Aster Chair for Medical Research
Executive Vice President for Research
Director, Blood Research Institute
BloodCenter of Wisconsin
Associate Dean for Research
Medical College of Wisconsin
Milwaukee, Wisconsin

Sidney W. Whiteheart, PhD
Professor
Department of Molecular and Cellular Biochemistry
University of Kentucky
Lexington, Kentucky

Peter Wiedemann, MD
Professor of Ophthalmology
Klinik und Poliklinik für Augenheilkunde
Leipzig, Germany

Björ Wiman, MD, PhD
Professor Emeritus
Department of Clinical Chemistry
Institution of Molecular Medicine and Surgery
Karolinska Institutet
Senior Physician, Retired
Department of Clinical Chemistry
Karolinska University Hospital
Stockholm, Sweden

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Lucia R. Wolgast, MD
Assistant Professor
Department of Pathology
Albert Einstein College of Medicine
Associate Director, Hematology Laboratories
Department of Pathology
Montefiore Medical Center
Bronx, New York

Angus K.T. Wong, BMedSc (Hons)
Thrombosis Laboratory
Australian Centre for Blood Diseases
Melbourne, Australia

Wenfeng Xu, PhD
Director
Molecular Immunology
Novo Nordisk Inflammation
Research Center
Seattle, Washington

Feng Ye, PhD
Postdoctoral Fellow
Department of Medicine
University of California San Diego
La Jolla, California

Shaojing Ye, PhD
Postdoctoral Student
Department of Molecular and Cellular
Biochemistry
University of Kentucky College of Medicine
Lexington, Kentucky

T.T. Yee, MD
Physician
Haemophilia Centre
Royal Free Hospital
London, United Kingdom

Bin Zhang, PhD
Assistant Professor
Genomic Medicine Institute
Cleveland Clinic Lerner Research Institute
Cleveland, Ohio

Lijuan Zhang, PhD
Zhufeng Professor
School of Medicine and Pharmacy
Ocean University of China
Qingdao, People's Republic of China

PREFACE

The preface to the first edition of *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* discussed the "hemostatic system" as the product of a multimillion year evolutionary process that has resulted in a finely balanced mechanism that is poised for hemostasis yet constrained to ensure the continuous flow of blood. The complexity of the system was illustrated by the factor VIII: von Willebrand factor (vWF) complex, a protein complex that serves as a nexus for platelet adhesion, blood coagulation, and endothelial cell function and whose dysfunction is a common cause of bleeding and perhaps thrombosis as well.

As with the first edition, the sixth edition of *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* "reviews the biochemistry and physiology of hemostasis, as well as the pathophysiology of thrombosis, and considers the diagnosis and treatment of both."

However, over the three decades since the publication of the first edition, an exponential growth in the breadth and scope of new information together with increasing demands of clinical practitioners has mandated substantive changes in the book's content and organization, an evolution in its own right. Hemostasis has been dissected down to the atomic level and the sequencing of the human genome is providing new opportunities to understand the molecular basis for thrombotic disorders that remain the major causes of morbidity and mortality in our current society. Thus, the sixth edition is a distinctly different product in comparison with the first edition. We have striven to assemble a textbook that is current and complete, giving both basic and clinical scientists access, in a single place, to current concepts about hemostasis and thrombosis. We humbly acknowledge that the current edition will be quickly outdated as the acquisition of knowledge continues at a rapid pace beyond its publication. Indeed, it is tantalizing to consider how subsequent editions over the next 30 years will reshape our views about hemostasis and thrombosis. We hope that thoughtful readers will discern the gaps in our present knowledge and devise ways to close them.

We have taken to heart the insightful comments of reviewers of prior editions and have added or altered whole sections of the textbook to make it more accessible, useful, and informative. To start, the sixth edition has an entirely new editorial group, with each member bringing outstanding talent and expertise to the book's composition. The new editors include (in alphabetical order) William C. Aird (Harvard Medical School), who has created entirely new sections on vascular biology and the endothelium and who has himself recently published a large volume on *Endothelial Biomedicine* (Cambridge University Press); Joel S. Bennett (University of Pennsylvania), who has made seminal contributions to our understanding of platelet biochemistry and who has reorganized the basic and clinical sections related to platelets; Sam Schulman (McMaster University), who has wide experience in all aspects of both thrombosis and bleeding disorders and who is at the forefront of clinical trial assessments of new anticoagulants; and Gilbert C. White, II (BloodCenter of Wisconsin), who is widely knowledgeable in coagulation and platelet physiology and the clinical aspects relating broadly to these fields. Victor J. Marder (David Geffen School of Medicine at UCLA) is the lone editor to have participated in all of the previous five editions and who is fortunate to have the broad and deep expertise of the new editors in conceiving and constructing the sixth edition. All of the editors contributed to all aspects of the volume, having both overlapping and complementary experience and expertise in coagulation, platelet physiology, vascular biology, and clinical aspects of venous, arterial, microvascular, and complex hematologic disorders that have become the routine in outpatient and inpatient medical management. The principles described in this book are now widely applied to virtually every discipline in medicine, notably but not at all limited to neurology, cardiology, pulmonology, orthopedic and vascular surgery, oncology, and radiology.

The sixth edition comprises 132 chapters (two pairs of chapters of closely related content are indicated as A and B). Entirely new sections of importance have been added, including those that consider fundamental science and methods related to every aspect of hemostasis and thrombosis, vascular biology and primary disorders of blood vessels, and bleeding or thrombotic manifestations in systemic conditions in which the hemostatic system is secondarily perturbed. While the book has been expanded and reorganized to more broadly cover the field, it is not meant to serve as a practical guide for all aspects of diagnosis and treatment. Rather, it is designed to provide a conceptual framework for approaching complex issues in hemostasis and thrombosis, for rationally considering the science, and for logically applying reasoned insights to clinical problems. Ideally, the information will be applied in a productive manner to translate science to important new clinical breakthroughs, as has already occurred in the 30 years since the publication of the first edition.

On a technical note, the sixth edition is being published as a single volume in four-color format throughout, which represents an improvement over previous editions. Purchasers of the book will be provided access to an electronic version that allows downloading of all graphics for academic purposes. For clarity and brevity, each chapter is limited to 20 references in the printed version. The full list of references may be accessed in the electronic version.

In conclusion, it is the editors' hope that readers will find that the sixth edition of this long-standing primary reference addresses the myriad of scientific and clinical questions emerging from this exciting and still growing field of inquiry.

Victor J. Marder
William C. Aird
Joel S. Bennett
Sam Schulman
Gilbert C. White, II

FROM THE PREFACE TO THE FIRST EDITION

The hemostatic system has evolved over millions of years from a much simpler system. In the limulus, a 400-million-year-old living fossil, the entire hemostatic system is contained in one cell (the amebocyte) that, in response to endotoxin elaborated by a vibrio, engulfs the organism and forms a coagulant from intracellular constituents. This single cell can be viewed as a progenitor of the platelet and the leukocyte, serving both hemostatic and inflammatory functions. The hemostatic system that evolved in humans features extracellular coagulation proteins, many of which are still present in platelets and endothelial cells, and has, therefore, conserved a function and structural interrelationship of plasma proteins and cellular elements.

Hemostasis and Thrombosis reviews the biochemistry and physiology of hemostasis, as well as the pathophysiology of thrombosis, and considers the diagnosis and treatment of both. The difficulty of separating one part of the system from another is illustrated by considering one of the best known hemostatic proteins, factor VIII, which is currently regarded as a complex of two molecules—von Willebrand protein and procoagulant factor VIII. Von Willebrand protein is synthesized in endothelial cells and megakaryocytes and is secreted into the plasma, while the coagulant factor VIII molecule is synthesized in other cells, possibly under the control of the von Willebrand protein. The latter influences platelet adhesion to exposed subendothelium and probably to other cells, while factor VIII increases the rate of factor X activation and thrombin formation, which, in turn, stimulates platelets to undergo the release reaction and to aggregate and convert fibrinogen to fibrin. An obvious requirement for hemostasis, being deficient in the coagulant portion in classic hemophilia and in both the coagulant and platelet cofactor portions in von Willebrand disease, the factor VIII complex is probably also intimately associated with pathologic thrombus formation. Thus, an understanding of the role of factor VIII requires a detailed knowledge of its interactions with platelets, vascular wall cells, and other plasma proteins, as well as the influence of rheologic factors on these reactions, as manifest in the opposing mechanisms of hemostasis and thrombosis.

This textbook is directed at all who wish to explore this fascinating area of biology in greater depth. We hope that it will prove to be a useful reference for the student, the physician, and the scientist who is interested in laboratory or clinical aspects of these disorders and who may wish to obtain a comprehensive discussion and complete reference source. Many authors have contributed to *Hemostasis and Thrombosis*, and some overlap of information was inevitable, but this was not actively avoided. Therefore, we hope that each chapter is complete in its own right, concentrating on either basic aspects or clinical applications of a disorder or relevant cellular or protein components.

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The editors wish to express their appreciation to Dr. Robert W. (Bob) Colman for his foresight and efforts in the genesis of *Hemostasis and Thrombosis: Basic Principles and Clinical Practice*. It was his concept of a comprehensive text encompassing the breadth of basic and clinical science of this field that was responsible for the first five editions of *Hemostasis and Thrombosis* and that is the inspiration for the sixth. Bob has been an important contributor to many aspects of the field in studies that have spanned surface activation of coagulation; the role of lipids, membrane receptors, and phosphodiesterases in regulating platelet function; the structure and function of coagulation factor V; and the physiology of kinins, particularly with regard to inflammation and angiogenesis.

Since the first edition was published in 1982, *Hemostasis and Thrombosis: Basic Principles and Clinical Practice* has stood as the preeminent standard. Although much of its success can be attributed to wise choices of authors, the skill of the editors in choosing authors and in organizing their contributions into a coherent text is equally important. In addition to Bob Colman, six others have participated in editing the previous five editions of the text. Therefore, the present editors wish to acknowledge the vital efforts of Jack Hirsh and Edwin Salzman, who along with Victor Marder coedited the first three editions, and Alex Clowes, Jim George, and Sam Goldhaber, who participated in subsequent editions. Their expertise, energy, and creativity have provided the foundation upon which we have based the sixth edition.

We note with sadness the passing of Edwin Salzman in 2011 after a prolonged and difficult illness. Ed was a unique individual and a wonderful colleague whose contributions to understanding and treating thrombotic diseases and the nuances of platelet function will be long remembered.

Finally, GCW, SS, WCA, and JSB wish to express their great appreciation to Victor Marder for his leadership during the development of the 6th edition. His organizational skills, dedication to detail, and commitment to quality were inspiring and have made this edition what it is. We thank him for inviting us to be a part of this edition and making us part of the *Hemostasis and Thrombosis* tradition.

Victor J. Marder
William C. Aird
Joel S. Bennett
Sam Schulman
Gilbert C. White, II

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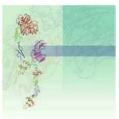

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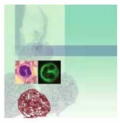

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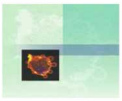

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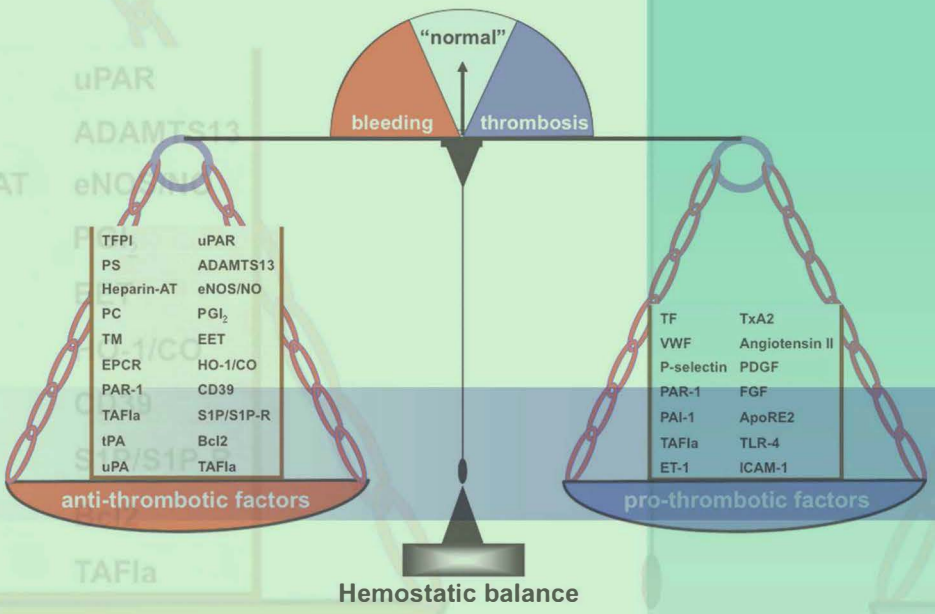

PART I

Introduction to the Field of Hemostasis and Thrombosis

CHAPTER

1

The Field of Hemostasis and Thrombosis: Selected Translational Achievements

VICTOR J. MARDER, WILLIAM C. AIRD, JOEL S. BENNETT, SAM SCHULMAN, AND GILBERT C. WHITE, II

INTRODUCTION

As with any field of active scientific inquiry, the field of "Hemostasis and Thrombosis" has experienced its own evolution of thoughts and concepts, and the ever refining of the details of existing information. Given the enormous worldwide impact of hemostatic and thrombotic disorders, the intense focus of scientists on the field is justified, even essential. The modern era of coagulation could arguably be dated from 1905, when Morawitz proposed that four ingredients are involved in clot formation.[1] By this formula, fibrinogen (factor I) is converted to fibrin by thrombin, produced from prothrombin (factor II) by the action of "thrombokinase" (factor III) and calcium (factor IV). The designations factors I, II, III, and IV were preserved in the nomenclature consensus agreement of 1959,[2] which brought some semblance of order to the plethora of synonyms used for each real or implied clotting participant reported by that time. Observations regarding thrombotic disorders in the venous circulation were reported independently, and one could date the origin of this discipline to the late 19th century with the reports of Virchow (1856)[3] and Trousseau (1865).[4] Virchow's many and dramatic breakthrough observations include the now-obvious connection between venous thrombosis of the lower extremity with pulmonary embolism,[3] although the often-cited "Virchow's triad" of causes for venous thrombosis may actually not belong to his credit alone.[5] Trousseau brought to our attention the association of carpal spasm with tetany, but more memorably, the association of malignancy with venous thrombosis.[4,6]

RECOGNITION OF THROMBOSIS AND HEMOSTASIS AS A UNIFIED FIELD OF SCIENTIFIC INQUIRY

Despite the slow pace of discovery during the first half of the 20th century, there were a number of important and remarkable observations. Three separate discoveries of potential therapeutic agents are representative. First is the serendipitous purification of an anticoagulant from liver in 1916 by Jay McLean, a Johns Hopkins medical student,[7,8] named "heparin" by his faculty mentor William Howell.[9] Second is the serendipitous finding[10] by Tillett and Garner in 1933 to 1934 of a clot-lysing ingredient in Streptococcal broth, termed fibrinolysin.[11–12] Third is the detective work that solved the mystery of hemorrhagic disease in cattle caused by spoiled (moldy) sweet clover hay[13], leading ultimately to the purification of dicoumarol by Campbell and Link in 1941.[14,15] Translation of these observations to the clinic was likewise a slow process; it took 20 years until heparin was tested in a relevant animal model,[16] 14 years for a coumarin agent to be applied as a rodenticide,[17] and 12 years for fibrinolysin (Streptokinase) to be used in animals[18] and 16 in humans but for the lysis of pleural adhesions, not vascular thrombosis.[19, 20] Concurrent with these discoveries, inquiry into the components of blood coagulation began to reveal the complexity of this process. Historical reviews detail these events,[21–25] but suffice it to say that patients with distinct deficiencies were identified in a flurry of discovery in the 1940s and 1950s, following work on antihemophilic factor (factor VIII) in 1937 by Patek and Taylor.[26] The full cast of clotting factors was then identified, mostly through clinical comparisons and the differential correction of abnormal blood coagulation in the laboratory. In this way, patients were found who were deficient in "proaccelerin" (factor V) by Owren,[27] "serum prothrombin conversion accelerator" (factor VII) by Alexander,[28] "Christmas factor" (factor IX) by groups led by Aggeler,[29] Biggs,[30] and Schulmann,[31] "plasma thromboplastin antecedent" (factor XI) by Rosenthal et al.,[32] "Hageman factor" (factor XII) by Ratnoff,[33] "Stuart-Prower factor" (factor X) by Hougie et al.,[34] and "fibrin stabilizing factor" (factor XIII) by Duckert,[35] some 17 years after its biochemical identification by Robbins[36] and by Laki and Lorand.[37]

These observations were facilitated by the development of simple, yet incredibly important, bench technology for measuring blood coagulation, namely, the prothrombin time devised by Quick in 1935 (the "Quick time")[38] and the partial thromboplastin time by Langdell, Wagner, and Brinkhous in 1953.[39,40] With the development of platelet aggregometry by Born and by O'Brien in 1962,[41,42] the field of platelet physiology and pathology took a major leap forward[21] beyond the 19th century description of a role for platelets in clot and thrombus structure,[43–45] and the early 20th century descriptions of Glanzmann thrombasthenia,[46] von Willebrand disease,[47] and Bernard-Soulier syndrome.[48]

The expanding number of hereditary bleeding disorders and the vexing problem of competing terminology for the same clotting component contributed to a complexity that even to this day, frightens the medical student and humbles the young scientist upon entering the field. The nomenclature issue was resolved by the committee,[2] providing for simplified nomenclature for the bewildering array of blood clotting components, using Roman numerals for each factor. More importantly, clarity to the question of how the factors interact to initiate clotting was provided in 1964 via the brilliant syntheses of the science by Ratnoff and Davie[49] of the "waterfall" and by Macfarlane[50] of the

"cascade" of biochemical events. Admittedly, these explanations were not without need for modification.[22,23] For example, it was necessary to account for thrombin activation of factors V and VIII,[51] for their function as cofactors and not enzymes,[52] and for the activation of factor IX by the "extrinsic" system factor VII: tissue factor complex.[53] Still, the schemes devised by Ratoff and Davie and by Macfarlane had a profound impact on our concepts of blood coagulation and provided a framework for a remarkable, ever-accelerating application of new scientific technology to the "field."

One might assume that the "field" of hemostasis and thrombosis existed from the start. However, a formal designation of a discipline for the study of both bleeding and clotting disorders was glaringly absent until the mid-1900s, and many basic and clinical research groups focused uniquely on the diagnosis and treatment of patients with hereditary bleeding disorders caused by clotting factor or platelet disorder, or on the pathology, treatment, and prevention of thrombosis, mostly in the venous circulation. Arguably, the field of Hemostasis and Thrombosis began with three organizational events, the first being the founding of the journal Thrombosis et Diathesis Haemorrhagica (TDH) in 1957, formally inviting scientific submissions in both thrombosis and bleeding. Second, the International Committee on Clotting Factors, upon completing its nomenclature goals, changed its focus and name in 1964 to the International Committee on Thrombosis and Hemostasis (ICTH). Third, the International Society on Thrombosis and Haemostasis (ISTH) was established in 1969, incorporating the ICTH as the Scientific and Standardization Committee and collaborating with the journal TDH as a major outlet for publishing reports and scientific studies. Now, many former Hemophilia Centers are termed Hemophilia and Thrombosis Centers, reflecting a broader scope of interest and cementing the collaboration of basic scientists working in the areas of vascular and cell biology, biochemistry, genetics, physiology, immunology, and pathology with clinical scientists with expertise in diagnosis, pharmacology, biostatistics, epidemiology, and public health. A major goal of the field is to apply basic principles to clinical practice, and notable achievements have been accomplished in the field of Hemostasis and Thrombosis.

OVERVIEW OF HEMOSTASIS AND THROMBOSIS

In a healthy individual with an intact vascular system, blood flow is unimpeded and the soluble and cellular components of the blood are balanced to maintain blood fluidity yet are poised for a rapid and effective response to vascular trauma (FIGURE 1.1). This balance is complex and multilayered but can be affected by confounding influences such as hereditary polymorphisms, inflammatory processes, malignancy, vascular injury, superimposed medical illness or surgical intervention, or drug exposure yet be primed for effective protection against excessive bleeding after vascular trauma.

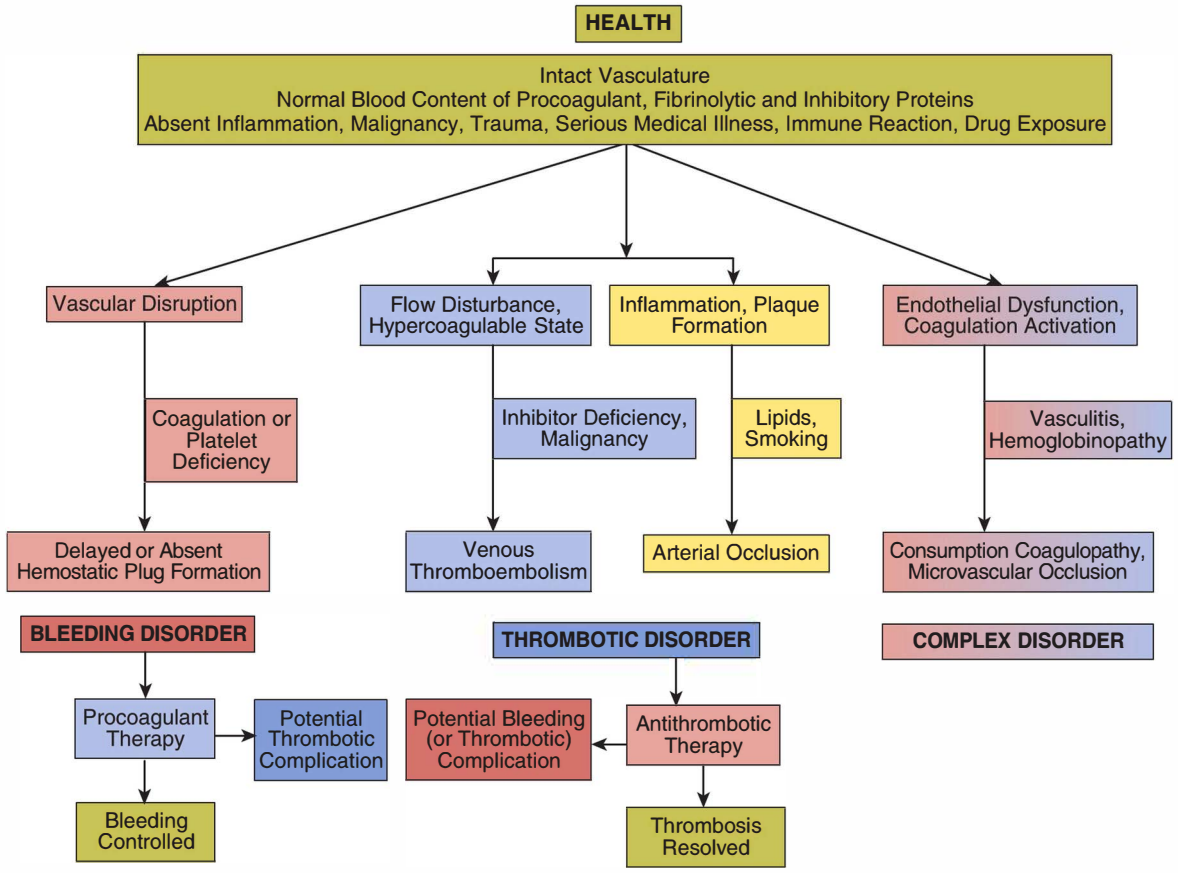

FIGURE 1.1 Schematic of bleeding and thrombotic disorders, contributing factors, and complications of therapy.

HEMOSTATIC DISORDERS

Hemostatic disorders may first become apparent after vascular injury such as during surgery or may seem to be "spontaneous" without evident injury, but following otherwise trivial activities such as walking or dental care (FIGURE 1.1, **left**). While the distinction between normality and a mild bleeding state is often problematic, as in the case of "simple" purpura, fully manifest bleeding disorders, regardless of the etiology, may be dramatic and life threatening. Based on the current state of knowledge and clinical experience, the diagnosis of a hereditary coagulation disorder is usually straightforward. Nonetheless, the relative contribution of coagulation factor deficiencies to bleeding varies, and in some individuals with abnormal *in vitro* coagulation tests, bleeding may be unusually absent (factor XII deficiency) and there may even be a risk of thrombosis (lupus anticoagulant). The hemorrhagic manifestations of thrombocytopenia and/or platelet dysfunction are distinctive, manifest as mucocutaneous bleeding due to small capillary leaks. Immune disorders such as acquired hemophilia due to autoantibodies against or thrombocytopenia due to the appearance of platelet autoantibodies can appear suddenly in a patient with no prior history of bleeding. Once a diagnosis is made, treatment is usually evident and effective, but the same cellular and protein components that are involved in hemostasis also promote thrombosis. Thus, in some instances, such as with the use of recombinant factor VIIa to arrest hemorrhage, treatment carries a potential complication of thrombosis.[51]

VENOUS THROMBOSIS

The predispositions for venous or arterial thrombotic disease differ markedly. A triad of conditions, generally attributed to Virchow,[5] consisting of the confluence of a systemic hypercoagulable state, disturbance of venous blood flow (stasis), and vascular injury makes for a perfect storm of venous thromboembolic (VTE) disease. For example, in 1959, Sevitt and Gallagher[52] highlighted the VTE risk that can complicate hip fracture in which hypercoagulability is induced by the release of bone marrow contents into the circulation, the fracture causes complete immobility, and there is associated venous damage due to the initial injury or surgical repair. However, not all three components of the triad are required to present an increased risk of VTE, as reflected by the 80% prevalence of VTE after total knee arthroplasty in the absence of DVT prophylaxis[53] or the smaller risk of VTE that accompanies ankle and even arthroscopic surgery.[54] In the latter instances, it would not be unreasonable to anticipate that additional factors, such as the absence of a natural anticoagulant inhibitor such as protein C (PC) or the presence of the factor V Leiden mutation, strongly impact on clinical outcome.[55–57,58] The balance of hemostasis and thrombosis is reflected in their respective hereditary predispositions of absent inhibitors of procoagulant proteins or coagulation inhibitors.

Options of the effective treatment of venous thromboembolism are expanding rapidly with the recent introduction of oral thrombin and factor Xa inhibitors to the standard regimen of coumarins and heparins.[59] Nonetheless, in converse to the treatment of bleeding disorders, (FIGURE 1.1, **bottom center**), treatment of VTE with anticoagulants carries a risk of a bleeding complication. Again, concomitant hereditary predispositions, in this case to bleeding, increase the risk of such a complication.

ARTERIAL THROMBOSIS

Arterial thrombosis is responsible for the most prevalent and lethal pathologic conditions in the world, myocardial infarction and ischemic stroke.[60] The pathogenesis of these arterial events is distinct from that in venous thrombosis and usually results from the rupture of an atherosclerotic plaque in epicardial coronary vessels and often, but not always, involves an upstream embolic event in ischemic stroke. Thrombi in the vicinity of a ruptured atherosclerotic plaque have a prominent platelet component, initiated by exposure of blood to tissue factor on the plaque surface and in plaque debris. This results in a "white thrombus," composed predominantly of platelets and fibrin, although a retrograde red thrombus composed of red cells and fibrin may form when arterial blood flow is blocked. The "white thrombus" is analogous to the primary hemostatic platelet plug that forms after vascular trauma, a clear example of the coalescence of cellular and coagulation responses in physiologic and pathologic conditions. Predispositions to arterial thrombosis are distinct from those that predispose to venous thrombosis, being primarily those influences that promote arthrosclerosis, and less commonly resulting from inflammation (vasculitis) or immune damage as in the antiphospholipid syndrome (APLS). Less often, arterial occlusion can result from the same pathophysiology as VTE, as after embolization of an atrial thrombus in a patient with atrial fibrillation or from paradoxical embolization in a patient with a patent foramen ovale.

Because platelets are the predominant component of arterial thrombi, antiplatelet drugs are the agents of choice in treating these disorders. As with treatment for VTE disease, there is a risk of antithrombotic treatment inducing a hemorrhagic complication, with the added burden that the hemorrhage may be intracranial in a patient with an ischemic stroke. Adding complexity to treatment, we now appreciate that treatment can result in thrombosis as well as hemorrhage. Thus, the heparin-induced thrombocytopenia (HIT) that results from immune complexes containing antibodies against platelet factor 4 (PF4) can produce arterial and venous thrombi, and vitamin K antagonists (coumarins) can induce cutaneous microvascular thrombosis producing skin necrosis in a patient with inherited PC deficiency.[62,63]

COMPLEX DISORDERS

These disorders manifest both bleeding and thrombosis and emphasize the disparate properties of some coagulation proteins. Thrombin, the central player in the coagulation cascade, mediates feedback amplification of the "intrinsic" system by activating factor XI; activates platelets to expose phosphatidylserine, thereby accelerating the formation of factor Xa; activates factors V, VIII, and XI; activates factor XIII and the thrombin-activated fibrinolysis inhibitor making clots more resistant to plasmin lysis; and cleaves fibrinopeptides A and B from fibrinogen to induce fibrin formation. Thrombin also binds to thrombomodulin (TM) on microvascular endothelial cells and changes its character to activate PC. When bound to the endothelial cell activated protein C (APC) receptor and in concert with cofactor protein S (PS), APC proteolytically inactivates factors Va and VIIIa, thus effectively turning off the coagulation cascade. Accordingly, thrombin is not only the quintessential procoagulant enzyme but also the quintessential anticoagulant molecule.

The prototypical example of a complex disorder (FIGURE 1.1, **right**) is disseminated intravascular coagulation (DIC), most

commonly associated with sepsis and endotoxemia, in which there is activation of both platelets and coagulation.[64] This results in loss of endothelial antithrombotic functions, microclot formation, consumption of platelets and coagulation factors, microvascular occlusion causing organ failure, and bleeding. The difficulty in recognizing and managing DIC derives in part from the variable multi-organ dysfunction that can be mimicked by other pathologic syndromes. For example, thrombotic thrombocytopenic purpura, initially described by Moschowitz in 1924, results from the failure to cleave ultra-large high molecular weight von Willebrand factor (vWF) multimers because of an inherited or acquired ADAMTS 13 deficiency. This results in platelet thrombi in the microcirculation of the brain, kidney and even heart, lungs and liver.[65] Thus, at one extreme are the abnormal or absent vWF proteins that cause the hemorrhagic syndrome of von Willebrand disease,[66] while at the other extreme is the persistence of ultra-large multimers on the endothelial surface[67] that are decorated with platelets and lead to microvascular occlusion.

Multiple small and medium-sized vessels can be occluded as part of specific disorders, such as sickle cell disease, polyarteritis, and the APLS. APLS in particular may present with complex features, including non-bacterial thrombotic endocarditis, systemic activation of coagulation, embolic phenomena, placental ischemia, as well as the lupus inhibitor that prolongs the PTT and prothrombin time (INR). While patients with APLS may also have a specific coagulation factor inhibitor that causes bleeding,[68] the inhibitor actually represents a risk for the thrombotic, not hemorrhagic, manifestations.[69] Thus, patients with APLS can present a dilemma for clinicians as patients could simultaneously manifest both bleeding and thrombosis. Additional examples could include patients with the Budd-Chiari syndrome (hepatic vein thrombosis) portal hypertension and bowel ischemia who also have active bleeding from esophageal varices or patients with postoperative hip or knee replacement patient and significant bleeding into joint prosthesis, but also having a proximal DVT and PE.

SELECTED TRANSLATIONAL ACHIEVEMENTS IN THE FIELD OF HEMOSTASIS AND THROMBOSIS

Translational advances made by the "clotters" have had an immense impact in medicine. A limited number of highly selected translational achievements are described below and reflect the broad application of their insights on medical practice beyond the field of hemostasis and thrombosis.

NEW ORAL ANTICOAGULANTS

Heparin and coumarin-based vitamin K antagonists have been the mainstays of thrombosis treatment and prevention for more than half a century, and efforts to improve on their safety, efficacy, and especially their pharmacologic limitations, have led to development of some very inventive agents. Examples of new directions in anti-coagulant therapy include the strict anti-thrombin leech salivary gland extract, hirudin,[70] and its recombinant or synthetic derivatives,[71] and short peptide inhibitors of thrombin such as argatroban,[72] which remain in clinical use as intravenous antithrombotic agents for patients with HIT. In 1992, Bode et al.[73] at the Max-Planck Institute for Biochemistry reported the crystallographic solution of the 3-dimensional configuration of thrombin with a resolution of 1.9 Å and revealed the active site pocket in complex with D-Phe-Pro-Arg CH_2Cl and other non-peptidic molecules. This observation paved the way for the design of highly-specific thrombin inhibitors with perfect fit into the serine protease pocket. Drug development at the Swedish company Hässle (subsequently Astra and then Astra-Zeneca) led to the first orally available thrombin inhibitor melagatran (and its prodrug ximelagatran).[74] The concept applied to the design of these synthetic agents was to mimic the structure of fibrinopeptide A and, as opposed the slow-tight binding of another prototype (efegatran), melagatran demonstrated a high association rate constant indicating that it would rapidly inhibit thrombin.[75] Gustafsson et al. pioneered the preclinical development and clinical trials with melagatran,[76,77] showing that the drug had similar efficacy and bleeding risk as low molecular weight heparins and warfarin for prophylaxis against VTE,[78,79] prophylaxis against stroke in atrial fibrillation,[80,81] and treatment of VTE.[82,83] Notwithstanding the liver toxicity that appeared during the phase III clinical trials and led to the withdrawal of the drug in 2006, the concept of an oral anticoagulant that avoided the multiple drug interactions and which did not require the careful laboratory monitoring used for coumarin agents, was correct. Direct-acting thrombin inhibition, not requiring activation of the serpin antithrombin (AT), is effective and with a bleeding risk similar or less than with conventional anticoagulants. A favorable risk/benefit-ratio was confirmed with the successor to melagatran, dabigatran, in a large trial in patients with atrial fibrillation, showing that reduction of ischemic stroke can be achieved simultaneously with reduction of intracranial bleeding.[84] Currently, more than 500,000 patients are receiving long-term dabigatran for stroke prophylaxis. New small molecule anticoagulants that bind in the factor Xa serine protease pocket have also been developed, including rivaroxaban, apixaban and edoxaban and advanced clinical trials show impressive efficacy and safety results in stroke prevention in atrial fibrillation,[84,85,86] in DVT prophylaxis and treatment,[87–89,90,91] and in acute coronary syndromes.[92] It now appears that we have entered a new age of anticoagulant pharmacotherapeutics that will decrease our reliance on parenteral heparins or the oral coumarins.

ABCIXIMAB, THE FIRST THERAPEUTIC MONOCLONAL ANTIBODY

The saga of the development of a chimeric human/mouse anti-$\alpha_{IIb}\beta_3$ monoclonal Fab fragment into the anti-thrombotic agent abciximab over a period of nearly 40 years is a superb example of the elegant translation of basic studies of hemostatic mechanisms into a successful and widely used medication. In the late 1950s and early 1960s, platelets were found to aggregate when exposed to stimuli such as the adenine nucleotide ADP, a phenomenon that was missing from the platelets of patients with the bleeding disorder Glanzmann thrombasthenia.[93] Further, platelet aggregation was noted to require the additional presence of the plasma protein fibrinogen, an observation that would prove to be the key to understanding the mechanism of platelet aggregation.[94]

With the development of the methodology to separate proteins by molecular weight, Phillips et al.[95] found that platelets

from patients with thrombasthenia lacked glycoproteins, that were subsequently designated glycoproteins IIb and IIIa. Shortly thereafter, Bennett and Vilaire[96] and Marguerie, Plow, and Edgington[97] demonstrated that platelets contain specific receptors for fibrinogen that bound fibrinogen only following platelet stimulation by agonists such as ADP. Moreover, these receptors were missing from thrombasthenic platelets.[96] Taken together, these observations provided an explanation for the functional defect in thrombasthenia and suggested that the proteins missing from thrombasthenic platelets, a heterodimer composed of GPIIb and GPIIIa, represented the fibrinogen receptor. More importantly, the data provided a testable hypothesis[96] for the mechanism of platelet aggregation: binding of dimeric fibrinogen molecules to the 50,000 to 80,000 GPIIb/IIIa molecules on one activated platelet to the same number of GPIIb/IIIa molecules on adjacent activated platelets crosslinked the platelets into macroscopic hemostatic aggregates.

A variety of techniques were applied to confirm this hypothesis, including the production of monoclonal antibodies in mice directed against the human GPIIb/IIIa heterodimer. A number of these antibodies prevented fibrinogen binding to activated platelets, reproducing the thrombasthenic phenotype using normal platelets.[98,99] The implication of these results did not escape the notice of Coller and collaborators at Centocor who posited that such an antibody could be engineered into a drug that might be useful in preventing the platelet thrombi responsible for acute coronary syndromes and stroke. To avoid antibody binding to the platelet FcγRIIa receptor, they converted the monoclonal antibody (designated 7E3) to Fab fragments.[100,101] Then, to minimize the antigenicity of the murine molecule, they replaced the framework of the mouse Fab with that of human IgG. The resulting chimeric molecule, abciximab (c7E3, ReoPro), inhibits agonist-stimulated fibrinogen binding and platelet aggregation *in vitro*, binds tightly to GPIIb/IIIa with a dissociation constant of ≈ 5 nM, and binds to the vitronectin receptor $\alpha_v\beta_3$ and the leukocyte integrin $\alpha_M\beta_2$ as well.[102]

An elegant series of experiments in animals confirmed the hypothesis that abciximab could inhibit platelet-mediated thrombosis *in vivo*[103,104] and the clinical efficacy of abciximab as an antiplatelet agent in humans was confirmed in four pivotal clinical trials in patients undergoing percutaneous coronary intervention (PCI) for acute coronary syndromes.[105] In 1994, abciximab was approved by the FDA for the prevention of ischemic complications of angioplasty and subsequently, for PCI with stents and as medical therapy for unstable angina.

THE CLONING OF FACTOR VIII AND THE DEVELOPMENT OF RECOMBINANT PRODUCTS

The cloning of the F8 gene and the development of safe factor VIII concentrates for the treatment of hemophilia A was a major translational advance in the field of blood coagulation in the past 40 years. The urgency for the development of recombinant products was driven by the discovery that HIV-1 was transmitted by blood products and was especially concentrated during the manufacture of pooled plasma derived clotting protein concentrates.[106,107] The gene for factor VIII was cloned in 1984 by two groups—scientists at Genetics Institute working with scientists at the Imperial College of London and other institutions and with support from Baxter Healthcare, and scientists at Genentech working with colleagues at UCSF, Mayo Clinic and other institutions with support from Bayer Pharmaceuticals. This illustrates the considerable strength that an academic-industry collaboration can bring to a task. The publication of the F8 gene and structure by these two groups appeared in back-to-back articles in *Nature*.[108,109,110,111] At the time, F8 was by far the largest gene cloned and it remains one of the largest. Following the cloning of the F8 gene, the race was on to develop recombinant forms of factor VIII. Just 3 years later, in March 1987, the first infusion of recombinant factor VIII was administered.[112] Recombinant factor VIII preparations were co-licensed by Baxter Healthcare and by Bayer Pharmaceuticals and these products quickly gained market-share against plasma-derived products. The production of recombinant factor IX followed,[113] as did recombinant factor VIIa,[114] a product that has proven safe and effective in the treatment of patients with factor VIII and IX inhibitors, as well as other bleeding disorders. Additional products now include recombinant vWF,[115] recombinant factor XIII,[116] and recombinant ADAMTS13.[117] The process of improving the properties of factor concentrates continues; for example, the elimination of all traces of human protein in an effort to minimize contamination of blood-borne infections[118] and the production of molecular variants to prolong the half-life of infused factor and reduce the number of injections needed for prophylaxis (see Chapter 79). Recombinant products are not without side effects, the most important of which is the occurrence of thrombotic complications after infusing an activated enzyme such as factor VIIa,[119] but this potential is well within the risk to benefit equation considering its broad application in serious hemorrhagic situations, including battlefield wounds. A question also remains as to whether recombinant factor VIII products are more prone to induce inhibitors than plasma-derived products,[120] but this appears less likely with recent information that implicates the potential role of factor VIII haplotypes.[121]

To date, it is estimated that more than 50 billion units of recombinant clotting factor products have been produced since their introduction; current annual sales of recombinant factor VIII exceed $1.5 billion per year, while sales of recombinant factor VIIa and recombinant factor IX are $750 million and just under $500 million, respectively. Most importantly, no new cases of human immunodeficiency or hepatitis virus transmission have been reported since the introduction of recombinant products.

The significance of the development of recombinant clotting factors cannot be underestimated. Not only did it provide a safe and effective method of treatment for hemophilia and other bleeding disorders, but it helped spawn and validate the field of biotechnology. It showed that recombinant therapeutics could be generated in large enough amounts to supply the whole disease population, that good manufacturing processes and reproducibility could be developed, and that the products could be successful pharmaceutics. Recombinant factor VIII was one of the early recombinant proteins, along with insulin, interferon α and γ, granulocyte colony stimulating factor (G-CSF), and erythropoietin. In many ways, these first recombinant products paved the way for biologics, in general. These were among the first proteins to go through FDA approval and they therefore set the standard for expectations regarding analysis. But it did more than this. By providing large amounts of purified factor VIII, it fostered previously unapproachable research on the structure

and function of factor VIII. It led to better understanding of the role of factor VIII in the coagulation process. It facilitated studies aimed at gene therapy for hemophilia. It also enabled current work aimed at generating imuteins (mutant proteins) with improved clotting factor properties—for example, ones that have a prolonged time in circulation or enhanced activity or reduced antigenicity.

DISCOVERY OF VASCULAR ENDOTHELIAL GROWTH FACTOR

It has long been recognized that tumors are associated with increased blood vessel growth. In 1971, Judah Folkman, a pediatric surgeon-scientist at Children's Hospital in Boston, published a seminal paper in which he suggested that the identification of key molecules mediating tumor angiogenesis might lead to new anti-angiogenic therapies that would starve a tumor.[122] Folkman's hypothesis was followed by decades of intense research that has culminated in the introduction of a number of anti-angiogenic medications in clinical practice.

In 1979, Harold Dvorak at Harvard Medical School demonstrated that tumors become enveloped in fibrin-gel within hours of implantation,[123] that a hepatocarcinoma tumor cell line secretes a vascular permeability factor (VPF), and that fibrin gels implanted into guinea pigs induce an angiogenic response. Thus, Dvorak established a pathophysiologic link between increased vascular permeability, neoangiogenesis, and stromagenesis in tumor growth. In 1983, the Dvorak team purified VPF from the hepatocarcinoma cells, and showed that the 34 to 42 kDa protein was distinct from other known permeability factors such as prostaglandins, kallikrein, leukokinins or histamine. When injected into guinea pig skin, VPF caused a rapid, transient increase in microvascular permeability without causing endothelial cell damage or mast cell degranulation, antihistamines did not block its action, and antibody against purified VPF neutralized virtually all of the permeability-increasing activity.[124] Dvorak went on to show that a broad spectrum of tumor cells from several species, including humans, secrete the same VPF.[125] He proposed that the permeability activity might be important for tumor growth. In collaboration with the Monsanto Company, Dvorak sequenced the NH2-terminal amino acid sequence of tumor-secreted VPF that had been purified to homogeneity.[126]

In 1989, Napoleone Ferrara, working at Genentech Inc. in San Francisco, purified a heparin-binding factor from pituitary follicular cells which was a potent mitogen for vascular endothelial cells, naming the protein vascular endothelial growth factor (VEGF).[127] That same year, he reported the cDNA sequence for bovine and human VEGF.[128] In back-to-back articles in *Science*, Daniel Connolly from Monsanto Company, who had shown that VPF was also a potent endothelial cell mitogen, reported the cloning of the gene.[129] VEGF and VPF proved to be identical. In collaboration with others at Genentech, Ferrara developed a murine anti-human VEGF monoclonal antibody with potent anti-tumor effect in xenograft models,[130] followed by development of the antibody bevacizumab (Avastin) that binds and neutralizes all human VEGF-A isoforms. Based on positive phase III trials, Avastin received FDA approval for colorectal cancer treatment in 2004, and another Genentech anti-VEGF antibody (ranibizumab, Lucentis) was FDA approved in 2006 for the treatment of wet age-related macular degeneration.

Dvorak went on to show that VEGF/VPF is widely overexpressed not only by many malignant animal tumors and human cancers, but also by epithelial and inflammatory cells in healing wounds and chronic inflammatory diseases. This process is highly adaptive in the case of wounds, and is shut off once healing has occurred, as VEGF/VPF levels return to normal. In contrast, tumors continue to produce large amounts of VEGF/VPF, promoting further blood vessel and tumor growth, prompting the comment that tumors behave as "wounds that fail to heal."[131] The discovery of VEGF/VFP stands as a seminal contribution in the field of vascular biology, with >37,000 articles related to this protein having been published, dealing not only with its permeability-enhancing and pro-angiogenic functions, but also with its critical role in embryonic development, inflammation, pulmonary pressure homeostasis, neural and vascular patterning, and general endothelial physiology.

THE PROTEIN C ANTICOAGULANT SYSTEM AND THROMBOPHILIA

Natural coagulation inhibitors protect against inappropriate activation of coagulation in the circulation.[132] The first example of an inherited deficiency was described by Egeberg in 1965[133] for AT; patients with partial deficiency (65% to 80%) manifesting a 5- to 10-fold increased risk of VTE disease,[134] and patients with complete deficiency presenting a significant risk of fetal demise.[135] While hereditary AT deficiency occurs in 0.02% of the general population,[134] other risk factors for VTE disease are in aggregate >100-fold more prevalent. Identification of the PC anticoagulant system, we resulted predominantly from the efforts and insights of Stenflo, Lollar, Owen, the Esmons (Chuck and Naomi), Dahlbäck, and Bertina. Esmon and Dahlbäck have recorded their personal recollections in the discovery of TM[136] and the FV Leiden mutation,[137] as well as general reviews of the PC pathway and thrombophilia.[138–140]

The sequence of discoveries that led to identification of the PC anticoagulant system started with the description of a new vitamin K-dependent protein by Stenflo in 1976.[141] "PC" was the third peak eluted from an absorption column, over which Stenflo had passed a barium sulfate extract of bovine plasma; the protein resembled other vitamin K-dependent proteins in its content of γ-carboxyglutamic acid residues, but its function was unknown. Esmon et al.,[142] working with Stenflo, Suttie and Jackson, showed that PC is a precursor of a serine esterase that bound to phospholipid, but that "thrombin and factor Xa could not activate PC," and that it did "not seem to be necessary for blood coagulation." In 1976, Kisiel, Ericsson and Davie[143] demonstrated that PC could be activated by Russell's viper venom or trypsin, and in 1977, Kisiel et al.[144] showed that PC was activated by thrombin and that APC acted upon factor V, observations that were confirmed and extended in 1979 by Walker, Sexton and Esmon.[145]

As described by Esmon and Owen,[136] interest turned to the endothelium as a participant in thrombin regulation, following the *in vitro* demonstration of thrombin binding to cultured endothelial cells.[146–148] Lollar and Owen[149] performed *in vivo* rabbit studies and *ex vivo* isolated rabbit heart perfusion studies.[150] Radio-labelled DIP-thrombin was cleared in remarkably rapid fashion, 90% at 1 minute, in the vascular bed of multiple organs after aortic injection and in the lungs after intravenous injection. They concluded that thrombin clearance was achieved by

saturable, high affinity binding sites in a reversible manner and that active thrombin was released from these sites after rapid neutralization by AT. Studies in the isolated heart using plasma-free buffer (no plasma inhibitors) produced the same clearance results as in the whole rabbit model, allowing them to propose the presence of thrombin binding sites on the "endothelial cell surface."[149]

In seminal experiments performed by the Owen:Esmon team, they infused both thrombin and PC into the isolated rabbit heart and recovered "potent anticoagulant activity," 20,000-times that achieved in solution. This suggested the presence of a cofactor on the vascular endothelium that catalyzed the reaction.[151] The cofactor, later called TM, was isolated from rabbit lung by Naomi Esmon, Whyte Owen and Chuck Esmon[152] and was shown to increase PC activation by 1,000-fold over thrombin alone. The Esmons went on to show that TM binding of thrombin changed the character of the enzyme, eliminating its procoagulant activation of platelets[153] and its ability to clot fibrinogen.[154] Coupled with the effect of activated APC to prolong a clotting time, it was then understood that the TM interaction converted thrombin to an anticoagulant protein.

The anticoagulant effect of APC occurs when it inactivates factor Va,[154,155] an activity promoted by the cofactor, PS.[156] Further, PS along with unactivated factor V work in concert with APC to degrade factor VIIIa.[157,158] Only free PS acts as the cofactor, as it is regulated by interaction with C4b-binding protein.[159] Thus, similar to thrombin, factor V has both procoagulant and anticoagulant effects (Janus-faced), as it is a procoagulant cofactor for factor Xa in the prothrombinase complex and an anticoagulant cofactor with PS and APC to inactivate factor VIIIa.[160] The complexity of the system is further illustrated by the potentiation of PC activation by the endothelial cell PC receptor.[161,162]

The key clinical aspect of the PC anticoagulant pathway[139] as it relates to hereditary thrombophilic states, is illustrated by patients with deficiencies of PC and PS, but it even more notable in mutation of the target protein, factor Va. As described by Dahlbäck,[137,163] detailed studies linking families with a thrombotic tendency to the curious phenomenon of "APC resistance"[57,164,165] were eventually traced to a mutation in factor V in which Arg506 is replaced by glutamine (G to A in nucleotide 1691).[55,56,58] The mutation at this site and at a second APC cleavage site (amino acid 306)[155] render the factor Va substrate resistant to APC action, thus precluding a physiologic limit to coagulation, and explaining the thrombophilic tendency of patients carrying these mutations.[164] Whereas PC and PS deficiencies account for 0.2% and 0.03% to 0.13% of thrombophilias in the general population, the factor V "Leiden" mutation is by far the commonest hereditary predisposition, present as carriers in 3% to 7% of the white population.[134]

The progression of events, from discoveries of new vitamin K-dependent proteins that were not procoagulants, to indirect evidence of endothelial-mediated activation of PC by thrombin, very rapid clearance of thrombin by the microcirculation, purification of TM, study of APC action on factor Va for its anticoagulant function, cofactor roles for PS and intact factor V, and the mutational analysis of a factor V protein that is resistant to APC, have allowed the field of thrombosis management to reach a new level of understanding and practice.

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CHAPTER

2

Molecular Evolution of the Vertebrate Blood Coagulation System

MARIANNE A. GRANT AND WILLIAM C. AIRD

Most multicellular organisms have evolved hemostatic mechanisms to prevent the loss of fluid and promote blood vessel repair following injury. In invertebrates, hemostasis is mediated by blood cell (hemocyte) aggregation and/or gelatinization of hemolymph. In vertebrates, blood clotting involves both cells (thrombocytes in nonmammalian vertebrates and platelets in mammals) and a highly conserved network of protease reactions that ultimately lead to thrombin-catalyzed conversion of a soluble plasma protein, fibrinogen, into an insoluble, polymeric fibrin clot. Hemostasis in vertebrates is balanced by a number of important natural anticoagulant pathways.

An interesting question is *when* did the clotting cascade evolve? Previous studies have demonstrated that thrombin-catalyzed conversion of fibrinogen to fibrin occurs in all extant vertebrates, from jawless fish to mammals, but is conspicuously absent in invertebrates. These findings indicate that the clotting cascade evolved during an extremely narrow window of time (a mere 50 to 100 million years) in the ancestral vertebrate following the divergence of the protochordates (urochordates and cephalochordates) some 500 to 600 million years ago. A related question is *how* did the clotting system evolve? The answer, of course, is through natural selection of heritable traits that conferred a survival advantage. However, any such explanation must take into account not only the short time period during which the vertebrate clotting mechanism appeared, but also the fact that virtually every component of the clotting cascade is important and that some factors depend on others for their activity. As we will discuss in this chapter, the explanation appears to involve a combination of gene duplications, exon shuffling, and functional diversification. Finally, we must ask *why* did the clotting cascade evolve in the first place? At first glance, the response seems straightforward enough: to stem the loss of blood at sites of injury in animals with a highly pressurized closed circulation (i.e., vertebrates). However, there is now increasing evidence that coagulation may have played a critical early role in innate immunity.

The goal of this chapter is to review various evolutionary questions related to the clotting cascade. We begin by introducing basic principles of evolutionary biology. Next, we briefly review the mammalian clotting cascade and reframe the various components in terms of their protein domains or modules, which serve as a substrate for natural selection. After a brief discussion of comparative hemostasis, we provide a summary of what is known about the clotting cascade in extant vertebrates and invertebrates. Finally, we draw on these data to propose a scheme that explains the evolutionary origins of the clotting system.

PRINCIPLES IN EVOLUTIONARY BIOLOGY

Terminology

An important challenge in uncovering evolutionary histories is to reconstruct the past based on evidence from the present. Unfortunately, there is little in the fossil record that sheds light on the origins of hemostasis. Rather, we are left with comparative analyses of morphology, function, and molecular sequences. A central assumption in comparative anatomy and physiology is that two structures that look or act alike are closely related. However, that is not always the case. Structures may look similar simply because they have evolved similar adaptations. This is termed *convergent evolution*. An example is seen in the case of the horseshoe crab (*Tachypleus tridentatus*), which contains a clotting system comprised of a protease cascade of three serine protease (SP) zymogens and a clottable protein (coagulogen). However, these components arose independently of the vertebrate clotting proteins. Among other things, they lack γ-carboxylated glutamic acid (GLA) domains, epidermal growth factor (EGF)-like domains, and kringle domains, which promote essential interactions of vertebrate clotting factors. Conversely, *divergent evolution* describes dissimilar phenotypes that share a common ancestor. The interpretation of molecular phylogenies for gene sequence divergence is similarly wrought with limitations. It is widely believed that the vertebrate genome has undergone two whole-genome duplication (WGD) events in addition to many local duplications, translocations, gene conversions, and gene loss. Thus, it is important to distinguish between *orthologous genes* (orthologs) and *paralogous genes* (paralogs). Orthologous genes in different species have evolved directly from a common ancestral gene, whereas paralogs have evolved from a common ancestral gene through duplication and have diverged in sequence and function from the parent gene through mutations and selection or drift. Stated another way, orthology is the relationship where sequence divergence follows speciation, whereas paralogy is defined as that condition where sequence divergence follows gene duplication.

Methods

To establish the evolutionary history of the coagulation cascade, we must turn to modern-day animals (especially early diverging vertebrates) and examine how their blood clots and/or determine whether their genome contains sequences for the various clotting factors. Functional studies of nonmammalian vertebrates have routinely employed the same clotting time assays that are used in humans, including whole-blood clotting time, prothrombin (PT) time, and activated partial thromboplastin

time (aPTT). However, the absence of factor-deficient plasma from many of these species has precluded the use of mixing studies to measure individual clotting factor levels. Functional studies of blood clotting in early vertebrates are further hampered by inter- and intraspecies variations in clotting times, small volumes of available blood (e.g., in small fish such as the zebrafish), and the nonuniform plasma composition and body temperatures of poikilotherms.

Protein purification has been used to isolate fibrinogen in different species. However, such a strategy is not feasible for many of the SPs, which circulate at trace levels. With the advent of polymerase chain reaction (PCR), it has been possible to use degenerate primers (based on multiple sequence alignment of available gene sequences) against specific domains of clotting factors to clone the cDNA for SP clotting factors. More recently, searching for DNA sequences (orthologs) has been facilitated by the availability of the whole-genome sequence of the sea squirt (*Ciona intestinalis*), amphioxus (*Branchiostoma floridae*), puffer fish (*Fugu rubripes*), and other higher vertebrates. Complete genome sequence is not available for lamprey or hagfish. However there is a lamprey trace database maintained by the NCBI and EBO. Once DNA sequences are available from multiple species, it is possible to align sequences and construct phylogenic trees, which are diagrams that depict the lines of evolutionary descent of different species, organisms, or genes from a common ancestor. Sequence similarities and phylogenic trees based on aligned sequences provide important information about when clotting factors arose during evolution. In addition to analyzing DNA sequences, it is valuable to examine gene organization. For example, the occurrence and arrangement of catalytic and peripheral domains yields clues about gene duplication and exon shuffling. Similarities in intron–exon structures are characteristic of orthologous genes. The presence of two similar genes (paralogs) on the same chromosome is a clue that they originated from local duplication, rather than genome-wide duplication.

Taxonomy

Studies on the evolution of the clotting cascade have focused on the vertebrate–invertebrate divide, namely the chordates. Chordates form a phylum of animals that includes vertebrates and closely related invertebrates. There are three subphyla: urochordates or tunicata (composed of more than 3,000 species and represented by the sea squirt), cephalochordates or lancelets (composed of ~29 species and represented by amphioxus), and the extant vertebrates including jawless fish or cyclostomes (hagfishes and lampreys) and jawed vertebrates or gnathostomes (composed of ~53,000 species) (FIGURE 2.1). Urochordates and cephalochordates are referred to as basal chordates, or protochordates. Among other features, they have a notochord that persists throughout life but do not have backbones. Traditionally, cephalochordates were considered to represent the closest living relatives of vertebrates, with tunicates representing the earliest chordate lineage. However, recent evidence indicates that urochordates are more closely related to vertebrates than are cephalochordates.[1,2] Jawless fish are the earliest appearing vertebrates. The two extant genera are hagfish and lamprey. Although morphologic and molecular phylogenic studies have yielded conflicting results, it is believed that hagfish and lamprey are not monophyletic, but rather represent separate divergences, with hagfish being more primitive.[3] The jawed vertebrates include cartilaginous fish (also termed elasmobranchs), the bony fishes (also termed teleosts), amphibians, reptiles, birds, and mammals.

Mechanisms

It is widely accepted that the vertebrate genome has undergone multiple rounds of WGD (reviewed in Ref.[4]) (FIGURE 2.1). Although the timing and the genomic scale of such duplications remain poorly understood, the favored hypothesis is that the first round occurred in a common ancestor of jawed and jawless vertebrates (~630 to 565 million years ago), with the second round occurring in a common ancestor of the jawed vertebrates (~565 to 525 million years ago). An additional WGD may have occurred in an ancestor of the majority of ray-finned fish some 350 to 320 million years ago. The idea of genome duplication has been strengthened by the observations that model vertebrates often have multiple duplicate genes (paralogs) corresponding to a set of invertebrate orthologs.[5,6] When a gene is duplicated, one of the copies is redundant and escapes purifying selection. Natural selection cannot operate on idle proteins, but only on those that are being used. Thus, most gene duplications from WGDs are rapidly lost (this accounts for why the genome of sea squirt is smaller [330 Mb] than that of amphioxus [540 Mb]). Rarely, the mutations may provide the gene with a new function. Indeed, duplicate functional modules arising from WGDs are likely to have played a critical role in enhancing morphologic and functional complexity during vertebrate evolution.[4,7,8]

Although it is widely accepted that large regional or WGD events contributed to the structure of vertebrate genomes,[9–11] local duplication and translocation have certainly contributed to the evolution of multigene families on separate chromosomal regions. Domain shuffling (insertion) is another important mechanism for the evolution of novel genetic material. Since the domains are often correlated with exon boundaries, exon shuffling is considered to be the driving force behind domain shuffling.[12] In this way, different domains of modular proteins may have evolved in unrelated protein families, and then been subsequently fused to previously existing domains.[13] Other mechanisms of domain shuffling include simple fusion of genes and recruitment of mobile elements.

OVERVIEW OF MAMMALIAN BLOOD COAGULATION

Mammalian blood coagulation is a complex, balanced process initiated and regulated by a networked series of protein reactions and interactions that form positive and negative feedback loops. In humans, blood coagulation involves more than two dozen extracellular proteins, almost half of which are members of the SP family. Blood coagulation in mammals (FIGURE 2.2) is initiated by the exposure and binding of factor VII (FVII) to membrane-bound (cell surfaces in the subendothelial matrix) tissue factor (TF). Bound TF activates FVII to the protease FVIIa generating a TF–FVIIa protease complex that can proteolytically activate FIX and FX, producing proteases FIXa and FXa, respectively. In the absence of its activated cofactor FVa, FXa generates trace amounts of thrombin by the proteolytic processing of PT. This thrombin cleaves FXI to FXIa.

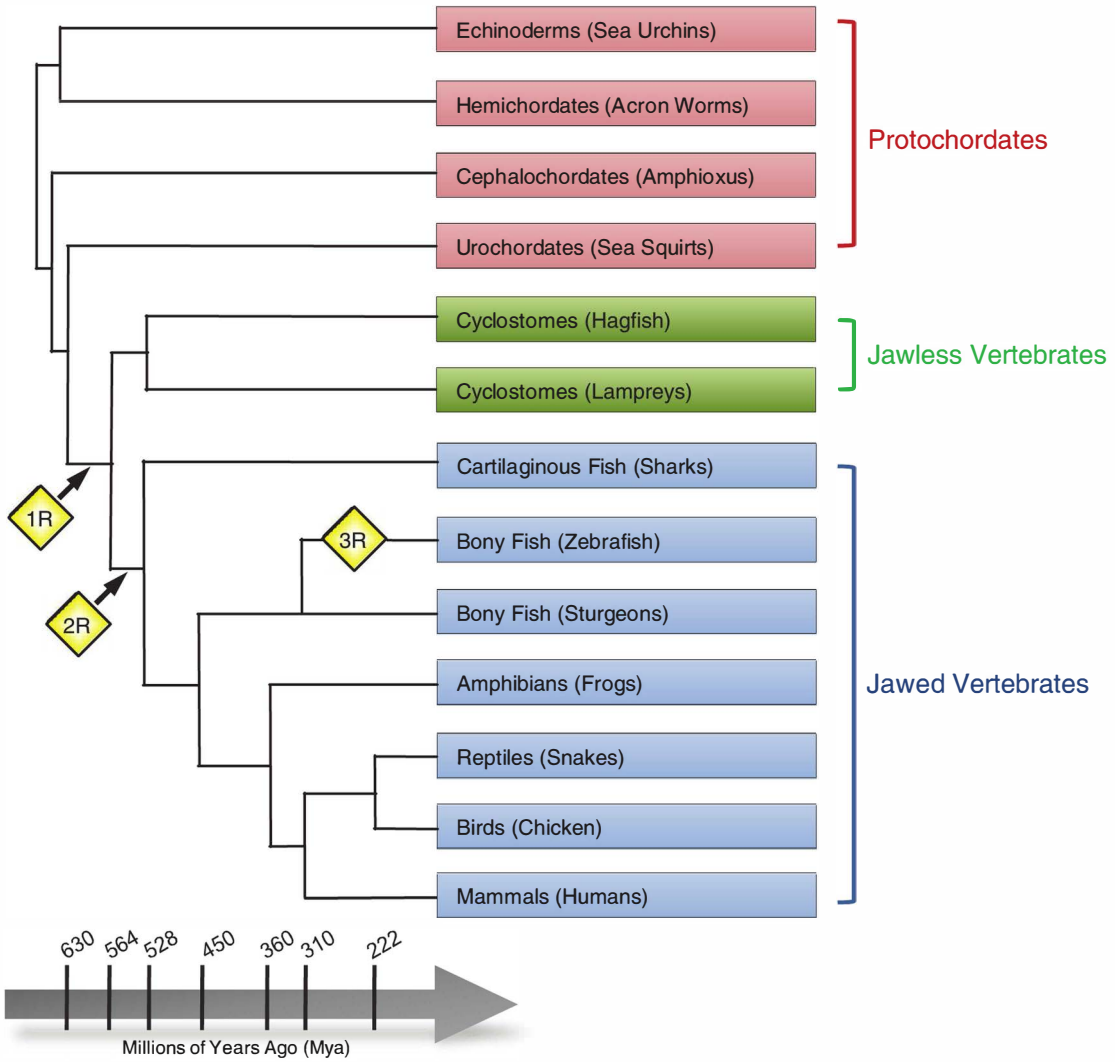

FIGURE 2.1 A phylogenetic tree representation of WGD during evolution. The points in evolutionary time where WGD events have been hypothesized to occur are indicated by *yellow diamonds*. The first round of WGD (*1R*) is thought to have occurred at the stage of divergence between urochordates and cyclostomes. The second WGD round (*2R*) is estimated to have occurred after the divergence of jawless fish (cyclostomes) and jawed vertebrates. A third round of WGD (*3R*) that is hypothesized to be a lineage-specific or fish-specific genome duplication is shown during divergence in the bony fish lineages.

FXIa then cleaves FIX to FIXa, which along with FVIIIa cleaves larger amounts of FX to FXa. Although insufficient to initiate significant fibrin polymerization, trace amounts of thrombin formed in the "initiation" stage of coagulation are sufficient to proteolytically activate FV and FVIII in a positive feedback loop. Greater amounts of activated FVa and FVIIIa function as cofactors for FXa and FIXa, respectively, forming complexes that amplify the generation of thrombin during the "propagation" stage, which leads to the generation of the large amounts of thrombin required to convert fibrinogen to fibrin. These reactions occur on the surface of platelets that are anchored to the subendothelial matrix *via* collagen and von Willebrand factor, which themselves activate platelets along with the newly generated thrombin.

Thrombin also proteolytically activates the anticoagulant pathway (see FIGURE 2.2). Thrombin forms a complex with the endothelial thrombomodulin (TM) receptor, and by an allosteric mechanism its substrate specificity is altered. The procoagulant substrates of thrombin, including FV, FVIII, and fibrinogen, are no longer efficiently proteolyzed. Instead, the thrombin–TM complex activates protein C (PC), generating activated PC (APC), which then associates with its protein S (PS) cofactor to rapidly inactivate procoagulant cofactors FVa and FVIIIa. Thrombin-TM-mediated activation of PC is further augmented 10- to 20-fold if PC is bound to another receptor, termed endothelial protein C receptor (EPCR).[14] The procoagulant process of "propagation" described above occurs independently of the TF–VIIa complex, which is rapidly inactivated

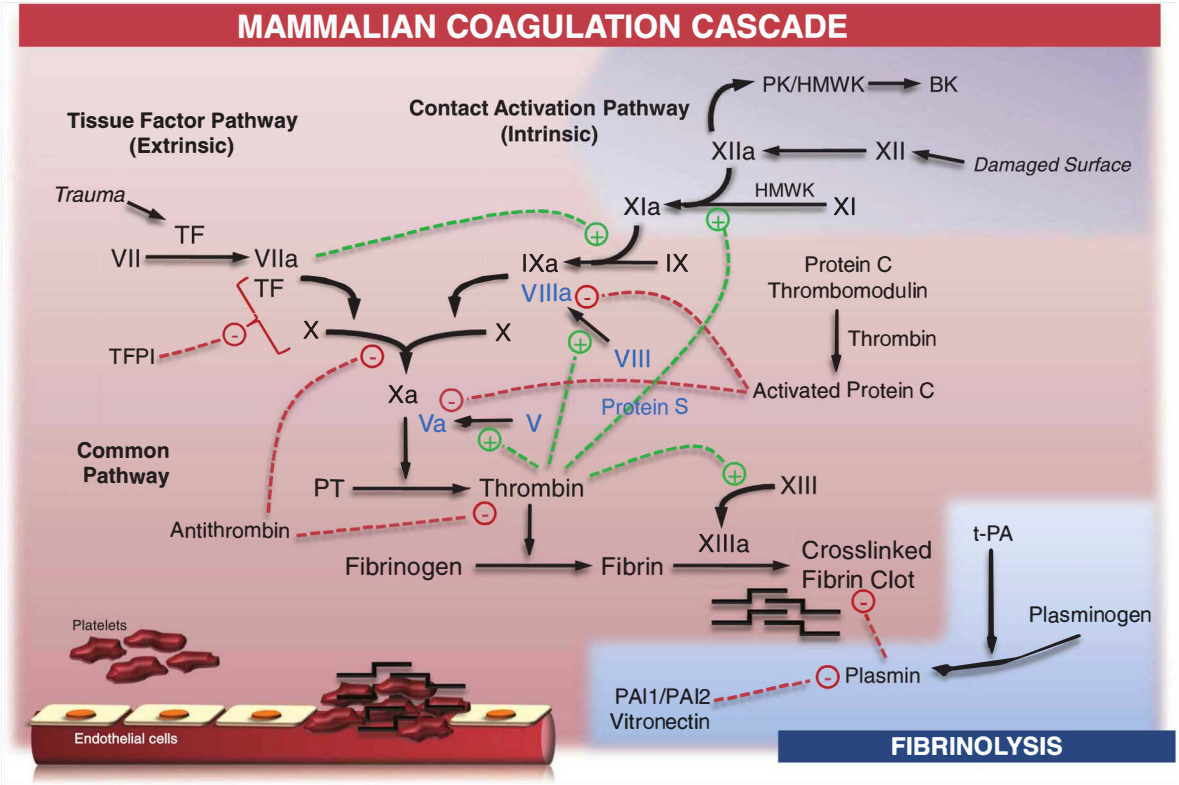

FIGURE 2.2 Schematic representation of coagulation in mammals. *Solid black arrows* represent the cascade of reactions of zymogen activation to active enzyme (suffix a) and the active complex formations driven by protein–protein interactions of factors in the system. Stimulatory actions are indicated with *green dashed lines* (+). Inhibitory actions are indicated with *red dashed lines* (–). PT, prothrombin; TF, tissue factor; TFPI, tissue factor pathway inhibitor; PAI 1 and PAI 2, plasminogen activator inhibitors 1 and 2 ; t-PA, tissue-type plasminogen activator; PK, prekallikrein; HMWK, high molecular weight kininogen; BK, bradykinin.

by the tissue factor pathway inhibitor (TFPI). Thrombin, FIXa, and FXa are specifically inhibited by antithrombin (AT), heparin cofactor II (HC II), and Z-dependent protein inhibitor/protein Z (PZ) complex. The fibrin clot is lysed in fibrinolysis (see FIGURE 2.2) by plasmin, which is generated from plasminogen by cleavage with tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). This reaction is subsequently inhibited by two plasminogen activator inhibitors, namely plasminogen activator inhibitor (PAI)-1 and PAI-2.

The contact system is composed of three proteins: FXII, prekallikrein (PK), and high molecular weight kininogen (HMWK) (reviewed in Ref.[15]). The intrinsic or contact pathway is initiated when FXII is autoactivated upon exposure to negatively charged surfaces (including kaolin, silicone rubber, polyethylene, celite, and glass), forming FXIIa. FXIIa converts FXI to FXIa, which in turn converts FIX to FIXa. FXII also activates PK, forming plasma kallikrein that can reciprocally activate more FXII and liberate kinins (e.g., bradykinin) from HMWK.[15] Kinins are small peptides that bind to receptors on many cell types, leading to an inflammatory response, including vasodilatation and vascular permeability. Although FXII promotes surface-activated clotting *in vitro*, deficiencies of the contact system proteins (if one excludes FXI) are not associated with clinical bleeding. Under *in vivo* conditions, FXI is activated by thrombin and not by FXIIa.

CLOTTING FACTORS, CELL SIGNALING, AND INFLAMMATION

In addition to their role in coagulation, several components of the clotting cascade mediate cell signaling. For example, the structural protein fibrinogen has been shown to activate inflammatory cells *via* distinct ligand–receptor interactions (reviewed in Ref.[16]). SPs bind to protease-activated receptors (PARs). PARs belong to the family of 7-transmembrane G-protein–coupled receptors. There are four known members (PAR-1, -2, -3, and -4), each of which is activated by proteolytic cleavage of its *N*-terminal domain. Thrombin binds and activates PAR-1 (and to a lesser extent PAR-3 and PAR-4). FVIIa-TF and FXa signal through PAR-2. In this way, initiation of coagulation is mechanistically coupled with cell-signaling events, which typically contribute to a proinflammatory phenotype (reviewed in Ref.[17]). In contrast, APC activates PAR-1 and when bound to EPCR is cytoprotective. APC exerts pleiotropic effects not only on endothelial cells, but also on neuronal cells and innate immune cell populations. Other natural anticoagulants, including AT and TFPI, also inhibit inflammation *via* cell-signaling–dependent mechanisms.[18,19]

GENE ORGANIZATION AND PROTEIN DOMAIN STRUCTURE

The clotting proteins contain genetically autonomous units (domains or modules), which are linked together to form unique domain architectures.[13] In many cases, the domains are encoded by separate exons. Among these domains are SP catalytic domains and subsidiary (noncatalytic, regulatory) domains that mediate biologic specificity of the enzymes through protein–protein interactions and localization of the clot to sites of injury. For example, GLA domains and discoidin domains serve to anchor proteins to platelets at the sites of wounds. Kringle, EGF, and plasminogen-apple-nematode (PAN) domains mediate protein–protein interactions necessary for the ordered assembly of proteins in the clotting cascade. It has been proposed that these various nonregulatory domains represent genetically autonomous units (miniproteins) that have undergone shuffling (e.g., fusion to proteases) during evolution.[13] The relationship between SP paralogs can be inferred by the kinds and arrangements of secondary domains associated with the catalytic protease domains. Indeed, the similarities in gene organization and protein domain structure among the major mammalian blood procoagulant, anticoagulant, and clotting cofactors suggest that that they evolved through the duplication and functional diversification of different gene families (FIGURE 2.3).[13] Three of these families are discussed in turn.

The GLA-EGF1-EGF2-Serine Protease Family

Vitamin K–dependent SPs are comprised of a GLA-EGF1-EGF2-SP domain structure common to FVII, FIX, FX, PC, and PZ. In contrast to these latter proteases, PT has two kringle (K) domains in place of the EGF domains. The EGF-like domain is a short sequence containing six cysteine residues, which have been shown to be involved in disulfide bonds. The main structure is a two-stranded beta-sheet followed by a loop to a *C*-terminal short two-stranded sheet. Similarly, the kringle domain has a unique characteristic triple-loop fold structure, whose conformation is defined by three pairs of cysteine disulfide bonds, a number of hydrogen bonds, and small pieces of antiparallel beta-sheet. (Kringle domains are also found in hepatocyte growth factor, apolipoprotein a, u-PA, t-PA, and plasminogen.) Analysis of amino acid sequence and codon usage at highly conserved residues associated with the active site function in the SP domain distinguishes PT from FVII, FIX, FX, and PC and suggests that thrombin was the ancestral blood coagulation enzyme.[20,21,22] With the exception of PT, the genes encoding vitamin K–dependent factors of coagulation have virtually identical exon/intron distributions.

The A1-A2-B-A3-C1-C2 Cofactor Family

A1-A2-B-A3-C1-C2 domain structure is common to cofactors FV and FVIII, which are members of the ferroxidase family of proteins. FV and FVIII share sequence identity with the copper-binding plasma protein ceruloplasmin (CP) and the enterocyte transmembrane protein hephastin (HP), which have a common A1-A2-A3 domain structure. FV and FVIII differ from CP and HP in having two discoidin domains (C1 and C2) at their *C*-terminal ends. The discoidin domains of FV and FVIII bind to thrombocyte surfaces to orient the cofactors normal to the surface where they serve as binding cofactors for FX or FIX, respectively, and allow for optimal conversion of PT to thrombin. In FV and FVIII, there is an additional domain between domains A2 and A3, designated the B domain, which is released following thrombin activation. The B domain has no known structural homologs and is dispensable for procoagulant activity. The primary sequences of human FV and FVIII are 40% identical except in the B domain where there is no homology.

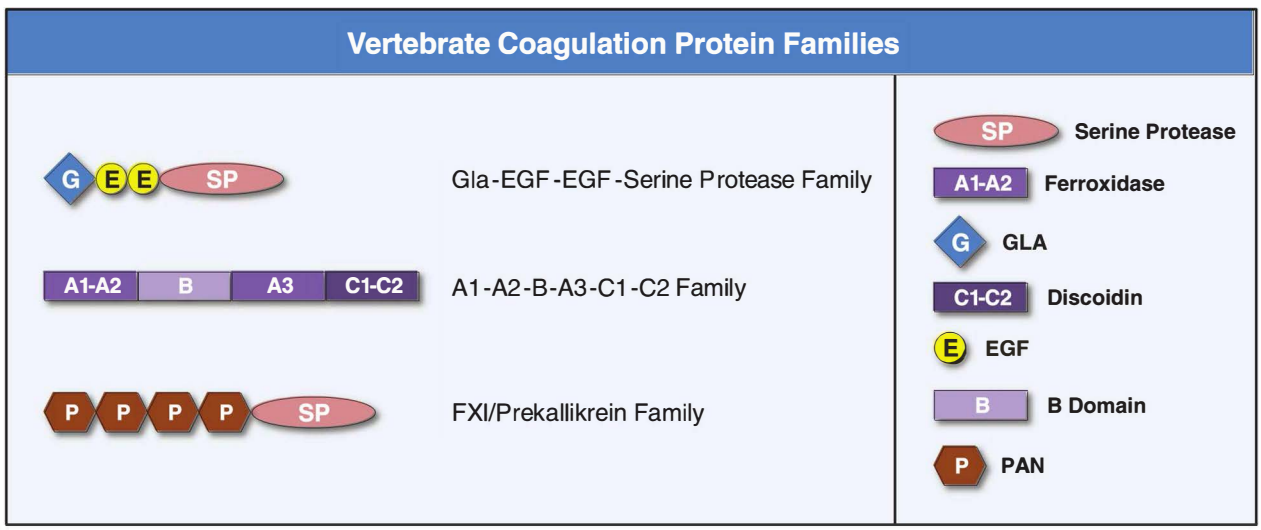

FIGURE 2.3 Schematic representation of the three major vertebrate coagulation protein families. The GLA-EGF-EGF-SP family comprises FVII, FX, FIX, PC, and PZ. The A1-A2-B-A3-C1-C2 protein family includes cofactors FV and FVIII. The PAN-SP family consists of FXI and PK. Domain representations are illustrated on the *right*. Epidermal growth factor (EGF)-like, discoidin (C), ferroxidase (A), γ-carboxy glutamic acid (GLA), plasminogen-apple-nematode (PAN), serine protease (SP), and B (B) domains.

The FXI/Prekallikrein Family

FXI and PK are closely related SP proteins. The most notable feature of FXI and PK is a series of four disulfide-constrained approximately 90-amino acid repeats initially called apple domains. More recently, these domains have been assigned to the larger domain superfamily known as the PAN module superfamily.[23] FXI is a disulfide-linked dimer that circulates in blood as a complex with HMWK.[24] PK, the zymogen of the protease α-kallikrein, is a monomeric homolog of FXI with the same domain structure that also circulates in complex with HMWK.

COMPARATIVE FUNCTIONAL STUDIES OF BLOOD COAGULATION

The horseshoe crab has a hemolymph coagulation system. However, it uses different enzymes and structural proteins to form a clot compared with vertebrates. In other words, arthropod hemolymph clotting factors are not orthologs of vertebrate blood clotting factors. Indeed, fibrin clots have not been observed in any protochordate (the closest relatives of the vertebrates). In the sea quirt, hemostasis is mediated by circulating cells called hemocytes, which become clumped at sites of injury.[3,25] Amphioxus does not have circulating blood cells and appears to lack clotting.[3,25]

The earliest animals to exhibit fibrin clots are the fishes. The first systematic study of fish coagulation was published in 1962 by Russell Doolittle.[26] Blood and plasma were collected from lamprey, dogfish shark (an elasmobranch), blackfish (a teleost), and human. Whole-blood clotting time (blood collected directly into glass tubes without anticoagulant) was fast in blackfish (less than a minute), very slow in dogfish, and weak/poorly organized in lamprey even after 24-hour incubation at room temperature. Addition of lamprey tissue extract to the sample resulted in rapid clot formation within 1 minute. These findings led to the conclusion that lamprey lack an efficient intrinsic pathway. Addition of high concentrations of calcium resulted in accelerated clotting in dogfish and blackfish, but reduced clotting efficiency in human plasma. Plasma clotting in all fish was greatly accelerated by addition of aqueous extracts of homologous tissue (TF source). Although the effect demonstrated relative species specificity, even lamprey skin extract accelerated the clotting of human plasma and bovine brain extracts accelerated the clotting of lamprey plasma. Taken together, this early study suggested that blood coagulation in fish is fundamentally similar to clotting in mammals, with the major difference lying in the intrinsic pathway.

A subsequent report confirmed poor whole-blood clotting of lamprey blood.[27] Consistent with an absence of an intrinsic pathway, the aPTT was >120 seconds. Surprisingly, the PT time with bovine thromboplastin (tissue extract) was shorter than it was with lamprey skin extracts. Elasmobranch blood also revealed long whole-blood clotting times, although these were highly variable between specimens. As with lamprey, the aPTT was more than 120 seconds. Comprehensive analyses of multiple species of bony fish revealed highly variable clotting times. Most of the fish clotted in times close to the human range. Homologous tissue extracts had variable thromboplastic effects.

A previous study of blood from multiple elasmobranch species showed very long clotting times in glass tubes (in some cases exceeding 48 hours).[28] These data were used to conclude that cartilaginous fish lack an intrinsic clotting mechanism. Additional calcium (up to a certain concentration) resulted in a marked shortening of the clotting time. Beyond that concentration, the clotting time became prolonged again. The clots that formed lysed rapidly. Clot lysis was prevented by the addition of epsilon-amino caproic acid. In assays of the extrinsic pathway, tissue extract from skate accelerated skate clotting time (but not that of human plasma). Human brain extract accelerated human but not skate clotting times.

More recently, hemostatic parameters have been measured in zebrafish.[29] Zebrafish thromboplastin was found to clot zebrafish but not human plasma, and rabbit thromboplastin resulted in only partial clotting of zebrafish plasma. Thus, these findings confirmed the species-specific nature of the TF–VII interaction. In addition, the aPTT was measurable (using a rabbit partial thromboplastin reagent), indicating the presence of an intrinsic cascade. This latter result is surprising based on the clear absence of contact factors in zebrafish. Warfarin dissolved directly into the tank water resulted in dose-dependent prolongation of PT time and aPTT. Addition of unfractionated heparin to zebrafish plasma resulted in dose-dependent prolonged aPTT.

Blood clotting in fish is highly dependent on environmental variables. For example, when fish that live in estuarine waters were exposed to normal fluctuations in temperatures and salinity, their whole-blood clotting varied widely.[30] Similarly, in reptiles, which also lack an intrinsic system, clotting times are influenced by changes in temperature and pH that reflect variations associated with normal life activities.[31]

In summary, these and other studies suggest that all groups of fish (cyclostomes, elasmobranchs, and teleosts) generate thrombin through pathways involving vitamin K–dependent factors, exhibit FXIII-dependent fibrin crosslinking, and mediate fibrinolysis that is inhibited by the same factors that inhibit fibrinolysis in mammals.[26,32,33]

COMPARATIVE MOLECULAR STUDIES OF BLOOD COAGULATION

The evolutionary appearance of various genes associated with the process of blood clotting can be inferred by examining the genomes of various classes of extant vertebrates. The availability of whole-genome sequence databases from a variety of vertebrates has provided an opportunity to answer the question of whether all vertebrates employ the full repertoire of factors involved in mammalian clotting. Below we review the biochemical evidence and evidence from molecular cloning and DNA sequence analysis (of genomic and expressed sequence tag (EST) databases) for the major blood coagulation proteins in vertebrates. In the first part, we review these data from the standpoint of different animal groups (FIGURE 2.4). In the second part, we reframe the findings according to the individual clotting factors (FIGURE 2.5). Thus, some overlap between the two sections is unavoidable. However, we find both approaches to be complementary. Together, they provide a strong foundation for formulating plausible evolutionary scenarios to account for the evolution of blood coagulation network by a gradual step-by-step Darwinian process.

From the Perspective of Animal Groups

Basal Chordates (Sea Squirt and Amphioxus)

Two protochordate genomes have been fully sequenced to date, the urochordate or ascidian called the sea squirt (*C. intestinalis*) and the cephalochordate called amphioxus or lancelet

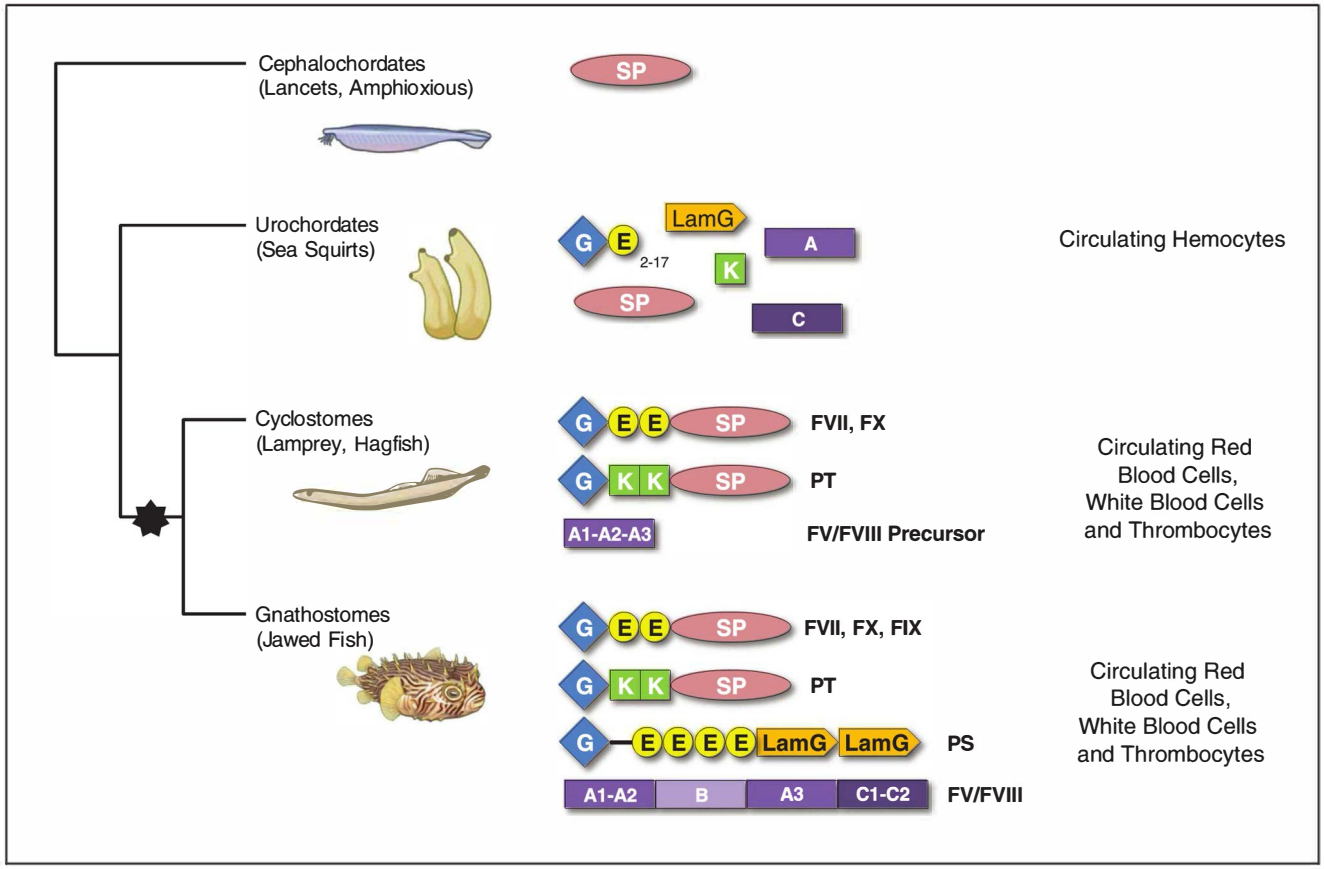

FIGURE 2.4 A phylogenetic tree representation of coagulation factor domain precursors and coagulation factor gene families in early chordates. Shown are the coagulation gene families with characteristic domain structure found in bony fish (gnathostomes) and lamprey (cyclostomes), as well as coagulation factor precursor domains found in sea squirt (urochordates) and amphioxus (cephalochordates) genome sequences. The type of circulating cells identified in each vertebrate class and the coagulation factors that have been identified in each vertebrate class are described on the *right*. The (*) symbol represents the evolutionary time point where members of the GLA-EGF-EGF-SP, GLA-K-K-SP, and triplicated A domain–containing protein families emerged from ancestral SP precursors. GLA (G), kringle (K), EGF-like (E), laminin G (LamG), serine protease (SP), ferroxidase (A), discoidin (C), and B (B) domains.

(*B. floridae*). Recent whole-genome sequence analysis of the sea squirt has revealed the presence of proteins containing many of the same domains that occur in vertebrate clotting factors, including GLA, EGF, kringle, and V/VIII A and C domains. However, none of these modules is physically linked to an SP domain, arguing against the existence of any genuine orthologs for the coagulation factor genes.[25] In contrast, they almost always occur in association with multiple EGF domains (in the case of the tunicate, *Halocynthia roretzi*, one GLA domain-containing protein was found to be associated with a receptor kinase[34]). Interestingly, one of these proteins is followed by two laminin G domains.[25] This sequence of GLA-EGF-laminin is similar to that found in PS. A recent search of the *C. intestinalis* genome also revealed a candidate predecessor of FV/FVIII.[25] It has three ferroxidase A domains similar to those found in CP/HP as well as two discoidin domains, as observed in vertebrate FV and FVIII. There are several genes for fibrinogen-related domains (FREDs) in the genomes of both the sea squirt and amphioxus.[3] In addition, a putative fibrinogen ancestral gene was found. Although this gene shares many of the same domains and structure as vertebrate fibrinogen, its sequence shows that it cannot possibly be a substrate for thrombin or FXIII.[25] In summary, protochordates, and for that matter all invertebrates studied to date, lack orthologs for any of the vertebrate clotting factors. However, the *C. intestinalis* genome contains the catalytic and accessory domains that ultimately comprise vertebrate clotting factors.

Jawless Fish (Hagfish and Lamprey)

The genome of jawless fish has unique features. For example, most lamprey species have more than 150 chromosomes, and genomic sequences reveal an extraordinarily high GC content in protein-coding regions, which probably reflects highly biased codon usage in these animals.[35] The study of clotting factors in jawless fish is mostly limited to lamprey.[36,37] A combination of protein purification, degenerate PCR-based cloning, DNA sequencing, and searches of genomic databases has revealed the presence of a clotting cascade that is decidedly simpler than in mammals. Early studies identified the PT and fibrinogen genes and proteins,[38,39] indicating that the final step of blood clotting has been conserved throughout vertebrate evolution. The fact that homologous

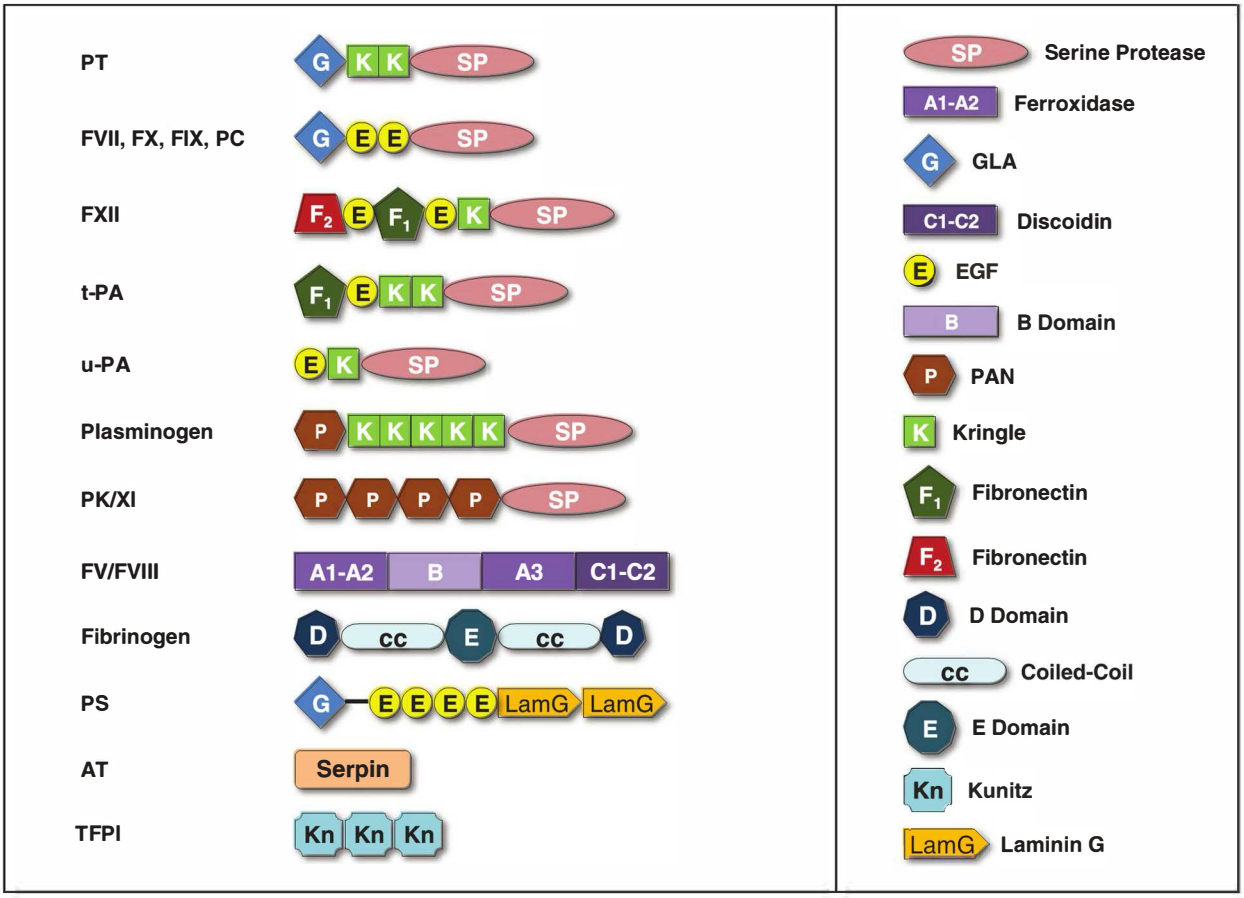

FIGURE 2.5 Schematic representation of the domain content of coagulation factors. Identification of the individual domain representations is shown on the *right*.

tissue extract promotes clotting of lamprey blood in a PT assay indicates the presence of TF. Lamprey has three factors VII (the sequence of one of these predicts that it is inactive) and two factors X (one appears to be inactive).[37,40,41] Sequence analysis indicates that the duplication of FX occurred well after the divergence of the lamprey. Thus, lampreys have an intact extrinsic pathway. In contrast, efforts to clone FIX have failed. While searches of the two B domains from FV and FVIII against lamprey trace database did not yield significant hits, there were a large number of hits with the A domain. The majority of these were similar to CP/HP. However, one was closer to V/VIII than to CP/HP and may represent an ancestral V/VIII gene. Lampreys have a simple HMWK, but lack other components of the contact system, including FXI, FXII, and PK.[37] On the fibrinolytic and anticoagulant side, lamprey liver EST analyses have also suggested the presence of PC, TFPI, TM, t-PA, and plasminogen.[37] There is no evidence for AT. However, a possible gene for HC II is present.[37]

It should be emphasized that in most cases, the presence of a procoagulant or anticoagulant is based on EST analyses. With few exceptions, the full-length clones are not available, and a functional role for many of these putative clotting factors remains to be established. As discussed earlier, hagfish are now considered to be more primitive than lamprey. Thus, it would be useful to know whether the same complement of clotting factors is present in hagfish or whether they have an abbreviated repertoire. Recent work in our own laboratory has revealed the presence of genes for PT, FVII, FX, plasminogen, and TFPI in *Myxine glutinosa*. Despite extensive studies using degenerate PCR primers against multiple domains, we have not identified FIX or FXI. In summary, lamprey and hagfish possess a similarly abbreviated clotting cascade that is limited to the extrinsic pathway alone. They have a plasmin-based fibrinolytic system, and natural anticoagulants against the extrinsic pathway (TFPI), cofactors (TM-PC), and SPs (HC II).

Bony Fish

The most complete information about the coagulation cascade in bony fish is from puffer fish (*F. rubripes*). Two major studies have examined the genome of the puffer fish for the presence of putative clotting factors. One such study revealed the presence of a single gene for PT, three for FVII (only one of which is predicted to be functional), two for each of FIX (both of which may be functional) and PC (one of which is predicted to be inactive), and one for FX, FV, and FVIII.[42] In another study, orthologs were identified for each of the five vitamin K–dependent SPs (one PT, four FVII, two FIX, one FX, and PC).[41] Similarities in the sequences of the two FIX genes suggest the occurrence of a gene duplication, leading to two homologs after fish diverged from the lineage leading to tetrapods. As in humans, FX and FVII are adjacent on the same chromosome, consistent with a previous

local gene duplication. Also identified were genes for TF (only 34% identity with human TF, but identical intron–exon structure), fibrinogen, the A chain of FXIII, FV, FVIII, plasminogen, t-PA, u-PA, AT, TFPI, TM, and PS. There was no evidence for FXI, PK, FXII, or PAI-1.

HMWK is present and is more complex than that of lamprey. However, as with jawless fish, the contact system (FXI, FXII, and PK) is missing in puffer fish. Since they lack FXI, it is likely that FIX in fish is activated by FVIIa. Consistent with the lamprey data, puffer fish have the fibrinolytic components (u-PA, t-PA, and plasminogen); TM and PC; and TFPI. However, they also have evidence for PS and AT. In summary, teleosts have evolved FIX and a more complete set of cofactors (FV, FVIII, and PS). AT also makes its first appearance in bony fish. Since they lack PK, another activating protease likely serves to generate kinins.

Amphibians and Birds

Contact series proteases made their first appearance in tetrapods; FXII first appears in amphibians. Interestingly, the predecessor of mammalian FXI and PK also appears first in amphibians. Birds lack both FXI and FXII.

From the Perspective of Individual Clotting Factors

Tissue Factor

TF is a transmembrane glycoprotein composed of two fibronectin type III domains, which are very common in vertebrate proteins, a membrane-spanning domain for cell surface attachment, and a short cytoplasmic tail. Protein sequence comparisons show that TF belongs to a large group of proteins called cytokine receptors. Cytokine receptors can be divided into families on the basis of their sequences and overall domain structures. TF belongs to the class 2 group of cytokine receptors and shares a similar arrangement of fibronectin and membrane-spanning domains with various cytokine receptors in the same class (e.g., the interferon receptor, IFN-γR). Similarity in domain structure and sequence identity among these varied proteins is highly suggestive of common ancestry. Searches of the sea squirt genome have identified two potential class 2 cytokine receptors.[41] Sequence comparisons suggest that these genes expanded into a very large number of paralogs during early vertebrate evolution. Many of these receptors play roles in the innate immune response against bacteria and viruses.

Evidence for TF-initiated coagulation in fish was established in 1962 from biochemical studies in which aqueous extracts of homologous tissue greatly reduced plasma clotting times.[26] These and subsequent studies demonstrated TF activity in jawless fish (lamprey and hagfish), cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals. TF has proven difficult to clone or identity in genome databases, because it is a rapidly changing gene (mammalian proteins are only 60% to 70% identical to one another). A single gene was found for TF in sequence database searches of the puffer fish (*F. rubripes*) genome.[41] The putative protein is only 34% identical with human TF, but the five introns are situated at the same positions in the puffer fish and human genes. The molecular cloning of TF has also been reported from the rainbow trout (*Oncorhynchus mykiss*).[43] Comparisons of mammalian and teleost TF amino acid sequences show high conservation of residues identified as critical for function in human TF.

Prothrombin

Initial evidence for PT in jawless fish was demonstrated by functional studies in lamprey in 1962.[33] Thereafter, amplification and sequence analyses of PT-encoding cDNAs from numerous vertebrate species were reported, and full-length gene sequences from mammals, birds, bony fish, lamprey, and hagfish have been characterized.[38] The PT amino acid sequence is highly conserved between hagfish and humans (hagfish 50% sequence identity; 69% sequence similarity with human). The presence of the PT and fibrinogen genes and proteins in cyclostomes (lamprey and hagfish) indicates that the final step of blood clotting has been conserved throughout vertebrate evolution.

It is widely believed that the SPs evolved from an ancestral trypsin-like protease containing a signal peptide and a zymogen peptide domain. Sequence and species comparisons indicate that PT was the ancestral blood clotting protein. It is unique among the vitamin K–dependent proteases in that it has two kringle domains following the GLA domain, while in the other proteases there are two EGF domains. The origin of PT has been debated.

Phylogenetic analyses of the vitamin K-dependent clotting factors suggest that the ancestral trypsin-like SP had a signal and a zymogen peptide domain.[13] Before the divergence of vitamin K–dependent SPs and the fibrinolytic enzymes, an EGF domain was likely inserted into the ancestral protease. Subsequent insertion of another EGF domain and the GLA domain occurred in the ancestral blood coagulation enzyme. PT diverged from the ancestral EGF-SP in a subgroup that included PT, PC, FIX, FX (and FVII), and the EGF domains were replaced by kringle domains from another subgroup that included u-PA, t-PA, and plasminogen.[13] Elsewhere, it was proposed that PT was derived from a K-K-K-K-SP domain-containing ancestor.[3] According to this scenario, duplications of the kringle domain-containing PT gene would have led to the appearance of an ancestral gene for FVII, FX, and eventually FIX. In this case, there would have been considerable domain shuffling in the early stages that resulted in the replacement of kringle domains (characteristic of PT) with EGF domain-containing factors (characteristic of FVII, FX, and FIX). Still another group posited that PT arose independently from other vitamin K–dependent factors by way of a GLA-EGF-laminin G (LamG)-type domain-containing predecessor (that also gave rise to PS).

Fibrinogen

Mammalian fibrinogen is a disulfide-linked hexamer ($\alpha_2\beta_2\gamma_2$), containing three pairs of three chains, encoded by separate genes, alpha, beta, and gamma. Fibrinogen-related proteins have been found in mollusks, echinoderms, and insects, where they participate in the immune response. These findings suggest that FREDs evolved before the divergence of the protostomes and deuterostomes.[21] Initial demonstration that jawless fish (lamprey and hagfish) have fibrinogen came from functional studies by Doolittle et al.[44,45] Fibrinogen was purified from the plasma of lamprey and shown to have the same subunit structure as that found in mammals. Orthologs of the fibrinogen genes, alpha (denoted alpha-1), beta, and gamma, have been reported from lamprey, *Petromyzon marinus*,[39] and lamprey, *Lethenteron japonicum*,[37] as well as a separate gene encoding a minor form of the α-chain, denoted alpha-2.[39] In puffer fish, the β- and γ-chains of fibrinogen are 58% and 52% identical

to their human counterparts. The α-chain in puffer fish is less conserved. The fibrinogen gamma (γ)-chain gene produces two gene products from alternative (or non) splicing, γ and γ′ respectively. The γ′-chain of human fibrinogen serves regulatory roles in the binding of FXIII by fibrinogen[46,47] and in the binding of thrombin by fibrin.[48,49] Sequence analysis of fibrinogen γ sequences from lamprey to human indicates that the γ′ sequence is directly descended from a primitive unspliced version of the γ gene. The intron involved in this particular γ-chain splicing did not appear until the evolution of reptiles and birds.[3,50] The results suggest that the regulation of clotting in the manner of FXIII binding to fibrinogen and by thrombin binding to fibrin is a phenomenon that appeared relatively late in the evolution of the vertebrate coagulation process.

Factor VII, IX, X, and Protein C

Separate liver lamprey EST analyses identified one FVII gene sequence and two putative FX sequences in *P. marinus* and *L. japonicum*, respectively.[36–38] In the case of both *P. marinus* and *L. japonicum*, one of the FX gene products lacks key residues in the activation sequence and ought not to be active as proteases. To date, despite searching with FIX sequences from a wide variety of species, no evidence for a FIX sequence in lamprey has been found. Additionally, another cDNA clone from lamprey EST analysis encoded an SP domain similar to that of human PC; however efforts to clone portions of the gene beyond the SP domain failed, and thus the entire domain content of the gene sequence remains unclear.[37] Four genes were found for FVII (42% to 44% identity) in puffer fish at sites adjacent to each other and to a gene for FX; however, one of the homologs has an active site mutation in the catalytic SP region. The single FX gene identified in puffer fish is 41% identical to human FX. FIX first appears in teleosts. Indeed, as described earlier, there are two FIX genes in puffer fish, both of which may be functional. The proteins are 51% identical with each other and share greater similarity with one another than to mammalian FIX sequences, suggesting that gene duplication giving rise to the two homologs occurred after teleost fish diverged from the lineage leading to tetrapods.

The origins of FVII, FIX or FX, and PC, relative to PT, are debated. It is not clear whether they arose from a common ancestral protease. In any case, it has been proposed that the ancestral vitamin K–dependent (Gla-EGF-EGF-SP)—whether or not it arose independently of PT—initially duplicated (a WGD) (FIGURE 2.6), giving rise to PC and the ancestral FVII/FIX/FX gene.[42] The next gene duplication gave rise to FVII and the

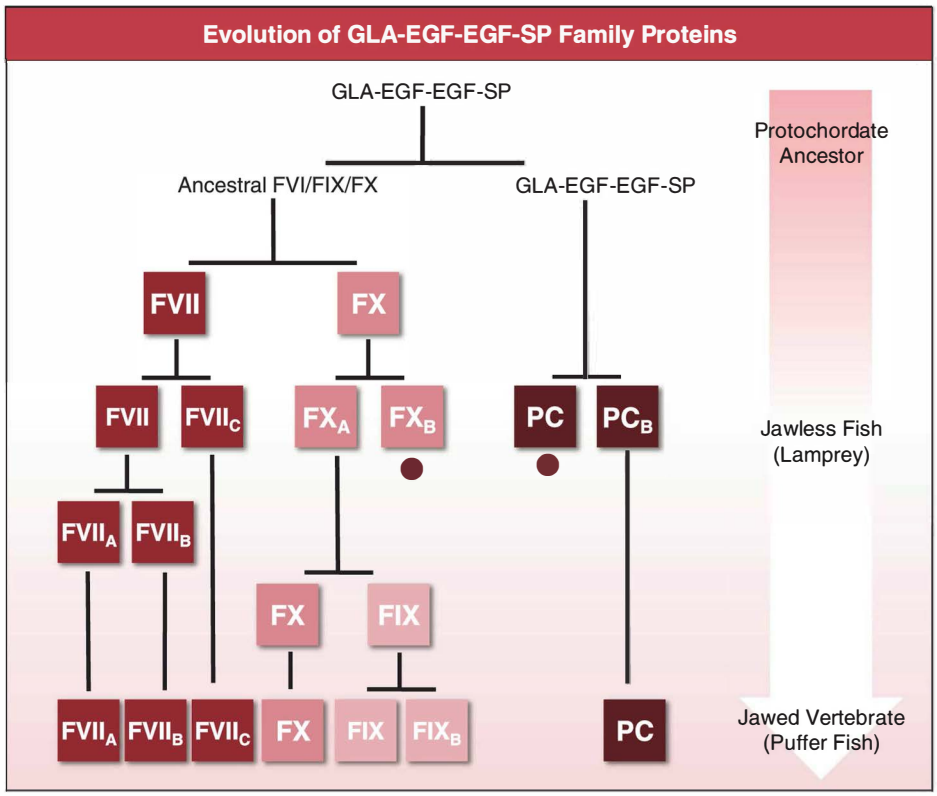

FIGURE 2.6 A scheme for the evolution of the blood coagulation GLA-EGF-EGF-SP family in early vertebrates based on sequence phylogenetic analyses. Initial gene duplication of an ancestral GLA-EGF-EGF-SP gave rise to two lineages, one giving rise to FVII and FX *via* an ancestral FX/FVII, the other giving rise to PC. The FVII/FX lineage is thought to have undergone additional rounds of local and genome-wide duplications to give rise to the two FXs and three FVII genes identified in the lamprey (jawless fish). Gene loss (X) of one of the FX genes and further gene duplication is thought to have given rise to the three FVII genes, one FX gene, and two FIX genes identified in the puffer fish (bony fish). The PC lineage is thought to have undergone genome-wide duplication and subsequent gene loss (X).

ancestral FIX/FX gene (see FIGURE 2.6). It is interesting to note that the FX and FVII genes are chromosomally adjacent to each other in puffer fish and humans (in contrast, the genes encoding for FIX, PC, FV, and FVIII are located on separate chromosomes in humans). This finding suggests an evolutionary origin of FX and FVII genes from a gene-duplication event on a single chromosome, which generated FVII and FIX/X only a short time before the last common vertebrate ancestor emerged. A third gene duplication (a WGD) gave rise to FIX and FX (see FIGURE 2.6). There would, of course, have to be some gene loss to account for the present repertoire of SPs.

Factor V and Factor VIII

As discussed earlier, FV and FVIII consist of three A domains and two *C*-terminal discoidin domains. There is a long, extended region between the second and third A domains, called the B domain.[51] Searches against the B domain in lamprey failed to yield significant hits, whereas a search against the A domain revealed a possible single precursor/predecessor of FV/FVIII (this finding is consistent with the presence of FX, but absence of FIX in lamprey). In contrast, in puffer fish, genes for FV and FVIII were readily identified, which encode for large proteins composed of three A domains and two *C*-terminal discoidin domains (41% and 42% identity with human orthologs, respectively).[42] The B domain between the second and third A domains varies greatly between puffer fish and human proteins (FV B domain, 16% sequence identity; FVIII B domain, 6% identity), consistent with large variations observed between the B domains of FV and FVIII from any species. These findings suggest that there has been very little selective pressure to conserve amino acid sequences in the B domains. The B domain in human FV and FVIII is encoded by a single large exon. It has been postulated that these exons arose through gene insertion and that the exons encoding the B domains of both FV and FVIII are derived from a single primordial cofactor gene (in which case the lack of current sequence homology between the two cofactors would be due to divergent evolution). FV and FVII are closely related to CP and HP (which share an A1-A2-A3 domain structure) (FIGURE 2.7). All four triplicated A domain–containing proteins appear to have evolved from a common ancestral gene through two serial gene-duplication events. It has been proposed that two first rounds of WGD generated ancestral FV/FVIII and CP/HP genes from an ancestral gene with an A1-A2-A3 structure.[42] Since both FV and FVIII have the domain structure A1-A2-B-A3-C1-C2, gain of B and C domains likely occurred before a second gene-duplication event, which gave rise to all four genes (FV, FVIII, CP, and HP)[42] (see FIGURE 2.7).

Factor XIII

FXIII is a transglutaminase that circulates in the plasma as a heterotetramer of two catalytic A subunits and two carrier B subunits (2A and 2B). Upon activation by thrombin, FXIIIa catalyzes the formation of γ-glutamyl-ε-lysyl amide crosslinks between adjacent fibrin molecules to form a mechanically stable, insoluble clot. The A chain of FXIII was identified in sequence analysis of the puffer fish whole genome, but a gene for the FXIII accessory B chain was not found.[41] In mammals, the B chain is composed of 10 "sushi" domains, also known as complement-control modules or β_2-glycoprotein domains. These domains are quite common in mammalian proteins where they occur in various components of the complement system. Sushi domains are also common in the puffer fish genome. However, no gene encoding for a sushi domain arrangement corresponding to that found in the FXIII accessory B chain was identified.

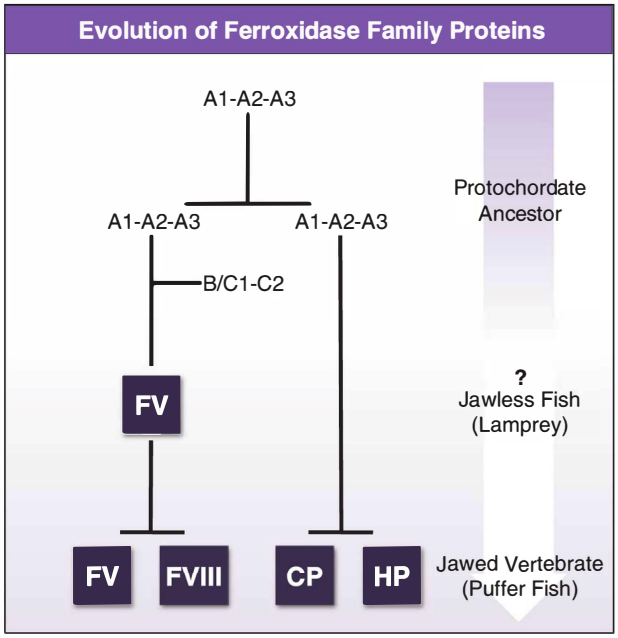

FIGURE 2.7 A scheme for the evolution of the blood coagulation ferroxidase domain family in early vertebrates based on sequence phylogenetic analyses. Initial gene duplication of an ancestral triplicated A domain-containing (A1-A2-A3) gene gave rise to two lineages, one lineage giving rise to coagulation cofactors FV and FVIII identified in jawed vertebrates and the other lineage giving rise to ceruloplasmin (CP) and hephastin (HP) proteins.

The Contact System

HMWK is present in all vertebrates. HMWK in the lamprey is highly simplified. The complexity of its structure has increased during evolution (e.g., compared with fish, HMWK in birds and mammals is composed of additional domains).[25] A functional role for a kinin–kininogen system in jawless fish is supported by a study in hagfish in which bradykinin promoted vasorelaxation of precontracted mesenteric arteries.[52] In contrast to HMWK, the SPs of the contact system (FXII, FXI, and PK) evolved only later with the tetrapods. FXII has a similar domain structure as hepatocyte growth factor activator (HGFA), and these two genes are thought to represent paralogs. Genes for HGFA, but not FXII, are present in lamprey and puffer fish.[53] FXII has been found in the genome of a reptile (Carolina green lizard) and is present in frog, platypus, and opossum genomes.[3] FXII is absent in birds, suggesting that birds have lost the gene for FXII over the 300 million years since the divergence of birds from mammals.[54] Interestingly, some marine mammals (whales, dolphins, and porpoises) have also lost the gene for FXII.[55] These animals diverged from other mammals 60 million years ago. The loss of FXII in these animals has left as its trace a corresponding pseudogene.

FXI and PK have similar DNA sequences and domain arrangement and are thus considered to be recently derived paralogs. The most notable structure of these proteins is the presence of a series

of four disulfide-constrained 90- to 91-amino acid repeats originally called apple domains, but now referred to as PAN domains. The apple/PAN domains of FXI and PK are believed to have derived from the *N*-terminal domain of plasminogen and hepatocyte growth factor.[53] A single paralog of FXI and PK occurs in frog, chicken, and platypus (a monotreme, which diverged from other mammals 160 million years ago), suggesting that it first appeared in early tetrapods.[53] FXI first makes its appearance in the opossum, which has two distinct genes for FXI and PK.[53] (The aPTT is much faster in opossum compared to human.) Thus, the FXI/PK precursor underwent gene duplication early in mammalian evolution, following the divergence of the monotremes from other mammals. Evidence suggests that PK was the ancestral gene for FXI/PK. In fact, no gene for FXI has been found in a nonmammalian species. Jawed fish lack the three contact factor proteases PK, FXII, and FXI. However, in contrast to jawless fish, they have FIX. In summary, the contact phase of clotting is a relatively late invention. It appears that the inflammatory response initiated by kinins evolved independently of the pathways leading to fibrin formation. The two systems became linked after the appearance of tetrapods.[25]

The Fibrinolytic System

t-PA has two kringle domains, while plasminogen contains five such domains. t-PA and plasminogen occur as early as the jawless fish. It is believed that fibrinolytic and blood coagulation enzymes originated from the same ancestral SP.[13] Following the divergence of these two families, a kringle domain was inserted into the common ancestor for plasminogen and plasminogen activators. The kringle domain underwent internal quintuplication (these were transferred to PT). After the divergence of u-PA and t-PA, a finger module (F) (related to fibronectin fingers) was inserted into t-PA and the kringle domain was duplicated.

Thrombomodulin

TM is an intronless gene. From an evolutionary standpoint, evidence suggests that intronless genes in mammals are more rapidly evolving. TM belongs to the family of C-type lectin-like domains, which bind to protein ligands rather than to carbohydrates, and are involved primarily in immune surveillance. C-type lectin-like domains are found in *Drosophila* and *Caenorhabditis elegans* proteins. TM shares similar domain composition and organization with C1qRp, a molecule involved in leukocyte adhesion and complement activation. In humans, TM and C1qRp colocalize to the same region of chromosome 2. Recent analysis of the NCBI Trace sequence database for lamprey (*P. marinus*) identified a potential sequence for TM, which contains a homologous *N*-terminal lectin domain and five EGF domains. This lamprey TM sequence is only 30% identical to human TM; however, as in other vertebrates (humans and puffer fish) the sequence is intronless.[36] Two intronless genes for TM were found in the puffer fish genome (29% and 37% sequence identity to human TM).[41] These two genes were found adjacent to each other. A similar arrangement is seen in the human genome where the intronless TM lies next to a related intronless gene (an accessory protein for complement factor C1Q).[56]

Antithrombin

AT belongs to the superfamily of SP inhibitors (serpins), which also includes α_1-antitrypsin and plasminogen activator inhibitors 1 and 2. Serpins have been identified in prokaryotes, plants, animals, and viruses. However, they have undergone extensive gene duplication and structural/functional divergence. Vertebrate serpins, including AT, appear to have arisen from a common invertebrate serpin that was intracellular rather than extracellular. AT has been cloned and sequenced from a wide variety of nonmammalian vertebrates. A gene for AT was found in puffer fish and zebrafish.[40,41] In both cases, the putative protein is 55% identical with the human protein. A search of the lamprey (*P. marinus*) genome draft assembly identified a sequence for an ortholog of HC II but no AT sequence.[36] The cDNA for HC II with one serpin (SP inhibitor) domain was identified in cyclostomes by liver EST analysis in lamprey (*L. japonicum*).[37] However, because of the simple domain organization and the presence of other coagulation genes or noncoagulation genes sharing the same domain, the identification is tentative. HC II has been cloned in multiple teleosts (Ref.[57] and references therein). In summary, it appears that HC II arose in a common ancestral vertebrate, whereas AT arose following the divergence of the jawless fish. Interestingly, AT-activatable heparan sulfate emerged some 250 million years earlier, suggesting this heparan sulfate proteoglycan initially served a nonanticoagulant function.

Protein S

PS has a Gla domain followed by four EGF-like domains and the sex hormone–binding globulin-like region consisting of two laminin G (LamG)-type domains. Sequence analysis of a liver EST from lamprey (*L. japonicum*) failed to reveal evidence for PS.[37] However, a putative PS gene was identified in the puffer fish genome with a similar intron–exon structure that encodes a protein with 48% sequence identity and 70% similarity with human PS. The putative PS gene, like its human counterpart, is situated near a similar homolog (a growth arrest–specific gene). Interestingly, a GLA domain–containing protein has been identified and cloned from the urochordate sea squirt, *C. intestinalis*, which resembles an elongated version of the PS prototype.[58] The GLA domain of this sea squirt protein, Ci-Gla4, is separated from the first EGF domain by a short putative disulfide loop—a situation analogous to PS where the GLA domain is separated from the first EGF domain by a thrombin-sensitive, disulfide-linked loop, noted as the thrombin-sensitive region (TSR). The potential common evolutionary origin of Ci-Gla4 and PS is further suggested by the finding that both disulfide loops are encoded by single exons. The central region of Ci-Gla4 has 17 EGF domains in tandem followed by two laminin G-like domains analogous to the domains at the *C*-terminus of PS.

Tissue Factor Pathway Inhibitor

TFPI is a Kunitz-type multivalent SP inhibitor. The Kunitz/bovine pancreatic trypsin inhibitor (BPTI) protease inhibitor family is found in a wide variety of animals, including in the venom of snakes. The prototypical Kunitz-type inhibitor is BPTI. TFPI has three Kunitz domains. Based on phylogenetic trees, the ancestral Kunitz-type inhibitor was predicted to have arisen about 500 million years ago. Recent analysis of the NCBI Trace sequence database for lamprey (*P. marinus*) identified a sequence for TFPI.[36] The lamprey sequence is 45% identical to human TFPI. The EST analysis of lamprey, *L. japonicum*, also identified a potential cDNA for TFPI containing a three-Kunitz domain organization.[37] TFPI has been cloned from carp and zebrafish,[59] with amino acid sequences of 30% and 28% identity

to human TFPI, respectively. Two genes for TFPI were identified in the puffer fish genome, just as is the case in humans. The amino acid sequence comparison best-match among these was 44% identical to the human TFPI sequence. Although functional data are presently lacking, these findings suggest that TPFI arose in the ancestral vertebrate.

Protease-Activated Receptors

PARs are G-protein–coupled receptors that play key roles in hemostasis, thrombosis, vascular development, and the inflammatory response. Four PARs (1 to 4) have been identified in mammals. Homologs of mammalian PARs 1 to 3 have been discovered in zebrafish.[60] The human and mouse PARs 1 to 3 genes are clustered in a small region (200 kb) of a single chromosome, PAR-1 and PAR-3 being separated by the IQGAP2 gene. PAR-2 is flanked by corticotropin-releasing hormone binding protein (CRHBP). Orthologs for PAR-1 and PAR-3 in zebrafish are also separated by IQGAP2 on the same chromosome. However, in the zebrafish, PAR-2 (in conjunction with CRHBP-2) is located on a different chromosome. These findings suggest that the zebrafish PAR-2 underwent tandem duplication and was translocated, along with CRHBP, away from PAR-1 and PAR-3 to a different chromosome.

PUTTING IT ALL TOGETHER

Comparative physiology, biochemistry, and DNA sequences allow for several predictions related to the evolution of the vertebrate clotting system. First, the absence of clotting factors in cephalochordates and tunicates and their presence in jawless fish suggest that the system evolved in an ancestral vertebrate some 500 to 600 million years ago. Second, although the basic clotting system (thrombin-mediated cleavage of fibrinogen) appeared over an extraordinarily short period of time (some 50 to 100 million years), the complex mammalian version did not arise in one fell swoop, but rather evolved over time. The extrinsic system appeared first, followed by FIX and then the contact system. Not surprisingly, the fibrinolytic system evolved with fibrinogen from the outset. The importance of anticoagulant mechanisms from the dawn of vertebrate lineage is evidenced by the presence of TFPI, HC II, and TM/PC (and possibly PS) in jawless fish. It seems that AT arose somewhat later. Finally, there is unlikely to be an extant vertebrate species with a more rudimentary clotting system than lamprey or hagfish. This leaves us to speculate on how the system might have arisen in the ancestral vertebrate (FIGURES 2.8 and 2.9).

The central feature of vertebrate clotting is the thrombin-catalyzed conversion of fibrinogen to fibrin. Based on molecular phylogenetic studies, thrombin was the first clotting SP to appear. It is unlikely that thrombin and fibrinogen appeared at the same time. Rather, they each evolved separately. Thrombin probably evolved first as a mediator of PAR-dependent cell signaling. Indeed, a critical role for PT in cell signaling during development is supported by knockdown studies in mice and zebrafish embryos.[61,62] It has been proposed that TF in the ancestral vertebrate directly activated thrombin, which then promoted PAR-mediated cell clumping.[3] A GLA domain could have helped to keep the evolving thrombin protease (or thrombin-like protease) localized on the surface of thrombocytes.

The subsequent appearance of fibrinogen would have allowed thrombin to broaden its attack, generating a more durable clot composed of fibrin.[3] Fibrinogen may have had an original, yet separate role apart from the coagulation system in cell–cell interactions, a property of many proteins with FREDs. Perhaps an early FXIII-like transglutaminase crosslinked fibrinogen to form a polymeric clot in a manner independent of

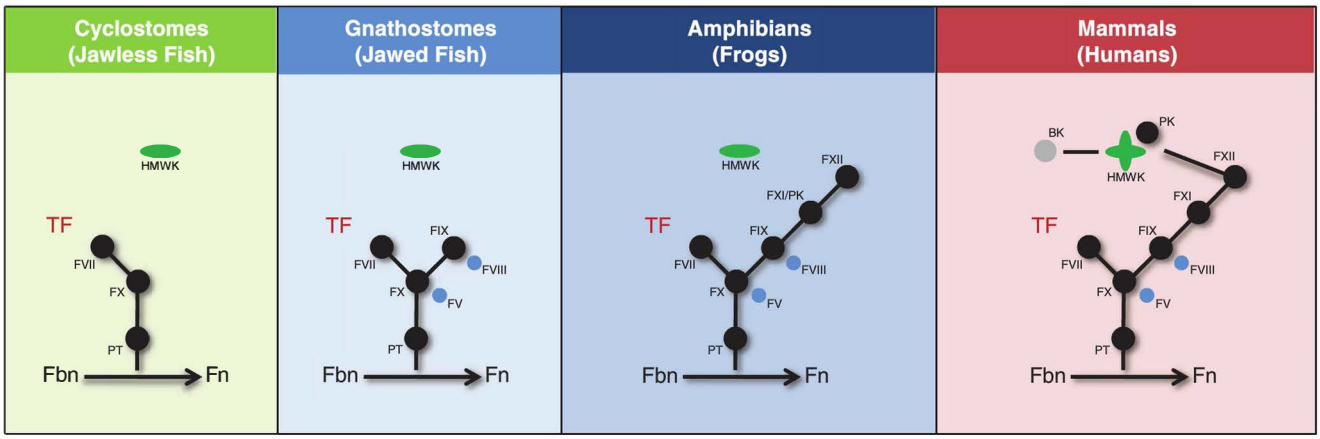

FIGURE 2.8 Increasing complexity of the coagulation system during vertebrate evolution. HMWK (*green oval*) is present in all vertebrates, though the complexity of its structure is increased in birds and mammals (indicated by a change in shape). The blood coagulation system in jawless fish generates fibrin (Fn) from fibrinogen (Fbn) through a simplified set of factors: TF and the SPs FVII, FX, and PT (*black circles*). Cyclostomes lack FIX and FVIII, and the presence of an ancestral FV/FVIII cofactor is not yet conclusive. In jawed fish, the cascade expands to include FIX (*additional black circle*). Both cofactors FV and FVIII (*blue circles*) are both present. In amphibians, factors of the contact system emerge, namely a single FXI/PK type factor and FXII (*additional black circles*). In mammals, duplication of the FXI/PK precursor gene results in separate genes for FXI and PK following divergence of the monotremes from other mammals. FXII cleaves PK to kallikrein; kallikrein hydrolyzes HMWK to bradykinin (BK, *grey circle*), thus connecting the clotting cascade with the kallikrein–kinin system.

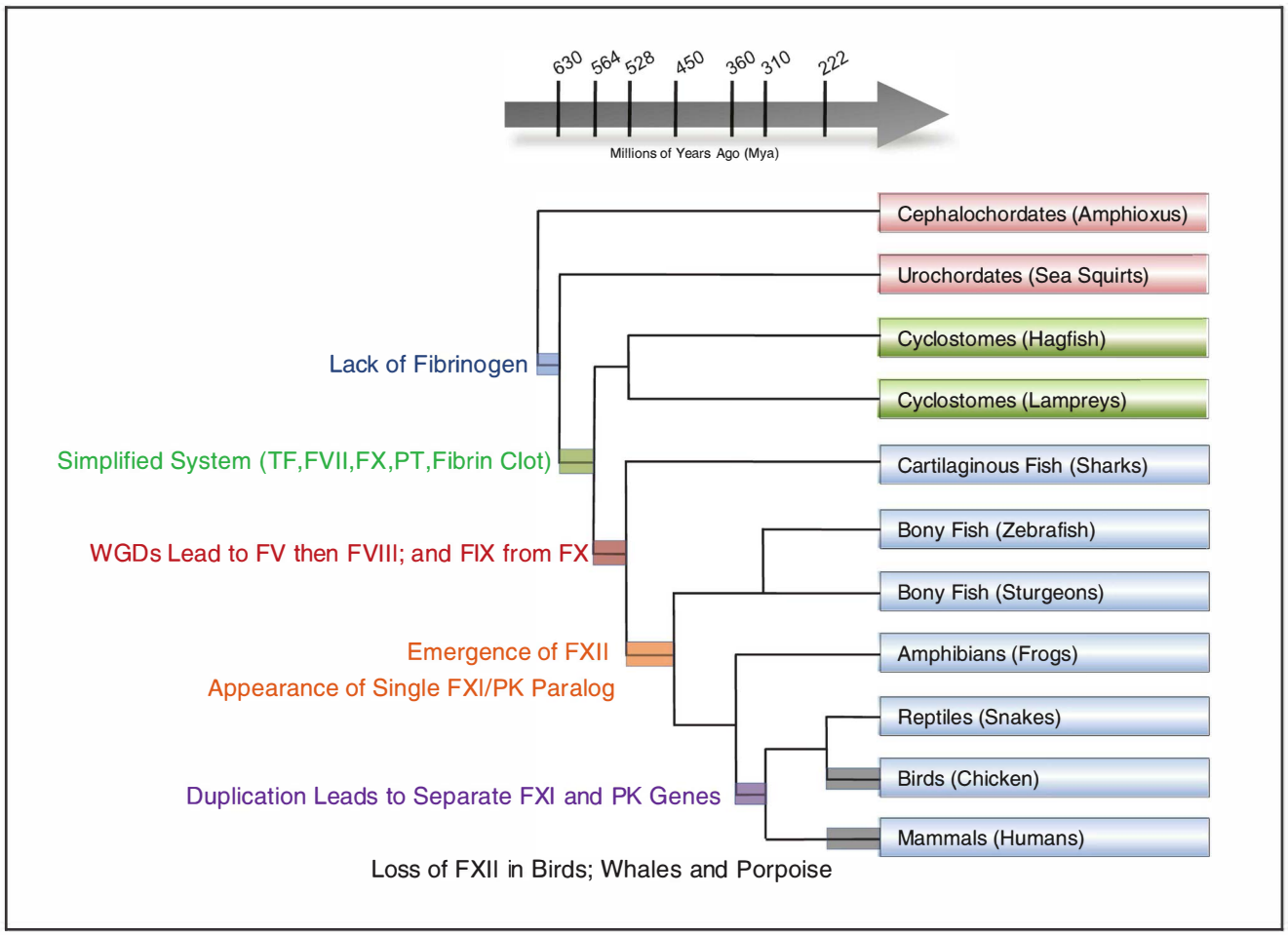

FIGURE 2.9 A phylogenetic tree representation of key evolutionary steps in the emergence of blood coagulation. Time periods of gene gain and gene loss are shown by *shaded boxes* on the evolutionary timeline.

thrombin-like activation. This scenario has similarities to the system that evolved independently in crustaceans like horseshoe crabs and lobsters, in which crosslinking enzyme leaks from damaged cells and causes a hemolymph protein to polymerize into a gel.

PT then underwent a gene duplication. The new gene (ancestral FX) would have undergone amino acid substitutions (e.g., to the substrate recognition pocket), resulting in a change in its substrate from fibrinogen to PT itself. There is some evidence that a precursor for FV/FVIII also arose around the same time, serving as a cofactor for FX. The ability of FX to activate PT released the dependence of PT on TF for its activation. A local gene duplication of FX resulted in the appearance of FVII only a short time before the last common vertebrate ancestor emerged (again, this duplicated gene underwent functional diversification with alteration of its substrate specificity, in this case from PT to FX). Thus, at the dawn of the vertebrates, blood coagulation was represented by the extrinsic cascade (TF-FVII-FX-PT-fibrinogen) alone. The next major change was the addition of FIX in the teleost lineage. This was accompanied by the appearance of distinct FV and FVIII genes. The contact system SPs (XII, XI, PK) appeared only with the tetrapods. The increasing complexity of the clotting cascade likely reflected the increasing demands of hemostasis in a wider spectrum of environments.[3] By enhancing the amplification property of the coagulation response, the addition of new proteases may have provided the necessary protection for animals with higher pressured cardiovascular systems and higher metabolic rates.

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CHAPTER

3 Molecular and Clinical Genetics

LESLEY EVERETT, KARL DESCH, AND DAVID GINSBURG

Keeping up to date with the remarkable progress in human genetics and the rapidly changing genomic laboratory toolbox poses a significant challenge for hemostasis physicians and scientists. This chapter provides a review of fundamental concepts in molecular genetics and an overview of key genetic technologies.

GENOME STRUCTURE AND DNA SEQUENCE VARIATION

The first draft of the human genome was published in 2004, resulting from a publicly funded, international effort (The Human Genome Project) utilizing Sanger sequencing.[1] The human genome is packaged into 23 pairs of chromosomes, including 22 pairs of autosomes and 1 pair of sex chromosomes (XX or XY), with each chromosome containing a single double-stranded DNA molecule. Human chromosomes range in length from 51 to 249 megabases (Mb), with an average length of about 135 Mb.[2] The complete haploid human genome is approximately 3 billion base pairs (bp), with 6 billion base pairs for the full diploid genome. In addition to the 23 pairs of nuclear chromosomes in any human cell, there also exists a small mitochondrial chromosome (mtDNA molecule) that is present in each mitochondria and thus in multiple copies in the cytoplasm of each cell. The mtDNA molecule is only 16.6 kilobases (kb), a tiny fraction of the total human genome size.[2] Nevertheless, mutations of mtDNA cause a variety of human diseases and are also implicated in aging and age-associated disease.[3]

Only approximately 1.5% of the human genome corresponds to protein-coding sequence (~21,000 genes), while the remaining intergenic DNA is composed of repetitive sequences, control regions, and large sections of the genome with unknown function. Human protein-coding genes show enormous variation in size and organization. The average length of a gene is 53.5 kb, but genes can range in length from several hundred bp up to 2,400 kb (dystrophin).[2] Gene length in nucleotides typically correlates with the size of the encoded protein, but this in part depends on the number and size of *introns* and *exons* in each gene. An intron is a portion of a gene that is transcribed into RNA, but does not code for amino acids and is removed by splicing to generate the mature messenger RNA (mRNA) that is translated into protein. All but a very small number of human protein-coding genes contain introns. In contrast, exons are the portions of a gene that are retained in the mature mRNA. Though all the DNA sequences that encode amino acids are contained in exons, noncoding exons also exist, and these contain the 5′ and 3′ untranslated regions (UTRs) of each mRNA.

The average number of exons per gene is 9.8 (range 1 to 363), and the average exon size is 288 bp (range <10 bp to 18.2 kb). The distribution of genes along and between chromosomes is not uniform. Some chromosomal regions are densely populated with genes, while other regions (often referred to as "gene deserts") are gene poor. At the chromosomal level, some chromosomes have substantial regions of tightly packed (heterochromatic), transcriptionally silent DNA; these regions generally contain many repetitive DNA sequences and very few genes.

In addition to the coding exons, another 3% to 5% of the genome is composed of highly conserved noncoding sequences. These regions are thought to contain important regulatory sequences that may play critical roles in vertebrate development, regulation of transcription, DNA replication, and chromosome pairing and condensation.[4,5]

DNA Sequence Variants

Despite vast phenotypic variation in the population, human beings are genetically very similar. One individual human genome differs from another by approximately 1 nucleotide in every 1,000 bp (0.1%). Thus, each haploid human genome of 3×10^9 bp harbors approximately 3,000,000 DNA variants, most of which are rare and predominantly found in noncoding DNA such as introns and intergenic regions.

DNA sequence variants are classified by their frequencies in the population (common or rare) and by their nucleotide composition (single-nucleotide variants [SNVs] or more complex structural variants).[6] Some sequence variants are associated with human disease, while other sequence variants are "silent" or nonpathogenic.

All DNA Sequence Polymorphisms are Due to Mutations, but not All Mutations Result in Polymorphisms

A *mutation* is any change of an ancestral base-pair sequence to a different nucleotide. Rare DNA sequence variants may be unique to an individual or a family. In contrast, *polymorphisms* are common differences in DNA sequence, strictly defined as having a frequency ≥1% for the less common allele(s). Thus, all polymorphisms are due to mutations, but not all mutations result in polymorphisms.

A mutation may be (a) a single base-pair substitution, (b) a deletion or insertion of 1 or more base pairs (*indel*), or (c) a larger deletion, insertion, or other rearrangement of genetic material. Mutations may be neutral (i.e., cause no observable change in phenotype or functional disruption) or deleterious (disease causing). In humans, new mutations occur approximately every 1×10^{-8} bp/generation, which corresponds to approximately 60 new genetic mutations per individual.

However, >98.5% of mutations occur in introns or intergenic regions (noncoding regions), so it is difficult to assess the consequence and implication of these mutations for human health and disease susceptibility.

Most well-characterized disease-causing mutations occur in exons or in exon/intron junctions. DNA mutations may disrupt normal gene expression or function in several ways (See FIGURE 3.1 and Chapter 4). *Point mutations* are the replacement of a single-base nucleotide with another nucleotide. Some point mutations within the coding sequence do not alter the encoded amino acid (*synonymous substitution*) and have no effect on the function or expression of the gene. However, a subset of synonymous mutations may alter the structure, function, and expression level of proteins via other mechanisms.[7] *Missense mutations* (*nonsynonymous substitutions*) are changes in the DNA sequence that cause one encoded amino acid to be changed to a different amino acid, thus changing the overall peptide sequence of the gene product. Some missense mutations are deleterious to protein function, while others may be neutral.

Nonsense mutations are another type of nonsynonymous substitution, changing a specific amino acid codon to a stop codon and thereby producing a shortened protein product. *Frameshift mutations* result from the insertion or deletion of a number of nucleotides (not divisible by 3) that alters the normal protein-coding *reading frame*. The reading frame of a DNA or RNA molecule refers to the sequence of three-letter codons that can be translated into amino acids. A frameshift mutation changes the amino acid sequence downstream of the mutation, often resulting in a premature stop codon. Of note, small insertions/deletions (*indels*) of a multiple of three nucleotides will insert or delete one or more amino acids, but are not frameshift mutations because they do not alter the reading frame.

Finally, *splice-site mutations* result in an altered RNA sequence by changing the specific site at which splicing of an intron takes place during mRNA processing. Splice-site mutations may lead to the production of aberrant proteins. However, mutations far from a splice site may also affect splicing via alternate mechanisms[8] or may be implicated in abnormal polyadenylation of mRNAs, RNA stability, or transcript processing.[2] An important cellular mRNA surveillance mechanism known as *nonsense-mediated decay* (NMD) results in the instability of mRNA-containing nonsense mutations, thereby reducing the expression of truncated, potentially harmful proteins.[9]

Mutations that functionally disrupt protein-coding genes, known as *loss-of-function (LOF) mutations*, are surprisingly common in the human genome. LOF variants have historically been assumed to cause disease, as is the case for many Mendelian diseases. However, recent large-scale sequencing projects have revealed that apparently healthy individuals harbor at least 100 LOF variants in their genomes, including approximately 30 in the homozygous or compound heterozygous state.[10]

Some LOF variants that are common in healthy individuals have been known and well characterized for years, such as the O allele of the blood group ABO antigen locus, and other variants that contribute to variable drug-metabolizing capacity between individuals. Nevertheless, the finding that healthy individuals carry dozens to hundreds of seemingly benign LOF variants came as a great surprise to the scientific community. Even more startling is the mounting evidence that gene disruption may actually be beneficial in some cases.[10]

It is possible for one disease to result from many different types of mutations in a given gene. For example, over 1,000 unique mutations causing hemophilia A are reported in the worldwide hemophilia database (HAMSTeRS).[11] As a result of its X-linked inheritance, hemophilia A is observed with a high frequency in the human population (1:5,000 male newborns worldwide) (Chapter 51). As Haldane predicted in 1935, one-third of males with a lethal X-linked disorder should represent *de novo* somatic genetic mutations in eggs or sperm,[12] based on the assumption that one-third of lethal X-linked mutations are carried in males and will be lost in each generation. Thus, most hemophilia A mutations are expected to be on average only a few generations

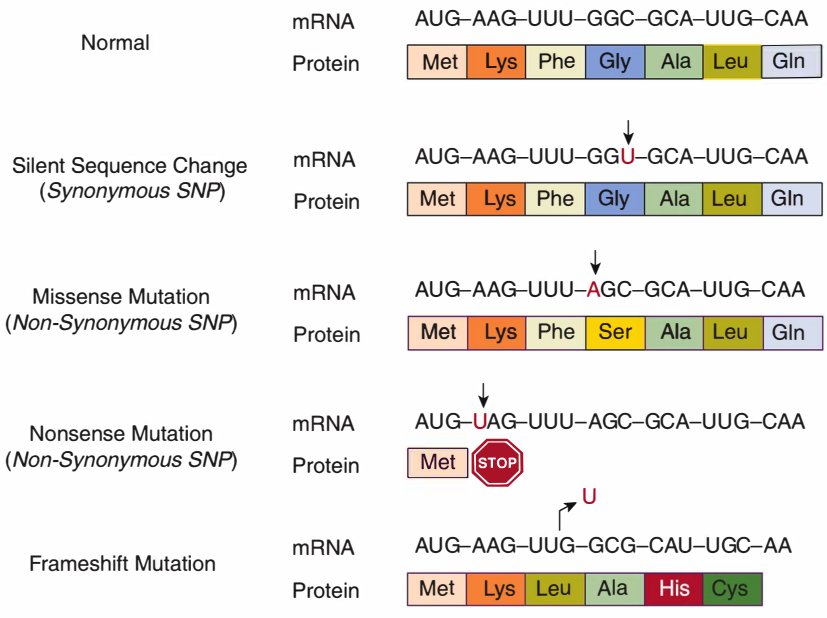

FIGURE 3.1 Examples of different classes of mutation.

old. Many of these mutations occur at CpG dinucleotides, which are known hotspots for mutation in mammalian genes.[13,14]

Mutations occurring outside of coding regions, such as in regulatory regions or in splice-site consensus sequences, may alter gene transcription or lead to alternative mRNA transcripts, respectively. Of historic interest, by analyzing DNA recovered from skeletal bone specimens of the Romanov family, a mutation in the *F9* gene predicted to alter RNA splicing was identified as the cause of the "Royal Disease" transmitted from Queen Victoria to the Royal families of Europe, thereby determining that this disease was hemophilia B, not the more common hemophilia A.[15]

Finally, in addition to smaller coding and noncoding mutations, structural rearrangements comprise another important class of genetic mutations, including deletions, insertions, inversions, and chromosomal translocations. Such larger genomic disruptions have been observed to cause hemophilia A, including Line 1 retrotransposon insertion.[11,16] Of particular note, the recurrent intron-22 inversion in the *FVIII* gene accounts for approximately 35% to 45% of all severe hemophilia A cases.[17] Duplications or deletions of large segments of DNA termed *copy number variations* (CNVs) are surprisingly common in the human genome. CNVs ranging in size from thousands to millions of base pairs may be present in anywhere from 0 to 2 or more copies when comparing one individual to the next.[18] CNVs can encompass entire genes or groups of genes, leading to gene dosage imbalance. Healthy individuals harbor multiple CNVs, which likely contribute to normal trait variation, though a subset of CNVs are associated with disease or disease susceptibility. Until recently, structural variants were technologically difficult to detect and characterize, as compared to single-nucleotide substitutions. Common structural variants are now estimated to involve between 9 to 25 Mb (0.5% to 1%) of the genome.[6]

Genetic Polymorphisms

As defined above, the formal definition of a polymorphism is any sequence or trait for which the less common or minor form(s) exhibit a population frequency of ≥1% (Table 3.1). These variants are considered to be *common* in the population. Common variants are not generally deleterious or associated with disease, which is consistent with the expectation that deleterious mutations would not typically be expected to reach a frequency of ≥1% in the population. *Single-nucleotide polymorphisms* (SNPs) represent positions in the genome where two or occasionally three alternative nucleotides are common in the population. Though the term "SNP" is typically used to refer to all single-nucleotide DNA variants, even those with minor allele frequencies ≤1%, the latter should more correctly be identified as SNVs. Unlike common polymorphisms with relatively high population frequencies, variants occurring at a frequency <0.01 in the population are considered to be rare. These variants are also known as "private" polymorphisms because they are often restricted to specific pedigrees in a population.

Of note, the terms *polymorphism* and *neutral variant* are often erroneously used synonymously. However, rare variants can be neutral and common polymorphisms can be deleterious (Table 3.1). In clinical practice, rare sequence changes in a disease gene in a patient can often only be described as "unclassified variants" or "variants of unknown significance." The relative frequency of neutral, near-neutral, and nonneutral genetic variants remains to be precisely determined (Table 3.2).[19]

Balanced polymorphisms represent a special class of genetic variant observed under balancing selection. In this case, an individual who is heterozygous at a particular genetic locus has a greater fitness or survival advantage compared to either type of homozygous individual, a phenomenon known as *heterozygote advantage*. For example, individuals with sickle cell trait (heterozygote carriers of the HbS mutation) are asymptomatic with normal life expectancy, though they are resistant to *Plasmodium falciparum* malaria,[20] which is endemic in West Africa. A balance exists between selection against individuals who suffer from sickle cell anemia (homozygous for HbS) and selection for heterozygous HbS carriers who are resistant to malaria.

Table 3.1

Classification of DNA sequence variants

Variant Frequency	<1%	≥1%
"Normal"	Rare variant or "private polymorphism"	Polymorphism
"Disease"	Disease-causing sequence variant	Common disease-causing variant For example, FV Leiden (2.5% allele frequency) and the common Δ508 cystic fibrosis mutation (2% allele frequency)

Databases Catalog Common Genetic Variation

The International HapMap Project is a publicly available catalog of common genetic variation in the human population. By collecting SNP data from hundreds of individuals, investigators can predict the likelihood of neighboring SNPs being inherited together. This analysis takes advantage of the fact that discrete blocks of genetic sequences tend to be inherited together more often (or less often) than expected by chance, given their individual allele frequencies in the population. This type of statistical association between genetic alleles is known as *linkage disequilibrium (LD)*, and it typically results from the maintenance of ancestral *haplotypes* in the population. Haplotypes are a series of specific adjacent SNP alleles that are inherited together because they have not yet been broken up by meiotic recombination. Such patterns of SNPs that are strongly correlated with each other across individuals form a limited number of relatively small haplotype blocks (10 to 50 kb).[21] Haplotype blocks thus provide important clues for understanding the evolutionary and demographic history of human populations.[22,23]

Several known genetic mutations, including the FV Leiden mutation and the prothrombin G20210A mutation, arose from a single mutational event on a distinct founder haplotype.[24,25] Haplotype blocks allow for the construction of genomic maps in populations sharing a geographic or ethnic background.[26] The original 270 HapMap samples were derived from

Table 3.2

Glossary of terms

Allele	One of two or more versions of a gene.
Allelic heterogeneity	A single disorder, trait, or pattern of traits caused by different mutations within the same gene.
Alternative splicing	More than one mRNA may be generated from the same gene by use of different exons, resulting in the generation of related proteins from one gene, often in a tissue- or developmental stage–specific manner.
Codominance	The phenotype of the heterozygote individual includes elements of both homozygous phenotypes (the discrete contributions of both parental alleles are visible in the phenotype).
Complex genetic traits	Result from the combined effects of many genetic loci and environmental factors (multifactorial).
Copy Number Variant (CNV)	A DNA sequence (ranging from thousands to millions of base pairs) that is present at variable copy number among individuals within a population, as compared to a diploid reference genome.
Epigenomic modifications	Chemical marks, such as DNA methylation and histone acetylation, that mediate the dynamic regulation of gene expression by influencing which genes are expressed and which are repressed.
Exome	The complete sequence of all exons in the human genome, comprising about 1.5% of the entire genome.
Exon	The portions of a gene that are retained in the mature mRNA. Though all the DNA sequences that encode amino acids are contained in exons, noncoding exons also exist, and these contain the 5′ and 3′ UTRs of each mRNA.
Frameshift mutation	Results from the insertion or deletion of a number of nucleotides (not divisible by 3) that alters the normal protein-coding reading frame.
Gene	The fundamental physical and functional unit of heredity. A gene is an ordered sequence of nucleotides located in a particular position on a particular chromosome that encodes a specific functional product (i.e., a protein or RNA molecule).
Genome-wide association studies (GWAS)	Utilize large samples of unrelated individuals to identify population-wide statistical associations between disease phenotypes and tested genotypes.
Genetic heterogeneity	Different genetic variants underlying the same phenotype or trait in different individuals or families (includes locus and allelic heterogeneity).
Genome browser	A database of genome information with graphical user interfaces.
Haplotype	A series of specific adjacent SNP alleles that are inherited together because they have not yet been shuffled by meiotic recombination.
Incomplete penetrance	A subset of individuals inheriting a disease-causing mutation exhibit no symptoms of the disease.
Indel	Insertion or deletion of a few nucleotides.
Intron	A portion of the gene that is transcribed into RNA but is removed by splicing to generate the mature mRNA that is translated into protein.
LD	A statistical association between genetic alleles such that discrete blocks of genetic sequences tend to be inherited together more often (or less often) than expected by chance.
Locus	The chromosomal location of a gene or a specific genetic sequence.
Locus heterogeneity	The same genetic disorder, trait, or pattern of traits that can be caused by mutations in one of a set of two or more genes.
Mendelian	A monogenic trait or disease whose pattern of inheritance suggests it is caused by variation at a single genetic locus—a monogenic character.

Table 3.2

Glossary of terms *(Continued)*

miRNA	Small RNA molecules that are processed from small hairpin RNA (shRNA) precursors that are produced from miRNA genes. miRNAs are 21–23 nucleotides in length, and through the RNA-induced silencing complex, they target and silence mRNAs in a sequence-specific manner.
Missense mutation	Change in the genetic sequence that causes one encoded amino acid to be changed to a different amino acid.
Modifier gene	Genes acting together with environmental and stochastic effects to modulate the effect of a major Mendelian gene.
Mutation	Any alteration in a gene from its ancestral state—may be disease-causing or a benign variant.
Next-generation sequencing (NGS)	New DNA sequencing methods based on massively parallel sequencing of thousands of DNA sequences simultaneously.
Nonsense-mediated decay (NMD)	A cellular mRNA surveillance mechanism used to detect nonsense mutations and to prevent the expression of truncated or potentially harmful proteins.
Nonsense mutation	A change from an amino acid codon to a stop codon.
Nonsynonymous mutation	A mutation that changes the encoded amino acid sequence to another amino acid or a stop codon.
Phenocopy	An individual with a phenotype (often environmentally induced) mimicking one usually produced by a specific genotype.
Point mutation	The replacement of a single-base nucleotide with another nucleotide.
Polymorphism	Any sequence or trait for which the less common or minor form(s) exhibit a population frequency of ≥1%.
Positional cloning	Using knowledge of chromosomal location to identify a disease gene.
Proteome	The set of proteins expressed by the genetic material of an organism under a given set of environmental conditions.
Quantitative trait	Many complex traits and diseases are quantitative, meaning that the observable human trait or disease varies over a continuous (and measurable) range of phenotypes.
RNA-seq	Transcriptome profiling technique that uses deep-sequencing technologies to provide a precise measurement of levels of transcripts and their isoforms.
Semidominance	Also known as incomplete dominance, this occurs when the phenotype of the heterozygote is an intermediate of the phenotypes of the two homozygous genotypes.
SNP	A position in the genome where two or occasionally three alternative nucleotides are common in the population, with the less common or minor form(s) exhibiting a population frequency of ≥1%. In common usage, often referred to as SNVs.
SNV	Similar to an SNP, but includes those with minor allele frequency <1%.
Synonymous mutation	A genetic mutation that does not alter the encoded amino acid sequence.
Transcriptome	The total mRNA content of a cell at a given time.
Variable expressivity	A wide range in disease severity or other features of the phenotype among individuals inheriting the same disease-causing mutation.

Caucasians of European ancestry, Yoruba people from Nigeria, and Asian individuals of Japanese and Han Chinese origin and included data for over 3.1 million SNPs. Currently in Phase III of the study, HapMap now includes data from 1,301 samples taken from 11 different global populations, enabling detailed study of common and rare genetic variation in diverse human populations.[27]

Another useful source of sequence variation data is the single nucleotide polymorphism database (dbSNP), a free public archive cataloging genetic variation within and across different species. In contrast to the HapMap Project, dbSNP contains information about SNPs as well as several other types of sequence variants from multiple species. The most recent dbSNP release (Build 135, November 2011) includes a total of 436,271,275 SNPs from 102 different organisms.[27] Finally, the database of genotypes and phenotypes (dbGAP) reports the results of studies investigating the interaction of genotype and phenotype, including data from genome-wide association studies (GWASs), medical sequencing, and other molecular diagnostic assays (Table 3.3).

Table 3.3

Other useful resources and web sites

General Resources

Resource	Description	URL
All about the Human Genome Project	General information sheet about the Human Genome Project	http://www.genome.gov/10001772
The 1,000 Genomes Project	Public effort to provide a comprehensive resource on human genetic variation	http://www.1000genomes.org
NIH Genetics Home Reference	Guide to understanding basic genetics concepts, genetic conditions, and additional genetics resources	http://ghr.nlm.nih.gov/

Genome Browsers

Database	Source	Description	URL
Ensembl	Wellcome Trust Sanger Institute/ European Bioinformatics Institute	Genome databases for vertebrates and other organisms presented with a convenient user interfaces	http://www.ensembl.org
NCBI map viewer	US National Center for Biotechnology Information		http://www.ncbi.nlm.nih.gov/mapview/
UCSC Genome Browser	University of California at Santa Cruz		http://genome.ucsc.edu

General Nucleotide Sequence Databases

Database	Source	Description	URL
GenBank	National Center for Biotechnology Information	Annotated collection of all publicly available DNA sequences	http://ncbi.nlm.nih.gov
dbSNP	National Center for Biotechnology Information	Archive for genetic variation within and across different species	http://www.ncbi.nlm.nih.gov/projects/SNP/
dbGAP	The database of Genotypes and Phenotypes (NCBI)	Archive of study results for interactions between genotype and phenotype	http://www.ncbi.nlm.nih.gov/gap
HapMap	The International HapMap Association	Identifies and catalogs genetic similarities and differences in humans	hapmap.ncbi.nlm.nih.gov http://snp.cshl.org/abouthapmap.html
ENCODE	Encyclopedia of DNA elements/ NHGRI	Effort to identify all functional elements in the human genome	http://www.genome.gov/10005107

General Protein Sequence Databases

Database	Source	Description	URL
UniProt	UniProt Consortium	Comprehensive database of protein sequence and functional information	http://www.uniprot.org/
SWISS-PROT	Swiss Institute of Bioinformatics	High-quality annotated and nonredundant protein sequence database	http://ca.expasy.org/sprot

Table 3.3

Other useful resources and web sites *(Continued)*

Diseases and Clinical Genetics Resources

Database	Source	Description	URL
Online Mendelian Inheritance in Man (OMIM)	US National Center for Biotechnology Information	Comprehensive database of human genes and genetic phenotypes	http://www.ncbi.nlm.nih.gov/omim
Human Gene Mutation Database (HGMD)	Institute of Medical Genetics in Cardiff	Published mutations causing or associated with human inherited disease	http://www.hgmd.cf.ac.uk/ac/index.php
Genecards	The Human Genome Compendium	Web-based cards integrating automatically generated information on human genes	http://www.genecards.org/
GeneTests/The Genetic Testing Registry	NIH	Information on genetic testing and its use in diagnosis, management, and genetic counseling	http://www.ncbi.nlm.nih.gov/sites/GeneTests/
HAMSTeRS	The Haemophilia A Mutation Database and Factor VIII Resource Site	General resource for *FVIII* genetic variation	http://hadb.org.uk/
Pharmacogenomics Factsheet: One size does not fit all—the promise of pharmacogenomics	NCBI	A basic primer on pharmacogenomics and its implications for clinical medicine	http://www.ncbi.nlm.nih.gov/About/primer/pharm.html

Gene Expression Profiling Databases

Resource	Description	URL
The Genotype-Tissue Expression (GTeX) Project (NIH Roadmap Initiative)	Large-scale study of human gene expression and regulation in multiple tissues to establish correlation between genotype- and tissue-specific gene expression levels	http://nihroadmap.nih.gov/GTEx/index.asp
Mammalian Gene Collection (NHGRI)	Sequence-validated full-length protein-coding cDNA clones for most known human, mouse, and rat genes	http://mgc.nci.nih.gov/
Mouse Transcriptome Project (NHGRI)	Database and public repository of gene transcripts for many mouse tissues (microarray, NGS, and other high-throughput functional genomic data)	http://www.ncbi.nlm.nih.gov/projects/geo/info/mouse-trans.html

Many methods are now available to detect DNA sequence variants, though most are now being supplanted by direct DNA sequencing. As explained later in the chapter, recent advances in sequencing technologies now enable the sequencing of entire exomes (the total complement of expressed exons in a genome) and whole genomes.

IDENTIFYING HUMAN DISEASE GENES

Human genetic diseases fall into two broad categories: (a) monogenic disorders, which are typically characterized by Mendelian inheritance patterns (e.g., hemophilia, von Willebrand disease [vWD]) or (b) multigenic, complex diseases in which multiple risk—conferring alleles in many genes cumulatively contribute to the disease (e.g., diabetes, heart disease). Human Mendelian traits give characteristic pedigree patterns that are often easily recognizable, including autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive, and Y-linked inheritance patterns. Rapid progress has been made in identifying the genes responsible for monogenic diseases, with about 3,000 now catalogued in the Online Mendelian Inheritance in Man (OMIM) database.[28] The identification of causative genes for complex diseases has proved difficult, but new methods and technologies are facilitating rapid progress in this area.

Mendelian Genetics

Mendelian traits and diseases can be dominant, recessive, or codominant. Almost all Mendelian diseases can be inherited maternally or paternally, except for sex-linked disorders, imprinting disorders, and mitochondrial genetic disorders (mitochondria are exclusively maternally inherited).[29] The terms *dominant* and *recessive* are properties of the traits, not the genes themselves. A trait is *dominant* if it is evident in an individual who is heterozygous for the causal mutation at the causative locus, and it is *recessive* if mutation in both maternal and paternal alleles at the locus is required to elicit a phenotype. Glanzmann thrombasthenia and Bernard-Soulier syndrome are both autosomal recessive conditions and are two of the most clearly defined platelet functional disorders at the molecular level.[30,31] Only individuals who are homozygous for the deleterious mutations are affected by disease, with heterozygous "carriers" of each mutation unaffected.

In complete dominance, the phenotype of the heterozygote is indistinguishable from that of the homozygote. Such disorders are quite rare and typically result from gain-of-function or dominant-negative mutations. Though a homozygote has never been reported, the rare disorder α_1-antitrypsin Pittsburgh (α_1-AT-P) might be expected to exhibit complete dominance. This mutation (conversion of methionine to arginine at residue 358 [Met358Arg]) converts α_1-AT into a potent thrombin inhibitor resembling antithrombin III.[32]

Incomplete dominance, also known as *semidominance*, occurs when the phenotype of the heterozygote is an intermediate of the phenotypes of the two homozygous genotypes. For example, homozygous hereditary protein C deficiency results in neonatal purpura fulminans (Chapters 81 and 98), whereas heterozygous individuals may exhibit venous thrombosis with typical onset in the third to fourth decade of life.

Finally, in *codominance*, the phenotype of the heterozygote is the same as both homozygous phenotypes (the discrete contributions of both parental alleles are visible in the phenotype). ABO blood type is an instructive example; the I^A and I^B alleles are codominant in producing the AB blood group phenotype because both the A-type and B-type antigens are produced.

A genetic mutation may lead to different genetic disease phenotypes in different individuals. If a subset of individuals inheriting a disease-causing mutation exhibit no symptoms of the disease, this is referred to as *incomplete penetrance* (vWD and FV Leiden are well-known examples in hemostasis). Similarly, a wide range in disease severity or other features of the phenotype among individuals inheriting the same disease-causing mutation is referred to as *variable expressivity*. For example, the same platelet disorder might manifest with variable tendencies toward mucosal bleeding, menstrual bleeding, or epistaxis in different individuals. Although Mendelian disorders are typically due to mutations at a single genetic locus, incomplete penetrance and variable expressivity may result from the action of other genes, termed *modifier genes*,[33] acting together with environmental and stochastic effects. It is also possible for a member of an affected pedigree to exhibit manifestations of disease, yet lack the disease genotype segregating through the pedigree. For example, consider a family with one or more individuals exhibiting easy bruising and frequent nosebleeds, without inheriting the familial vWD mutation. These individuals are known as *phenocopies*, and their bleeding phenotypes may result from any number of causes unrelated to the familial VWD mutation.

Linkage Analysis for Genetic Mapping of Mendelian Traits

The genetic loci underlying most Mendelian disorders were historically identified by careful genetic and phenotypic analysis of affected family pedigrees. Through a process known as *linkage analysis*, the gene responsible for an observable phenotype or disease of interest is "mapped" to a specific chromosomal segment.[2] Mapping a disease locus requires the genotyping of hundreds to thousands of markers spanning the genome in both affected and unaffected family members. Pedigrees are then checked to identify specific genetic markers that are coinherited with the disease. An informative genetic marker in close physical proximity to a disease-causing allele is "linked" to that allele, serving as a proxy for the "presumed" nearby deleterious sequence variant. Following initial linkage studies, a process known as *positional cloning* (locating the precise position of a the disease-causing gene) has enabled the identification of many genes encoding important proteins in the coagulation system, as well as thousands of other known pathologic and nonpathologic Mendelian traits.[28]

There are several barriers to successful positional cloning. Large pedigrees are generally required to achieve statistical significance and to identify a gene's chromosomal location with high resolution. Other factors, including *incomplete penetrance, phenocopies, locus heterogeneity*, and genotyping errors can also limit the power of linkage analysis. Locus heterogeneity occurs when a single disorder or trait is caused by mutations in genes at two or more different chromosomal loci. For example, mutations in the *LMAN1* or *MCFD2* genes cause the same rare autosomal recessive bleeding disorder, combined deficiency of coagulation Factor V and Factor VIII (F5F8D).[34] In contrast, *allelic heterogeneity* refers to the existence of many different mutations within the same gene in unrelated people with the same phenotype, as described above for *FVIII* mutations in hemophilia A.

Complex Genetic Traits

Most common human genetic diseases are not monogenic and do not exhibit Mendelian inheritance patterns. Rather, they result from the combined effects of many genetic loci and environmental factors. Hence, these phenotypes are termed *complex* or *multifactorial*. Complex diseases include thrombophilia, hypertension, coronary artery disease, diabetes, obesity, cancer, and many others.

Many complex traits and diseases are quantitative, meaning that the observable human trait or disease varies over a continuous (and measurable) range of phenotypes. Genetic factors known as *quantitative trait loci* (QTLs) are thought to influence or contribute to this interindividual variation in observable quantitative traits.[35] Blood pressure and von Willebrand factor (vWF) levels represent quantitative, continuous traits. vWD may be a Mendelian disease, but plasma vWF levels represent a widely varying quantitative trait in the population.

Genome-Wide Association Studies

Although linkage studies are well suited to detect variants with large effects and high penetrance, they often fail to detect variants of small effect. Most complex traits are determined by environmental factors combined with small effects of multiple genes. To overcome the limitations of linkage analysis, GWAS and candidate gene association studies have served as the primary analytic methods for investigating complex disorders.

GWAS[36] utilize large samples of unrelated individuals to identify population-wide statistical associations between disease phenotypes and tested genotypes. GWAS is based upon the assumption that unrelated individuals with similar phenotypes also share the same common genetic risk variants, which they inherited from a distant common ancestor. GWAS involve genotyping a large number of SNPs to identify loci that are more or less common in people with the disease as compared to those without the disease. It is presumed that the SNPs found more commonly in diseased individuals are in linkage disequilibrium with the actual risk variants. Just as in linkage analysis, an SNP found to be associated with disease in a GWAS is merely a physical marker for the presumed disease-susceptibility variant. The specific SNP itself does not necessarily directly contribute any risk of disease, but rather serves as a proxy for a nearby causative variant.

The power of GWAS to detect a low-effect locus depends upon the number of individuals studied, the allele frequency of the tagging SNP, and the allele frequency of the actual causative genetic variant. Hundreds to thousands of individuals with and without a trait or disease of interest are typically recruited, and each participant is genotyped for up to a million or more SNPs and structural variants.

Allele frequencies at each of the genetic markers are then correlated with trait variation (disease or no disease, or level of the quantitative trait) in the population-based sample. Since the disease-causing genetic variant is assumed to be within a conserved haplotype, genetic markers on this haplotype will be closely associated with the disease-causing variant (i.e., the marker and variant are in LD) and will be more commonly observed in disease subjects than in healthy controls.[36,37] The results of this type of analysis are typically presented as a *Manhattan plot*, a visual scatter plot with genomic coordinates displayed along the x-axis and a measure of statistical significance for each SNP displayed on the y-axis. An example of a GWAS to identify common susceptibility risk variants for venous thrombosis is shown in FIGURE 3.2. Since heterozygosity for FV Leiden is associated with a sevenfold increased risk of venous thromboembolic (VTE) disease[38] and non-O blood group carriers are also more predisposed to VTE than are O carriers,[39] the large GWAS signals at these loci are not surprising.

GWAS have now been completed for most common human diseases and many related traits, including cardiovascular disease, inflammatory bowel disease, asthma, neuropsychiatric disorders, rheumatologic disorders, cancers, and common traits including obesity, height, and hair color. As of mid-2011, the National Human Genome Research Institute has cataloged 1,449 published GWAS reporting statistically significant genetic associations (P values $\leq 5 \times 10^{-8}$) for 237 traits, and this list is updated weekly with new reports (FIGURE 3.3).

Several notable GWAS have identified variants that confer a substantial risk of disease, including a common variant in the complement factor H (CFH) gene, which is associated with a two- to fourfold increased risk of age-related macular degeneration (AMD), accounting for 20% to 50% of AMD risk.[40] Prior to this surprising report, CFH and other members of the complement pathway had no known association to AMD. Similarly, GWAS identified several loci associated with high levels of HbF,[41] including variants near the *BCL11A* gene leading to reduced *BCL11A* expression. Subsequent studies demonstrated that *BCL11A* is a key determinant of the fetal to adult hemoglobin switch, a major modifier of disease severity in sickle cell disease and severe β-thalassemia, and a novel therapeutic target for the treatment of these diseases.

More commonly, however, GWAS have identified many susceptibility factors of small biologic effect and limited heritability, meaning that a significant proportion of the genetic determinants of most complex diseases remain to be discovered. For example, GWAS have successfully identified >50 risk loci for type 2 diabetes, though these common genetic variants have only explained a portion of heritability and add little predictive value to existing conventional clinical risk factors.[42] Similarly, genetic factors are responsible for approximately 80% of variation in platelet count, a highly heritable trait.[43] Despite this large heritable contribution, a recent meta-analysis of data from over

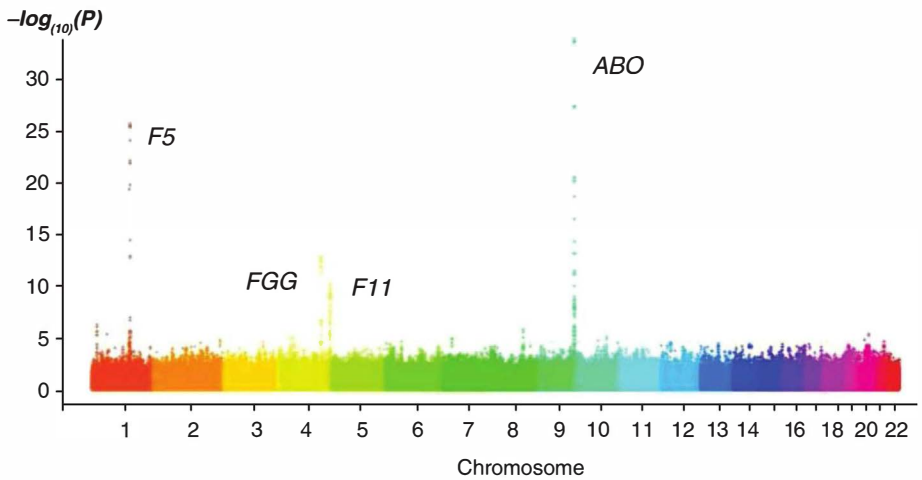

FIGURE 3.2 Thrombosis GWAS Manhattan plot of the association results from the combined analysis of two imputed GWAS data sets for 2,475,305 SNPs. No novel loci associated with venous thrombosis were identified. Through imputation analysis, only 99 SNPs reached genome-wide significance, and all of these SNPs were located within the *ABO, F5, FGG, or F11* loci. (Adapted from Germain M, Saut N, Greliche N, et al. Genetics of venous thrombosis: insights from a new genome wide association study. *PLoS One* 2011;6(9):e25581, with permission.)

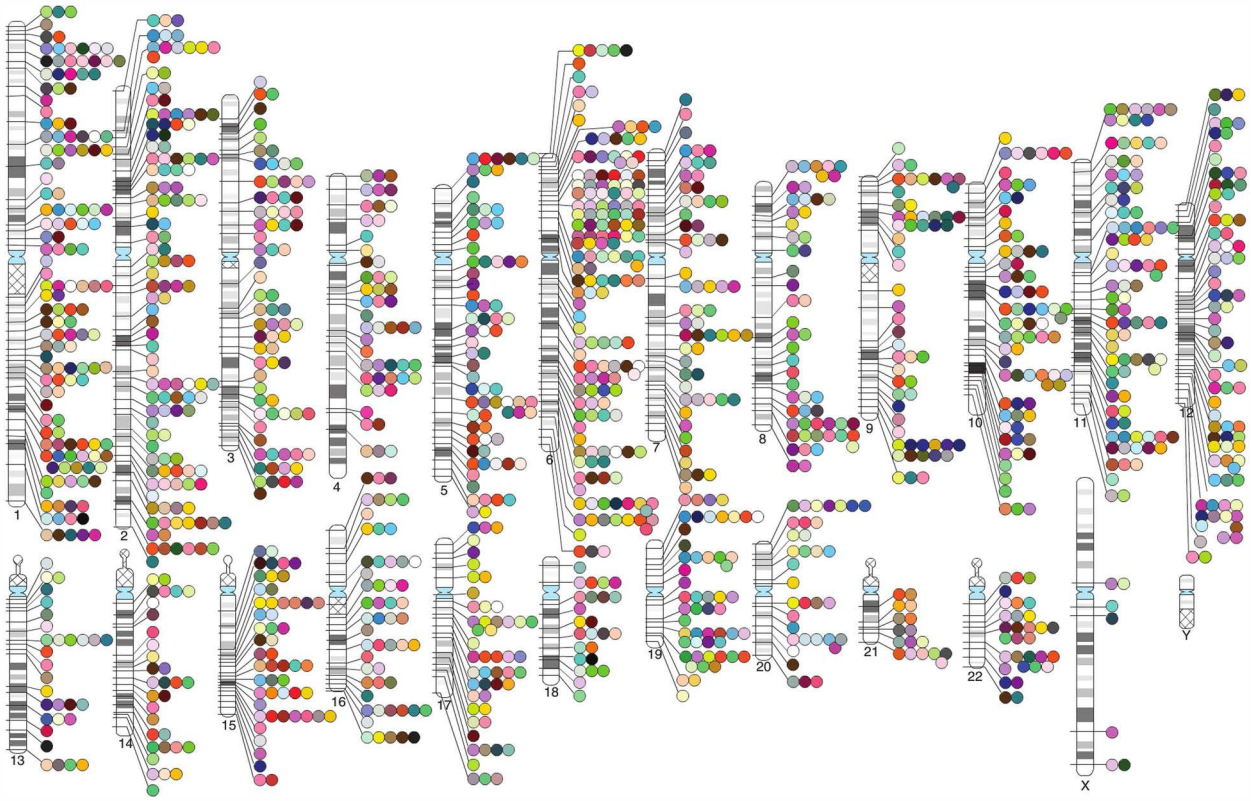

FIGURE 3.3 Published genome-wide associations through June, 2011. As of June, 2011, the National Human Genome Research Institute had cataloged 1,449 published genome-wide association studies demonstrating statistically significant genetic associations ($p \leq 5x10\text{-}8$) for 237 traits on all 22 autosomal chromosomes and on the X-chromosome. Each circle represents a unique reported association, and different colors correspond to different diseases.

66,000 individuals identified 68 genomic loci that cumulatively only account for 4.8% of the phenotypic variance in platelet count and 9.9% in mean platelet volume.[44] The remaining so-called missing heritability of these complex disorders could be due to the cumulative effect of multiple variants not detectable by current GWAS methods.[45,46] Of particular note, the contributions of most of these common variants of small effect size are not generally clinically useful for diagnosis, prognosis, or choice of therapy.[47] Advances in human genetics/genomics may eventually explain much of this missing heritability, with important implications for medical management.

Most Previously Reported Single-Gene Association Studies are Wrong

Single-gene association studies are based upon the expectation that an informative genetic marker located nearby a candidate gene should be more common in patients than in healthy controls. This approach has several inherent biases and shortcomings, including false-positive associations from multiple observations, as well as variable effects of population substructure.[47,48]

Very few of these previous reports have been confirmed by GWAS, indicating that they likely represent false-positive statistical errors. Instructive examples in hemostasis and thrombosis include the hundreds of candidate gene association studies performed to analyze the common plasminogen activator inhibitor 1 4G/5G and *MTHFR* gene polymorphisms for their roles in thrombosis, hypertension, coronary artery disease, and many other diseases. Despite the large number of reports, neither of these variants has been detected in any of the multiple well-powered GWASs for these same conditions.

Distinguishing Between Disease-Causing Mutations and Neutral Sequence Variants is Often Difficult

In addition to individual human genes, it is now possible to sequence entire exomes or genomes to identify genetic mutations. This raises the important questions of which mutations are disease causing and which are not? Distinguishing between disease-causing mutations, incidental gene-altering mutations, and neutral sequence variations is a critical clinical problem. The presence of widespread genetic diversity as well as LOF alleles in healthy individuals poses a major challenge for analysis of human genomes, particularly for clinical genome-sequencing studies.[49] For example, simply identifying a novel variant in a suspected gene in a patient does not establish causality. A rare variant in the *FVIII* gene of a hemophilia A patient that is absent from a set of healthy controls could be a neutral "private polymorphism" or a disease-causing mutation. While some changes in genetic sequence lead to obvious gene disruption (e.g., large deletions, rearrangements, frameshifts, or nonsense mutations), the impact of missense mutations is often unclear. Classifying the latter mutations often requires functional analysis of the encoded gene product. Though computational programs can estimate the functional consequence of genetic mutations,[49,50] these predictions are of limited clinical utility. In the absence of detailed functional

Table 3.4

Commercially available NGS platforms

Sequencing Platform	Read Length	Throughput	Sequence by Synthesis Method
Roche/454	400–600 bp	500 Mb/run	Pyrosequencing
Illumina HiSeq/Solexa	100 bp	200 Gb/run	Reversible terminators
ABI/SOLiD	50–100 bp	>100 Gb/run	Sequencing by ligation
Danaher/Dover/Azco Polonator	26 bp	8–10 Gb/run	Sequencing by ligation
Helicos Biosciences	25–55 bp	21–37 Gb/run	True single-molecule sequencing
Ion Torrent	>400 bp	>1 Gb/run	Sequence by synthesis on a semiconductor chip
Pacific Biosciences (PacBio RS)	>1,000 bp	~10 Mb/run	Single-molecule sequencing by "strobe sequencing"
Complete Genomics	Current: Short reads (70 bp) 2012: Long-fragment reads (LFR) (up to 10,000 bp)	>70 Gb/run	Current: Ligation-based chemistry LFR: will provide the ability to determine strand-specific sequences and enable whole-genome haplotyping.

Adapted from Metzker ML. Sequencing technologies—the next generation. *Nat Rev Genet* 2010;11(1):31–46.

analysis, missense mutations discovered in clinical testing must often be classified as "variants of unknown significance."

NEXT-GENERATION SEQUENCING

New DNA sequencing methods known as "*next-generation sequencing*" (NGS), or massively parallel DNA sequencing, were developed in the early to mid-2000s as a major advance over traditional Sanger sequencing.[51,52] High-throughput NGS generally involves a process of DNA template preparation, sequence generation, genome alignment, and assembly. Similar to Sanger sequencing, many NGS methods employ a "sequence-by-synthesis" approach, recording DNA sequences as each new nucleotide is incorporated into a growing DNA strand. These methods have dramatically improved sequencing speeds and high-throughput capacity, and they have lowered the cost of sequencing on a per base-pair basis. A number of NGS platforms are available commercially, each with slightly different profiles for base read length, error rate, throughput capacity, and method (Table 3.4). NGS technologies continue to improve rapidly, though significant limitations remain, including a relatively high (1%) error rate, requiring a high level of redundancy (often in the range of 50 to 100×) to distinguish true sequence variants from sequencing errors.

NGS technologies are already widely used for rapid genome sequencing[53,54] and for detailed analysis of transcribed mRNAs extracted from different cells and tissues. Massively parallel sequencing also plays a central role in new methods for the study of DNA–protein interactions (ChIP-seq, methyl-seq, and DNase-seq), defining genome-wide-binding patterns for transcription factors and other important regulatory molecules.[55,56]

Rapid advancements in DNA sequencing technologies have made the long-awaited "$1,000 genome" an imminent reality.[57] Improvements in NGS technology throughput have dramatically outpaced the rates of growth in computing and data processing capacity (FIGURE 3.4). Despite major advances in the

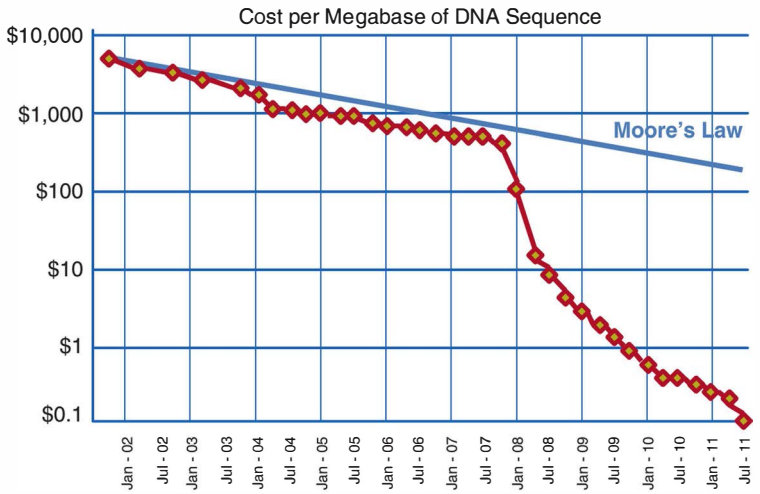

FIGURE 3.4 Massively parallel sequencing costs relative to Moore's law. As modeled by Moore's Law, historical trends in computing hardware, data processing, and memory capacity show that the capabilities of many digital electronic devices improve at roughly exponential rates. NGS sequencing technologies have outpaced advances in computing and data processing power.

computational analysis of large sequencing datasets, future developments in DNA sequencing technology are likely to increase the need for even more powerful computational platforms.[58]

NGS Technologies and Monogenic Diseases

The availability of NGS has enabled new approaches to the search for novel disease-causing mutations in diseases for which the responsible gene is unknown. Exome sequencing specifically targets the approximately 1.5% of the genome containing approximately 80% of the disease-causing mutations for Mendelian diseases.[59,60] The remaining approximately 20% of Mendelian disease mutations not detected by exome sequencing likely lie in introns or intergenic regulatory sequences, or are simply missed because only approximately 90% of the exome is efficiently captured and sequenced by currently available exome-sequencing platforms.

Exome sequencing has successfully identified mutations responsible for monogenic disorders that would previously have required large pedigrees for linkage analysis and positional cloning. For example, sequencing the exomes of just four affected individuals from three independent families was sufficient to identify the gene responsible for Miller syndrome, a rare Mendelian disorder of (formerly) unknown etiology.[61] Similarly, exome sequencing identified *NBEAL2* as the causative gene for gray platelet syndrome, a recessive platelet disorder characterized by mild thrombocytopenia with large platelets and lack of α-granules.[62]

Whole-genome Sequencing for Complex Disorders

Whole-genome sequencing will likely provide the best means of identifying rare causal variants and filling in the "missing heritability" currently observed in GWAS. It will permit the identification of less common variants that may have relatively large effects on disease risk.[63] Already, whole-genome sequencing for structural variants such as CNVs has led to the delineation of new microdeletion and microduplication syndromes.[64] As NGS costs continue to fall, whole-genome sequencing will become an increasingly routine part of genetic and clinical evaluation, bringing with it the challenges of storing and interpreting tremendous volumes of data.

Perhaps no discovery or technologic advance has transformed genetics research more dramatically than NGS. These powerful new techniques are used to address a myriad of biologic questions with unprecedented implications for clinical medicine and population biology. NGS has been the cornerstone for a number of large-scale projects to explore the range of genetic variation across the world's population, including the 1,000 Genomes Project, which now includes data from 2,500 individuals representing 27 different populations.[65] All data generated are publicly available and are being incorporated into other resources such as the Human Gene Mutation Database (HGMD) (Table 3.3) in order to improve annotations of gene models and disease-associated sequence variants.

STUDYING GENE FUNCTION IN THE POSTGENOME ERA

NGS technologies have enabled a shift in the scope of genetic research from the study of individual genes and their products to global, genome-wide analyses in which many or all gene products are studied simultaneously, an approach sometimes referred to as *functional genomics*.

Transcriptome Data Informs Gene Function

Although the genome itself is virtually identical in most nucleated cells of an organism, gene expression patterns vary dramatically among different tissues and cell types. Differential tissue expression patterns are mediated through the tight regulation of DNA transcription, RNA processing, protein synthesis, and posttranslational modification. The mRNA *transcriptome* reflects the results of DNA transcription, RNA processing, and RNA turnover, and therefore represents the fraction of the whole genome that is transcribed into mRNA (<5% in humans). Similarly, the *proteome* is the combined output of the transcriptome followed by mRNA translation, posttranslational processing (including protein cleavage and modification), and protein turnover.

Quantitative Transcriptomics by Microarray Hybridization or Massively Parallel Sequencing

Genome-wide mRNA expression analysis was previously performed exclusively by DNA *oligonucleotide microarrays*, also known as gene chips or DNA chips.[66] Microarrays are small slides with individual DNA oligonucleotides representing sequences of interest (probes) attached to the solid surface. Probes may be short sections of genes or other DNA elements used to capture and hybridize cDNA molecules in a biologic sample. Microarrays are thus used to measure the expression levels of large numbers of genes simultaneously by differentially labeling sample mRNAs with fluorescent tags and quantifying their relative hybridization to the microarray.

Numerous DNA microarrays are commercially available, many of which are capable of detecting expression of all approximately 21,000 human or mouse genes. Others are used for targeted research initiatives, such as "metabolomics" microarrays that include sequences corresponding to all the known genes involved in metabolic pathways. Hybridization-based microarrays only detect the expression of known transcripts that were purposefully included on the array. Hence, rare transcripts or alternatively spliced mRNA variants are generally missed.

RNA-Seq Reveals the Complexity of Transcriptomes

RNA-seq, otherwise known as massively parallel cDNA sequencing, represents a major advance in gene expression analysis.[67] Compared to microarrays, this technology does not have an upper limit for quantification, and it provides a much expanded dynamic range of expression levels over which transcripts can be detected (>9,000-fold in a recent *Saccharomyces cerevisiae* experiment[68] and spanning a range of five orders of magnitude for analysis of 40 million mouse sequence reads[69]). RNA-seq analysis also enables the identification and quantification of novel transcripts and alternatively spliced forms, as well as the comprehensive, detailed analysis of transcription initiation start sites. Furthermore, it improves the detection of alternatively spliced transcripts and gene fusion transcripts in physiologically normal and disease settings. For example, RNA-seq analysis of platelets from a patient with gray platelet syndrome identified abnormal transcripts resulting from a splice-site mutation (FIGURE 3.5), contributing to the identification of *NBEAL2* as the responsible gene.[70] The large amount of tissue-specific gene expression profiling data that have been generated through microarray and RNA-seq studies has been systematically catalogued in a number of databases (Table 3.3).

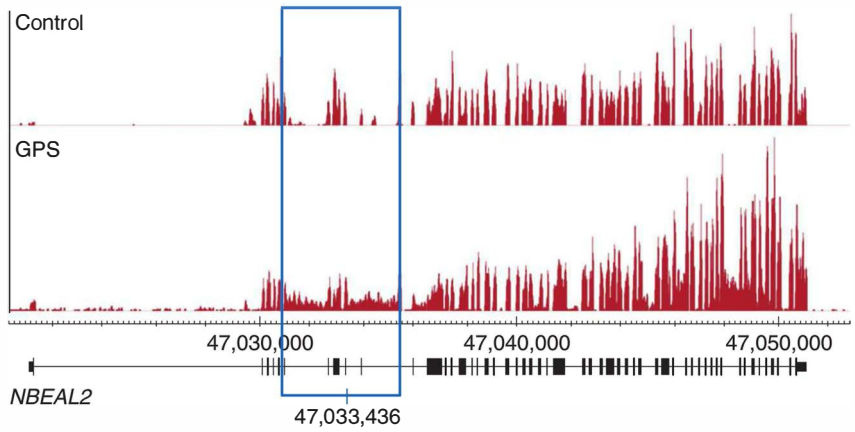

FIGURE 3.5 Abnormal sequence reads in *NBEAL2* transcripts in platelet RNA from an individual with GPS. *NBEAL2* transcripts expressed in platelets isolated from a healthy donor (top) and a patient with grey platelet syndrome (bottom). The red box outlines a region where introns are abnormally retained in *NBEAL2* transcripts from GPS platelets.

Genomes and their Messenger Molecules are Highly Dynamic

Gene expression and transcriptome studies have revealed that genomes exist in highly dynamic states, with numerous regulatory elements and reversible DNA modifications. *Epigenomic modifications* are chemical marks, such as DNA methylation and histone acetylation, which mediate the dynamic regulation of gene expression by influencing which genes are actively expressed and which are repressed. Epigenomic modifications serve as an additional set of instructions and regulatory control for the cell's transcriptional machinery, although they are not part of the actual double-stranded DNA helix. Nevertheless, they may be passed on during cell division from a mother cell to its daughter cells, and in some instances, from one generation to the next.[71,72]

In addition to mRNAs, several other classes of RNAs exist that also play important regulatory roles in the cell.[73] For example, *microRNAs (miRNAs),* a class of RNAs first discovered in plants, add another layer of gene expression regulation. miRNAs modulate protein expression primarily by degrading mRNA or repressing translation by recognizing specific complementary sequences in the target mRNA. Current estimates suggest that more than 60% of human protein-coding genes contain miRNA target sites in their 3′ UTR.[73] miRNAs play important roles in hematopoiesis, including platelet production and activation,[74] the pathogenesis of vascular disease,[73] and presumably many other disorders.

THE ROLE OF MODEL ORGANISMS IN GENETIC RESEARCH

Model organisms represent a powerful research tool for studying the pathophysiology of human disease. Laboratory mice are a widely utilized mammalian genetic model, in large part because they reproduce quickly and because the mouse genome is relatively easy to manipulate.[2] Recent progress has also markedly expanded the toolkit for manipulation of the rat genome, particularly for use in cardiovascular disease research.[75]

Essentially any gene of interest can be inserted into the mouse or rat germline (transgenic animals) or inactivated (i.e., "knocked out") by replacement or disruption of an endogenous gene. Even a specific mutation can be introduced into an endogenous gene in its natural location by creating a "knock in" genetic allele. As a result of this technology, thousands of genetically distinct mouse models are currently available, including unique inbred strains and genetically engineered mutants mimicking many human diseases. These mice are cataloged and available for purchase from The Jackson Laboratory (JAX), the UC Davis Knockout Mouse Project (KOMP), and The European Conditional Mouse Mutagenesis Program (EUCOMM) (Table 3.5). Mouse models have proved to be extremely useful for the study of hemostasis and thrombosis, with genetically deficient mice generated for most known human coagulation factors.[76]

Chemical mutagenesis is another powerful tool for the generation of new animal models, a process in which experimental animals are exposed to DNA-damaging chemicals and screened for resulting phenotypes.[77] This technique has been used to identify genes involved in megakaryocyte and platelet development, among many others.[78,79] *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis has also been used to elucidate pathways in blood and vessel development in zebrafish[80] and to identify modifier genes that lessen the severity of thrombosis in mice carrying Factor V Leiden and tissue factor pathway inhibitor mutations.[33]

PERSONALIZED MEDICINE

GWAS have identified many disease genes and susceptibility alleles, generating much new information with potential clinical importance. However, the synthesis and reduction of this information to practice has not been straightforward.[47] Genetic testing in approved clinical testing laboratories is available for approximately 3,000 single-gene disorders. The result of some genetic tests provides information that clinicians can immediately act upon, thus significantly altering medical management; examples include newborn screening programs and genetic testing for some cancer predisposition syndromes. In contrast, some genetic tests are informative but have no real impact on medical management (e.g., Huntington disease, Alzheimer disease, etc.).[47] The clinical utility and predictive value for many of the remaining genetic tests is often unclear.

For example, genetic factors account for approximately 60% of the overall risk of VTE. Though FV Leiden contributes approximately 25% of the overall attributable risk, the lifetime penetrance of VTE is still only 10% in FV Leiden individuals, and no specific VTE prophylactic treatment is indicated for asymptomatic subjects. In addition, following an initial episode of VTE, the recurrence rate does not differ significantly between patients with and without this common polymorphism. Thus,

Table 3.5

Model organism resources

Database or Resource	Description	
Mouse Genome Informatics—The International Database for the Laboratory Mouse	Integrates genetic, genomic, and biologic data for mouse models of human disease	http://www.informatics.jax.org/
JAX	Performs mammalian genetics research and provides scientific services and model organisms to researchers	http://www.jax.org
UC Davis KOMP	Comprehensive public resource of mouse embryonic stem cells containing a null mutation in every gene in the genome	http://www.komp.org
EUCOMM	Generation and distribution of up to 12,000 conditional mutations across the mouse genome in mouse embryonic stem cells	http://www.eucomm.org
North American Conditional Mouse Mutagenesis Project (NorComm)	Development and distribution of mouse embryonic stem cell lines carrying single gene trapped or targeted mutations	http://www.norcomm.org
NHGRI Knockout Mouse Fact Sheet	General information about the generation and use of knockout mice	http://www.genome.gov/12514551
Flybase (*Drosophila*)	Flybase Consortium genome browser	http://flybase.org
SGD (*Saccharomyces*)	Yeast genome browser	http://yeastgenome.org
Wormbase (*C. elegans*)	Wormbase Consortium genome browser	http://wormbase.org
ZFIN (Zebrafish Information Network)	University of Oregon-based zebrafish genome browser	http://zfin.org

FV Leiden testing is not generally indicated on clinical grounds, since current treatment recommendations are not altered by the results of the test.[81]

Despite remarkable progress in identification of genes responsible for most known single-gene disorders, only a small subset of these discoveries have resulted in direct improvements in therapies to treat these diseases.[47] However, some of the most notable exceptions have been in the thrombosis and hemostasis fields, including the successful development of recombinant protein product replacement for hemophilia A and B, and the use of recombinant tPA to treat pathologic thrombosis.

Similarly, the practical implementation of modern pharmacogenomic discoveries to guide choice and mode of therapy has also been disappointing. Some of the most attractive opportunities in pharmacogenetic testing have been identified for hemostatic therapies, notably to guide treatment with the anticoagulant warfarin and the antiplatelet drug clopidogrel.[82]

Though common variants in the *CYP2/C9* and *VKORC1* genes account for approximately 30% of the interindividual variability in response to warfarin, prompting the FDA to include this information in the warfarin package inserts, such testing has not yet been shown to significantly improve clinical outcomes in warfarin-treated patients and is not widely used in clinical practice.[83] Similarly, while several trials are underway to demonstrate the potential utility of platelet reactivity testing for personalized antiplatelet therapy, genetic testing for *CYP2/C9* genotype has not yet proven useful for treatment optimization in the clinic.[84]

DIRECT-TO-CONSUMER GENETIC TESTING

Direct-to-consumer (DTC) genetic testing for large panels of SNPs is now widely available over the Internet, offering customers risk prediction for a broad range of common diseases, including thrombosis and coronary artery disease.[85] However, the changes in risk conferred by these SNPs are generally very modest, and often dwarfed by other easily measured clinical factors such as body–mass index and family history. In addition, risk prediction is highly inconsistent across providers.[86] Considering that some of the testing panels do include large-effect genetic risk factors, consumers may not always receive adequate genetic counseling in conjunction with DTC, and the impact of this testing on the subsequent interactions of these consumers with their physicians is potentially problematic.[87,88]

GENE THERAPY

Since alteration or mutation of genes often results in the synthesis of dysfunctional proteins, the identification of the defective gene for approximately 3,000 Mendelian disorders has raised considerable hope for direct correction of these conditions by *gene therapy*. Potential approaches include insertion of the normal gene into a nonspecific location within the genome to replace a defective gene, or repair of the abnormal gene. Though early progress was disappointing, including unfortunate adverse events,[89] recent success in treating rare immunodeficiency disorders and hemophilia has highlighted the promise and future

potential of this technology, including a recent successful report of adenovirus-associated vector-mediated gene transfer in hemophilia B.[90] In addition, though not generally considered gene therapy, recombinant protein therapeutics (tPA, *FVIII*, IX, etc.) have played a central role in the treatment of hemostasis disorders and will continue to be a mainstay of therapy.

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CHAPTER

4 Coagulation Enzymology

SRIRAM KRISHNASWAMY

THE CONTEXT

Blood coagulation is initiated by the exposure of tissue factor to flowing blood following vascular damage.[1,2] A series of highly specific proteolytic activation steps ensue to convert an inactive circulating precursor to an active product. The product in turn participates in a subsequent activation reaction (FIGURE 4.1). Thrombin, formed as a result of these reactions, is the key effector product of coagulation as it is the proteinase responsible for the cleavage of fibrinogen to form the insoluble fibrin meshwork. It also activates platelets, other blood cells, and the endothelium by cleavage of the protease-activated receptors. Thrombin is a self-limiting product. Early in the coagulation response, it functions as a positive feedback effector. It greatly increases flux through the cascade by activating cofactors and factor XI necessary for the earlier steps of coagulation. Later in the response, it acts as a negative feedback effector by binding thrombomodulin (TM) and catalyzing protein C activation, which in turn proteolytically inactivates V and VIII. Their inactivation reduces flux toward thrombin formation. Active thrombin and the other proteinases of coagulation are eliminated by reacting with circulating serine proteinase inhibitors. As a result of this central regulatory role played by thrombin and the critical role played by proteinase inhibitors, the fate of active thrombin formed following the initiation of coagulation exhibits a transient profile, in which the profile for fibrin formation is dictated by the amplitude and timing of the peak as well as its integrated area, reflecting the total amount of active thrombin produced (FIGURE 4.2).

THE PROBLEM

Regulated enzymic function plays a dominant role in modulating the hemostatic response to vascular damage. Proteinase specificity reflects the ability of any one enzyme to selectively act on its cognate substrate in the complex milieu of blood. This becomes an especially important aspect of coagulation enzymology because the coagulation proteinases are trypsin-like enzymes belonging to the S1 family of subclan PA(S) of serine endopeptidases.[3] Their catalytic domains are highly structurally homologous to each other and to the digestive enzymes chymotrypsin and trypsin, the archetypic members of the clan.[3] The nature of the problem is highlighted by the fact that while trypsin can, in theory, act to cleave after any lysine and arginine in any protein, the homologous coagulation proteinases act with marked specificity to cleave after just one or two arginines in a limited subset of structurally related proteins. Thus, rigidly limited proteinase specificity lies at the heart of the hemostatic response. Flowing blood also constrains coagulation enzyme function to a physical regime that differs markedly from those encountered in the "test tube" where formalisms deal with circumstances in which substrates can be quantitatively converted to product. Instead, normal hemostasis derives from activation reactions that occur at a reactive patch, representing the area of vascular damage, to which substrates are constantly being delivered by flowing blood and products are washed away. Membrane-dependent enzyme function localizes many of the coagulation reactions to the site of vascular damage to which inert precursors are delivered by flowing blood. Products that escape the reactive patch are rapidly inactivated downstream by proteinase inhibitors. This chapter focuses on the unique strategies employed by the enzymes of blood coagulation to achieve these essential aspects of function.

PROTEINASE FORMATION

As defined in subsequent chapters, specialized structural domains present in the NH_2-terminal half of each of these zymogens contribute to a variety of aspects of zymogen and resulting proteinase function, including membrane binding by the vitamin K–dependent zymogens. However, the zymogens all possess a (chymo)trypsinogen-like domain at their respective COOH-termini that is converted to the catalytic domain by proteolytic activation.[4,5] Similarity between the catalytic domains of the different enzymes has been established by an abundance of high-resolution x-ray structures. Based on this identity, Bode et al.[6] introduced a uniform strategy of numbering residues in the catalytic domain or its precursor. Topologically equivalent and conserved residues within the family are numbered using the sequence numbers for the equivalent residues in chymotrypsinogen (e.g., I_{c16}, chymotrypsinogen numbering denoted by the subscripted c). When insertion loops unique to the individual coagulation proteins are encountered, they are denoted alphabetically, resuming with the equivalent numbering at the end of the insertion (e.g., P_{c204}-F_{c204A}-N_{c204B}-N_{c205}). This provides a uniform and facile approach for considering the structural correlates of catalytic function in this family of enzymes.

Proteinase formation results from cleavage of the peptide bond following R_{c15}, regardless of other cleavages that may occur in the processing of any particular zymogen by its cognate enzyme. This cleavage reaction produces a new *N*-terminus with variations on the sequence I_{c16}-V_{c17}-G_{c18}-G_{c19} seen in trypsin. The nascent sequence inserts in a sequence-specific manner into an *N*-terminal binding cleft to form a key salt bridge with D_{c194}. The term "Molecular Sexuality," coined by Bode, provides a graphic description of the sequence-specific nature of the insertion process.[7,8] Proteinase formation is impaired in mutants bearing an altered nascent sequence in spite of cleavage following R_{c15}.[9,10]

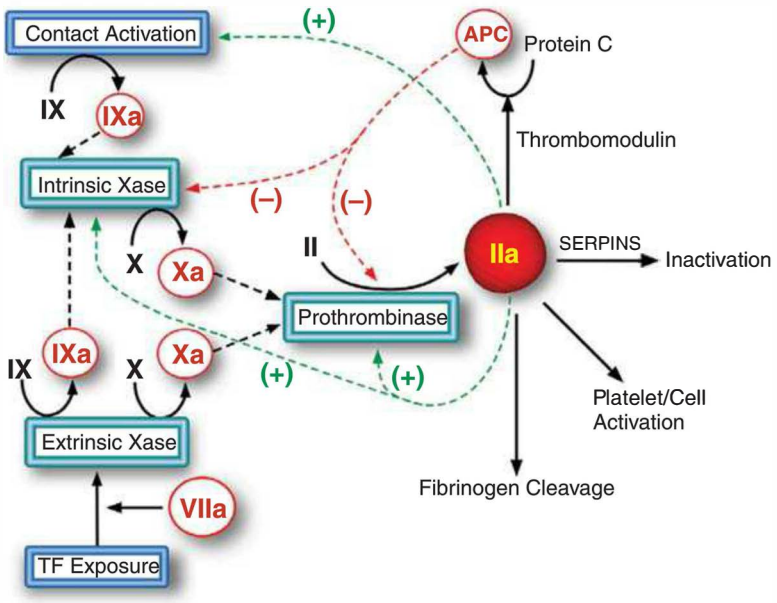

FIGURE 4.1 The blood coagulation cascade. The scheme illustrates zymogen activation steps in the pathway for thrombin formation. The positive feedback reactions of thrombin are illustrated by the *green arrows* and the negative feedback reactions that result from the action of activated protein C are shown by *red arrows*.

Conversely, trypsinogen can be conformationally activated even without cleavage by the addition of a I-V dipeptide but not V-I.[7] Ion pairing is associated with changes in four activation domains (Table 4.1) that are flanked by highly conserved glycines, referred to as glycine hinges.[11] Changes in these activation domains about the glycine hinges lead to optimization of the primary specificity pocket for substrate binding and a flip in the G_{c193} amide bond to form the oxyanion hole necessary for catalysis. These transitions instill proteolytic activity in the product by enhancing the ability of the active site to engage the substrate and by increasing catalytic power. Depending on proteinase, changes in other regions important for ligand binding may also result.

Relatively subtle differences in the main chain traced in x-ray structures of the zymogen and the corresponding proteinase yield the impression that the zymogen to proteinase transition is accompanied by subtle changes in protein structure. However, the effect of cleavage and salt bridge formation is primarily reflected in the ordering of the activation domains, which are otherwise disordered in the zymogen.[12] Thus, very significant ordering effects result from proteinase formation and an associated 10^4-10^5-fold increase in catalytic activity. Since maturation of the proteinase is rooted in conformational transitions that follow irreversible cleavage of the zymogen, it seems reasonable that formation of the fully stabilized proteinase is irreversibly favored following cleavage. However, this idea is not consistent with observations made in early studies of chymotrypsin[13] and studies of VIIa,[14,15] and is increasingly questioned in the case of thrombin.[12,16,17] Conversely, while the uncleaved zymogen is prevented from making the conformational transitions required for proteinase formation, evidence from the related proteins of fibrinolysis suggests that the uncleaved protein can be imbued with catalytic activity as a result of contributions of alternate side chains in the vicinity of D_{c194}.[18] Thus, although the relationship

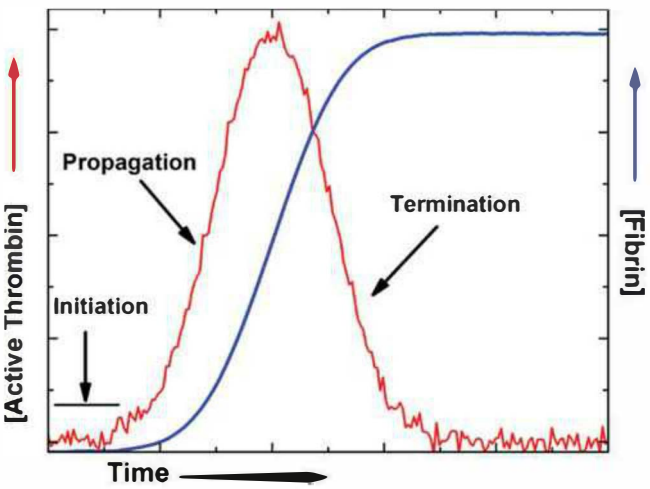

FIGURE 4.2 Expected fate of active thrombin following initiation of coagulation. The regulated hemostatic response results in the transient formation of active thrombin. Positive feedback reactions of thrombin produced at low levels in the initiation phase lead to amplified thrombin generation during the propagation phase. Negative regulation by activated protein C and inactivation by serpins predominate late in the response leading to depletion of active thrombin. The expected course of fibrin formation is also illustrated.

Table 4.1
Activation domains of zymogen activation

Domain	Functional Correlate
c16-c21	*N*-terminal insertion region
c142-c152	Autolysis loop
c184-c194	Region encompassing bottom of specificity pocket, oxyanion hole, and ion pair partner.
c216-c223	Residues framing entrance to S1 site

between cleavage following R_{c15} and proteinase formation is tacitly assumed to be absolute, it need not be so. Ligand-dependent effects on the various transitions along the pathway of conversion of zymogen to proteinase may represent an underappreciated but prevalent strategy for enzyme regulation in hemostasis.

CATALYTIC SPECIFICITY

Specificity for peptide bond hydrolysis results from a catalytic triad of side chains provided by H_{c57}, S_{c195} and D_{c102} that are precisely oriented at the active site.[19] A charge transfer complex is formed by the abstraction of a proton from S_{c195} by H_{c57}, which is in turn stabilized by long-range electrostatic interactions with D_{c102} (FIGURE 4.3). Precise orientation of the scissile bond in the substrate allows covalent nucleophillic catalysis at the carbonyl group by electronegative S_{c195} to yield a tetrahedral oxyanion intermediate. The tetrahedral intermediate is stabilized by electrostatic interactions with the oxyanion hole. Transfer of a proton to the tetrahedral adduct by the action of H_{c57} as a general acid catalyst releases the COOH-terminal fragment, while the NH_2-terminal fragment acylates S_{c195} and remains covalently tethered to the enzyme. Water diffusing into the active site is oriented through orbital steering by H_{c57}, which then acts as a general base catalyst to abstract a proton allowing for the hydrolysis of the acyl-enzyme intermediate. The resulting tetrahedral intermediate is also stabilized by the oxyanion hole before it collapses to release the NH_2-terminal fragment, returning the catalytic triad to its initial state for the next round of catalysis. These steps outline the range of strategies in chemical catalysis employed by serine proteinases to catalyze peptide bond hydrolysis.

BINDING SPECIFICITY

Peptide bond cleavage requires the precise positioning of the polypeptide substrate to subtend the carbonyl group of the scissile bond to the catalytic residues within the active site cleft. Binding specificity that arises from such positioning drives the ability of proteinases within this structurally homologous family to act with high selectivity on their cognate protein substrates. This is accomplished by structures on the proteinase complementary to structural features on the substrate. Selectivity of the coagulation proteinases for cleavage following arginine indicates that they are trypsin-like with a binding pocket optimized to accommodate this side chain leading to the presentation of the scissile bond to the catalytic residues. Because the coagulation enzymes do not cleave after all arginines, additional residues must also contribute to substrate-binding specificity. Drawing from studies with the less specific proteinases in this family, concepts are grounded in the idea that two to four residues preceding the scissile bond are major determinants of binding specificity with their side chains engaging complementary sites in the enzyme. The terminology of Schechter and Berger[20] is followed, where substrate side chains are numbered S1, S2, S3, and so on extending *N*-terminal to the scissile bond and S1′, S2′, S3′, and so on extending in the *C*-terminal direction. Sites on the enzyme complementary to these substrate side chains sites and flanking the catalytic site are labeled P1, P2, P3 or P1′, P2′, P3′ and so on. Ideas regarding the determinants of substrate specificity have historically been developed from work done with peptidyl substrates with readily measureable leaving groups (FIGURE 4.4). As a result, and because of high homology with trypsin, the principal focus has been on the role played by the S residues and P sites in determining substrate specificity with less emphasis on the S′ residues and P′ sites.

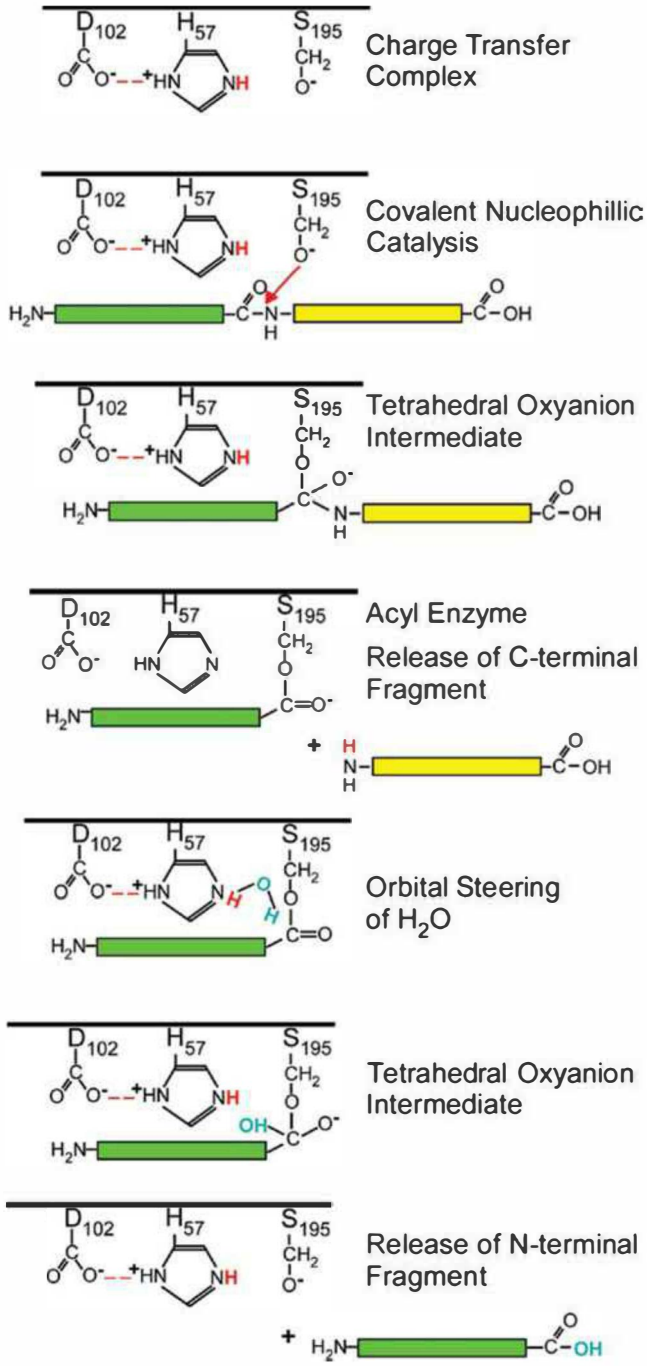

FIGURE 4.3 Peptide bond hydrolysis catalyzed by serine proteinases.

KINETICS OF ENZYME ACTION

Cleavage of a typical peptidyl substrate by a coagulation proteinase requires binding of the substrate to complementary sites on the enzyme to form a Michaelis complex (ES). Positioning

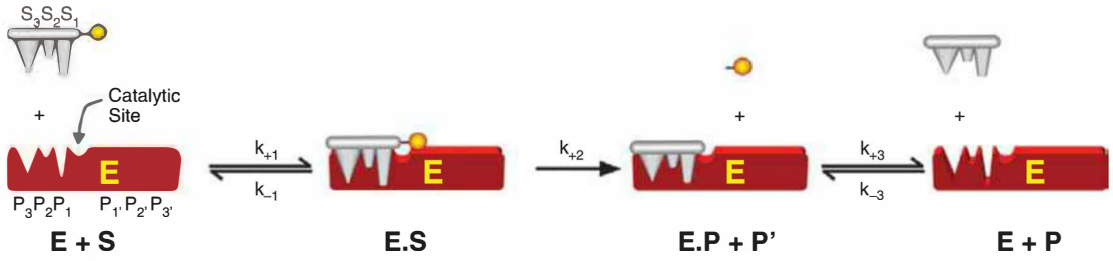

FIGURE 4.4 Kinetic scheme for peptidyl substrate binding and cleavage.

of the scissile bond at the catalytic triad leads to catalysis and release of the chromophore, followed by reversible peptide dissociation and a new round of catalysis. This represents a straightforward platform for the further consideration of the kinetics of enzyme action from simple measurements of the rate of product formation. Despite its simplicity, the scheme yields stiff differential equations that typically require single turnover studies on the millisecond timescale for the resolution of all stepwise rate constants.[21] Although approaches have been presented to solve these rate constants from simple measurements of the rate of product formation at different viscosities, the extraordinary level of data precision required for reliable conclusions makes this approach impractical.[22] Consequently, a meaningful quantitative analysis from simple measurements of the rate of substrate consumption and of product formation requires simplifying assumptions: (a) [E] « [S]. This is an appropriate and reasonable condition, given the catalytic power of the typical enzyme, (b) the initial velocity assumption requires that initial rates are measured before there is significant substrate consumption or product formation. This principally allows product inhibition effects to be ignored, although such effects need to be accounted for in the case of some tight-binding substrates/products, and (c) either the rapid equilibrium or the steady state assumption. In the former case, it is assumed that the binding and dissociation of substrate is very rapid relative to the rate constant for catalysis. In the latter, it is assumed that the system is in steady state during the rate measurement and distributions between the various enzyme species are invariant with time. This yields the familiar equation widely attributed to Michaelis and Menten,[23] wherein the constants take on different meaning depending on the assumptions that apply:

$$v = \frac{dP}{dt} = -\frac{dS}{dt} = \frac{Vmax.S}{Km + S}$$

$$\text{Where, } V_{max} = E.k_{+2} = E.k_{cat} \text{ and } K_m = \frac{k_{-1}}{k_{+1}} \text{ (Rapid Equilibrium)}$$

$$\text{or } K_m = \frac{k_{-1} + k_{+2}}{k_{+1}} \text{ (Steady State)}$$

E, S, and P refer to the total concentrations of enzyme, substrate, and product, k_{+2} (kcat) is the rate constant for catalysis, V_{max} is the maximum rate of product formation at infinite S, and K_m is the Michaelis constant. In the limiting case that ES and E are in rapid equilibrium, K_m reflects the equilibrium dissociation constant (K_d) for substrate binding, but can be significantly larger than K_d in the steady-state case. Therefore, K_m is a measure of the affinity of the enzyme for substrate with smaller values of K_m reflecting tighter binding. At fixed E, the dependence of rate on S is defined by a rectangular hyperbola and Km is the concentration of S at which $v = 0.5\ V_{max}$. When acylation limits the rate constant for catalysis, the leaving group and peptidyl product are produced at an equal rate. When deacylation is rate limiting, the leaving group is produced more rapidly than the peptidyl product. Substrate species that display the latter properties are employed in burst kinetic reactions to determine the concentration of active proteinase.[24]

In the free-energy landscape for the reaction (FIGURE 4.5), the ES complex represents a ground state that needs to overcome the free-energy barrier imposed by the transition state before it can be converted to product. Thus, kcat is inversely proportional to the magnitude of the free-energy barrier ($\Delta G^{\ddagger}$) separating the ground and transition states. Within this framework, an increased kcat requires that $\Delta G^{\ddagger}$ be reduced by stabilizing the transition state and/or destabilizing the ground state. Both ground and transition state effects are accommodated in the catalytic efficiency term (k_{cat}/K_m), which represents the overall second order rate constant for the reaction. In the screening of different substrates with any particular enzyme, preference for a particular substrate is implied by a larger value for catalytic efficiency (also referred to as specificity constant). Considerations developed from peptidyl substrate cleavage studies continue to dominate ideas underlying substrate specificity, and analyses using different peptidyl substrates

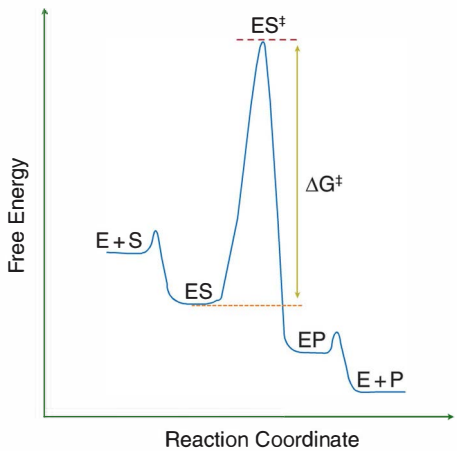

FIGURE 4.5 Free energy diagram for peptidyl substrate hydrolysis by serine proteinases.

continue to represent a primary avenue for the investigation of the substrate preferences of the various coagulation proteinases.

LIMITATIONS OF PEPTIDYL SUBSTRATE STUDIES

Oligopeptidyl substrates are microscopic in relation to the protein substrate and thereby interact in a limited way with the active site of the proteinase. While the protein substrates of the coagulation proteinases must bind to the active site before they are cleaved, these substrates approximate the size of the enzyme and could potentially engage the enzyme far more extensively, with such interactions contributing in a major way to binding specificity. Thus, while kinetic measurements with peptidyl substrates are facile and widely employed, they routinely fail to provide an explanation for cleavage site specificity within the protein substrate.[25–27] This limitation is widely evident in the coagulation proteinases, where extended interactions between enzyme and substrate play a fundamental role in determining macromolecular substrate specificity.

PAN-SPECIFICITY OF THROMBIN

Thrombin is an atypical coagulation proteinase in that it cleaves numerous substrates in reactions that are essential for clot formation and its regulation. Although the S2-S1-S1′ sequence is P-R-S at several cleavage sites in its different substrates, a single consensus pattern flanking the S1 arginine fails to emerge. Early studies with thrombin revealed that proteolysis in its catalytic domain could greatly reduce its ability to cleave fibrinogen without affecting the rate of peptidyl substrate hydrolysis.[28] Dissociation of the two functions provided an early indication that the basis for macromolecular substrate specificity of thrombin lies in enzymic structures beyond those relevant to the hydrolysis of peptidyl substrates. A series of elegant studies of the mechanism of inhibition of thrombin by hirudin from the laboratories of Stuart Stone and Jan Hofstengee established the central role of a basic region distant from the catalytic site in this high-affinity inhibition reaction.[29–33] Subsequently, ample biochemical and structural evidence has established the central role played by this anion-binding exosite 1 (ABE1) region (FIGURE 4.6) in engaging acidic regions found in several thrombin substrates distant from the scissile bond. In these cases, substrate binding to ABE1 likely guides and facilitates active site engagement of structures surrounding the cleavage site leading to catalysis. The interaction with ABE1 can be a major determinant of binding affinity, as well as specificity, since proteolysis of the loops that constitute ABE1 or its occlusion by ligands can abrogate cleavage of substrates that engage thrombin in this way.[28,34,35] These ideas provide a partial accounting for the strategies employed by thrombin to achieve pan-specificity without strict conservation of residues flanking cleavage sites in its various substrates.

ENZYME COMPLEXES OF COAGULATION

While thrombin acts on a range of protein substrates, the other proteinases of coagulation exhibit far more restricted substrate specificity. In addition, many of these reactions are catalyzed by enzyme complexes that assemble from membrane-dependent interactions between a proteinase and a cofactor protein. Thus, there is no basis, *a priori*, to presume that strategies utilized by thrombin for substrate recognition would apply to other reactions.

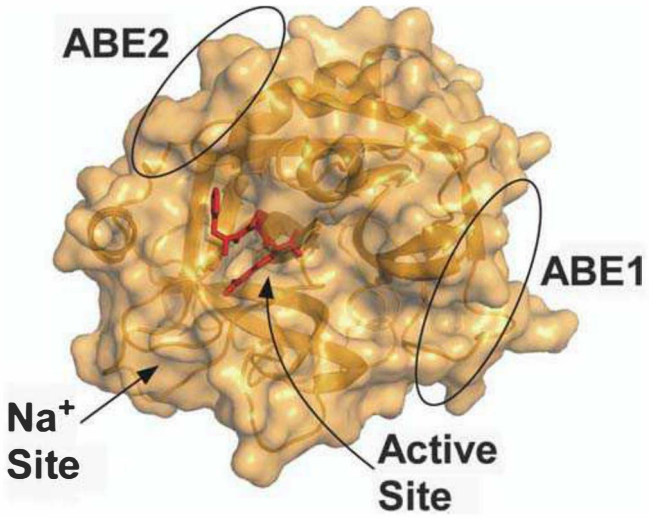

FIGURE 4.6 X-ray structure of thrombin. Structure of thrombin modified by D-FPR-chloromethyl ketone (1PPB) is shown as a rendered surface in the standard orientation.[6] The active site is highlighted by the bound inhibitor shown as *red sticks*. The positions of anion-binding exosite 1 (ABE1) and exosite 2 (ABE2) are indicated as is the Na^+ binding site.

The prothrombinase complex, the enzyme complex that converts the zymogen prothrombin to thrombin, is considered an archetype for the other complexes of coagulation. This is appropriate because of the wealth of biophysical and enzymologic information delineating function and the reversible binding interactions that lead to complex assembly and substrate recognition.[1,4]

Prothrombinase is composed of the serine proteinase, factor Xa, bound to factor Va, its protein cofactor, on a membrane surface (FIGURE 4.7). Although complex assembly *in vivo* requires exposing phosphatidylserine on the outer membrane of activated blood cells such as platelets, and possibly also the endothelium, most of the quantitative information in this system has been developed in defined systems with purified proteins and synthetic membranes containing phosphatidylcholine and phosphatidylserine (PCPS). While factor Xa is a fully competent serine proteinase, its ability to cleave prothrombin is minor in comparison to the rate observed following its incorporation into prothrombinase. Indeed, based on rates, the concentration of thrombin formed in 1 second by Xa saturably bound to Va and membranes would require approximately 5 days by the equivalent concentration of Xa in solution. Thus, the assembly of Xa into prothrombinase is required for rapid thrombin formation following initiation of coagulation, and it is prothrombinase rather than free Xa that is considered the physiologically relevant catalyst for rapid prothrombin activation following vascular damage. The other enzyme complexes are composed of homologous constituents that assemble in an analogous way with comparable functional consequences.

The functional consequences of the reversible interactions of Xa with membranes and Va are evident from steady-state kinetic constants for "prothrombin activation" determined using Xa in

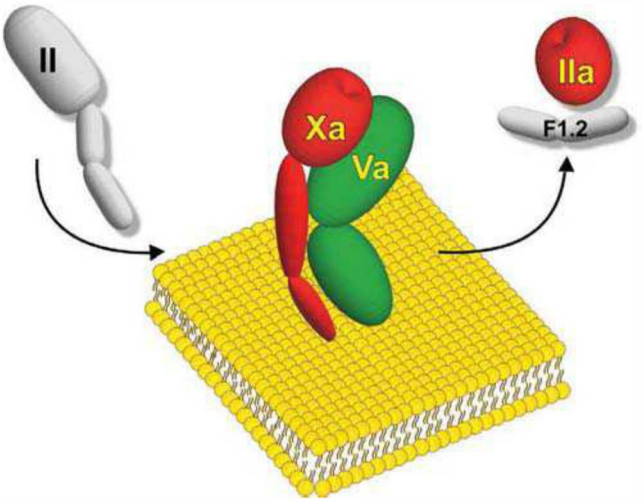

FIGURE 4.7 Schematic representation of prothrombinase. The complex assembles through membrane-dependent interactions of the proteinase Xa with the cofactor protein, factor Va, on membranes containing phosphatidyl-L-serine. The complex converts the zymogen, prothrombin (II) to thrombin (IIa) with the release of the *N*-terminal propiece (F12). Catalytically active species are colored *red*.

solution compared with Xa saturably bound to membranes or Xa saturated with Va and membranes (Table 4.2). Binding of Xa to membranes decreases the apparent affinity of the proteinase for prothrombin, while addition of saturating concentrations of cofactor does not affect Km further, but yields a large improvement in the rate constant for catalysis. At physiologic concentrations of prothrombin (1.4 μM), these kinetic constants reveal that the minimal activity of Xa in solution is increased approximately 500,000-fold upon its incorporation into prothrombinase. These types of observations are generalizable to the other complexes of coagulation. They also exhibit comparable changes in steady-state kinetic constants attributable to membrane binding and cofactor binding. Because these binding interactions are reversible, valid comparisons of the type shown here require knowledge of the bound concentrations of the proteinase in the various binary and ternary mixtures from individual binding constants established by independent physical measurement. Incomplete consideration of the degree of saturation of enzyme components can lead to incorrect conclusions and is probably the root of the persistent idea that prothrombinase assembled on platelets is a far superior catalyst to the complex assembled on synthetic membranes.[36,37] Indeed, the reverse is probably true because of nonproductive interactions of enzyme components with the platelet surface.

Table 4.2

Steady-state kinetic constants for prothrombin activation[a]

Enzyme Species[b]	K_m (μM)	V_{max}/E_T (s^{-1})	Relative Rate[c]
Xa	84	0.01	1
Xa/PCPS	0.7	0.05	203
Xa/Va/PCPS	0.4	108	512,000

[a]Kinetic constants for bovine prothrombin cleavage by bovine Xa alone,[102] or for the activation of human prothrombin by human enzyme constituents in the presence of membranes.[103]
[b]The enzyme species correspond to Xa alone, Xa saturably bound to membranes (Xa/PCPS) and Xa assembled into prothrombinase (Xa/Va/PCPS) using saturating concentrations of membranes and Va.
[c]Relative rates at 1.4 μM prothrombin calculated from the steady-state kinetic constants.

MEMBRANE EFFECTS

The ability of proteinase, cofactor, and substrate to bind reversibly to membranes forms the basis for the ability of membranes to affect the reaction rate. Membrane binding and the resulting enhanced function of the membrane-bound enzyme play primary roles in localizing enzyme function and facilitating product formation at the site of vascular damage. With respect to prothrombinase, one major function of the membrane surface is to enhance the thermodynamics and kinetics of enzyme complex assembly.[4] Because Xa and Va interact weakly in solution (K_d ~ 1 μM), complexation in solution is expected to be negligible at the nanomolar concentrations of these proteins produced in blood following initiation of coagulation.[38] Membrane-dependent complex assembly proceeds through the individual interactions of Xa (K_d ~ 100 nM) and Va (K_d ~ 3 nM) on a contiguous membrane surface expressing phosphatidylserine (FIGURE 4.8).[39] As reversible membrane binding is mediated by a specific domain in each protein, the proteins are bound to the surface in an oriented fashion and are constantly dissociating and being recaptured by the surface.[40,41] Thus, their diffusional translation along the membrane surface occurs under conditions that approximate two-dimensional space leading to greatly accelerated and oriented collisional encounters between the two interacting partners. The resulting binding interaction between membrane-bound Xa and

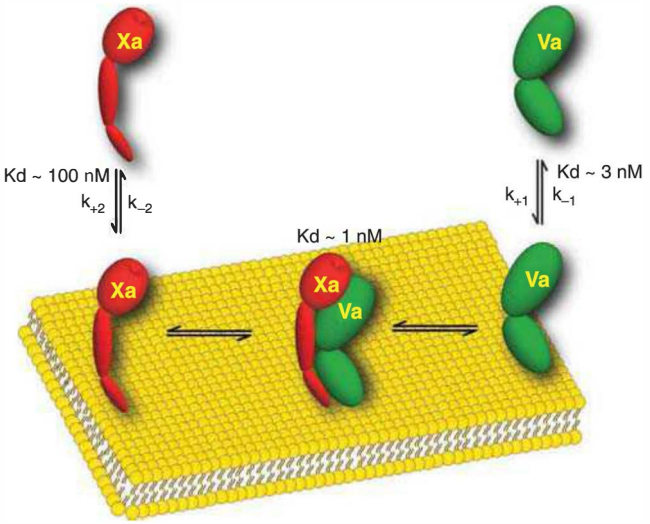

FIGURE 4.8 Membrane-dependent assembly of prothrombinase. Xa and Va bind separately to a contiguous membrane with the noted equilibrium dissociation constants. The membrane-bound proteins then interact efficiently with nanomolar affinity on the membrane surface despite the fact that the solution-phase protein–protein interaction is of micromolar affinity.

Va (K_d ~1 nM) is 1,000-fold tighter than the protein–protein interaction in solution.[42] Furthermore, linkage between protein–protein and protein–membrane interactions further enhances overall affinity, permitting the enzyme to assemble efficiently at picomolar concentrations, much lower than would be predicted from simple knowledge of the individual binding constants.[42]

Similarly, the binding of prothrombin to membranes through its *N*-terminal 4-carboxyglutamic acid-containing domain allows for the accelerated delivery and oriented presentation of the substrate to the membrane-assembled enzyme. This accounts for the major effect of membranes on the apparent affinity of the enzyme complex for the substrate. Apparent affinity is an appropriate term because this effect partly arises from delivery phenomena in step(s) preceding the encounter of substrate with the enzyme.

COFACTOR EFFECTS

Since factor Va increases the kcat for the reaction, it follows that the cofactor must reduce $\Delta G^{\ddagger}$ by changing the catalytic site of Xa within the complex to favor the transition state or by destabilizing the Michaelis complex. It is a long-held view that the cofactor proteins function by modulating the active site of their partner proteinases.[43,44] However, the enhancing effects of Va on catalysis by Xa are limited to observations made with prothrombin and its derivatives and not with peptidyl substrates or transition state-like irreversible inhibitors even bearing the same peptidyl sequence preceding the scissile bond in the protein substrate.[45] This repeats the theme introduced earlier in the chapter with thrombin and indicates that the ability of the cofactor to enhance reaction rate derives from features of the protein substrate that are not approximated by peptidyl substrates that are microscopic in comparison and target the catalytic site of the proteinase in a limited way. It also follows that mechanisms driving macromolecular substrate specificity of the enzyme complex and those underlying cofactor functions cannot be treated separately.

PROTHROMBIN CLEAVAGE

A complicating feature of prothrombin activation derives from the fact that thrombin is produced following two cleavages in the substrate. As a result, there are two possible pathways for prothrombin activation with two possible intermediates (FIGURE 4.9). In either pathway, thrombin is formed following two consecutive enzyme-catalyzed reactions.[46] Thus, kinetic constants that phrase the problem are obtained by varying the substrate for the first reaction and measuring the rate of product formed from the second. Mechanistic ideas grounded in such kinetic constants, which cannot be interpreted in the traditional way, require major reconsideration. Interpretations are further complicated by the fact that the cleavage pathway is dependent on the composition of the catalyst. The reaction proceeds mostly via the formation of prethrombin 2 in the absence of Va and essentially entirely via the formation of meizothrombin when Xa is saturably incorporated into prothrombinase (FIGURE 4.9).[46–48] The rate-enhancing effects of Va are also associated with a change in mechanism, precluding sensible interpretations of how the cofactor might function from measurements that do not take this into account. Such considerations might seem to dwell on the minutiae of a peculiarly complicated case. However, proteolytic processing at multiple sites, sometimes in a seemingly ordered fashion, is commonplace in coagulation and is reflected in the activation of factors IX, XI, V, and VIII, cleavage of fibrinogen, and the proteolytic inactivation of Va and VIIIa.[4]

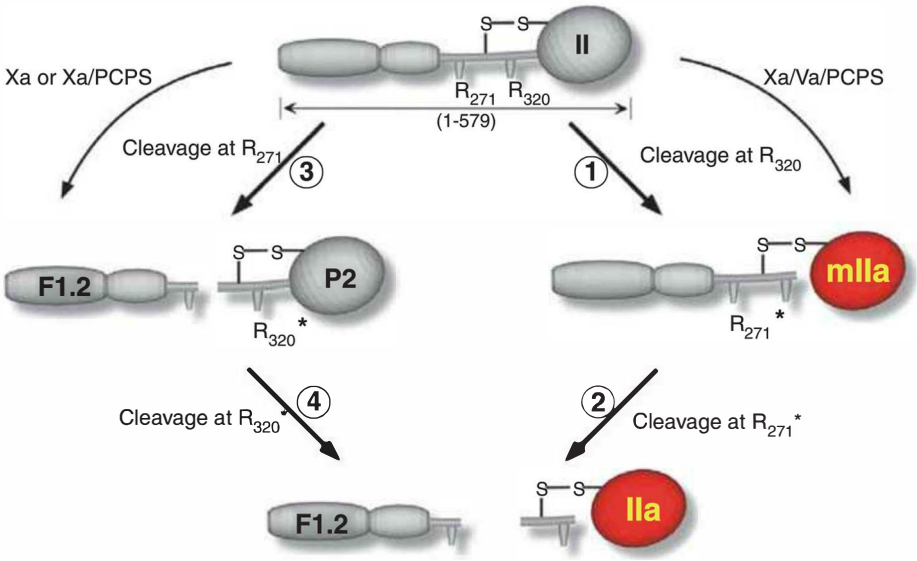

FIGURE 4.9 Pathways for prothrombin cleavage. Thrombin (IIa) formation requires cleavage of prothrombin (II) at R_{271} and R_{320} (numbered sequentially from the mature *N*-terminus of prothrombin). Cleavage through steps 3 and 4 yields the zymogen prethrombin 2 (P2) and the propiece fragment 1.2 (F1.2) as intermediates. This pathway predominates when the catalyst is Xa or Xa bound to membranes (Xa/PCPS). When the catalyst is prothrombinase (Xa/Va/PCPS), the reaction proceeds essentially exclusively through steps 1 and 2, yielding meizothrombin (mIIa) as an intermediate. Functional catalytic domains are colored *red*.

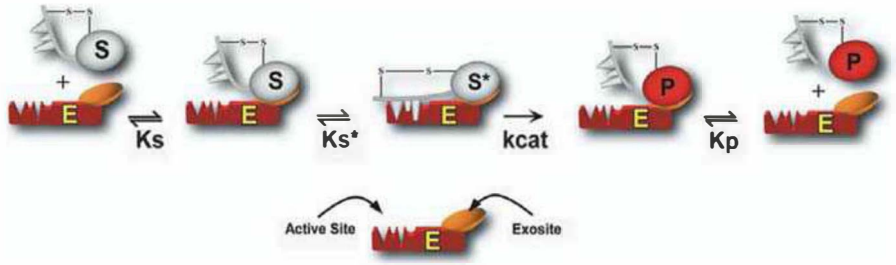

FIGURE 4.10 Kinetic pathway for protein substrate cleavage by prothrombinase. Prothrombinase (E) is represented in stylized fashion to highlight the active site of Xa within the catalyst as well as extended surfaces (Exosite) that contribute to substrate binding. Initial binding of the protein substrate (S) occurs through the exosite (Ks) followed by engagement of substrate structures at the active site (Ks*) leading to cleavage to yield product (P). The cleaved product also interacts with the enzyme through exosite binding (Kp).

MACROMOLECULAR SUBSTRATE RECOGNITION BY PROTHROMBINASE

An analysis of prothrombin cleavage, suitable for reliable interpretation, can be obtained from studies of the four individual half reactions, utilizing intermediates as substrates, and by studies of recombinant prothrombin variants in which one of the two cleavage sites has been rendered uncleavable.[46] For each half reaction, protein substrate recognition proceeds through an equivalent multistep binding pathway (FIGURE 4.10). Initial binding occurs through exosite interactions that are independent of the active site of the enzyme and at least partly involves the *C*-terminal region of the substrate distant from the scissile bond.[49–52] Exosite docking is followed by active site engagement of structures flanking the scissile bond followed by catalysis. The product bound to the exosite of prothrombinase dissociates before a new round of catalysis. This pathway, initially elucidated using the cleavage of prethrombin 2 by prothrombinase as a model reaction, has since been shown to apply to each half reaction of prothrombin activation.[46] Each substrate for the four half reactions and the three possible products bind in an equivalent manner to the exosite, allowing them to bind prothrombinase in a mutually exclusive manner.[46] Because active site engagement follows exosite docking, it now occurs in an intramolecular rather than bimolecular interaction. The individual half reactions can adequately be described by the Michaelis-Menten equation, with new insights provided by the more complex nature of the kinetic constants, derived for illustrative and simplifying purposes using the rapid equilibrium assumption.

$$Km_{obs} = K_{S\cdot}\frac{K_{S\cdot}}{\left(1+K_{S\cdot}\right).}\,k_{cat,obs} = \frac{k_{cat}}{\left(1+K_{S\cdot}\right).}\frac{k_{cat,obs}}{Km_{obs}} = \frac{k_{cat}}{K_{S\cdot}K_{S\cdot}}$$

While the apparent affinity of the enzyme for substrate results from both exosite binding and active site engagement, the apparent rate constant for catalysis also contains the unimolecular binding constant for active site engagement. Thus, large improvements in $kcat_{obs}$ can result from modulation of active site engagement without necessarily stabilizing the transition state or destabilizing the ground state. Since the protein substrate engages the active site of Xa with poor affinity (Table 4.2), the Dock-and-Lock strategy employed by prothrombinase allows exosite binding to efficiently drive an otherwise unfavorable reaction. Thus, it is exosite binding and not active site engagement that dominates the affinity of prothrombinase for its macromolecular substrate.

EXOSITE BINDING AND ENZYME SPECIFICITY

The active site of factor Xa, whether or not assembled into prothrombinase, is permissive and accepts a wide range of peptidyl substrates.[26] This observation has been puzzling, considering the narrow specificity with which this proteinase acts on prothrombin following the sequence Glu/Asp-Gly-Arg.[4] Such seemingly anomalous behavior is readily reconciled when the predominant contribution of exosite interactions in driving substrate binding is accounted for. This concept has been tested with recombinant prothrombins in which the cleavage site residues are replaced with sequences found preceding cleavage sites in the other coagulation zymogens not otherwise cleaved by prothrombinase. These prothrombin variants are readily cleaved by prothrombinase with the same Km_{obs} but with varying values for $kcat_{obs}$.[53] Replacement of the P1 arginine with glutamine yields an essentially uncleavable form. Lack of substrate cleavage in the glutamine variant arises from a defect in $kcat_{obs}$, rather than affecting binding affinity dominated by exosite interactions. This illustrates how specificity for prothrombin arises from the restricting effects of exosite binding that only allow prothrombinase to cleave substrates that can engage the enzyme in this way, despite the fact that the active site of Xa can readily accommodate sequences found flanking the activation sites of the other coagulation zymogens. It demonstrates experimentally that although the subsequent active site engagement step is required for catalysis, this unimolecular binding interaction contributes to $kcat_{obs}$ rather than macromolecular substrate affinity.[53] This clearly sets apart the strategy employed to achieve binding specificity for the protein substrate from that for peptidyl substrates. Consequently, it is not surprising that studies with peptidyl substrates have uniformly failed to provide insights into the function of the enzyme on its protein substrate.

The ability of prothrombinase to selectively act on prothrombin rather than the other coagulation zymogens represents but one level of enzymic specificity. The second reflects the ability of the enzyme complex to discriminate between the two cleavage sites within the substrates. Thus, despite the fact that both cleavage sites are accessible to added proteinase, prothrombinase is

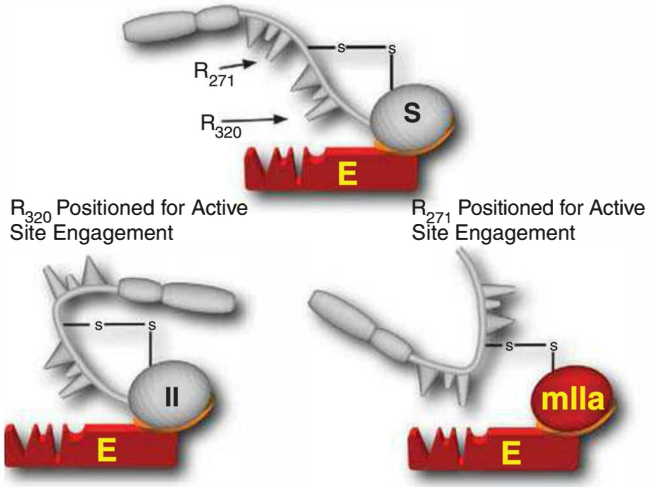

FIGURE 4.11 Exosite-dependent tethering of substrate to enzyme. Ordered cleavage of prothrombin results from the geometric constraints associated with exosite tethering. When the substrate is the zymogen, the R_{320} site is optimally positioned to engage the active site of the enzyme while R_{271} is distant. Following cleavage at R_{320}, otherwise distant R_{271} is brought into the vicinity of the active site to permit its engagement.

essentially only able to cleave following R_{320} in prothrombin. The action of prothrombinase following R_{271} requires prior cleavage at R_{320} and the formation of meizothrombin (FIGURE 4.9). The basis for this discrimination lies in geometric constraints enforced by exosite-dependent tethering of the substrate to the enzyme (FIGURE 4.11). On the basis of modeling and the available structures of prothrombin derivatives, the two cleavage sites are expected to be approximately 40 Å apart.[54] Exosite tethering of prothrombin geometrically constrains the presentation of the substrate, such that structures flanking the R_{320} scissile bond are positioned to preferentially engage the active site of Xa within prothrombinase.[10,55] The R_{271} site is distant, does not effectively engage the active site, and is cleaved poorly. Conversion of the zymogen to proteinase following initial cleavage at R_{320} provides the appropriate structural changes in the substrate, termed ratcheting, to bring the once distant R_{271} site into the vicinity of the active site for binding and cleavage. The essential role for the zymogen to proteinase transition in the presentation of individual cleavage sites for active site engagement is documented by modulation of the pathway for prothrombin cleavage via stabilizing prothrombin in a proteinase-like state or by abrogating the conformational transition to proteinase following cleavage at R_{320}.[10,55]

Discrimination between the two cleavage sites is not absolute and the R_{271} site in prothrombin can indeed be cleaved, but at a 30-fold lower rate.[10] The finite rate of cleavage at R_{271} likely reflects a low probability of capture of the otherwise distant site because of flexibility in the exosite-tethered substrate.[55] This requires consideration of a third level of specificity with broad relevance to proteinases in general. If the catalytic site of Xa within prothrombinase is accepting of a wide range arginine-bearing sequences and the substrate is tethered in a flexible way, why is the substrate not cleaved at other sites? The answer lies in competition between the preferred sites, in terms of geometry as well as affinity, for engaging the active site over other structures.[56] As a result of this competition, cleavage at the suboptimal sites is minimized and only revealed following mutagenesis to eliminate the preferred cleavage sites.[56]

The structural basis for exosite-dependent substrate binding to prothrombinase is not unambiguously assigned, as in the case of thrombin (see above). Current ideas suggest that extended surfaces in both Xa and Va likely contribute to tether the substrate to the enzyme.[57–59,60] By definition, however, membrane binding by the substrate, which mediates its oriented presentation to the enzyme, constitutes another essential component of exosite-mediated substrate recognition. Thus, protein–protein and substrate–membrane interactions contribute to exosite tethering of the substrate. The available evidence suggests that perturbations in any of these interactions will impact the ability of prothrombinase to discriminate between the two cleavage sites by altering the constraints on enzyme-bound substrate. This consideration may bear on some of the disagreements in the field or on the idea that activated platelets, likely providing subsaturating levels of membranes for prothrombin binding, exhibit an altered pathway for prothrombin cleavage in comparison to synthetic membranes.[61,62]

PROTEINASE ALLOSTERY

Ideas centered on the allosteric modulation of proteinase function feature prominently in considering the fundamental role played by cofactor proteins in coagulation enzymology.[4,63] However, there are substantial difficulties in ascribing functional importance to relatively small or subtle changes in proteinase active site function typically associated with cofactor binding.[45,64,65] This is less of a problem in the case of VIIa for which peptidyl substrate hydrolysis is significantly increased upon tissue factor binding.[66–69] Although the 20 to 30-fold increase in peptidyl substrate cleavage is small in comparison to the improvements in X or IX activation, a series of studies have shown that VIIa is zymogen-like despite cleavage following R_{c15} with the nascent *N*-terminus accessible to solvent.[14] Binding to tissue factor is associated with salt-bridge formation arising from the allosteric stabilization of VIIa in the proteinase-like form.[14,15] While M_{c164} is considered the key residue for transmission of allosteric effects arising from tissue factor binding, recent studies have shown analogous stabilization of proteinase-like VIIa by antibodies targeting different regions of the proteinase domain.[15,70]

Allostery is considered an important determinant of the ability of TM to redirect thrombin toward its anticoagulant function.[71] Binding of thrombin to TM yields a large increase in protein C activation with a concomitant loss in function toward its procoagulant substrates.[71–74] This switch in specificity results partly from TM binding to ABE1 with nanomolar affinity, thereby preventing binding of procoagulant substrates that employ interactions with ABE1 to achieve binding specificity.[75] The TM–thrombin complex is most likely an efficient protein C-activator because of optimized ground state interactions permitting its productive binding to the active site of thrombin, which is otherwise nonproductive in the absence of the cofactor.[76–78] The central role of allostery in this switch has been investigated extensively by mutagenesis and structural work, backed by a voluminous literature on the subject (see ref.[79] for a recent review on the subject). Numerous different side chains have been proposed as important for the allosteric transition of procoagulant thrombin to the anticoagulant form. Mutations at these sites yield species considered anticoagulant thrombins with a preference for protein C activation.[80] Na^+

binding to a specific site found in thrombin (FIGURE 4.6) but not in the zymogen is proposed to be essential for promoting allosteric transitions between a putatively inactive E* form, a Na^+-free slow form with preference for the anticoagulant substrate, and a Na^+-bound fast species with procoagulant function.[79] These forms, with varying substrate specificity and revealed by a bewildering range of mutations, have yielded variously distorted structures proposed to represent these specifically regulated states of thrombin.[81–86] Nevertheless, the logical basis for the proposal that Na^+ binding is among the most critical modulators of thrombin function is no longer tenable based on binding constants determined at physiologic temperature and pH.[16]

LIGAND-DEPENDENT REGULATION OF THROMBIN ZYMOGENICITY

A more general framework for the further consideration of thrombin allostery derives from the initial suggestion, subsequently confirmed by thermodynamic and NMR studies, that thrombin can exist in zymogen-like and proteinase-like states depending on its ligation state.[12,16,17] Substrates and inhibitors that ligate the active site and bind the zymogen poorly, if at all, shift the equilibrium in favor of the proteinase-like state (FIGURE 4.12). This is also true for Na^+ binding because the site for this cation is optimally formed in the proteinase but not zymogen.[87] The proteinase-like state is also favored by the binding of ligands to ABE1, which bind more tightly to the proteinase than to pro-ABE1 in the zymogen.[88] In contrast, the propiece F12 binds ABE2 present on the zymogen more tightly than in the proteinase,[16,89] and shifts the equilibrium in favor of the zymogen-like states of thrombin (FIGURE 4.12). Thus, the distribution of thrombin between zymogen-like and proteinase-like states is determined by the complement of ligands bound to these different sites. Although a two-state model (FIGURE 4.12) is sufficient for the formal consideration of such ligation effects,

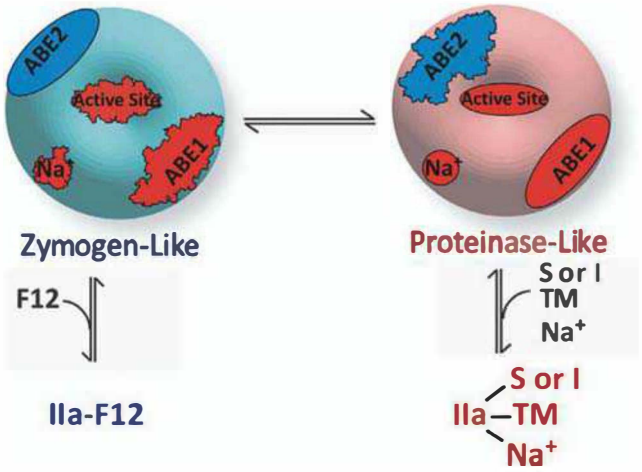

FIGURE 4.12 Ligand-dependent interconversions of thrombin. The active site, Na^+ site and ABE1 are shown as distorted in the zymogen-like form but optimally configured in the proteinase-like form. The reverse is true for ABE2, which is optimally formed in the zymogen-like species and distorted in the proteinase-like form. The distribution of thrombin between these states is determined by opposing effects of ligand binding at these different sites.

the NMR and thermodynamic studies support the ability of thrombin to be reversibly distributed among a range of states with varying zymogen-like or proteinase-like character.[12,16] The basis for this lies in the complete thermodynamic additivity of the various transitions in the activation domains that result from cleavage following R_{c15}.[16] Thus, any destabilizing mutation or removal of Na^+, which is part of the structure of the folded proteinase, would favor zymogen-like forms. The resultant enzyme will exhibit an apparently altered specificity profile dependent on the extent that the tested substrate shifts the equilibrium to favor proteinase. Because one of the activation domains forms the oxyanion hole, the altered specificity profile could be reflected in changes in K_m, k_{cat}, or both.

Major destabilizing mutations, for example, the mutation of a highly conserved disulfide bond,[90] would be expected to cause a large shift in equilibrium to the left, preventing significant rescue by weak ligands or substrates such as fibrinogen. The resulting enzyme would appear to possess little procoagulant function. Because of the much higher affinity for TM binding, the cofactor could partially or fully drive the enzyme to the proteinase-like state. Because TM also blocks the procoagulant function of thrombin, full or partial rescue by TM will yield the appearance of normal or selectively retained anticoagulant function. This provides an accounting for how the pro- and anticoagulant functions of thrombin can be seemingly dissociated by a number of strategies. Accordingly, distorted structures of mutant thrombins considered to reflect specific regulated states are rectified by inhibitors targeting the active site or by ligands directed toward ABE1.[82,91]

THE OTHER COAGULATION PROTEINASES

Growing evidence implicates the strategies outlined for the function of thrombin and prothrombinase as prevalent and applicable to the other reactions of coagulation. The membrane-dependent function of prothrombinase closely parallels the function of the intrinsic Xase.[92] However, it does take on a different character for the extrinsic Xase and for the thrombin–TM complex because the cofactors are transmembrane proteins that are irreversibly anchored in the membrane. In these cases, phosphatidylserine is not absolutely required to facilitate enzyme complex assembly as the soluble extracellular domains of both TF and TM can bind with good affinity to their corresponding enzymes.[66,93] The presence of phosphatidylserine does further enhance function toward their respective membrane binding substrates by facilitating substrate delivery to the membrane-anchored enzyme.[94,95]

Exosite-dependent substrate recognition plays an important role in the function of the extrinsic Xase, intrinsic Xase, as well as IX activation by Xia.[92,94,96] Both the extrinsic Xase and XIa catalyze the cleavage of two bonds in IX, although details of the pathway for ordered cleavage differ for the two catalysts, perhaps reflecting differences in strategies employed for exosite tethering of the substrate and the fact that XIa is dimeric.[97–99] In the case of the extrinsic Xase, exosite-dependent binding of X and Xa to the enzyme complex is an essential feature of its regulation by tissue factor pathway inhibitor (TFPI). TFPI bound to Xa either prior to its dissociation from the enzyme complex or following its release utilizes exosite tethering interactions between Xa and the VIIa/TF complex to achieve rapid and high-affinity inhibition of the enzyme complex. High affinity arises from the summed energetic contributions of the interaction of TFPI

with VIIa and the exosite-dependent binding of Xa to the complex, leading to the essentially irreversible inhibition of VIIa/TF function.[100] A comparable scaffolding strategy to achieve either substrate or product-dependent inhibition of VIIa/TF is also seen in the action of nematode anticoagulant peptide C2.[57,101] In both cases, exosite-dependent tethering of the natural substrate or product, provides for specificity and enhanced energetics for inhibition, despite the fact that either inhibitor can only weakly inhibit VIIa/TF directly.

Ligand-dependent enzyme regulation, by mediating transitions between zymogen-like and proteinase-like forms, has come to the fore in the case of thrombin and has long been known to represent a distinctive feature of VIIa/TF function.[12,15,16] It is presently unclear whether such ideas apply widely to the other reactions of coagulation and whether they reflect a prevalent mechanism by which cofactors may exert their effects. Given their high structural homology, it stands to reason that a variable level of zymogen-like character in the cleaved proteinase is likely to persist in the coagulation proteinases. This suggests new targeting strategies for therapeutic gain wherein proteinase activity can be enhanced or inhibited by tight-binding ligands that can influence the equilibrium distribution between zymogen-like and proteinase-like forms.

PERSPECTIVES

The enzymology of the serine proteinases of coagulation is dominated by the strategies employed by structurally homologous enzymes with similar active sites to act with marked and defined specificity on their respective protein substrates. The underlying theme behind each of these strategies lies in the fact that the substrates approximate the size of the proteinase and are macroscopic in relation to the catalytic site, allowing for extended interactions that lie at the heart of specificity and function. These ideas undoubtedly underlie the function of homologous families of catalysts, such as protein kinases and phosphatases, in signaling pathways also relevant to hemostasis and that are required to act with specificity on their respective protein substrates in reactions that are further modulated by other binding partners.

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CHAPTER

5 Cell Biology, Protein Processing, and Cell Signaling

RANDAL J. KAUFMAN AND LAURA POPOLO

In order to perform their multiple functions, eukaryotic cells have evolved an internal structural organization that consists of several organelles and an intricate network of protein filaments, called the cytoskeleton, which determines cell shape and regulates its dynamic changes. Organelles are membrane-bound compartments that play fundamental functions in cellular physiology. The *nucleus* hosts the DNA and is the site where the genetic information is transcribed into RNA molecules that are processed, transported to the cytoplasm, and translated into proteins, the final executors of the genetic program of a cell. The *mitochondria* are numerous in a cell and responsible for the production of most of the ATP molecules derived from the catabolism of organic compounds but are also involved in Ca^{2+} signalling and cell death pathways. *Lysosomes* and *peroxisomes* play specialized functions in the digestion of macromolecules and oxidative and detoxifying reactions. The *endoplasmic reticulum* (ER) is the site for the synthesis of lipids and proteins destined to be part of the cell membranes or secreted into the extracellular space. Proteins exit the ER and enter the *Golgi apparatus* that is a central sorting and processing station where proteins are packaged into vesicles for transport to their final destination.

Due to the complexity of such an internal organization, the elucidation of the molecular mechanisms regulating the synthesis, processing, and transport of proteins to the various intracellular compartments has fascinated cell biologists for years. Studies on protein secretion have taken advantage of the powerful genetic approaches applied to yeast, a lower eukaryote. A striking evolutionary conservation in the basic components of protein trafficking and processing was found between yeast and mammalian cells.

This chapter summarizes the basic principles of mammalian protein synthesis and processing with a focus on the secretory pathway. Many human proteins that circulate in the blood are secreted and extensively modified by specialized cells and play important functions in human physiology. The findings of many years of research provide the basis for our understanding of the molecular mechanisms of cell biology and how malfunctioning of these processes contributes to human diseases including some hematological disorders.

PROTEIN SYNTHESIS AND MATURATION

Protein Synthesis and Regulation

In eukaryotic cells, nuclear protein-encoding genes are transcribed into pre-mRNA molecules (also named heterogeneous-nuclear) that are transported to the cytoplasm after complex processing. In the cytoplasm, mRNAs become templates for translation, a highly energy-consuming process. Beyond 80S ribosomes and aminoacyl-tRNAs, several soluble factors and GTP are required for the initiation, elongation, and termination (also named release) steps of protein synthesis. Accordingly to the stage of translation in which they transiently associate with the ribosomes, the soluble factors are named eIFs (initiation factors), eEFs (elongation factors), and eRFs (releasing factors) with the prefix e- indicating eukaryote. Several ribosomes that are simultaneously engaged in the translation of the same mRNA form a polyribosome (or polysome). Polysomes are either free in the cytosol or bound to the surface of ER membranes.

Translation is also an important target of regulation. Several examples of regulatory mechanisms that control the expression of specific mRNAs in response to a given cue have been reported. The expression of some proteins involved in iron metabolism is regulated at the level of translation initiation or stability of mRNAs.[1,2] Besides specific mechanisms, global translational control exists. A transient arrest of translation is often required to overcome various kinds of stress. For instance, the sudden onset of an unfavorable condition, such as amino acid starvation, heat shock, accumulation of unfolded proteins in the ER, or lack of heme in reticulocytes, triggers a rapid halt in global protein synthesis. This regulation is mediated by specific sensor protein kinases that phosphorylate the alpha subunit of heterotrimeric initiation factor eIF2, a G-protein, on residue Ser51. eIF2α phosphorylation prevents the GDP–GTP exchange on eIF2 because the guanine nucleotide exchange factor (GEF) eIF2B is sequestered by tight binding to the phosphorylated form of eIF2. The GTP-bound form of eIF2 becomes insufficient for the formation of the ternary complex (eIF2-GTP-Met-$tRNA_i$), and protein synthesis slows down. In contrast, ligands that activate signal transduction pathways promoting growth rapidly stimulate protein synthesis. Insulin signalling exemplifies a growth promoting pathway that, through the protein kinase mTOR, activates components of the translational machinery including eIFs and eEFs.[3,4]

Another site of protein synthesis is mitochondria that possess a genetic system and independent mRNA translation machinery. However, mitochondrial protein synthesis, which is from bacterial origin and occurs on 55S ribosomes present in the matrix, is responsible for the synthesis of only a small set of highly hydrophobic proteins that reside within the mitochondrion inner membrane. The rest of the mitochondrial proteins are nuclear encoded, translated on cytoplasmic ribosomes, imported from the cytoplasm, and sorted to the outer membrane, inner membrane, or matrix.

The broad spectrum of functions typical of a specialized cell of metazoans depends on a defined set of proteins that is referred to as the "proteome." The proteome is specific for a

particular physiological cellular condition or stage of development. Furthermore, many proteins are modified by covalent attachment of chemical groups that contribute to increase the complexity of proteins produced by a cell. The analysis of the proteome and its modifications, which is carried out by a huge armamentarium of technologies that were developed in recent years, highlights the qualitative and quantitative changes occurring in pathological conditions with respect to the normal and greatly contributes to the identification of the molecular mechanisms fundamental to the basis of human disease.

Protein Sorting in the Cytoplasm

The great majority of cellular proteins are synthesized on "free" ribosomes in the cytosol and then transported to their eventual destination. Short linear sequences (or three-dimensional patches of particular amino acids) and their cognate receptors govern the sorting events. The sorting decision occurs after approximately 30 amino acids of the nascent polypeptide emerge from the ribosome. In the absence of an "ER signal sequence," most often found near the amino-terminal end of the protein, translation is completed in the cytosol. The protein can either stay in the cytosol or be posttranslationally (after synthesis) transported into one of the organelles (FIGURE 5.1). Alternatively, if the protein does contain an amino-terminal signal sequence, the nascent peptide–ribosome complex is docked on the membrane of the ER and the emerging polypeptide is extruded cotranslationally (during synthesis) into the lumen of this organelle. This marks the entry of the protein into the "secretory pathway," a route that comprises the rough ER-Golgi-plasma membrane-lysosomes and through which a protein can reach the cell surface (FIGURE 5.2). The sorting signals to the various organelles are listed in Table 5.1.

Most of the proteins synthesized on "free" polysomes remain in the cytosol as cytosolic or "soluble" proteins. These include enzymes involved in glycolysis, signal transduction (e.g., soluble protein kinases or phosphatases), factors of the translation machinery, and components of the cytoskeleton. Different mechanisms operate to posttranslationally translocate the cytosolic proteins into the nucleus, mitochondrion, and peroxisome.[5–7]

The nucleus not only imports proteins but also exports proteins to the cytoplasm, and in this sense, the transport in this organelle is bidirectional and regulated. As shown in Table 5.1, nuclear localization (NLS) and nuclear export (NES) signals were identified and shown to be highly selective for nuclear proteins.[8,9] Some proteins that shuttle from the nucleus to the cytoplasm contain both signals.

Protein Folding and Maturation

In order to become a functional protein, the nascent polypeptide chain must collapse into a conformation that is dictated primarily by the amino acid sequence (primary structure). Although this process can occur spontaneously at very low protein concentrations in a test tube, emerging polypeptides require assistance to fold in the highly crowded environment of the cytosol as the protein concentration is approximately 200 to 300 mg/mL. In fact, inappropriate interactions, misfolding, or aggregation occur under these conditions. Thus, nascent chains are guided through their maturation by molecular chaperones that also prevent inappropriate aggregation. The population of chaperones that assist folding in the cytosol is distinct from those that operate within the ER or mitochondria. Most molecular chaperones are members of the heat-shock protein (Hsp) family, so called because their expression is induced by briefly heating cells to 42°C. In general, chaperones attach to nascent polypeptide chains and initiate the folding process even before translation is completed. As the nascent polypeptide is being extruded from the ribosome, it is bound by chaperones that

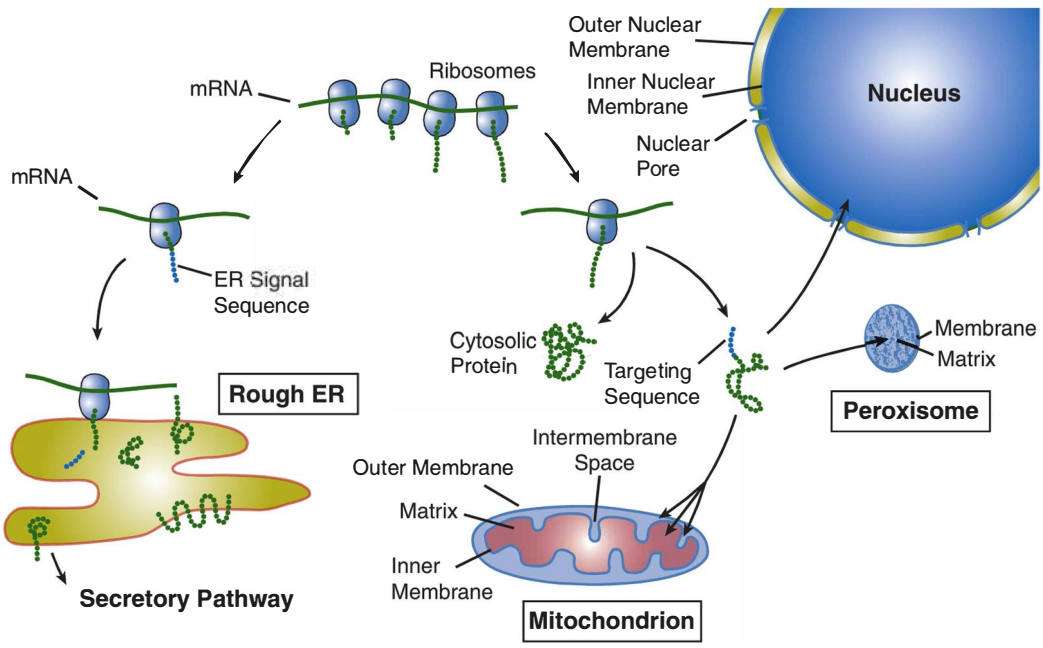

FIGURE 5.1 Trafficking of newly synthesized proteins in a eukaryotic cell.

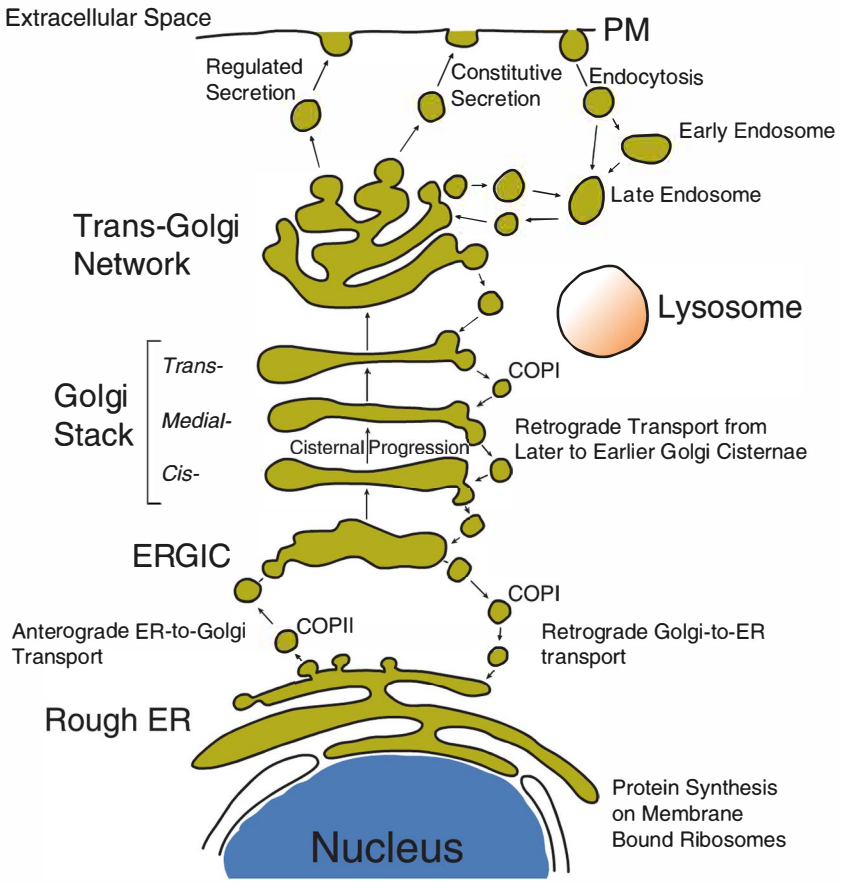

FIGURE 5.2 The secretory pathway. The diagram depicts the membrane-enclosed compartments of the secretory pathway and the endocytic pathway in mammalian cells.

Table 5.1

Examples of import signals

Organelle	Signal Location[a]	Example
Posttranslational Uptake		
Nucleus	Internal	SP**KKKRK***V**E* (import; NLS of SV40 large T antigen)
		KR-spacer (PAATKKAGQ)-**KKKK** (import, bipartite NLS of nucleoplasmin)
		LQLPP*LE***R**LTL*D* (export, NES of HIV-1 Rev)
Mitochondrion	*N*-terminal	MLGI**R**SSV**K**TCF**K**PMSLTS**KR**L (iron-sulfur protein of complex III)
Peroxisomes	*C*-terminal	**K**ANL (PTS1, human catalase)
	N-terminal	**R**LQVVLG**H**L (PTS2, human 3-ketoacyl-CoA thiolase)
Cotranslational Uptake		
ER	*N*-terminal	MMSFVSLLLVGILFWAT*EAE*QLT**K***CE*VFQ (ovine lactalbumin)

NOTES: [a]Acidic residues (negatively charged) are in italic type; basic residues (positively charged) are in bold type. Amino acids: A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; **H**, histidine; I, isoleucine; **K**, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; **R**, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
ER, endoplasmic reticulum; HIV, human immunodeficiency virus; NLS, nuclear localization signal; NES, nuclear export signal; PTS1, peroxisomal targeting signal-1; PTS2, peroxisomal targeting signal-2; SV40, simian virus 40.

recognize short sequence motifs containing hydrophobic amino acids. By undergoing cycles of binding and release (linked to ATP hydrolysis), these chaperones assist the nascent polypeptide to attain its native conformation, one aspect of which is burying hydrophobic sequence motifs in the protein interior so that they no longer contact the hydrophilic environment of the cytosol. Some properly folded protein monomers are assembled with other proteins to form multisubunit complexes. This also is facilitated by chaperones. Considerable effort is continuously devoted to the understanding of the mechanistic basis of protein folding and chaperone function.

Ubiquitin Addition Marks Proteins for Degradation

Proteins that are not properly folded are targeted for degradation. Such proteins are marked for selective destruction and are degraded to amino acids that are recycled in synthesis of new proteins. Degradation of selected molecules is achieved in two major phases. First, the molecules are tagged with a 76-residue polypeptide, termed *ubiquitin*, which is covalently attached to the target, and sequential attachment of multiple ubiquitin molecules can form a long polyubiquitin chain. Second, the polyubiquitylated molecules are ferried to a cytosolic ATP-dependent protease complex, the 26S proteasome, for destruction. The degradation of some mutated proteins that cannot fold is essential for the cell to prevent accumulation of misfolded or aggregated proteins.[10] Indeed, there is increasing evidence that many human diseases are due to accumulation or aggregation of misfolded proteins.

In addition to carrying out the disposal of damaged and misfolded proteins, ubiquitylation is also responsible for regulating a wide array of cellular processes including cellular differentiation, tissue development, inflammatory responses, antigen presentation, cell cycle progression, and apoptosis.[11–14] This regulation is accomplished through the controlled destruction of selected key regulatory proteins.

Other forms of attachment of ubiquitin (mono- or polyubiquitylation) trigger other types of processes such as endocytosis and DNA repair. Recently, histones were found to be a target of monoubiquitylation that is implicated in the recruitment for nuclear processes such as transcription and DNA repair.[15]

Targeting of Cytosolic Proteins to Membranes via Lipid Attachment

Some cytosolic proteins require the covalent attachment of lipid moieties in order to be inserted into a membrane where they will exert their function. The three main types of lipid attachment are *N*-myristoylation, prenylation, and palmitoylation. *N*-myristoylation consists of the attachment of a fatty acid of 14 carbon atoms (myristic acid) to the second residue, an invariable glycine, after proteolytic removal of the *N*-terminal methionine. This process is predominantly cotranslational, mediated by soluble enzymes, and has a strict consensus sequence (M**G**XXXS/T). The replacement of glycine with alanine abolishes the membrane attachment and consequently prevents the function of the protein at its site of action. This modification occurs in important regulatory proteins such as the proto-oncogene c-Src that is a protein kinase involved in the control of cell proliferation. More recently, members of the Ras family of small GTPases, which act as molecular GDP/GTP switches in many cellular processes, were found to be myristoylated. Among them are the ADP ribosylation factors (Arfs) that regulate membrane traffic through the secretory pathway and are ubiquitously expressed from yeast to humans[16] (see below). The best-characterized member of the Arf family is the yeast Arf1 protein. However, the mechanism of myristoylation of Arfs is not yet fully understood.

Prenylation is a posttranslational process also catalyzed by soluble enzymes. It involves the attachment of a prenyl group—either a farnesyl or a geranyl of 15 or 20 carbon atoms respectively—onto soluble proteins that carry a CaaX motif (C is cysteine, a is an aliphatic amino acid and X any amino acid) located at the *C*-terminal end of the protein. A prenyl group is attached to the cysteine residue, and then the last three amino acids are proteolytically removed and the cysteine is methylated. The Ras proteins undergo this modification that is otherwise rather rare. If the cysteine residue is mutated, the function of Ras in the cell is abolished because it cannot interact with its effectors. Since Ras proteins are activated in some kinds of cancer, inhibitors of prenylation are potential anticancer drugs.

As for the previous two modifications, also the attachment of a palmitic acid (a 16-C atoms fatty acid) to the SH group of a cysteine plays a role in the association of some proteins to the cytosolic side of the plasma membrane (S-palmitoylation). Polytopic membrane proteins catalyze this reaction, implying that it occurs at the cytosol-membrane interface. Palmitoylation is reversible and is often coupled with either *N*-myristoylation or prenylation to regulate the strength of membrane interaction of soluble proteins. Palmitoylation is more than a simple sorting signal. It has been implicated in the process of protein trafficking between organelles, in the segregation or clustering of proteins in the membrane compartment, and its regulatory role is often associated with the reversibility of this modification.[17] It was also reported that covalent attachment of fatty acid to the amino acid in proximity of a transmembrane region provides an additional membrane anchor to the protein. One of such examples is the E2 glycoprotein of the Sindbis virus.

Posttranslational Modifications and Regulation of Protein Function

Many proteins are subjected to further processing in order to become active. Among the vast array of dynamic or persistent modifications of the amino acid backbone, the most common modifications essential for protein functionality are the phosphorylation of specific amino acids or the proteolytic cleavage of a region (propeptide) of the protein precursor.

Protein phosphorylation is a reversible covalent modification catalyzed by protein kinases that transfer the γ-phosphate from ATP to specific hydroxyl amino acids such as serine (95% of the phosphorylated sites), threonine (4%), or more rarely tyrosine (<1%). Protein kinases represent one of the largest families of proteins within the human genome counting about 700 members. The opposite reaction is catalyzed by protein phosphatases that hydrolyze the linkage between the phosphate and the amino acid. Protein phosphorylation influences protein conformation. Cycles of phosphorylation–dephosphorylation control the functionality of many soluble enzymes involved in key steps of metabolic pathways or of plasma membrane proteins such as membrane receptors that are usually phosphorylated in their cytosolic domain upon stimulation by an extracellular ligand. An additional role of phosphorylation of specific residues is to

create docking sites for the binding to other proteins. This occurs in many signal transduction pathways where extracellular signals trigger the phosphorylation of specific amino acids to create binding sites for adaptor proteins that in turn recruit other proteins and activate a downstream cascade of intracellular signalling that culminates in the activation of transcription of responsive genes.

Characterization of the "phosphoproteome" is important to identify new substrates of protein kinases or compare the profile of phosphorylated proteins in different physiological conditions.[18] This can open the possibility to study on a large scale the biology of the response to the activation or inhibition of specific cellular receptors.[18]

Proteolysis is a terminal modification that is often used to activate in a controlled and irreversible manner the precursors of proteins whose activity is potentially harmful. In general, proteases are synthesized as longer precursors (zymogens) that are cleaved to produce the active protein (see further proteolysis in the Golgi). Other mechanisms for regulating protein activity are the interaction with ions or small molecules, such as Ca^{2+}, cyclic AMP, or guanylic nucleotides or with other protein partners.

OVERVIEW OF THE SECRETORY PATHWAY IN ANIMAL CELLS

In the presence of a "signal sequence" for the import in the ER (see Table 5.1), proteins are translocated across (soluble and secretory proteins) or inserted into (transmembrane proteins) the membrane of the ER. After folding and partial maturation in the ER, cargo proteins are loaded into vesicles and transported to the Golgi where proteins fully mature. Specific signals cause proteins to return from Golgi to the ER or to be retained in the ER, Golgi, or plasma membrane. In addition, proteins may be targeted from the Golgi to lysosomes or be transported from the plasma membrane to endosomes (see FIGURE 5.2). Although the map of protein maturation from the ER through the Golgi complex and storage in granules for traffic to the cell surface was charted by George Palade in 1975 by using pulse-chase experiments and ultrastructural analysis by transmission electron microscopy, the molecular dissection of the process was achieved upon genetic analysis of yeast, a unicellular microorganism. Classical genetic approaches led to the isolation of temperature-sensitive yeast mutants that were defective in different steps of secretion at the restrictive temperature (*sec* mutants). Then, recombinant DNA techniques allowed the isolation of the corresponding wild type genes that allowed their mammalian homologues to be cloned and characterized. Many *SEC* gene products are conserved from yeast to mammals, and this underlines the importance of this pathway for the life of eukaryotic cells.

Protein transport through the secretory pathway is mediated by vesicles. Different set of structural and regulatory proteins control the fusion of the appropriate vesicles with the target membrane. Sorting motifs dictate the selective incorporation of cargo proteins into those vesicles and their delivery to their intended destination.

The Endoplasmic Reticulum

The ER is a single and continuous membrane network that is contiguous with the nuclear envelope and is responsible for synthesizing the massive amounts of lipid and protein used to construct the membranes of most cellular organelles. The

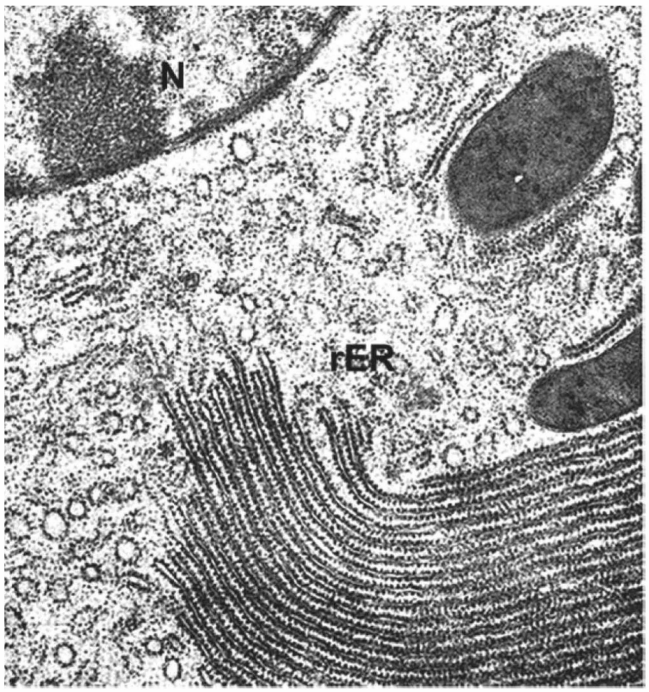

FIGURE 5.3 Electron micrograph of a pancreatic acinar cell. Long sacs are stacked in a regular array. The surface of the ER is studded with polysomes. N, nucleus; rER, rough endoplasmic reticulum.

ER comprises three interconnected domains: rough ER (rER), smooth ER, and ER exit sites. The rough ER is so called because it is studded with bound ribosomes that are actively synthesizing proteins (FIGURE 5.3). The ER-associated protein–ribosome complexes are very abundant, and the rER membrane is extremely expanded in cells producing high amount of secretory proteins. Among them, pancreatic exocrine cells that secrete large amounts of digestive enzymes every day or plasma cells that secrete thousands of antibody molecules (immunoglobulins) per second are the most remarkable examples.

The smooth ER lacks ribosomes, is not very abundant in most cells (except hepatocytes), and is thought to be the site of lipid biosynthesis and of cytochrome P450-mediated detoxification reactions. Finally, ER exit sites are specialized areas of the ER membrane where cargo is packaged into transport vesicles bound for the Golgi apparatus.

Translocation and Protein Processing in the ER

Incorporation of proteins into the ER occurs cotranslationally, a feature that distinguishes import into the ER from that into any other organelle. Nascent secretory proteins are earmarked for import early during their synthesis on cytosolic ribosomes by the presence of an amino-terminal "signal sequence" (see Table 5.1). The signal sequences have a length of about 15 to 30 amino acids, no conservation of primary sequence, and contain a hydrophobic core flanked by polar residues that preferentially have a short side chain in proximity to the cleavage site.

Cotranslational insertion of the nascent "preprotein" into the ER membrane involves the binding of ribosome to the

cytoplasmic side of the ER membrane and the passage of the nascent chain through a membrane channel that is called translocon. As the signal sequence is extruded from the ribosome, it is recognized by the *s*ignal *r*ecognition *p*article (SRP), a ribonucleoprotein, and this binding induces a temporary slowdown in translation.[19] The docking of ribosomes to the ER occurs by interaction of the SRP with the SRP receptor (SR) located in the ER membrane. Upon binding of GTP to both SRP and SR, the ribosome and the nascent chain are transferred to the Sec61 complex allowing translation to resume, and SRP is released to initiate a new cycle. Preproteins translocate through the Sec61 complex in the N to C direction. When approximately 20 largely apolar amino acyl residues enter the translocon, they are released sideways into the lipid bilayer. As the nascent polypeptide emerges from the luminal side of the translocon, its signal sequence is cleaved by a luminal signal peptidase.[20]

In the absence of specific sorting signals, a protein that is completely translocated into the lumen of the ER is secreted with the bulk flow in transit through the secretory pathway (default pathway). Evidence obtained both in yeast and mammalian cells suggests that different proteins are segregated in specific vesicles (see also below) suggesting the existence of parallel pathways of secretion. If a protein has hydrophobic domains (transmembrane domains, TMDs), it remains in the membrane and can assume different topologies. Notably, proteins with a single TMD or multiple TMDs can be oriented with the *N*-terminus end toward the ER lumen (Type I) or the cytosol (Type II), and this orientation will be maintained in the final destination taking into account that the lumen of ER is equivalent to the extracellular space. Sorting signals direct soluble and membrane proteins to the appropriate compartment of the secretory pathway.

Protein Maturation in the ER

Secretory proteins undergo folding in the ER where also a number of other processing reactions including disulfide bond formation, attachment of oligosaccharides (*N*-glycosylation), addition of glycolipids, and modification of specific amino acids take place. Protein chaperones are the key players for folding.[21] Chaperones like the Hsp70 family member, BiP, which is associated with the luminal face of the translocon, assist the folding of the nascent protein. Folding is completed posttranslationally and then oligomerization takes place. Different classes of chaperones are thought to act in sequence to achieve complete folding/assembly. In addition to chaperones, there are folding catalysts that increase the rate of protein folding by promoting reactions such as the formation of disulfide bonds or *cis–trans* proline isomerization that confer stability to the protein structure. Oxidoreductases play a relevant role in disulphide bond formation. The enzyme protein disulfide isomerase (PDI), an abundant ER protein, functions in the formation and isomerization (reshuffling) of disulfide bridges during protein folding in the ER. PDI1 is the best-characterized member of the PDI family that consists of 17 members that share at least one thioredoxin-like domain with the CXXC active site motif.[22] As shown in FIGURE 5.4, the oxidation of two thiols to produce a disulfide bond (S–S) in a substrate protein, concomitantly reduces two thiols of PDI that return to the oxidized state by another thiol-disulfide exchange catalyzed by ERO1, a membrane associated oxidoreductase. ERO1, which is a flavoprotein, returns to the oxidized state by transfer of electrons to molecular oxygen via its cofactor FAD. Glutathione buffers the system and reduces excessive or inappropriate formation of disulfide bonds (FIGURE 5.4).

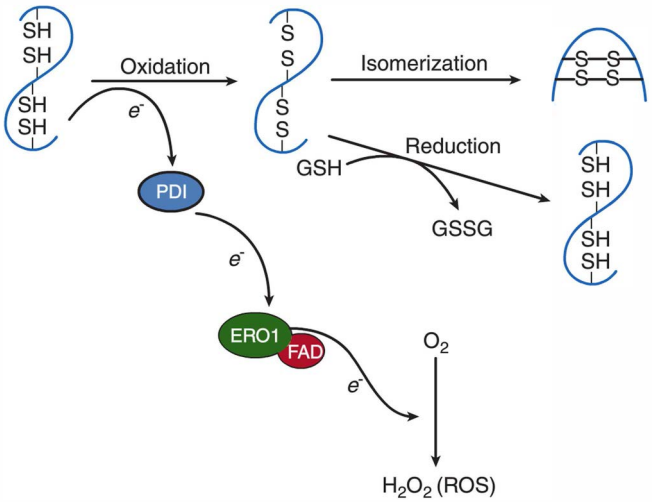

FIGURE 5.4 Disulfide bond formation in the ER. Proteins that fold in the ER acquire disulfide bonds through an oxidation process catalyzed by thiol-disulfide oxidoreductases. FAD, flavin adenine dinucleotide (cofactor of ERO1); GSH, glutathione; GSSG, oxidized form of glutathione; ROS, reactive oxygen species.

N-glycosylation is a biologically important protein modification that initiates in the ER (core-glycosylation) and is further completed in the Golgi apparatus. This process consists in the transfer *en-bloc* of a presynthesized "core" glycan chain from a lipid-linked donor (dolichol pyrophosphate) to the asparagine residue of a nascent protein in a consensus sequence *N*-[X]-S/T where X is any amino acid except proline.[23] Core oligosaccharide chains are composed of a glucose$_3$-mannose$_9$-*N*-acetylglucosamine$_2$ ($Glc_3Man_9GlcNac_2$) structure. The transfer is catalyzed by the oligosaccharyl transferase complex (OST) composed of multiple transmembrane subunits.[24,25] About 75% of the predicted consensus sequences of *N*-glycosylation site are occupied by sugar chains suggesting that steric factors also determine the recognition process by OST. After addition, *N*-linked glycans are processed in the ER where the glucose residues and one mannose residue are removed. This processing continues during their movement through the Golgi where final elaboration of oligosaccharide chains is completed (FIGURE 5.5).

N-glycans are important for the physical properties they confer and for many physiological purposes. Glycans protect proteins against denaturation and proteolysis and are abundant in hinge and spacers that are more susceptible for proteolytic attack. *N*-linked oligosaccharides confer hydrophilicity to the proteins and enhance their solubility. Moreover, *N*-glycans promote secretion and inhibition of *N*-glycosylation blocks secretion, whereas removal of single or multiple *N*-glycosylation sites by mutagenesis may reduce the efficiency of secretion. *N*-glycans can be enzymatically removed from the folded proteins without appreciable effects on protein structure and function. Although *N*-glycans are often not required for the enzymatic activity of many glycoproteins, there are glycans specifically required for the biological activity of some hormones and for mediating interaction with other proteins. Examples are erythropoietin,

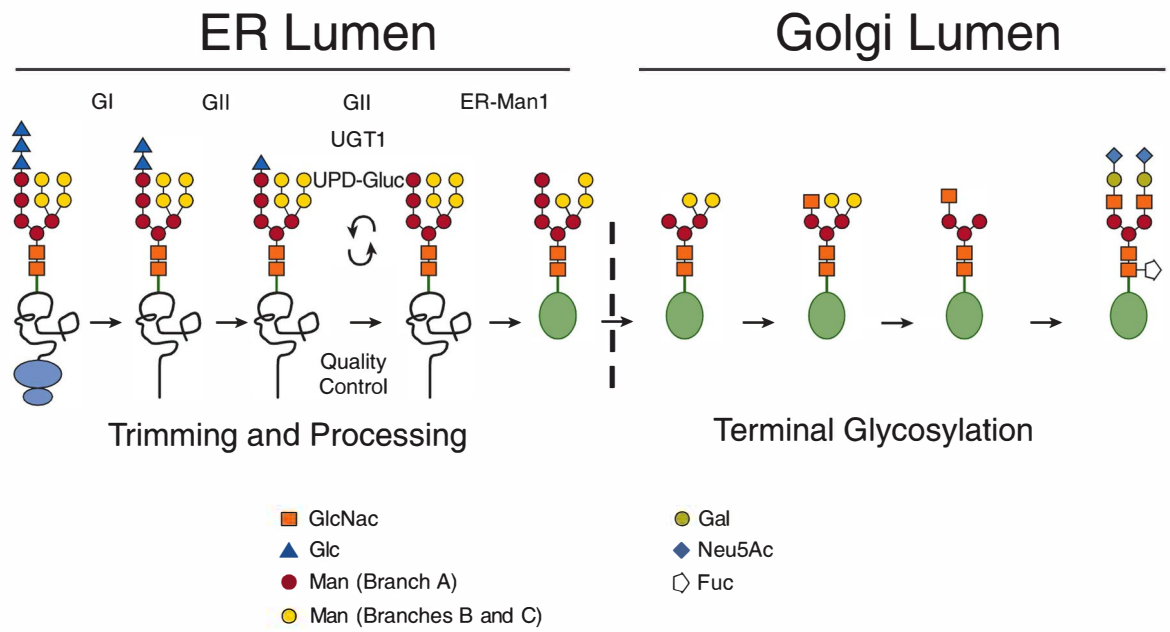

FIGURE 5.5 Processing of *N*-linked oligosaccharide chains in the ER and final maturation in the Golgi apparatus. Nascent glycopolypeptides undergo core *N*-glycosylation into the lumen of the ER. The processing of the *N*-linked chains is strictly coupled with folding and trafficking through the secretory pathway. Glucosidase I (GI) removes the outermost of the three terminal glucose residues. Then, glucosidase II (GII) removes the middle and the monoglucosylated glycoprotein enters the CNX/CRT cycle (not shown in the Figure, see text for detail). When the terminal glucose residue is removed by GII, the glycoprotein dissociates from CNX and CRT and leaves the ER if it is properly folded. The enzyme UDP-glucose: glycoprotein glucosyltransferase (UGGT, also named UGGT1 or UGT1) acts as a folding sensor and reglucosylates incompletely folded glycoproteins for another cycle of folding. The properly folded glycoprotein is represented by the green ellipse. Upon exit from the ER, ER-mannosidase I (ER-Man1) removes a mannose residue. Mannose trimming continues in the Golgi apparatus. Terminal glycosylation generates high-mannose, complex, or hybrid types of glycan structures (see FIGURE 5.7). The figure only shows a pathway leading to a biantennary complex-type glycan, but the number of antennae may vary up to six. In lysosomal soluble glycoproteins, the creation of a M6P signal targets the protein to the lysosome. (From *Angewandte Chemie International Edition*, Lehle L, Strahl S, Tanner W, vol. 45:6802–6818 ©2006 by John Wiley and Sons, with permission.)

the clotting factor IX, and the hormone chorionic gonadotropin that require particular *N*-linked chains for their function. The sugar moiety is required for proper secretion, for direct interaction with the cognate receptors, or to maintain the protein in an appropriate conformation for the interaction with the receptor. Moreover, decades of research have revealed that the carbohydrate moiety of glycoproteins modulate immunogenic properties of proteins and the mechanism by which they are cleared from the circulation. Factor VIII and von Willebrand factor (vWF) are relevant examples.[26]

In addition, several studies have outlined the important role of *N*-glycosylation in promoting folding. The addition of glycans may increase hydrophilicity of the folding peptide and thus prevent aggregation, influence the formation of secondary structures for steric effects and affect the disulfide bond formation. An indirect role concerns the interactions of glycans with specific chaperones with lectin-like properties to monitor folding.[23,27,28] In mammalian cells, core *N*-linked oligosaccharides are substrates for a complex chaperone system composed by the lectin chaperones calnexin (CNX) and calreticulin (CRT), ERp57 (an ER oxidoreductase), two glucose-removing enzymes (glucosidase I [GI] and glucosidase II [GII]), and one folding sensor endowed with reglucosylation activity (UDP-glucose: glycoprotein glucosyltransferase, UGT1). Proteins enter this system after they receive at least two *N*-glycan chains. GI and GII remove two terminal glucoses. The resulting monoglucosylated *N*-linked chain interacts with CNX/CRT and ERp57. Proteins that have not completed the folding process are detected by UGT1 and reglucosylated.[29] The addition of glucoses allows reassociation with the CNX/CRT chaperone system and a new cycle begins. If the protein is not folded after repetitive cycles, it is recognized and directed to degradation by ER-associated degradation (ERAD) (see further). Since the trimming of the glycans marks the disposal of these proteins, a "glycan-code" has been proposed to exist in the ER.[30]

In conclusion, folding requires sequential interactions with distinct chaperones and folding catalysts. An example is provided by major histocompatibility complex class I molecules that exploit both the CNX/CRT cycle and other devoted ER-resident molecules for their assembly.

Glycolipid Attachment

The glycosylphosphatidylinositol (GPI)-anchored proteins are a class of proteins that are usually localized in the outer side

of the plasma membrane.[31] The protein precursor has both a signal sequence and a *C*-terminal sequence that directs the attachment of a GPI anchor to the preprotein. These proteins lack a TMD, and the glycolipid functions as the only means for membrane anchoring. Defects in the human phosphatidylinositol glycan-A gene (PIG-A) involved in the first stage of GPI-biosynthesis is the cause for an unusual form of acquired hemolytic anemia that occurs in adults (paroxysmal nocturnal hemoglobinuria). This disease is due to the lack of expression of certain GPI-anchored proteins. An example of a GPI-anchored protein is the decay-accelerating factor (DAF) that down-regulates complement activation on "self" surface and protects red cells from its action. In the absence of DAF, there is excess reticulocyte hemolysis.

γ-Carboxylation of Glutamic Acid Residues

While glycosylation is a frequent modification in secreted proteins, other modifications are less common but are required for the function of some proteins. A specific set of proteins is posttranslationally modified by γ-carboxylation of glutamic acid residues (Glu).[32] This modification is catalyzed by a vitamin K–dependent γ-glutamyl carboxylase that converts selected Glu to γ-carboxylglutamic acid (Gla) in a ca. 45–amino acid peptide sequence (the Gla domain) that encompasses the *N*-terminal region of the target protein.[33] The Gla residues function to coordinate Ca^{2+} that is essential for the biological activity of multiple factors in blood coagulation.[34] These procoagulant factors are prothrombin and factors VII, IX, and X, as well as the anticoagulants protein C and protein S. Other substrates of the γ-carboxylase are less characterized except for the bone proteins osteocalcin and matrix Gla protein that both require γ-carboxylation for full activity.

γ-Carboxylase is an integral membrane glycoprotein localized to the ER.[35] Its obligate cofactor, reduced vitamin K (KH_2), is produced by the action of vitamin K–epoxide reductase (VKOR) that converts oxidized vitamin K to the reduced form (FIGURE 5.6). VKOR is a small transmembrane protein that is predicted to be localized in the ER as well.[36,37] The activity of VKOR is inhibited by warfarin, a potent anticoagulant compound. Similar to prothrombin and factor V–deficient mice, γ-carboxylase homozygous null mutants manifest dramatic developmental defects with partial midembryonic lethality and postnatal hemorrhage.[38] These findings suggest that components of the blood coagulation cascade are functionally critical substrates for γ-carboxylation.

β-Hydroxylation of Amino Acids

β-Hydroxylation of proline and lysine residues is another posttranslational modification that is typical of procollagen. In addition to this extracellular matrix protein precursor, a subset of secreted plasma proteins involved in blood coagulation, such as factor IX, contains epidermal growth factor (EGF) domains in which an aspartic acid or an asparagine are β-hydroxylated and converted in the ER into β-hydroxyaspartate (Hya) or β-hydroxyasparagine (Hyn).[34] The role of these modifications is uncertain. It has been proposed that formation of Hya negatively regulates fucosylation of the EGF domains.[34]

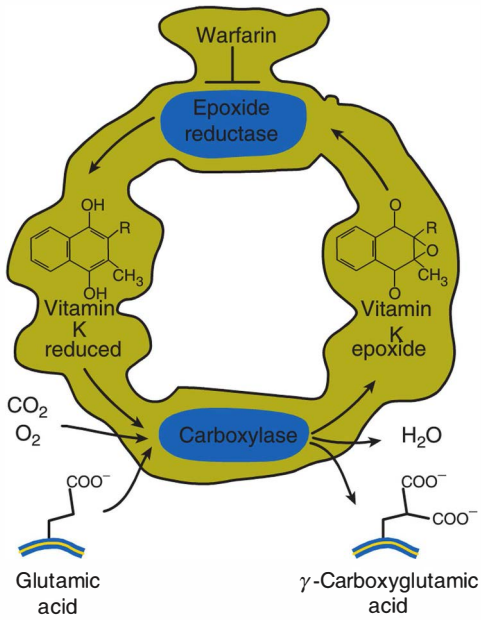

FIGURE 5.6 Protein γ-carboxylation is catalyzed by a vitamin K–dependent carboxylase. Several blood-clotting proteins contain glutamic acid residues that are converted to γ-carboxyglutamic acid by a carboxylase. This enzyme uses reduced vitamin K and oxygen to add a carbon dioxide molecule to the side chain of selected glutamic acid residues producing γ-carboxyglutamic acid. Vitamin K 2,3-epoxide is recycled to reduced vitamin K by vitamin K epoxide reductase, an enzyme inhibited by warfarin. (Adapted from Sadler JE. Medicine: K is for koagulation. *Nature* 2004;427:493–494.)

ANTEROGRADE TRANSPORT FROM ER TO GOLGI

The transport of proteins and lipids from the ER to the Golgi occurs through vesicles. On achieving transport competence, proteins are granted access to higher-ordered membrane domains termed ER exit sites. At ER exit sites, membrane-bound and -soluble proteins are concentrated into transport vesicles for trafficking to a network of smooth membranes called the *ER–Golgi intermediate compartment* (ERGIC). The COPII complex, composed of *co*at *p*roteins, concentrates and packages the cargo proteins into vesicles. COPII binds to cargo molecules either directly, if they span the membrane, or through intermediate cargo receptors and then provides some of the force that causes vesicle budding, thereby linking cargo acquisition to vesiculation.[39] The process has been dissected at a molecular level. On the cytosolic face of the ER membrane, the RAS protein SAR1 is activated by the ER localized GEF SEC12. SAR1 GTP assembles with the SEC23–SEC24 complex whose SEC24 subunit binds directly or through a membrane receptor to specific signals displayed by the cargo. This prebudding cargo complex recruits the outer layer SEC13–SEC31 complex leading to coat polymerization, membrane deformation, and COPII-vesicle formation.[39]

Receptor-Mediated Transport of Factors V and VIII in the Secretory Pathway

To date, the only well-characterized mechanism of cargo recognition in mammalian cells concerns two coagulation factors, factor V (FV) and VIII (FVIII).[40] Combined deficiency of FV and FVIII causes a bleeding disorder characterized by a reduced plasma level of these coagulation factors to 5% to 30% of normal. This reduction is due to a defective trafficking of FV and FVIII from the ER to the Golgi compartment.

One of two genes, *LMAN1* or *MCFD2*, is mutated in patients with combined deficiency of FV and FVIII. LMAN1, *l*ectin *ma*nnose-binding protein1 (also referred to as ERGIC-53), is a transmembrane protein with a *C*-terminal cytoplasmic tail containing an ER-exit-motif (two phenylalanine residues, FF). This motif allows the interaction of LMAN1 with the COPII-coat proteins. In addition, the luminal domain of LMAN1 recognizes mannose residues and binds MCFD2, a luminal protein, in a Ca^{2+}-dependent manner. The LMAN1–MCFD2 complex functions as a cargo receptor that recruits FV and FVIII into specific transport vesicles. The mutant forms of both LMAN1 or/and MFCD2 fail to recruit FVIII into the vesicles. Interestingly, the LMAN1–MCFD2 complex appears to be required only for the secretion of factors V and VIII, as there are no significant reductions in any other plasma proteins.[41]

Retrograde Golgi–ER Transport

ER resident proteins are selectively retained in the ER by both, the absence of export signals and the presence of ER retention signals. Soluble luminal ER residents are retained through a *C*-terminal ER tetrapeptide retention motif KDEL. Frequently, transmembrane proteins have either a *C*-terminal dilysine motif KKXX or an *N*-terminal diarginine motif XXRR, or variants thereof. However, it is more accurate to designate ER localization signals as "retrieval motifs" because proteins bearing these signals can transiently escape from the ER into the ERGIC, from which they are returned to the ER through the retrograde vesicular transport (FIGURE 5.2).

For the KDEL motif of luminal ER proteins, a specific retrieval receptor has been identified, first in yeast and then in mammals. The KKXX motif directly interacts with the COPI coat protein complex that is involved in retrograde transport from ER to the Golgi. Retrograde transport also serves to replenish the vesicle components lost as a result of anterograde (forward) transport. In conclusion, selective protein exit from the ER is achieved by monitoring/regulating (a) transport competence of nascent proteins, (b) capture of cargo in transport vesicles, and (c) protein retention/retrieval for ER-localized proteins.

INTRA-GOLGI TRANSPORT AND PROTEIN PROCESSING

The Golgi complex comprises a stack of flattened, membrane-bound cisternae that is highly dependent on microtubules for its integrity. The stack of cisternae can be subdivided into three parts referred to as *cis, medial*, and *trans* with the *cis* and *trans* sides facing the ER and the plasma membrane, respectively (FIGURE 5.2). Both the *cis* and *trans* faces are associated with tubulovesicular bundles of membranes. The ERGIC comprises the bundle on the *cis* side of the Golgi stack and is the site where incoming proteins from the ER are sorted into those directed for anterograde or for retrograde transport. The tubulovesicular bundle at the *trans* side is the *trans-Golgi* network (TGN, see FIGURE 5.2).

A major feature of the Golgi is polarity. The processing events are temporally and spatially ordered because the processing enzymes have a characteristic distribution across the Golgi stack. Among these enzymes are glycosyltransferases that sequentially modify *N*-linked chains. In the Golgi, additional types of modifications take place as for example protein *O*-glycosylation, phosphorylation of oligosaccharides, and sulfation of tyrosine residues (see further).

Retention of Resident Golgi Proteins

The continuous influx and efflux of proteins through the Golgi poses the question on how this organelle maintains its identity.[42] Extensive analyses have failed to reveal a clear retention motif enabling subdomain-specific retention of resident Golgi proteins. Two possible models have been proposed. The first is based on the finding that the TMDs of Golgi proteins are shorter than TMDs of plasma membrane proteins. These differences should allow a preferential interaction with the Golgi membrane lipid bilayer that is thinner than that of plasma membrane. The second postulates that proteins of a given subdomain of the Golgi membrane can aggregate into large detergent-insoluble oligomers as a way of minimizing lipid–protein contact. This would prevent the entry of proteins into the vesicles and thus their traffic to more distal cisternae. There is evidence in support of both models.

In general, a dynamic process of iterative rounds of retrograde and anterograde transport allow specific proteins, mainly glycosyltransferases, to maintain a compartment-specific distribution.

Protein Trafficking through the Golgi Apparatus

Cargo proteins exit the ER in COPII-coated vesicles that enter the ERGIC and are ultimately delivered to the *cis-Golgi* either in vesicles or along extended tubules. However, the means by which cargo proteins move across the Golgi complex from *cis* to *trans* remains controversial.[43] Two models have been proposed. The vesicular transport model contends that anterograde transport occurs in vesicles or tubules. The second suggests that there is a cisternal maturation. This alternative model proposes that Golgi cisternae are not fixed structures but move forward from the *cis* side to the *trans* side, generating an anterograde movement. As cisternae mature, resident Golgi proteins that belong to more *cis*-like cisternae must be selectively pinched off in vesicles and trafficked back to the *cis* side of the Golgi stack. This would occur by COP-I-mediated retrograde vesicular transport (FIGURE 5.2). Although it is presently unknown which of these models is correct, the majority of the experimental data support the cisternal maturation model.

Protein Modifications in the Golgi Apparatus

As mentioned above, a sequential series of additional carbohydrate modifications occur as proteins transit through the Golgi compartments. Mannose residues are removed by Golgi mannosidase I and II, and then glycosyltransferases catalyze the addition of complex sugars such as *N*-acetylglucosamine (GlucNac), fucose (Fuc), galactose (Gal), and *N*-acetyl neuraminic acid (sialic acid, Neu5Ac) in a spatial and temporal tightly regulated order (FIGURE 5.5). Three types of oligosaccharide structures can be generated: highly complex, high-mannose, and hybrid structures[44] (FIGURE 5.7).

In the Golgi also *O*-glycosylation occurs.[45,46] This post-translational modification involves the attachment of *N*-acetylgalactosamine (GalNAc) to the hydroxyl of serine or threonine residues through an *O*-glycosidic linkage. This reaction is carried out by UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl transferases (ppGalNAcTs) that comprise a striking redundant family of enzymes in eukaryotic cells. Target serine and threonine residues are often clustered together. Mucins represent abundantly *O*-glycosylated proteins and are used as model proteins for studies on protein *O*-glycosylation.

Tyrosine *O*-sulfation also occurs in the *trans*-Golgi network and is mediated by tyrosyl protein sulfotransferases that catalyze the transfer of a sulfuryl group (SO_3^-) from a donor 3'-phosphoadenosine 5'-phosphosulfate to specific tyrosine residues.[47] This modification occurs on many secretory proteins including a number of proteins that interact with thrombin, such as hirudin, a leech-derived anticoagulant, fibrinogen, heparin cofactor II, α-2-antiplasmin, vitronectin, bovine factor X, factor V, and factor VIII.[48]

Proteolytic Processing in the Golgi

Many secretory proteins are synthesized as larger precursors (preproproteins) that after translocation into the ER (proprotein) undergo further maturation by proteolysis in the late compartments of the secretory pathway. Among them are proteins that perform physiological important functions such as hormones, serum proteins, growth factors, and neuropeptides.[49] Consequently, this processing takes place in many different tissues. The proproteins are cleaved internally and the mature product/s result/s from the excision of specific segments. A family of Ca^{2+}-dependent serine endoproteases, called proprotein convertases (PCs), are responsible of the proteolytic processing. PCs hydrolyze the peptide bond on the carboxyl side of a pair of basic residues KK↓ or RR↓. Mammalian PCs are related to Kex2p, a Golgi subtilisin-like endoprotease required for processing of α-factor mating pheromone and killer toxin in the yeast *Saccharomyces cerevisiae*. Kex2p has been the first PC to be identified and was extensively studied taking advantage of genetic manipulation in yeast.[50]

PCs are themselves proenzymes. Mammalian PCs have a canonical signal sequence, a proregion, a catalytic domain, and a P domain but differ in the *C*-terminal region. The activity and localization of PCs are highly regulated. For instance furin/PC1,

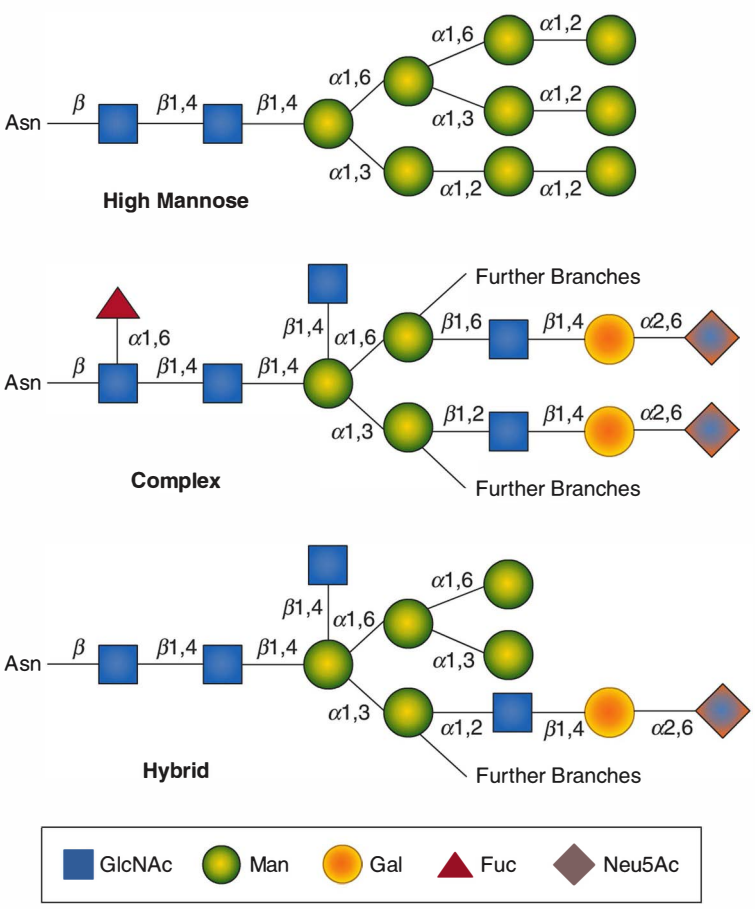

FIGURE 5.7 Scheme of the structure of high-mannose-, complex-, and hybrid-type *N*-linked glycans. (From Skropeta D. The effect of individual *N*-glycans on enzyme activity. *Bioorg Med Chem* 2009;17:2645–2653, with permission © 2009 by Elsevier Ltd.)

involved in the processing of proinsulin, becomes active by autocatalytic cleavage of the proregion that induces folding and exit from the ER. The prodomain remains attached noncovalently until the cleaved inactive enzyme reaches the Golgi where the more acidic pH (pH ~ 6.5) and also the Ca^{2+} enriched environment facilitates dissociation of the prodomain. A second cleavage within the prodomain precludes further inhibitory interactions resulting in full activation. PCs are usually retained in the TGN by virtue of their TM domains and cytosolic tails whereas nonanchored forms localize to secretory granules. In these locations these processing enzymes have access to the many proteins that move toward the cell surface via the constitutive or regulated branches of the secretory pathway. Insight into the catalytic mechanism of substrate specificity and cleavage came from the resolution of the crystal structure of Kex2p and furin.[50]

Another mechanism of proteolytic processing specific to the Golgi was recently identified. It consists of regulated intramembrane proteolysis of ER membrane precursors of transcription factors that are transported to the Golgi upon activation of a signalling pathway. Two remarkable examples are provided by ATF6 (see also section on unfolded protein response [UPR]), a latent precursor of a basic leucine zipper (bZIP) transcription factor, and the sterol-regulatory element binding protein. These proteins are transported from the ER to the Golgi in response to the activation of the UPR or to reduced sterol levels. In the Golgi membrane, proteolytic events catalyzed by two specific proteases (S1P and S2P) cleave the protein and release the soluble active transcription factor that migrates to the nucleus and activates the expression of specific sets of genes.[51]

Protein Assembly in the Golgi

Although the ER is the primary site for folding of secretory proteins, formation of disulfide bonds and multimer assembly for an essential hemostatic glycoprotein—vWF—undergo a remarkable conformational change in the Golgi.[52] The mechanism has probably evolved to bypass the limited capacity of cargo size for ER to Golgi transport vesicles. Sequential changes that occur in the ER, Golgi, and extracellular space were identified. The first assembly step occurs in the ER with "tail-tail" dimerization of vWF precursor. This event is coupled with the formation of disulfide bonds between the *C*-terminal domains of the protein. Then, vWF is transported to the Golgi where a proprotein protease, probably furin, cleaves the propeptide and additional intersubunit disulfide bonds form "head to head" between the *N*-terminal D3 domains.[53] Since the Golgi lacks known chaperones and has an acidic pH that inhibits disulfide bond rearrangement, vWF has evolved an *N*-terminal domain (propeptide) that functions as oxidoreductase at the low pH of the Golgi. More conformational changes occur in the extracellular environment to give origin to large multimers of vWF that are the most effective for hemostasis.[52]

GOLGI TO PLASMA MEMBRANE TRANSPORT AND ENDOCYTOSIS

Overview

The TGN is the station for intracellular sorting, where proteins bound for lysosomes or regulated secretory vesicles are separated from those entering the constitutive pathway of secretion leading to the plasma membrane (FIGURE 5.2). The secretion process is called exocytosis. The molecular basis for diversion of proteins into lysosomes and regulated secretory granules is described below.

Sorting into Lysosomes

Lysosomes are organelles with an acidic internal pH (5 to 5.5). They contain numerous hydrolytic enzymes designed to degrade proteins, carbohydrates, and lipids. In the Golgi soluble hydrolases are recognized by the presence of signal patches and are selectively marked for sorting into lysosomes by phosphorylation of their *N*-linked oligosaccharides that creates the mannose-6-phosphate (M6P) sorting signal. On arrival at the TGN, the modified hydrolase is bound by a cargo transmembrane receptor, the M6P-receptor (M6P-R), which delivers it first to a "late endosomal compartment," where the low pH releases the hydrolase from the M6P-R. Subsequently, the hydrolase is delivered to the lysosome, and the M6P-R is recycled to the TGN.

The motif responsible for targeting M6P-R to lysosomes is YSKV and is recognized by adaptins, multisubunit complexes (AP) that contribute to the assembly of clathrin on the cytosolic side of the vesicle. In this way, APs help the delivery to lysosomes by linking cargo acquisition to vesiculation. Thus, cargo recruitment occurs in a manner similar to the one described for the COPI- and COPII-coated vesicles except that the coat is clathrin. In addition to luminal hydrolases, lysosomes also contain a wide array of membrane proteins that are targeted to lysosomes via one of two consensus motifs: (a) YXXe, where X is any amino acid and e is any amino acid with a bulky hydrophobic side chain and (b) a leucine-based motif (LL or LI). Trafficking of these membrane-bound proteins to lysosomes is indirect, proceeding first to late endosomes or the plasma membrane prior to their retrieval to lysosomes. Failure to accurately target lysosomal hydrolases underlies Hurler syndrome and inclusion-cell (I-cell) disease. In I-cell disease patients, the hydrolases missing from the lysosomes are secreted from the cell and found in the blood.

Autophagosomes and Phagosomes

Lysosomes are also the organelles with which autophagosomes fuse. Autophagosomes are ER-derived organelles into which fragments of other cytoplasmic organelles or cytosolic components are engulfed. After fusion with the lysosomes, the contents of the autophagosome are degraded by digestive enzymes to recycle its constituents. Autophagy is activated in many stress conditions, such as nutrient or growth factor deprivation, hypoxia, oxidative stress, DNA damage, protein aggregation, damaged organelles, or intracellular pathogens.[54–56] Autophagy is a central process of the integrated cell response.[56] Recently, studies on autophagy have received significant attention after the discovery that this process is also required during development.[57] Notably, in mammals, autophagy is essential for the extensive cell reprogramming that occurs during development and cell differentiation. In early oocyte fertilization and early neonatal development, two massive waves of autophagy are triggered. Moreover, during erythroblast differentiation to erythrocytes, loss of organelles occurs. Although the implication of autophagy in this process is still uncertain, recent studies indicate that the mitochondrial clearance in reticulocytes is partly dependent on autophagy and defects in this process cause

reduction of mature erythrocytes (anemia) or compensatory expansion of erythrocyte precursors (reticulocytosis).[57]

Another organelle, the phagosome, derives from lysosomes. Microorganisms, like bacteria or fungi, are ingested by phagocytosis. The phagosome, into which a lysosome transforms to destroy an ingested pathogen, is a specific compartment of professional phagocytes such as macrophages, neutrophils, and dendritic cells that act as a first-line defense to pathogenic organisms. Phagosomes and autophagosomes are examples of the dynamic nature of the structural organization of the cell that changes to meet the needs of the organism.

Sorting into Regulated Secretory Granules

In regulated secretion, proteins are condensed into secretory granules that are stored and released to the plasma membrane after the cell has received an appropriate stimulus (FIGURE 5.2). The significance of this additional secretory system is to provide for rapid secretion of soluble components upon demand. Several examples are the secretion of hormones, neurotransmitters, and digestive enzymes.

After budding from the TGN, the granule proteins are concentrated (up to 200-fold in some cases) by selective removal of extraneous contents from clathrin-coated vesicles. Mature secretory granules are thought to be stored in association with microtubules until the stimulation of a surface receptor triggers their exocytosis. One example of stimulus-induced exocytosis is the binding of a ligand to the T-cell antigen receptor complex on a cytotoxic T lymphocyte. Conjugation of a cytotoxic T cell with its target causes its microtubules and associated secretory granules to reorient toward the target cell. Subsequently, the granules are delivered along microtubules until they fuse with the plasma membrane, releasing their contents for lysis of the target cell. Following release of the granule contents, the granule membrane components are internalized and transported back to the TGN, where the granule can be refilled with cargo proteins.

SPECIFICITY OF VESICULAR TARGETING

As described above, COPI and COPII vesicles transport material early in the secretory pathway whereas clathrin-coated vesicles transport material from the plasma membrane and Golgi. Coating proteins assemble at specific areas of the membrane in a process controlled by the coat recruitment GTPase. While SAR1 is responsible for COPII coat assembly at the ER membrane, ARF1 is responsible for the assembly of COPI coats and clathrin coats at Golgi membranes. Clathrin coat assembly at the plasma membrane is also thought to involve a GTPase, but its identity is unknown. These regulatory proteins also ensure that membrane traffic to and from an organelle is balanced.

After budding, vesicles are transported to their final destination by diffusion or motor-mediated transport along the cytoskeletal network (microtubules or actin) (FIGURE 5.8). The molecular motors kinesin, dynein, and myosin have been implicated in this process. The vesicles undergo an uncoating process before fusion with the correct target membrane. Both transport vesicles and target membranes display surface markers that selectively recognize each other.

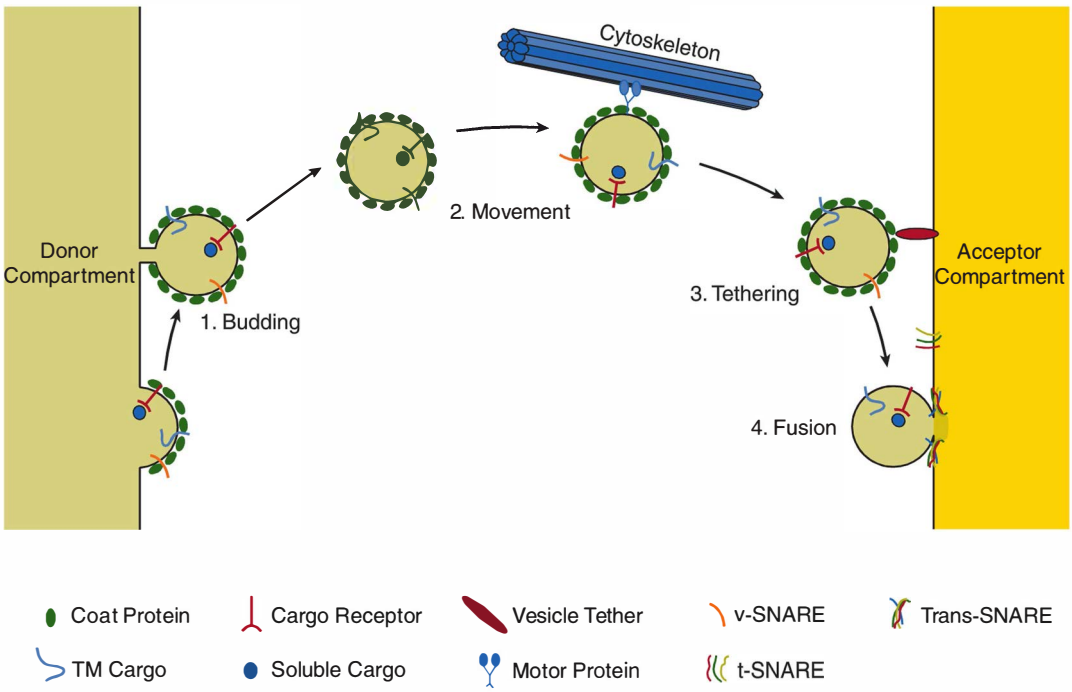

FIGURE 5.8 The essential steps in vesicle transport. The scheme represents: (*1*) budding and incorporation of soluble cargo into a vesicle; (*2*) movement of the vesicle toward the acceptor membrane by diffusion or with the aid of a cytoskeletal track; (*3*) tethering of the vesicle to the acceptor membrane by action of tethering factors in conjunction with Rab GTPases; and (*4*) fusion mediated by the assembly of a four-helix bundle between vesicle-associated and target-membrane SNAREs. (From Cai H, Reinisch K, Ferro-Novick S. Coats, tethers, Rabs, and SNAREs work together to mediate the intracellular destination of a transport vesicle. *Dev Cell* 2007;12:671–682, with permission © 2007 by Elsevier Ltd.)

Three classes of proteins assist the selectivity of vesicle docking and subsequent fusion: (a) a class of GTPases called Rabs, (b) protein complexes, called tethers, that together with Rabs facilitate the initial docking of the vesicles to the target membrane, and (c) complementary sets of *v*esicle SNAREs, v-SNAREs, [Soluble NSF (N-ethylmaleimide-sensitive factor) Attachment Protein Receptor], and *t*arget membrane SNAREs (t-SNAREs) that promote vesicle fusion[58,59] (see FIGURE 5.8).

PROTEIN QUALITY CONTROL IN THE SECRETORY PATHWAY

The main regulation of protein traffic through the secretory pathway occurs at the level of ER where newly synthesized proteins are subjected to quality control. As mentioned before, only properly folded proteins can exit this organelle and transit through the other compartments. Proteins that do not satisfy the necessary requirements are degraded and/or activate a complex response aimed to protect the cell from toxic effects of accumulation of abnormal proteins and to improve protein folding.

Targeting Proteins for Degradation in the ER

Improperly folded proteins are destroyed by ERAD.[60,61] A simple scheme of ERAD is shown in FIGURE 5.9. The recognition process is mediated by ER lectin-like proteins (not shown in the figure) that specifically recognize short glycan chains generated by the action of ER proteins named EDEMs (ER-degradation enhancing mannosidase-like).[62,63] The substrate for EDEMs are misfolded proteins that are probably recognized by BiP or by the CNX/CRT chaperone system due to their exposed hydrophobic patches of amino acids or sugar moieties, respectively. After retrotranslocation to the cytosol through a protein-conducting channel, luminal proteins are polyubiquitylated at the cytosolic face of the ER by the ER associated ubiquitin ligases.[64] The modified proteins are removed from the membrane by Cdc48, a member of the family of ATPases associated with diverse cellular activities (AAA^+) and directed to proteasomal degradation as occurs for misfolded cytosolic proteins.[64] Proteins that misfold their cytosolic, luminal, or membrane-spanning domains are directed to the ERAD-C, ERAD-L, or ERAD-M pathways, respectively.[65]

Another fate of misfolded proteins in the ER is entrapment in aggregates containing BiP. These aggregates are probably destroyed by autophagy. Since many diseases arise from aggregation of misfolded proteins, this area is the object of intense study.

The ER can cope through ERAD with many stress conditions. Thus, an efficient ERAD greatly contributes to homeostasis of the ER.

The Unfolded Protein Response

The homeostasis of the ER can be altered by several conditions that induce acute or chronic ER stress. The ER can be perturbed by accumulation of unfolded proteins that results from inhibition of *N*-glycosylation, high temperature, increased expression of proteins, expression of mutant proteins, Ca^{2+} depletion, and unfavorable growth conditions. Cells counteract such conditions by inducing the UPR.[66–68] The UPR is an adaptive response signaled through three ER-localized transmembrane proteins PERK, IRE1, and ATF6 to limit accumulation of unfolded protein through reducing protein synthesis, increasing the degradation of unfolded protein, and increasing the ER protein folding capacity. IRE1 is conserved in all

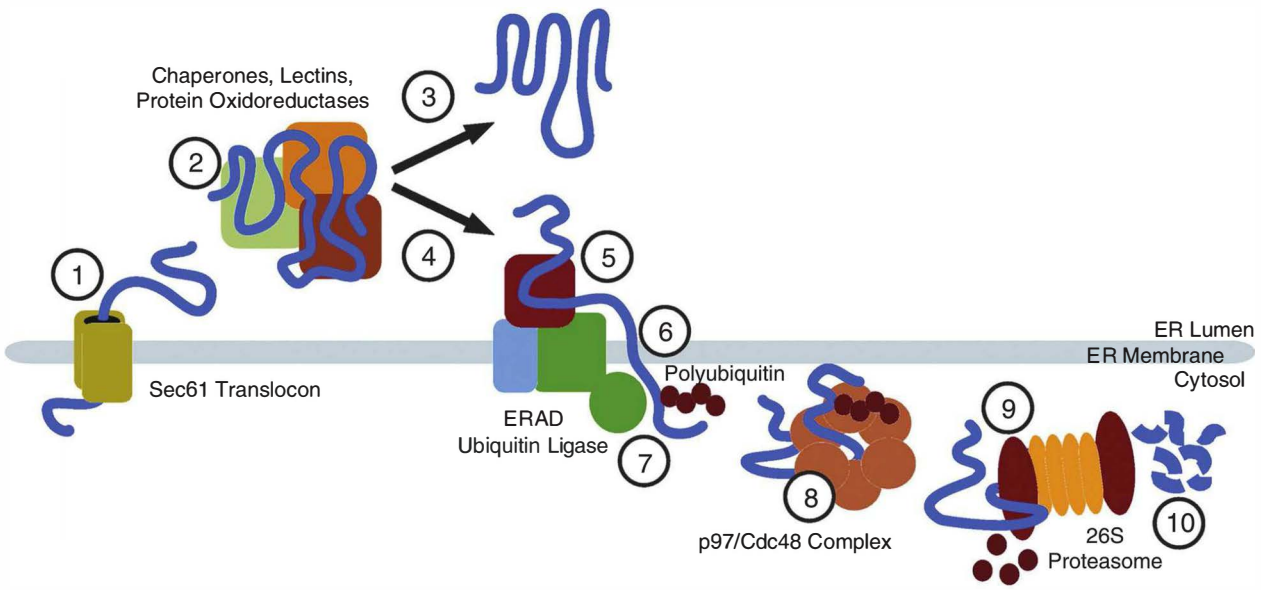

FIGURE 5.9 Model for ER-associated protein degradation (ERAD). Cotranslational translocation and folding of the nascent polypeptide (*1–2*). Attainment of native conformation and transport to the final destination (*3*). The misfolded protein is targeted to retrotranslocation (*4*). Retrotranslocation occurs by an unknown mechanism (*5* and *6*). Polyubiquitylation of the protein by an ER-membrane-bound ubiquitin ligase (*7*). A complex containing the AAA-ATPase p97/Cdc48 binds to the substrate (*8*) and promotes the transport to the 26S proteasome where the protein is degraded (*10*). (From Bagola K, Mehnert M, Jarosch E, et al. Protein dislocation from the ER. *Biochim Biophys Acta* 2011;1808:925–936, with permission © 2011.)

eukaryotic cells and has protein kinase and endoribonuclease activities that, upon activation, mediate unconventional splicing of a 26 base intron from XBP1 mRNA to produce a potent transcription factor. As mentioned above, ATF6 is a bZIP containing transcription factor that, upon accumulation of unfolded protein in the ER lumen, transits to the Golgi compartment where it is cleaved to yield a cytosolic fragment that migrates to the nucleus to activate gene transcription. Finally, PERK-mediated phosphorylation of eIF2 attenuates general mRNA translation; however, paradoxically, it increases translation of the transcription factor ATF4 mRNA to also induce transcription of UPR genes.[66] If the UPR adaptive response is not sufficient to correct the protein-folding defect, cells enter apoptotic death. Activation of the UPR and defects in the UPR are now known to be important factors that contribute to many disease processes ranging from metabolic disease, neurological disease, infectious disease, inflammation, and cancer.[69]

Glycoprotein Secretion for Therapeutic Use

The advent of the recombinant DNA technology has stimulated the production of many plasma glycoproteins designed for the treatment of hematological human disorders. It soon became clear that microorganisms (bacteria or yeast) are unsuitable for the production of large plasma glycoproteins with complex multidomain organization, and mammalian expression systems were preferred. Recombinant clotting factors VIII, IX, VIIa, and vWF were produced. In many cases, the recombinant protein preserves the same properties of the plasma-derived product although subtle differences can affect some important properties such as the half-life, interaction with other proteins, and immunogenicity. This can be due to differences in post-translational protein processing such as glycosylation (both *N*- and *O*-), γ-carboxylation, tyrosine sulfation, or proteolysis.[70]

Concluding Remarks

In the genome, about 30% of the predicted proteins have a signal sequence for entering the secretory pathway.[71] Blood contains many secretory proteins such as hormones, growth factors, coagulation factors, and proteins required for fibronolysis, inflammation, humoral immunity, etc. Blood specialized secretory cells secrete specific sets of proteins and also small molecules that altogether constitute the secretome. For instance, more than 300 different molecules are secreted by activated platelets that are involved in wound healing by acting on coagulation, stimulation of smooth muscle cell proliferation, and production of cytokines.[72] Research on protein secretion is very active not only for understanding the basic biology of secretory cells but also for the potential application of the discoveries in the design and development of new therapeutic targets and strategies.

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CHAPTER

6 Overview of the Immune System

ANDREW H. LICHTMAN

The immune system is a complex collection of molecules, cells, tissues, and organs whose major function is to protect hosts from microbial infection. This function is demonstrated by the fact that increased susceptibility to infections is the major consequence of deficiencies in one or more components of the immune system. In higher vertebrates, the immune system includes two closely interrelated subsystems that confer innate and adaptive immunity, respectively. Innate immunity, which is phylogenetically older, provides constant protection against microbial invasion at epithelial barriers, as well as rapid stereotypical responses of various cell types and molecules throughout the body to many different types of microbes. These rapid responses are critical for survival in a world teeming with microbes. The innate immune system also reacts to tissue injury even without infection, leading to sterile inflammatory responses. Adaptive immunity, which evolved later than the innate immunity, is more specialized and specific for different foreign antigens. Lymphocytes are central to all adaptive immunity, and include B lymphocytes that produce antibodies and T lymphocytes that have many different effector and regulatory functions.

Although the immune system is required for health, immune responses contribute to the primary pathogenesis or secondary manifestation of a large number of diseases. A normally functioning immune system may cause tissue injury and disease as a consequence of its activity in defending against infection. Deregulation of the immune system can lead to excessive responses to pathogenic or even nonpathogenic microbes (e.g., gut flora), as well as to environmental molecules (e.g., allergens). A normal adaptive immune system is generally tolerant of self molecules, but the mechanisms that maintain tolerance can fail, resulting in humoral or T-cell–mediated responses to host molecules and autoimmune disease.

An understanding of the immune system is relevant to the field of homeostasis and thrombosis for many reasons. Some examples are briefly considered here and are covered in more detail in other chapters. There are numerous bidirectional interactions between innate inflammatory responses and the coagulation system. Excessive innate immune responses that occur in sepsis are directly linked to impaired hemostatic functions of endothelial cells, and tissue-factor-dependent thrombosis. Coagulation factors bind to protease-activated receptors on platelets, mononuclear cells, or endothelial cells, thereby inducing expression of immunomodulatory cytokines. Platelets express some of the same adhesion and signaling molecules that are expressed by lymphocytes, dendritic cells (DCs), and phagocytes, which may lead to hemostasis-related complications of targeted immunotherapies. Antibodies produced in the setting of autoimmune disease may recognize platelets or phospholipids and thereby promote hemorrhagic or thrombotic disease. Arterial thrombosis is a frequent terminal event in immune/inflammatory-induced destabilization of atherosclerotic plaque and in transplant failure due to adaptive immune responses to alloantigens. The immune system is also the major barrier toward successful coagulation factor replacement therapy or repeated platelet transfusions. For all these reasons and others, a full understanding of hemostasis and thrombosis requires at least a basic understanding of the immune system.

CYTOKINES AND CHEMOKINES

Cytokines are soluble proteins that are intricately involved in all aspects of the immune system, including development and maintenance of lymphoid tissue architecture, cell trafficking, activation of all cellular components of innate and adaptive immune responses, and regulation of these responses. Because it is difficult to discuss any aspect of immunology without mentioning various cytokines, a brief background of these proteins is warranted at the beginning of this chapter. Cytokines are produced by and act on a wide variety of cell types, including but not restricted to cells whose main functions are related to immune defense. Most cytokines are produced transiently after newly induced gene transcription in recently activated cells. Cytokines bind to high-affinity receptors and usually work in a paracrine fashion and are therefore effective at very low concentration. Some cytokines have systemic effects, sometimes pathologic, and can be detected in the blood as markers of robust immune activity within tissues. Cytokines and their receptors can be grouped into families based on structural features that correlate with receptor signaling pathways,[1,2] such as the type 1 and type 2 cytokines and receptors, which signal by JAK-STAT pathways[1,3–5] and the tumor necrosis factor (TNF) superfamily cytokines and receptors, which are homotrimeric proteins, and signal by TRAF-NF-κB pathways.[6,7] Table 6.1 includes some basic information about selected cytokines that are mentioned in this chapter and play central roles in innate and adaptive immune responses. It is important to note that biologic reagents that block cytokine actions are proving to be some of the most successful therapeutic agents for immune-mediated diseases ever developed, such as anti-TNF regents now in wide use for the treatment of rheumatoid arthritis.

Chemokines are a subset of structurally homologous, approximately 8 Kd cytokines produced by many different cell types, which bind to G-protein–coupled receptors on leukocytes and regulate leukocyte migration and lymphoid tissue architecture.[8] Table 6.2 includes a selection of important chemokines and their receptors, many of which are mentioned in this chapter.

Table 6.1

Selected cytokines of innate and adaptive immunity

Cytokine	Cell Source	Cellular Targets and Effects
Cytokines of Innate Immunity		
Tumor necrosis factor (TNF)	Macrophages T cells	T cells, endothelial cells: activation (inflammation, coagulation) Neutrophils: activation Hypothalamus: fever Liver: synthesis of acute-phase proteins Muscle, fat: catabolism (cachexia) Many cell types: apoptosis
Interleukin-1 (IL-1)	Macrophages, endothelial cells, some epithelial cells	Endothelial cells: activation (inflammation, coagulation) Hypothalamus: fever Liver: synthesis of acute-phase proteins
Interleukin-12 (IL-12)	Macrophages, DCs	T cells: T_H1 differentiation NK cells, T cells: IFN-γ synthesis, increased cytotoxic activity
Type I interferons (IFN-α, IFN-β)	IFN-α: macrophages; IFN-β: fibroblasts	All cells: antiviral state, increased class I MHC expression NK cells: activation
Interleukin-10 (IL-10)	Macrophages, T cells (mainly regulatory T cells)	Macrophages, DCs: inhibition of IL-12 production and expression of costimulators and class II MHC molecules
Interleukin-6 (IL-6)	Macrophages, endothelial cells, T cells	Liver: synthesis of acute-phase proteins B cells: proliferation of antibody-producing cells
Interleukin-15 (IL-15)	Macrophages, others	NK cells: proliferation T cells: proliferation (memory CD8 cells)
Interleukin-18 (IL-18)	Macrophages	NK cells, T cells: IFN-γ synthesis
Interleukin-23 (IL-23)	Macrophages, DCs	T cells: maintenance of IL-17–producing T cells
Interleukin-27 (IL-27)	Macrophages, DCs	T cells: T_H1 differentiation; inhibition of T_H1 cells NK cells: IFN-γ synthesis
Cytokines of Adaptive Immunity		
Interleukin-2 (IL-2)	T cells	T cells: proliferation; apoptosis; regulatory T-cell development, survival NK cells: proliferation, activation B cells: proliferation, antibody synthesis (*in vitro*)
Interleukin-4 (IL-4)	$CD4^+$ T cells (T_H2), mast cells	B cells: isotype switching to IgE T cells: T_H2 differentiation, proliferation Macrophages: inhibition of IFN-γ-mediated activation Mast cells: proliferation (*in vitro*)
Interleukin-5 (IL-5)	$CD4^+$ T cells (T_H2)	Eosinophils: activation, increased production B cells: proliferation, IgA production (*in vitro*)
Interferon-γ (IFN-γ)	T cells (T_H1, CD8+ T cells), NK cells	Macrophages: activation (increased microbicidal functions) B cells: isotype switching T cells: T_H1 differentiation Various cells: class I and class II MHC molecules, increased antigen processing and presentation to T cells
Transforming growth factor–β (TGF-β)	T cells, macrophages, other cell types	T cells: inhibition of proliferation and effector functions B cells: inhibition of proliferation; IgA production Macrophages: inhibition of activation; stimulation of angiogenic factors Fibroblasts: increased collagen synthesis
Lymphotoxin (LT)	T cells	Recruitment and activation of neutrophils Lymphoid organogenesis

Table 6.1

Selected cytokines of innate and adaptive immunity *(Continued)*

Interleukin-13 (IL-13)	$CD4^+$ T cells (T_H2), NKT cells, mast cells	B cells: isotype switching to IgE Epithelial cells: increased mucus production Fibroblasts: increased collagen synthesis Macrophages: increased collagen synthesis
Interleukin-17 (IL-17)	T cells	Endothelial cells: increased chemokine production Macrophages: increased chemokine and cytokine production Epithelial cells: GM-CSF and G-CSF production
Interleukin-21	T cells (T_H17, T_{FH})	T cells: T_H17 differentiation B cell: affinity maturation and memory cell differentiation

Modified from Abbas, et al. *Cellular and molecular immunology,* 6ed. Saunders/Elsevier, 2010;Table 12-3, p 274:Table 12-5, p 290.

CELLS, TISSUES, AND CELLULAR TRAFFIC IN THE IMMUNE SYSTEM

The proper functioning of the immune system depends on many different cell types, which must recognize foreign substances or microbes in specialized lymphoid tissues and respond by performing effector functions that help destroy or remove the microbes.

Phagocytes

Neutrophils (polymorphonuclear leukocytes) and macrophages, the two major types of phagocytic cells in the body, are crucial effector cells for both innate and adaptive immune responses.[9] Detailed descriptions of these cell types are found in hematology and pathology textbooks (and in Chapter 47). Here, we will summarize their main features related to immune responses. Neutrophils are the most abundant leukocytes in the blood, but are not normally present in any healthy tissue. In responses to signals generated by microbial products and injured cells, neutrophils are rapidly recruited into tissue sites.[10] In contrast, resident macrophages are present in the most healthy tissues of the body at all times, but their numbers increase significantly in response to inflammatory stimuli, due to recruitment of blood monocytes that rapidly differentiate into macrophages once they leave the circulation.[11] The resident macrophages in normal tissues are most abundant in the connective tissues underlying epithelial barriers such as the dermis and lamina propria of the gut. Resident macrophages may assume specialized morphologies and functions in particular organs, such as the alveolar macrophages in the lung, Kupffer cells lining hepatic sinusoids, osteoclasts in bone, and microglia cells in the brain. Resident macrophages perform sentinel and first-responder functions, reacting to pathogens or tissue injury by secreting of cytokines and chemokines that promote further recruitment of blood leukocytes, including neutrophils, and blood monocytes.

A major function of both neutrophils and macrophages is the phagocytic ingestion and killing of microbes. Microbial killing is accomplished by generation of reactive oxygen and nitrogen species through the action of phagocyte-oxidase, myeloperoxidase, and inducible nitric oxide synthase, and by proteolysis of the ingested microbes by several different lysosomal enzymes. Neutrophils also produce cytokines and chemokines, which amplify inflammatory responses. Neutrophils are terminally differentiated, cannot divide, and live only a few hours within tissues. They undergo programmed cell death after ingesting microbes. The critical role neutrophils play in innate immunity is reflected by the devastating infectious consequences that are linked to severe reductions in the absolute blood neutrophil counts seen in leukemia and cancer patients. Tissue macrophages may live for years in tissues, and recently recruited macrophages may divide, and live for weeks to months. In addition to microbial killing, macrophages perform the important functions of ingesting dead cells, including neutrophils and tissue debris, and stimulating tissue repair through the production of cytokines and growth factors. Subsets of macrophages have been described that differ in inflammatory and tissue repair functions.[12,13]

Classically activated macrophages are induced by interferon-γ (IFNγ) secreting T cells, and perform proinflammatory and microbicidal functions. Alternatively activated macrophages are induced by interleukin (IL)-4- and -5-producing T cells, and produce anti-inflammatory cytokines (e.g., IL-10) and growth factors, which promote angiogenesis and fibrosis.

Natural Killer Cells

Natural Killer (NK) cells are bone marrow–derived cells of the innate immune system that arise from the common lymphoid progenitor but do not express highly diverse clonally distributed antigen receptors encoded by somatically rearranged genes, like B and T lymphocytes.[14,15] NK cells make up between 5% and 10% of blood lymphocytes, and are morphologically distinguished as $CD16^+$, $CD56^+$, and $CD3^-$ large granular lymphocytes. NK cells are activated by contact with target cells that are infected or otherwise stressed, leading to release of perforin and granzymes from cytoplasmic granules, which cause the apoptotic death of the target cells. This killing mechanism is identical to the way $CD8^+$ cytotoxic T lymphocytes (CTLs) kill targets, described later. NK cells also secrete interferon γ that promotes inflammation. NK cells distinguish healthy cells from infected or stressed targets by a variety of germline-encoded activating receptors that recognize stress-induced proteins, and inhibitory receptors, such as the killer Ig-like receptors, which recognize class I major histocompatibility complex (MHC) molecules that

Table 6.2

Selected chemokines

Chemokine	Original Name	Chemokine Receptor	Major Function
CC Chemokines			
CCL2	MCP-1	CCR8	Monocyte recruitment and endothelial cell migration
CCL3	MIP-1α	CCR1, CCR5	Mixed leukocyte recruitment
CCL4	MIP-1β	CCR5	T-cell, DC, monocyte, and NK recruitment; Receptor is a HIV coreceptor
CCL5	RANTES	CCR1, CCR3, CCR5	Mixed leukocyte recruitment
CCL11	Eotaxin	CCR3	Eosinophil, basophil, and T_H2 recruitment
CCL17	TARC	CCR4	T-cell and basophil recruitment
CCL19	MIP-3β/ELC	CCR7	T-cell and DC migration into parafollicular zones of lymph nodes
CCL20	MIP-3α	CCR6	T_H17 recruitment
CCL 21	SLC	CCR7	T-cell and DC migration into parafollicular zones of lymph nodes
CXC Chemokines			
CXCL1	GROα	CXCR2	Neutrophil recruitment
CXCL4	PF4	CXC3B	Platelet aggregation
CXCL5	ENA-78	CXCR2	Neutrophil recruitment
CXCL8	IL-8	CXCR1, CXCR2	Neutrophil recruitment
CXCL9	Mig	CXCR3	Effector T-cell recruitment
CXCL10	IP-10	CXC3, CXCR3B	Effector T-cell recruitment
CXCL11	I-TAC	CXC3	Effector T-cell recruitment
CXCL12	SDF-1$\alpha\beta$	CXCR4	Mixed leukocyte recruitment; CXCR4 is a HIV coreceptor
CXCL13	BCA-1	CXCR5	B cell migration into follicles
CX3C Chemokines			
CX3CL1	Fractalkine	CX3CR1	T-cell, NK cell, and macrophage recruitment; CTL and NK cell activation

Modified from Abbas, et al. *Cellular and molecular immunology*, 6th ed. Saunders/Elsevier, 2010;Table 12.4 p 280.

are present in most healthy cells. A lack of class I MHC, often termed "missing self," will result in NK cell activation due to a lack of inhibitory signals.

Dendritic Cells

DCs are ubiquitous bone marrow–derived cells that play a fundamental role in immune surveillance.[16–18] They are constitutively present in many tissues but most abundant beneath epithelial barriers (skin, bronchopulmonary, gastrointestinal, genitourinary) and in lymphoid tissues (lymph nodes, spleen). They are named for their morphology in tissues, where they typically display dendrite-like projections that interdigitate between other cells, a feature that maximizes their potential contact with pathogens. DCs perform effector functions of the innate immune system in response to microbial infection, and are essential for initiating adaptive immune responses involving T cells. Two main subsets of DCs, identifiable by cell surface molecules, are conventional DCs and plasmacytoid DCs (pDCs). Conventional DCs are phagocytic cells that take up pathogens or microbial products in infected tissues and transport them into draining lymph nodes for display to T lymphocytes. This function is called antigen presentation, which we will discuss in more detail later in the chapter. Plasmacytoid pDCs are so named because they look like antibody-producing plasma cells but are otherwise unrelated. pDCs respond to viral nucleic acids by secreting abundant type I interferons, which are cytokines that make cells resistant to viral replication.[19] Activation of pDCs by self nucleic acids and resulting abundant type I interferon secretion contributes to the pathogenesis of systemic lupus erythematosus.

Lymphocytes

Lymphocytes are the principal cells responsible for the defining characteristics of adaptive immunity, namely, extraordinary diversity of antigen specificities, specialization of functions that

adapt to different types of microbial infections, and memory. The majority of lymphocytes are located within lymphoid tissues and epithelial barrier tissue such as skin and gut. There is constant trafficking of between these tissues and the circulation, where they number 1–3 × 10^3/mm^3. In blood smears, resting lymphocytes appear as relatively homogeneous round cells with a high nuclear:cytoplasmic ratio and a diameter of approximately 7 μM. This uniform morphologic appearance does not reflect the existence of multiple lymphocyte subsets.

There are several subsets of lymphocytes (see Table 6.3). The two major types are B cells and T cells, typically distinguished by flow cytometric or immunohistochemical detection of lineage-specific surface molecules. B cells are the only cells that produce antibodies. Most B cells in blood and lymphoid tissues express membrane Ig, CD19, and CD20. Most T cells express CD3. Developmentally distinct subsets of B cells include follicular B cells, the most abundant and diverse type, and marginal zone B cells, which have a limited range of specificities. T cells have greater developmental and functional heterogeneity than B cells. The two numerically major subsets of T cells are distinguished by the expression of either CD4 or CD8, which correlate with helper or cytotoxic functions, respectively. Together $CD4^+$ and $CD8^+$ T cells account for approximately 85% to 90% of all T cells, and normally the $CD4^+$:$CD8^+$ T-cell ratio in blood and lymphoid organs is about 2. Among $CD4^+$ T cells, there are several functional subsets, including Th1, Th2, Th17, and regulatory T cells. Other numerically minor subsets of T lymphocytes cell are NKT cells and γδT cells.

Both B and T lymphocytes are also categorized based on their state of functional differentiation, which is related to their prior experience with antigen. B and T lymphocytes that have completed their maturation but have not ever been activated by antigen in the peripheral immune system are called *naïve lymphocytes*. Naïve B and T cells cannot perform protective functions, but they respond to antigen stimulation in lymphoid organs by clonal expansion and differentiation into *effector*

Table 6.3

Types of lymphocytes

Class	Functions	Antigen Receptor and Specificity	Selected Markers	Percent of Total Lymphocytes		
αβ T Lymphocytes				*Blood*	*Lymph node*	*Spleen*
$CD4^+$ helper T lymphocytes (T_H)	B-cell differentiation (humoral immunity) Macrophage activation (cell-mediated immunity)	αβ heterodimers Diverse specificities for peptide-class II MHC complexes	$CD3^+$, $CD4^+$, $CD8^-$	50–60	50–60	50–60
$CD8^+$ CTL	Killing of infected cells, killing of tumor cells	αβ heterodimers Diverse specificities for peptide-class I MHC complexes	$CD3^+$, $CD4^-$, $CD8^+$	20–25	15–20	10–15
Regulatory T cells (Treg)	Suppress other T cells (regulation of immune responses, self-tolerance)	αβ heterodimers	$CD3^+$, $CD4^+$, $CD25^{hi}$, $CD127^{lo}$	Rare	10	10
γδ T lymphocytes	Helper and cytotoxic functions (innate immunity)	γδ heterodimers Limited specificities for peptide and nonpeptide antigens	$CD3^+$, CD4, and CD8 variable			
B lymphocytes	Antibody production	Surface antibody Diverse specificities for all types of molecules	Fc receptors; class II MHC; CD19; CD20; CD21	10–15	20–25	40–45
NK cells	Killing of virus-infected or damaged cells (innate immunity)	Various activating and inhibitory receptors Limited specificities for MHC or MHC-like molecules	CD16 (Fcγ receptor)	10	Rare	10
NKT cells	Suppress or activate innate and adaptive immune responses	αβ heterodimers (Limited specificity for lipid-CD1 complexes)	CD16 (Fcγ receptor); CD3	10	Rare	10

(Modified from Abbas, et al. *Cellular and molecular immunology*, 6th ed. Saunders/Elsevier, 2010. Table 3.2 p 50).

lymphocytes, which are relatively short-lived cells that actively contribute to elimination of antigen. After most microbial antigen-specific B- and T-cell responses wane, a population of long-lived progeny of the expanded lymphocyte clones persists as *memory cells*, which mediate rapid and specialized responses to subsequent exposures to the microbial antigens. Memory lymphocytes can survive for many years, and probably undergo a low level of antigen-independent proliferation to maintain their numbers.[20–22] *In vitro* assays for recall responses to antigens after infection or immunization indicate that most memory T cells express the CD45RO surface protein, while naïve T cells, such as found in neonates, are mostly CD45RA$^+$. Memory T cells also express the IL-7 receptor. Effector T cells are usually localized within lymphoid organs or in infected tissues, and only rarely are they numerous in the blood, such as during acute EBV infections when they appear as lymphoblasts. Specific details of T- and B-cell antigen recognition, subsets, and effector function will be discussed later in this chapter.

Mast Cells, Basophils, and Eosinophils

These three cell types all have numerous cytoplasmic granules whose contents are released upon activation, and perform various proinflammatory and microbicidal functions.[23,24,25] Mast cells are bone marrow–derived, stationary tissue cells ubiquitously present adjacent to small blood vessels in most epithelial tissue. Their metachromatically staining granules contain several vasoactive amines such as histamine. Mast cells express high-affinity receptors for IgE antibodies, and cross-linking of the IgE by allergens causes activation of the cells, including granule release, and secretion of newly synthesized cytokines and eicosanoids. Mast cells are major contributors to allergic reactions and defense against parasites. Basophils share most of the properties just described for mast cells, except they are circulating cells of the myeloid lineage, which may be recruited to inflammatory sites. Their role in innate immunity is uncertain, in part because their numbers are low. Eosinophils are circulating granulocytes with eosinophilic granules containing major basic protein and proteolytic enzymes. Eosinophils are recruited to selective inflammatory sites, such as tissue with parasitic worm infections or sites of allergic inflammation. Their granule contents are toxic to helminths and are responsible for tissue damage and remodeling in chronic atopic diseases, such as asthma.

Lymphoid Organs and Tissues

Lymphocyte development, the process by which progenitor stem cells give rise to mature naïve lymphocytes, occurs in the primary or generative lymphoid organs, namely, the bone marrow and thymus. This process is distinct from differentiation of naïve lymphocytes into effector lymphocytes, which occurs in response to antigen recognition in secondary or peripheral lymphoid organs, including lymph nodes, spleen, and mucosal lymphoid tissues.

Bone Marrow and Thymus

The predominant sites of lymphocyte development are the bone marrow for B cells and thymus for T cells. The hematopoietic stem cell gives rise to a common lymphoid progenitor cell, from which both B- and T-cell lineages are derived.[26,27] The cytokine IL-7 is required for these early steps in lymphocyte development, and antigen receptors play important roles at later stages. Most of the steps of B-cell development take place in the bone marrow, but final differentiation into follicular B cells occurs in lymph nodes or spleen.[28] In contrast, the early progenitors of the T-cell lineage migrate to the thymus and T cells fully develop in that organ. The bone marrow is also the main site where long-lived antibody-producing plasma cells reside; these cells migrate to the marrow from secondary lymphoid organs.

The thymus is a prominent anterior mediastinal organ in children, which involutes at puberty. T-cell development does continue in adults, as shown in adult bone marrow transplant recipients who produce T cells derived from donor stem cells. The site of adult T-cell development is likely in small thymic remnants that persist after thymic involution. The thymic stroma is organized into an outer cortex and inner medulla. Developing T cells, called thymocytes, move from cortex into medulla as they mature. Thymic epithelial cells are stromal cells that produce cytokines such as IL-7 that promote T-cell development. These epithelial cells and bone marrow–derived DCs and macrophages display self-proteins, which positively or negatively select clones of developing T cells and thereby shape the repertoire of antigen specificities of mature T cells.[29] Further details of thymic structure and their relationship to T-cell development can be found in immunology textbooks.[30,31]

Lymph Nodes and Spleen

The lymph nodes and white pulp of the spleen serve as antigen-collecting sites for tissues and blood, respectively. Naïve B and T cells circulate through these organs and survey for the antigens they can recognize. A complex microarchitecture is maintained in both lymph nodes and spleen, such that B cells are localized in follicular structures and T cells are kept in separate interfollicular zones. This segregation is due to the action of constitutively secreted chemokines and different chemokine receptors on each type of lymphocyte. Lymphotoxins (LTs) produced by lymphoid tissue inducer cells are required for initial development of lymphoid tissues.[32,33] The chemokine CXCL13 and its receptor CXCR5 keep B cells in the follicles, and the chemokines CCL19 and CCL21 and their receptor CCR7 keep naïve T cells and DCs in the interfollicular zones.[34] Lymph nodes are encapsulated organs that are supplied with interstitial fluid (lymph) via afferent lymphatics, which empty into a subcapsular sinus. Some antigens are carried by lymph through specialized conduits composed of extracellular matrix proteins and lined by fibroblastic reticular cells (FRCs), into the interfollicular zones.[35] Resident DC antigen-presenting cells (APCs) take up the lymph-borne antigens in these conduits. DCs carrying antigens from the tissues are also delivered to nodes via the lymphatics and migrate into the interfollicular zones. Antigens in the subcapsular sinus gain access to underlying B-cell follicles with the help of macrophages. Lymph drains from lymph nodes via an efferent lymphatic, which may drain into another lymph node, or into larger lymphatics that eventually empty into the bloodstream via the thoracic duct. Naïve lymphocytes enter lymph nodes through an efferent artery and migrate out into the parenchyma through high endothelial venules (HEVs), which are located in the interfollicular zone where DCs are located. Interactions of DCs, T cells, and B cells during immune responses are described later in the chapter.

The immune functions of the spleen are to remove opsonized microbes and immune complexes from the blood, which is accomplished by macrophages lining the sinusoids in the red pulp, and to initiate adaptive immune responses to blood-borne

antigens, which occurs in the white pulp.[36] T cells in the white pulp are located in sheaths around small branches of the splenic artery, and B-cell follicles are arranged adjacent to the T-cell zones. FRC conduits like those in lymph nodes traverse the follicles. The anatomic arrangement of B cells and T cells in the splenic white pulp optimizes antibody responses.

Mucosal Lymphoid Tissues and Regional Immune Systems

Discrete collections of B cells, T cells, DCs, and macrophages found beneath mucosal epithelial barriers play important roles in the specialized immune responses that defend against microbes that traverse these barriers.[37] The best-defined mucosal lymphoid tissues are in the gut, and include Peyer patches in the lamina propria of the small bowel, and similar structures in the appendix and colon. Other mucosa lymphoid tissues are the tonsils and adenoids in the oropharynx, and lymphoid aggregates in the bronchial mucosa. These lymphoid tissues serve specialized functions, such as the generation of B cells that produce IgA antibodies, which are secreted into the lumen of the gut and defend against luminal pathogens. Another specialized function of these tissues is to sample antigens of commensal organisms that are present in enormous numbers outside the mucosal barriers and to induce regulatory mechanisms that prevent potentially harmful responses to those organisms. Immunologists often speak of "regional immune systems" with specialized functions, including the gastrointestinal-associated lymphoid tissues (GALT) and the skin immune system. In fact, based on numbers of lymphocytes located in these tissues, the skin and GALT represent the largest parts of the human adaptive immune system and defend against the largest and most common portals of microbial entry into the body.

Cellular Traffic in the Immune System

Microbial invasion and infections can occur anywhere in the body, and the innate immune system deals with this widespread unpredictable threat by the ubiquitous presence of immunocompetent cells in all tissues, and the rapid delivery of additional cells from the circulation. In contrast, the adaptive immune system produces small numbers of millions of different clones of lymphocytes before exposure to microbial antigens, but a particular microbe-specific clone must be activated by specialized APCs before an effective response can be generated. Thus, the initiation of adaptive immune response requires delivery of lymphocytes, APCs, and antigen to common sites within lymphoid tissues. Within lymphoid tissues, subsets of lymphocytes and APCs must be highly motile to maximize the chances of productive physical interactions. Basic features of whole-body and local immune cell trafficking are described below.

Leukocyte Interactions with Endothelial Cells and Migration from Blood into Tissues

Circulating leukocytes, including lymphocytes and myeloid cells, must interact with the endothelial lining of blood vessels in order to migrate into the tissue sites where they perform their effector functions.[38] This process is regulated by several factors, including flow characteristics of tissue microvasculature, adhesion molecules on both the leukocytes and endothelial cells, and chemokines produced by endothelial cells and tissue cells, which act on the leukocytes (see Table 6.2). Virtually all leukocyte extravasation occurs in postcapillary venules, which have an average diameter of 10 to 15 μM, and flow characteristics that promote margination of leukocytes to the periphery of the blood stream where they interact physically with endothelial cells. The adhesion molecules involved in the process include integrins on the leukocytes, selectins on the cell surfaces of leukocytes and endothelial cells, selectin ligands on leukocytes and endothelial cells, and integrin ligands on the endothelial cells. Chemokines involved in this process are displayed on the surface of endothelial cells and bind chemokine receptors on the leukocytes (see Table 6.2).[39] Extravasation of all types of leukocytes follows a sequential set of steps, referred to as the multistep adhesion cascade, during which leukocytes bind to endothelial cells at appropriate locations, resist the fluid shear stress caused by the blood flow, and exit the vessel. The specific chemokines and adhesion molecules will vary depending on the leukocyte type and tissue site. The sequence of the adhesion cascade begins with low-affinity adhesive interactions and leukocyte rolling on endothelium mediated by P- and E-selectin expressed on activated endothelial cells, which bind to glycoprotein selectin ligands on leukocytes, and L-selectin expressed on leukocytes, which bind ligands on endothelial cells. The next step is activation of leukocyte integrins by chemokines. Chemokines that are produced by phagocytes and endothelial cells in infected or damaged tissues, or are constitutively produced in lymphoid organs, are displayed on the luminal endothelial surface by heparan sulfate glycosaminoglycans, where they can bind to chemokine receptors on rolling leukocytes. Signals generated by the chemokine receptors induce conformational changes in the integrins that enhance their affinity for integrin ligands on the endothelial cells. The next step is stable arrest of leukocytes on endothelium mediated by activated integrins. Leukocyte integrins include leukocyte function-associated antigen (LFA)-1 and Mac-1, which bind to intercellular adhesion molecule (ICAM)-1 on endothelial cells, and very late activation antigen (VLA)-4, which binds to endothelial vascular cell adhesion molecule (VCAM)-1. High-affinity binding of integrins to their endothelial ligands results in cessation of rolling, firm adhesion, and resistance to dislodgement by fluid shear stress. Finally, the leukocytes transmigrate between endothelial cells and out into tissues, a process that requires leukocyte LFA-1 and endothelial ICAM-1, and CD31 expressed on both the leukocyte and the junctional membrane of endothelial cells. VE-cadherin and associated junctional proteins that bridge postcapillary endothelial cells must be temporarily modified to permit passage of the transmigrating leukocytes. Leukocytes may also pass through endothelial cells by a poorly understood process called transcellular migration. Antibody blockade of leukocyte integrins is in clinical use for treatment of inflammatory bowel disease and multiple sclerosis.

Migration of Granulocytes and Monocytes into Infected or Damaged Tissue

Myeloid cells, including granulocytes and monocytes, follow relatively simple traffic patterns after maturing in the bone marrow and entering the circulation. If they are recruited into a tissue inflammatory site, they perform their effector functions, and die at these sites by apoptosis within 1 to 2 days; they do not return to the blood. If they are not recruited into tissues,

blood neutrophils will become senescent in 4 to 5 days, at which time they are recruited into the bone marrow by the chemokine CXCL12, and undergo apoptosis. Blood monocytes live on average 8 to 10 days, but the mechanisms of their clearance are not well defined. Macrophages derived from monocytes may live for many days up to months.

Among blood leukocytes, neutrophils are the first and most numerous responders to infections and tissue injury.[10] Innate response to microbes and injured cells (described later in this chapter) induces the expression of the cytokines IL-1 and TNF by tissue resident macrophages, which stimulate endothelial cells in nearby postcapillary venules to upregulate Sho E-selectin, ICAM-1, and in some tissues VCAM-1. Innate immune responses to infection and tissue injury also induce the local expression of chemokines of the GRO family and CXCL-8, which bind to chemokine receptors on neutrophils, including CXCR1 and CXCR2. In addition, thrombin, generated by the coagulation cascades, acts via thrombin receptors on endothelial cells to mobilize P-selectin from Weibel-Palade bodies to the cell surface. These local responses to infection and injury promote the recruitment of neutrophils, via the multistep cascade, as part of the acute inflammatory response. The recruitment of eosinophils depends on selectins and integrins, but also requires local production of a combination of chemokines, including CCL7, CCL14, and CCL11, which bind to receptors expressed by these specialized granulocytes.

Monocytes are recruited along with neutrophils to sites of acute inflammation, but also to sites of effector T-cell activation and chronic inflammation. In both cases, the monocytes differentiate into macrophages. Monocyte recruitment into tissues involves rolling interactions mediated by endothelial P-selectin and its ligand PSGL-1 on the monocyte as well as L-selectin on the monocyte binding to ligands on endothelial cells. Integrin-mediated stable arrest of monocytes is mediated by VLA-4:VCAM-1 and LFA-1:ICAM-1 interactions. Blood monocytes are heterogeneous, and at least two populations can be distinguished by the chemokine receptors they express.[40] CCR2 expression defines monocytes that are rapidly recruited to sites of infection or injury, in response to the chemokine CCL2 (MCP-1). Another monocyte population lacks CCR2, but expresses CX3CR1, which binds to CX3CL1. Endothelial cells express membrane-bound CX3CL1, which mediates adhesion of $CX3CR1^+$ monocytes.

Lymphocyte Migration into and out of Lymphoid Tissues

Lymphocyte traffic throughout the body is more complex than that of myeloid cells, with a constant movement of cells between the blood and lymphoid tissues, and migration of effectors cells from lymphoid tissues via the blood to infected tissues.[41] These traffic patterns reflect the requirement that rare naïve lymphocytes of one clone and antigen specificity must continuously travel through lymphoid tissue sites of antigen collection in order for effective immune surveillance to take place. Furthermore, many types of effector lymphocytes generated from naïve cells in lymphoid tissues must be delivered by the blood to tissue sites of infection in order to perform their functions. Lymphocyte traffic is best understood for T cells, although some of the patterns and mechanisms are similar for B cells.

Lymphocyte recirculation refers to the movement of naïve lymphocytes from blood into secondary lymphoid tissues, especially lymph nodes, and then back into the blood.[41] Entry of naïve T cells into lymph nodes occurs through specialized postcapillary venules called high endothelial venules (HEVs) because of the tall appearance of their endothelial lining. HEVs express ICAM-1, and a ligand for L-selectin called peripheral lymph node addressin. HEVs also display the chemokines CCL19 and CCL21 made by the HEV endothelial cells and nearby stromal cells, respectively. Naïve T lymphocytes express ICAM-1, L-selectin, and the chemokine receptor CCR7, which binds CCL19 and CCL2. Thus naïve lymphocytes and HEVs have matching molecules that allow for efficient constitutive homing of the lymphocytes into the lymph node parenchyma. Importantly, HEVs are located in the T-cell zone of lymph nodes where DCs displaying antigen are also located. Naïve T cells do not express functional selectin ligands or chemokine receptors that bind inflammatory chemokines produced at sites of infection, and thus they do not enter these sites. If a naïve T cell does not become activated by antigen presentation within a few hours after entering a lymph node, it leaves via efferent lymphatics, and may move to an adjacent lymph node in the same chain. Eventually the naive T cells reenters the blood via the thoracic duct to begin the recirculation cycle over again. A single naïve lymphocyte may visit up to dozens of lymph nodes in a single day.

If a naïve T cell does become activated by antigen in a lymph node, it is retained, and undergoes proliferation and differentiation into effector T cells. Effector T cells do not express CR7 or L-selectin, but express other chemokine receptors and adhesion molecules that enable them to migrate differently from naïve lymphocytes. Some effector T cells that function as helper T cells for antibody responses express the chemokine receptor CXCR5, which binds CXCL13 and directs migration toward B-cell follicles. Other effector T cells will leave the node via efferent lymphatics, and enter the blood.

The exit of naïve and effector lymphocytes from lymphoid organs requires the expression of sphingosine-1-phosphate receptor 1 (S1PR1) and a stable low-to-high concentration gradient of its chemotactic ligand sphingosine-1-phosphate (S1P) between lymphoid tissues and the draining lymph or blood.[42] This gradient is maintained by S1P lyase degradation of S1P within the lymphoid tissues. Entry and exit of naïve and effector T cells from lymph nodes are regulated by dynamic changes in surface S1PR1 expression so that retention time within the lymph node is sufficient for naïve T-cell surveillance of APCs, and for clonal expansion and differentiation of effector cells. The immunosuppressive drug Fingolimod is an S1PR1 ligand that blocks S1P-regulated T-cell egress.

Lymphocyte Migration into Inflamed Tissues

Effector T cells that are generated in lymph nodes and then enter the blood express different combinations of chemokine receptors, integrins, and P- and E-selectin ligands, which promote their migration into different tissue sites of inflammation.[38] As is the case for neutrophils and monocytes, effector lymphocyte migration into these sites is promoted by local innate immune responses, which lead to upregulation of endothelial adhesion molecules, and the production of chemokines. Different subsets

of effector T cells have different migratory phenotypes, which are responsible for their selective migration into different types of inflammatory site tissues or dictate the timing of their appearance at a single site. For example, Th17 cells express CXCR6 and home to inflammatory sites where CCL20 is produced, while Th1 cells express CXCR3 and CCR5, and home to sites where CCL5 and CXCL10 (and other chemokines) are produced. Effector T and B cells may also selectively home to one anatomic location versus another, depending on where they were originally generated from naïve cells. For T cells, tissue-specific homing is imprinted by products of the antigen-presenting DCs from a particular tissue. For example, skin-derived DCs secrete vitamin D, which will instruct naïve T cells to differentiate into effector T cells that express CCR10 and E-selectin ligands that promote their homing back to the skin. Gut DCs secrete retinoic acid, which instructs naïve T cells to differentiate into effector T cells that express the $\alpha 4\beta 7$ integrin and CCR9 that promote their homing to the gut lamina propria.

INNATE IMMUNITY

The innate immune system is the collection of molecules and cells that provide early defense against a broad range of infectious organisms, before adaptive immune responses develop. Components of the innate immune system are present and functional at all times, and unlike adaptive immunity, responses are usually of similar magnitude and quality, regardless of prior history of exposure to the inciting microbe. The innate immune system recognizes and responds to only a limited number of commonly encountered molecular stimuli originating from microbes or damaged tissue cells, and cannot distinguish between and respond to millions of different foreign antigens as the adaptive immune system can. The microbial products that stimulate innate immunity are called pathogen-associated molecular patterns (PAMPs), and are typically molecules made by many species of the same class of microbes, and are essential for microbial survival, such as bacterial cell wall components or genomic nucleic acids. Hence microbes cannot evade innate immunity by loss of expression of PAMPs. Self-molecules that are only produced by and/or released from injured or stressed cells (which simulate innate immune responses) are called damage-associated molecule patterns (DAMPs). Innate responses to PAMPs and DAMPs are essential to prevent lethal infections from the ubiquitous microorganisms in our environment. Molecules induced by innate immune responses are also required, in addition to antigens, to activate naïve lymphocytes and initiate adaptive immunity. Thus adaptive immune responses only occur in response to two signals, namely, danger (indicated by the innate response) and foreign molecular structures (antigen).

Cells of the Innate Immune System

The cells of the innate immune system include phagocytic cells (neutrophils, macrophages, DCs), NK cells, and mast cells, which were described earlier in the chapter. In addition, epithelial barriers (skin and mucosal linings of the gastrointestinal, brochopulmonary, and genitourinary systems) are considered to be integral to the innate immune system. Mucosal epithelial cells secrete mucus, which impedes microbial contact with the barrier, as well as antimicrobial peptides, including defensins and cathelicidins, which damage bacterial membranes.

Cellular Pattern Recognition Receptors of the Innate Immune System

Many cell types express receptors that recognize PAMPs or DAMPs and transduce signals that activate the cells to produce proinflammatory and antimicrobial molecules.[43] As a group, these receptors are called pattern recognition receptors (PRRs), but PRRs include several types of receptors that are structurally distinct and recognize different classes of PAMPs or DAMPs. Phagocytes express the widest variety of these receptors, reflecting their principal function of ingesting microbes and damaged cells. Nonetheless, PRRs on barrier epithelial cells, lymphocytes, mast cells, endothelial cells, and other cell types all contribute to innate immunity. PRRs are located on the cell surface, in endosomal membranes and in the cytosol, which ensures that the innate immune system can respond to extracellular ligands, phagocytosed ligands, and ligands synthesized or delivered to the cytosol. The different types of PRRs are discussed below.

Toll-like Receptors

Toll-like receptors (TLRs) are a family of evolutionarily conserved homologous integral membrane proteins, widely expressed on many cell types, which recognize structurally diverse PAMPs and DAMPs, and engage signaling pathways that activate cells.[44] There are nine functional human TLRs, all of which are expressed as homo- or heterodimers (FIGURE 6.1). Each TLR chain includes an extracellular ligand recognition domain with multiple leucine-rich repeat motifs, and a cytoplasmic tail with a Toll/ IL-1 receptor (TIR) signaling domain, also found in the receptors for the cytokines IL-1 and IL-18. Some TLRs are expressed on the cell surface and recognize bacterial cell wall products such as lipopolysaccharide (LPS) (TLR4) and peptidoglycan (TLR 2), bacterial lipopeptides (TLRs 1, 2, 6), bacterial flagellin (TLR5), fungal beta glucans (TLR2), and host heat shock proteins (TLRs 2, 4). Some TLRs are located in endosomal membranes and recognize microbial (and sometimes host) nucleic acids including dsRNA (TLR3), ssRNA (TLR7), and demethylated CpG repeats (TLR9). Upon ligand binding to the TLRs, the TIR domains associate with either of two adaptor proteins, MyD88 or TRIF, which then engage downstream signaling molecules leading to cellular activation. There are two main pathways of cellular activation. The MyD88 pathway (TLRs 1, 2, 4, 5, 7, 9) activates the transcription factor NF-κB, which promotes expression of proinflammatory genes, including those encoding cytokines such as TNF and IL-1, chemokines, and endothelial adhesion molecules. Other molecules induced by the TLR-MyD88-NF-κB pathway include defensins in mucosal epithelial cells, and T-cell costimulators on DCs. TLR4 and TLR3 use the TRIF pathway, which leads to activation of interferon response factors (IRF3, 7), which in turn promote expression of type I interferons. These cytokines are secreted and act on other cells to induce an "antiviral" state, which is mediated by proteins that interfere with viral replication. TLR signaling also activates the AP-1 transcription factor, and there are crossover effects of MyD88 and TRIF pathways on IRF and NF-κB activation, respectively.

Cytosolic PRRs: RIG-like Receptors, NOD-like Receptors, and Inflammasomes

RIG (retinoic acid–inducible gene)-like receptors (RLRs), including RIG-I and MDA5 (melanoma differentiation–associated gene 5), are cytosolic receptors for viral RNAs, which

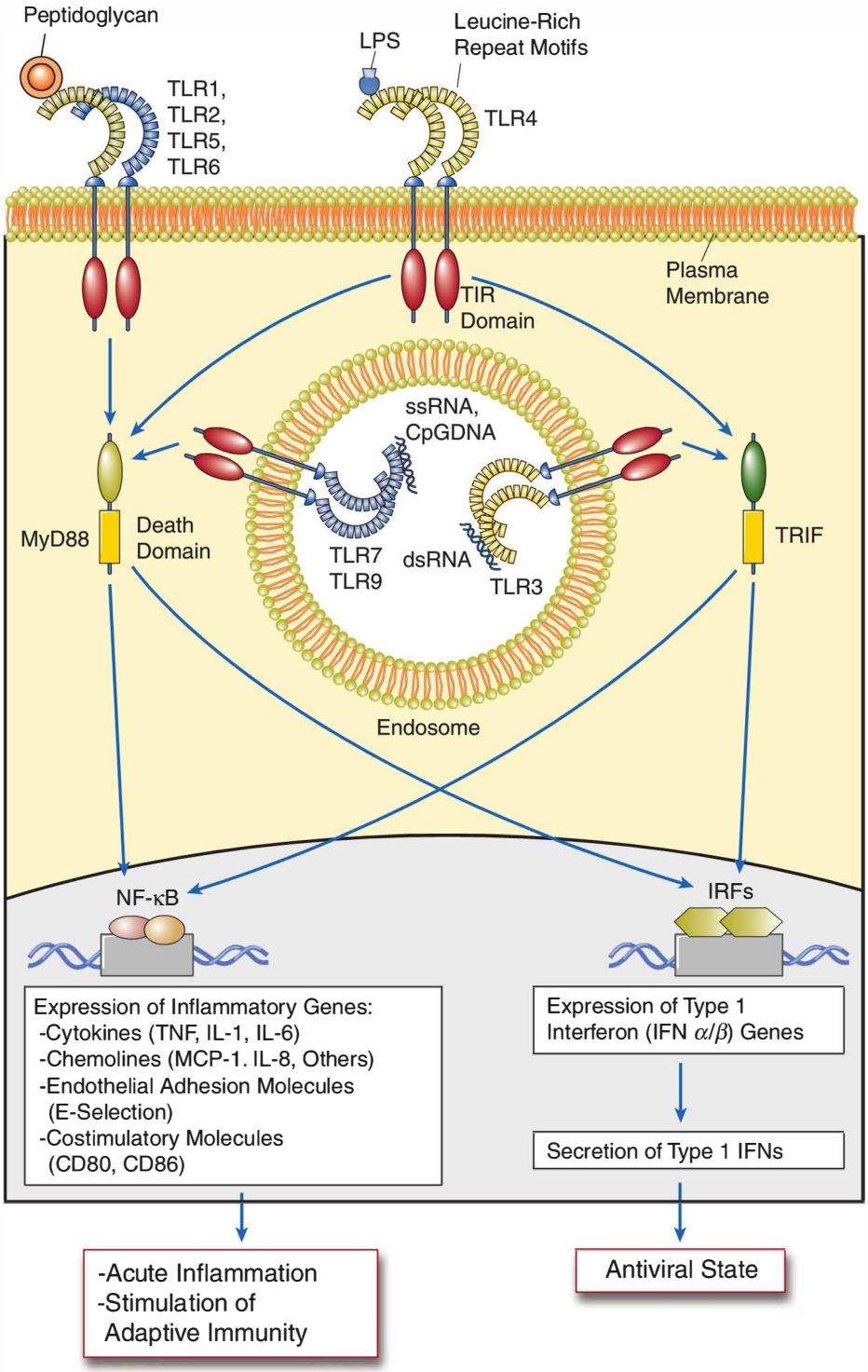

FIGURE 6.1 TLRs. TLRs are the major PRRs of innate immunity. They contain ligand binding domains with multiple leucine rich repeats, and cytoplasmic tails with TIR domains that interact with adaptor proteins. TLRs are expressed on the plasma membrane where they may bind extracellular PAMPs and on endosomal membranes where they recognize nucleic acids of microbes that have been phagocytosed by cells. TLRs 1, 2, 5, and 6 use the adapter protein MyD88 and activate the transcription factors NF-κB and AP-1, leading to inflammatory responses. TLR3 uses the adaptor protein TRIF and activates the IRF3 and IRF7 transcription factors, leading to type I interferon secretion and antiviral immunity. TLR4 can activate both pathways. TLRs 7 and 9 in the endosome use MyD88 and activate both NF-κB and IRF7 (not shown). Cells also contain cytoplasmic sensors of microbial infection (discussed in the text), including the NLRs and RLRs. (Modified from Abbas, et al. *Cellular and molecular immunology*, 7th ed. Philadelphia: Saunders/Elsevier, 2011; Figure 4.3 p. 62).

assemble signaling complexes that activate either NF-κB or IRFs, leading to inflammatory and antiviral responses (Hornung, 2010 #51).

NOD-like receptors (NLRs) are a large family of cytoplasmic proteins that function as PRRs for PAMPs and form signaling complexes that induce inflammatory responses.[45] The ligand recognition domain of these proteins contains leucine-rich repeats, similar to TLRs, and the signaling complexes that form upon ligand binding activate NF-κB. NOD-1 and NOD-2, two of the best studied NLRs, are expressed in the cytoplasm of phagocytes and mucosal epithelial cells, where they recognize peptidoglycans of bacterial cell walls. NOD-2 is particularly abundant in Paneth cells of the intestine, and its ligation in these cells induces secretion of defensins. Polymorphisms in the NOD-2 gene are associated with increased risk of inflammatory bowel disease.

Inflammasomes are NLR-containing multiprotein complexes that assemble in the cytoplasm of cells in response to various PAMPs and DAMPs and catalyze the posttranslational processing of pro-IL-1β and pro-IL-18 into active proinflammatory cytokines IL-1β and IL-18.[46] In the best characterized inflammasome, the recognition component is part of an NLR family protein called NLRP3. In response to cytosolic microbial products or endogenous molecules indicative of cell injury, the NLRP3 proteins undergo oligomerization and recruitment of adaptor proteins and the inactive precursor of the protease caspase-1. The recruited caspase-1 becomes autoactivated and is then able to generate active IL-1β and IL-18. Most inflammasome activity, and therefore most IL-1 secretion, is attributable to macrophages. The stimuli that activate the NLRP3 inflammasome include a diverse array of microbial structures, including flagellin, muramyl dipeptide, LPS, bacterial pore forming toxins, and microbial RNA. Sterile inflammatory responses after tissue infarction are driven by activators of the inflammasome that are released by necrotic cells.[47] In addition, a variety of DAMPs activate the inflammasome, including intracellular crystals of sodium urate and cholesterol, extracellular ATP, and decreases in cytosolic potassium ion. Excessive activation of the inflammasome and excessive IL-1 secretion can cause a pathologic inflammation and tissue injury. This occurs in response to various phagocytosed crystals such as asbestosis, or sodium urate when there is excessive cell death or altered uric acid metabolism, such as in gout. These crystals likely damage phagasome membranes, causing the release of substances into the cytosol that interact with NLRP3. Autoinflammatory diseases, such as familial periodic fevers, are due to genetic mutations in components of the NLRP3 inflammasome, and IL-1 receptor antagonists or anti-IL-1 reagents are effective in treating these disorders.

Other Cell Surface Pattern Recognition Receptors

A variety of other cell surface receptors play important roles in innate immune defense against microbes. Some of these receptors belong to the C-type lectin family, and recognize common microbial carbohydrate structures not found in mammalian cells.[48,49] The mannose receptor (CD206) on macrophages binds to terminal D-mannose residues on prokaryote carbohydrates, and promotes microbial phagocytosis. Dectin-1 and -2 are expressed by DCs and bind fungal β-glucan and high-mannose oligosaccharides, respectively. Ligand binding to dectins induces signals that alter the DCs so they promote helper T-cell differentiation into Th17 cells, which are particularly effective in antifungal adaptive immunity. Scavenger receptors on macrophages including SR-A and CD36 bind a wide variety of PAMPs, including LPS, lipoteichoic acid, nucleic acids, and fungal carbohydrates.[50] Scavenger receptors enhance phagocytosis of microbes, and CD36 is a coreceptor for TLR2/6 recognition of a variety of ligands. Neutrophils and macrophages express *N*-formyl Met-Leu-Phe receptors, which are G protein–coupled receptors (GPCR) that recognize bacterial *N*-formylmethionyl peptides. Signals induced by these receptors stimulate chemotaxis of the phagocytes.

Soluble Pattern Recognition Receptors and Effector Molecules of the Innate Immune System

The innate immune system utilizes several PRRs that are present in blood and extracellular fluids, in order to defend against extracellular microbes. Three major functions of these molecules are to opsonize microbes so they are readily ingested by phagocytes, to directly kill microbes, and to enhance inflammatory responses that deliver phagocytes and more soluble effectors molecules to sites of infection. The major features and functions of these soluble components of innate immunity are described below.

Natural Antibodies

Although antibody responses are generally considered a fundamental part of adaptive immunity, there are B lymphocyte subsets, such as marginal zone B cells, that produce antibodies with only a limited repertoire of specificities and without clear-cut exposure to foreign antigens. These natural antibodies are specific for PAMPs and DAMPs, and therefore are typical of other PRRs of the innate immune system. Most natural antibodies are of the IgM subclass, do not require T-cell help for their production, and bind carbohydrates or lipids. Many natural antibodies are specific for head groups of oxidized phospholipids, including lysophosphatidylcholine and phosphorylcholine, typically exposed on bacterial membranes and apoptotic cells.[51] The anti-ABO blood group natural antibodies, which recognize glycolipid blood group antigens on blood cells and endothelium, are the cause of thrombotic complications in the setting of allografts and transfusions.

Complement

The complement system includes plasma proteins that are activated to generate proteolytic cascades, yielding products with various proinflammatory and antimicrobial functions.[52] The central and most abundant complement protein is C3. There are three pathways of complement activation. The classical pathway is activated by immune complexes of IgM or IgG bound to antigen and by pentraxins. The lectin pathway is activated by microbial surface carbohydrates. The alternative pathway is activated by spontaneous hydrolysis of C3 at microbial surfaces (FIGURE 6.2). The recognition molecules that initiate the classical and lectin pathways are C1q and mannose-binding lectin (MBL), respectively. Each of the three pathways forms an enzymatic complex called C3-convertase that cleaves C3 to form C3a and C3b. C3a is a small peptide that binds a specific GPCR on phagocytes, mast cells and endothelial cells, and has chemotactic and proinflammatory effects. C3b covalently binds to microbial surfaces or antigen–antibody complexes, and acts as an opsonin

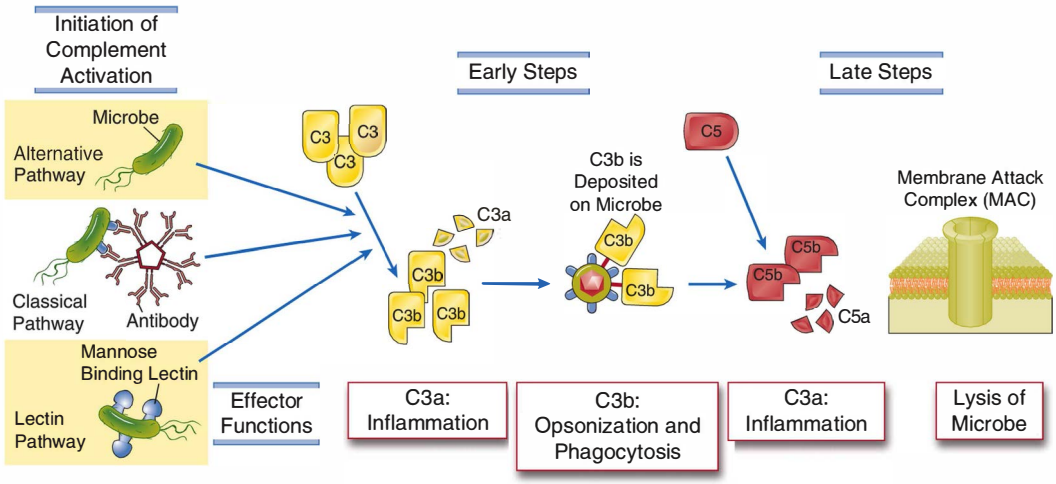

FIGURE 6.2 Pathways of complement activation. The proteolytic cascades of the complement system can be activated by antigen–antibody complexes binding to C1q (the classical pathway), by microbial carbohydrates binding to mannose-binding lectin (the lectin pathway), or spontaneously by C3 hydrolysis near microbial surfaces (the alternative pathway). All three pathways form C3 convertases that generate the opsonin C3b and the proinflammatory fragment C3a. C3b contributes to the formation of C5 convertase that generates the proinflammatory C5a fragments and initiates formation of the MAC that causes membrane damage and death of microbes or host cell membranes. (Modified from Abbas, et al. *Cellular and molecular immunology*, 7th ed. Saunders/Elsevier, 2011; Fig 4.9. p.73).

recognized by complement receptors on phagocytes. After C3b formation, all three pathways converge into a common terminal pathway that first generates a C5 convertase that cleaves C5 to form C5a and C5b. C5a binds to a specific GPCR on phagocytes, mast cells, and endothelial cells, and has similar but more potent proinflammatory effects as C3a. C3a and C5a peptides are called anaphylatoxins because of their ability to activate mast cells. C5a also has prothrombotic effects on the endothelium, including induction of tissue factor. C5b contributes to the common terminal complement pathway by nonenzymatically recruiting several lipophilic proteins named C6 through C9, which form a membrane attack complex (MAC). The MAC perturbs the integrity of some microbial membranes, and also damages host cell membranes in various inflammatory diseases. The complement system is crucial for effective innate immunity, as well as an effector mechanism of adaptive humoral immunity, and patients with genetic deficiencies in C3 are highly susceptible to various lethal bacterial infections.

Pentraxins, Collectins, and Ficolins

C-reactive protein (CRP), serum amyloid P (SAP), and pentraxin PTX3 are members of the pentraxin family of pentameric proteins.[53] They recognize phospholipids on microbes and apoptotic cells, and bind C1q to activate the classical complement pathway. CRP and SAP are acute phase reactants; their hepatic synthesis and plasma levels are upregulated by IL-6 and IL-1. For this reason, these pentraxins are clinically useful biomarkers of innate immune responses. PTX3 is made by neutrophils, DCs, macrophages, and endothelial cells in response to TLR ligands and cytokines.

Mannose binding lectin (MBL) and pulmonary surfactant proteins SP-A and SP-D are members of the collectin family of proteins. MBL binds terminal mannose and fucose residues on bacterial carbohydrates, and either activates the lectin complement pathway of or acts as an opsonin by simultaneously binding the C1q receptor on phagocyte membranes. Some MBL polymorphs are expressed at low levels in blood and are associated with increased susceptibility to infections. SP-A and SP-D opsonize microbes in the lung, promoting phagocytosis by alveolar macrophage. Ficolins are plasma proteins that bind to N-acetylglucosamine and the lipoteichoic acid in the cell walls of gram-positive bacteria. Ficolins act as opsonins or activate the classical pathway of complement.[54]

The Major Functions of the Innate Immunity

The components of the innate immune system described above perform three major functions. First they respond early to infection and cell injury by generating acute inflammation, characterized by increased delivery of plasma proteins and phagocytes to tissues. Complement, antibodies, other effector proteins, neutrophils, and macrophages work in concert to kill microbes and clear dead cells from the affected tissues. Second, some innate responses result in secretion of type I interferons, especially from pDCs, which induces an antiviral state in many cell types otherwise susceptible to viral infection. This function of the innate immune system occurs when viral nucleic acids ligate certain PRRs, such as TLR3 and RLRs. Third, innate immune activation of conventional DCs by TLR singling upregulates expression of various proteins involved in protein antigen processing and presentation to naïve T cells, thereby bridging innate immunity with adaptive immunity.

GENERAL FEATURES OF ADAPTIVE IMMUNITY

The major features of adaptive immunity that distinguish it from innate immunity are an enormous repertoire of specificities of its antigen-recognizing molecules, the inducible

specialization of effector mechanisms to best defend against different types of infections, and memory of first encounters with microbes, which enables more effective immunity to subsequent infections. The diversity of the repertoire of antigen specificities is based on the generation of large populations of B and T cells that express antigen receptors, but only a small number (~1,000) of which belong to a single clone and share the identical antigen specificity. These clones exist prior to foreign antigen exposure, and become activated (clonal selection) and increase in numbers (clonal expansion) when they recognize antigen.

Generation of Diversity of Lymphocyte Antigen Recognition Molecules

Lymphocyte antigen recognition is mediated by membrane receptors, including T-cell receptors (TCR) on T lymphocytes, and membrane immunoglobulin (antibody) on B lymphocytes. In addition, B lymphocytes that are activated by antigen secrete antibodies. The details of the structure of TCR and Ig molecules will be described later in the chapter. Suffice it to say that each receptor with a distinct specificity contains a different amino acid sequence in the antigen-binding region, and each receptor is encoded by a different gene. Since there are millions of different clones of lymphocytes, there are far more antigen receptor genes than the entire number of functional genes in the inherited human genome (~25,000). This tremendous diversity in protein sequence is achieved by somatic rearrangements, only in developing lymphocytes, of a more limited set of gene segments in the germline TCR and Ig loci.[55,56] A functional gene encoding an antigen-binding site for the polypeptides in Ig or TCR molecules is composed of DNA sequences called V (variable) and J (joining) segments. Two of the antigen receptor gene loci also have D (diversity) segments. There are many V, D, and J DNA segments that are spatially separated, and must be brought together by a process called V-D-J recombination, in order to form a functional gene. V-D-J recombination is catalyzed only in developing B and T cells, by the lymphocyte lineage-specific enzymes recombinase-activating gene-1 (RAG-1) and RAG-2,[57] in concert with more ubiquitous DNA repair enzymes. The result is the formation of functional genes that encode membrane immunoglobulin in B cells, or the TCR in T cells. Combinatorial diversity arises because the use of individual V, D, and J segments is random. For example, in the Ig heavy chain locus on chromosome 14 there are about 40 V genes, followed by approximately 25 D genes and approximately 6 J genes. Therefore, there are about 14,000 possible VDJ combinations that will encode heavy chains with antigen-binding sites. Junctional diversity is added because there are mechanisms in developing lymphocytes that add or delete nucleotides between the joining V, D, and J segments. Junctional diversity accounts for potentially millions of different variations in gene sequences in the regions of the antigen receptor genes that encode the antigen-binding sites of TCR and Ig molecules.

T-CELL–MEDIATED ADAPTIVE IMMUNITY

The major roles of T lymphocytes are defense against intracellular microbes, promotion and specialization of antibody responses to extracellular microbes, and regulation of adaptive immunity to prevent autoimmunity or excessive responses to microbes.

T-cell Antigen Recognition

T cells are defined by the expression of clonally distributed antigen receptors called TCRs. Most T cells express TCRs specific for a peptide in complex with an MHC molecule. Because MHC molecules are cell surface integral membrane proteins, T-cell antigen recognition only occurs by interaction with APCs, which display MHC–peptide complexes, and not by directly binding microbes or soluble microbial antigens. The major features of MHC molecules, the generation of peptide–MHC complexes by APCs, and TCRs will now be described.

MHC Molecules

MHC molecules have only one known function, which is peptide display on cell surfaces for T-cell immunosurveillance. In essence, MHC molecules alert the adaptive immune system of the presence of foreign proteins inside cells, and they may target immune responses against those cells. MHC proteins are encoded by highly polymorphic genes in the MHC locus on the short arm of the chromosome 6.[58,59] There are two major classes of MHC molecules and corresponding genes, called class I and class II MHC, which display peptides for recognition by $CD8^+$ or $CD4^+$ T cells, respectively. Three different types of human class I MHC molecules exist, called HLA (human leukocyte antigen)–A, HLA-B, and HLA-C, and there are three different types of human class II MHC proteins, called HLA-DP, HLA-DQ, and HLA-DR. The structure of a class I MHC molecules includes two noncovalently linked polypeptides, an α chain that is an integral membrane protein encoded by an MHC gene, and β_2 microglobulin, which is an entirely extracellular nonpolymorphic protein not encoded in the MHC locus. The *N*-terminal end of the class I MHC α chain forms a closed-ended peptide-binding groove, which accommodates peptides of 8 to 10 amino acids in length derived from other proteins inside the cell. Class II MHC molecules are composed of two noncovalently linked integral membrane proteins, called α and β chains, both encoded by polymorphic MHC genes. The N-terminal ends of both chains together form the class II MHC peptide-binding groove, which has a similar structure to that of the class I MHC β chain, but is open ended and accommodates longer peptides, usually 15 to 24 amino acids in length. Other than the length limitation, the peptide-binding grooves of class I and Class II MHC molecules are promiscuous for many different peptides, although there are constraints based on terminal amino acids that must serve as anchor residues and amino acid side chains that must fit into pockets in the floors of the grooves. Only a few peptides derived from any one protein will be able to stably bind in these grooves and be recognized by TCRs; these peptides are called the immunodominant epitopes of the proteins. As mentioned above, MHC genes are highly polymorphic. For example, there are over 1,000 HLA-A alleles, and over 1,500 HLA-B alleles. The polymorphic amino acid residues encoded by these alleles are concentrated in the peptide-binding grooves, and therefore each allelic form of an MHC molecule will be able to bind a different set of peptides. Consequently, the set of peptides that each individual in an outbred population will be able to display to T cells will be nonidentical, and individuals will vary in their ability to mount T-cell responses to different microbial proteins. However, the high degree of MHC polymorphisms ensures that an entire population will not succumb to a particular pathogen because of a total lack of antigen presentation.

As their name indicates, differences in MHC alleles between genetically nonidentical individuals account for the invariable T-cell–dependent rejection of allografts, in the absence of immunosuppression. Differences in the repertoire of peptides that can be displayed by different MHC alleles also likely explain the strong associations of many autoimmune diseases with particular MHC alleles.

Expression patterns of class I and class II MHC molecules reflect the different functions of T cells they display peptides to. All three types of Class I MHC molecules are simultaneously expressed on almost all nucleated cells in the body. Furthermore, both parental alleles of MHC genes are codominantly expressed, so that most cells express up to six different class I MHC proteins. $CD8^+$ effector T cells are the major defense mechanism against virally infected infections. Viruses can theoretically infect any nucleated cell, and all these cells can display viral peptides on I MHC molecules to $CD8^+$ T cells. All three types of class II MHC molecules are simultaneously expressed on a restricted set of cell types, including DCs, macrophages, and B cells. Some endothelial cells also express class II MHC, and expression may be induced on other cells types by the cytokine IFNγ. Like class I genes, both parental alleles of class II MHC genes are codominantly expressed, and because there are two HLA-DR β chain genes, up to eight different allelic forms of class II MHC molecules may be expressed on a single cell. $CD4^+$ T cells are key regulators of immune responses, and function to help other cell types perform effector functions. Given the potential for $CD4^+$ T cells to drive powerful and diverse adaptive immune responses, it is not surprising that only a limited number of cell types found in specific locations constitutively express class II MHC and are able to activate these T cells.

Antigen Capture, Processing, and Presentation

Conventional DCs are the major APCs responsible for activating naïve T cells. Immature DCs located within epithelial barrier tissues or within the parenchyma of other organs become activated by microbial PAMPs binding to TLRs and other PRRs. The DCs respond by internalizing microbes or microbial proteins, becoming motile, and entering draining lymphatics, which then carry them into lymph nodes. En route, the DCs mature into capable APCs by upregulating costimulatory proteins (described later), MHC molecules, and proteins involved in antigen processing. DCs also upregulate CCR7, the chemokine receptor that allows them to home to the same interfollicular T-cell zone of lymph nodes where naïve T cells are located. Blood-borne protein antigens may be taken up and processed by splenic macrophages and DCs. Antigen processing involves several sequential intracellular events in the APC, including the proteolytic degradation of proteins into peptides, delivery of the peptides to MHC proteins, assembly of peptide–MHC complexes, and expression of the complexes on the cell surface.[60] The details of these steps, which will not be described here, differ for class I and class II MHC. Importantly, the major determinant of whether a protein is processed for display by class I or class II MHC is the location of the protein in the cell. Cytosolic proteins are processed by the class I pathway, and would include most viral proteins that are synthesized in the rough endoplasmic reticulum of an infected cell, or other exogenously synthesized proteins that enter the cytoplasm via movement out of phagocytic vessels (so called cross-presentation). Therefore, viral proteins and proteins from microbes that survive by escaping from phagosomes into the cytosol will largely be displayed by class I MHC, and presented to $CD8^+$ T cells, which are adept at killing the infected cells. Proteins taken up by APCs through phagocytosis or endocytosis and end up in membrane-bound vesicles of the endosomal/lysosomal system are processed by the class II pathway. Therefore, peptide fragments of proteins from extracellular microbes will be displayed by class II MHC molecules and presented to $CD4^+$ T cells, which are adept at enhancing antibody responses and phagocyte activation to kill extracellular and phagocytosed microbes.

The antigen processing machinery does not distinguish between self and foreign proteins, and the vast majority of peptides displayed by class I or class II MHC molecules on the surface of cells are derived from normal self-proteins. A T cell can see and be activated by a foreign peptide–self-MHC complex even if it represents as little as 0.1% of the total MHC complexes on the APC surface. The fact that individuals usually do not react to the self-peptide–self-MHC complexes and develop autoimmunity is attributable to various mechanisms of T-cell tolerance, discussed later in this chapter.

T-cell Antigen Receptors

Over 90% of T cells in the blood and in lymphoid tissues express TCRs composed of a heterodimer of α and β polypeptide chains, which is specific for peptide–MHC complexes.[61,62] iNKT cells express $\alpha\beta$ TCRs with very little diversity that recognize lipid antigens displayed by the nonpolymorphic class I MHC-like molecule CD1. A small subset of T cells expresses a TCR composed of γ and δ polypeptides, which are structurally similar to α and β chains, but their specificities are poorly defined. Each chain of the common $\alpha\beta$ TCR includes a short *C*-terminal cytoplasmic tail, a transmembrane segment, a membrane proximal extracellular constant (C) region, and an N-terminal variable (V) region. The C and V regions have a compact three-dimensional structure first described in Ig molecules, called an Ig-fold or Ig-domain, which includes two adjacent β-pleated sheets connected by a disulfide bond. Proteins with Ig-folds belong to the *Ig superfamily*, and many molecules in the immune system are in this family, including MHC proteins, CD4 and CD8, to name just a few. The variable regions of the TCR are clonally distributed, meaning that one clone of T cells expresses a TCR with unique α and β amino acid sequences that differ from all other clones. The variable regions of both chains form the peptide–MHC binding site. Crystallographic analysis of the TCR structure reveals that the sequences that distinguish the variable region of one TCR α chain or β chain from another are concentrated within three hypervariable loops (also called complementarity determining regions) that connect adjacent strands of the β-pleated sheet of the V domains. The three hypervariable loops of both the α and β chains contribute to the specificity of one T-cell clone for a particular peptide–MHC complex.[63] Some of these loops contact polymorphic residues of the MHC molecules, and others contact side chains of the peptide within the peptide-binding groove of the MHC molecule. Every individual produces millions of different clones of T cells with unique TCRs. This diversity is generated by random somatic gene recombination mechanisms described earlier.

The TCR $\alpha\beta$ heterodimer is always expressed on the T-cell surface in association with a set of invariant signaling proteins, including ζ chains and CD3 proteins, to form the *TCR complex* (FIGURE 6.3). The cytoplasmic tails of these associated proteins

each contain immunoreceptor tyrosine-based activating motifs (*ITAMs*), which become phosphorylated on tyrosine residues when the TCR cell recognizes peptide–MHC on an APC. ITAM phosphorylation leads to a sequence of downstream signaling events that result in activation of the T cell.

Activation of Naïve T cells: Role of CD4/CD8 Coreceptors, Costimulators, and the Immune Synapse

In order for a naïve T cell to be activated by an APC it must engage in several molecular interactions with the APC in addition to TCR binding to peptide–MHC complexes. Effective TCR recognition of antigen also requires simultaneous binding of a coreceptor, either CD4 or CD8 on the T-cell surface with the peptide-displaying MHC molecule. CD4 and CD8 are Ig superfamily integral membrane proteins, expressed mutually exclusively on mature T cells, which bind to a nonpolymorphic region of the class II MHC β chain or class I α chain, respectively. A Src family protein tyrosine kinase called Lck is physically associated with the cytoplasmic tails of CD4 and CD8. Thus, binding of these coreceptors to the MHC molecules that are simultaneously recognized by a TCR brings Lck into the vicinity of the ITAMs in the cytoplasmic tails of the CD3 and zeta chains of TCR complex. Lck then phosphorylates the various substrates, including ITAMs, initiating the signaling cascade that will activate the T cell.

The signaling pathways downstream from the TCR are complex and best described in dedicated reviews[64–66] or textbooks.[30,31] A few major features are mentioned here because they are similar to activation pathways of other immune cells, including B cells and mast cells. Tyrosine phosphorylated ITAMs serve as docking sites for the cytoplasmic protein tyrosine kinase zeta-associated protein-70 (ZAP-70), which then becomes autoactivated and phosphorylates tyrosine residues on several adaptor proteins, including the membrane bound protein LAT. Phosphorylated LAT recruits various other signaling molecules that diversify and amplify downstream pathways, leading to activation of different transcription factors. Among these pathways is the phospholipase Cγ1-Ca^{2+}-calcineurin pathway that activates NFAT, and the Ras- and Rac-MAP kinase pathways, which lead to formation of the Jun-cFos containing AP-1 transcription factor. Antigen recognition by the TCR also activates PI-3 kinase, leading to Itk and Akt kinase activation. The net result of these signaling pathways and transcription factor activation is expression of IL-2 and its receptor, which promotes proliferation, expression of genes required for effector cell differentiation, and expression of antiapoptotic genes. An example of clinical pharmacologic blockade of TCR signaling is the use of cyclosporine and tacrolimus, which blocks calcineurin-dependent NFAT activation, in allograft patients.

In order to undergo productive activation, naïve T cells require additional signals provided by costimulatory molecules on the APC, which must bind to signaling receptors on the T cells at the same time as antigen recognition.[67,68] The expression of these costimulatory molecules on professional APCs, especially DCs, is induced by PAMP-mediated TLR signaling. The best characterized costimulatory molecules are CD80 (B7-1) and CD86 (B7-2), which bind to CD28, expressed on 90% of human $CD4^+$ T cells and on 50% of $CD8^+$ T cells. CD28 ligation activates PI-3 and Akt kinase-dependent signaling pathways, which synergize with TCR complex generated signals to enhance naïve T-cell proliferation, survival, and differentiation. Antigen recognition in the absence of costimulation can lead to T cell death or prolonged unresponsiveness (called anergy), which may protect against activation of self-reactive T cells. The immunosuppressive drug CTLA-4 Ig, which blocks B7-CD28 interactions, is approved for use to treat autoimmune diseases and prevent allograft rejection. Several other molecules on APCs have T-cell costimulatory activity, including ICOS-ligand (CD275) that binds to ICOS (CD278) on T cells, and LFA-2 (CD52) that binds to CD2 on T cells.

The molecular interactions between a T cell and an APC described above are facilitated by the formation of an immunologic synapse, which involves the close physical apposition of the plasma membranes of the two cells and the coordinated sorting of the relevant signaling proteins into centrally located lipid rafts and adhesion molecules to the periphery.[64] On the T-cell side of a typical immune synapse, the TCR complex, CD4 or CD8, and CD28 are centrally located, and LFA-1 is peripherally located. The respective ligands of these molecules on the APC are arranged accordingly. The immune synapse must be sustained for several hours in order for enough signaling to occur to activate the T cell, and prolonged synapse formation is also required for appropriate down-regulation of TCR signaling.

T-cell Clonal Expansion and Differentiation

In response to antigen and costimulatory signals delivered by DCs within secondary lymphoid organs, naïve T cells will undergo several rounds of proliferation to generate thousands of progeny T cells with the identical antigen specificity of the precursor naïve T cell. The magnitude of clonal expansion has been quantified during microbial infections by flow cytometry and the use of fluorescently labeled MHC-peptide tetramers, which bind to the TCRs of one antigen specificity. $CD4^+$ T cells expansion is typically in the range of $2–3 \times 10^4$-fold, while $CD8^+$ T-cell expansion during an acute viral infection is remarkably more than 10^5-fold. During clonal expansion, the T cells undergo differentiation into effector T cells, which have the ability to perform functions related to eliminating microbes. Effector differentiation is very much dependent on cytokines secreted by APCs or other cells in the lymph node microenvironment, as well as the antigen and costimulatory signals. $CD4^+$ T cells may differentiate into one of several subsets of helper T cells, described below (FIGURE 6.4), depending on the cytokines present, and $CD8^+$ T cells differentiate into CTL in response to IL-12 and type I interferons.

Effector T-Cell Subsets

Effector T cells may stay in lymphoid tissues where they were generated, or migrate to peripheral sites of infection. Like naïve T cells, effector T cells are activated by other cells presenting peptide-MHC antigen, and in response, they express surface or secreted molecules, which have various effects on the APCs or neighboring cells. $CD4^+$ T cells and $CD8^+$ T cells have distinct functions, and subsets of $CD4^+$ T cells are further specialized, based largely on the types of cytokines they produce.

CD4+ Helper T-cell Functions and Subsets

$CD4^+$ effector T cells are restricted to recognizing antigen on the limited set of cells that express class II MHC expressing cells, including DCs, macrophages and B cells. Many of the functions of effector $CD4^+$ T cells are to help these other cells perform

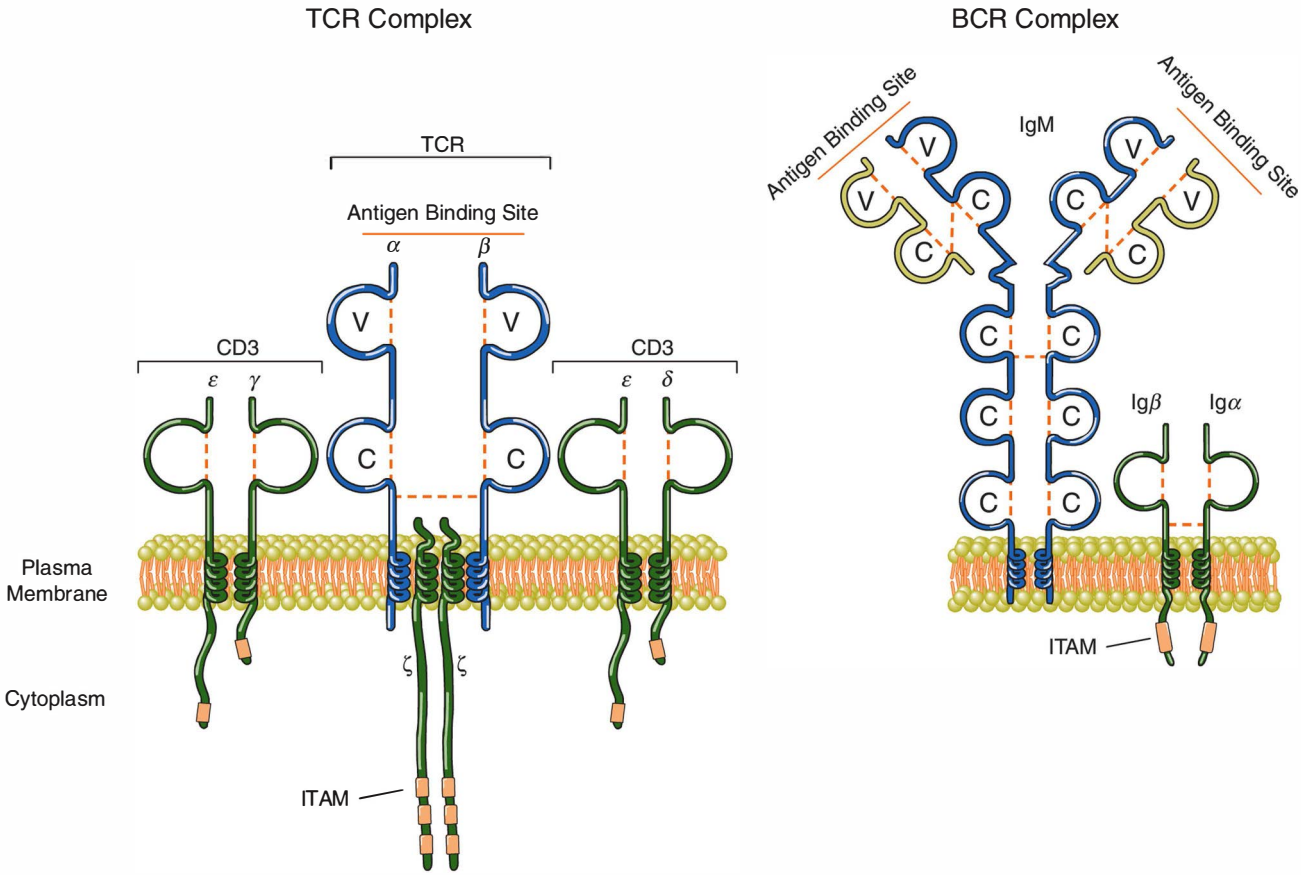

FIGURE 6.3 Antigen receptors of the adaptive immune system. An $\alpha\beta$ T-cell receptor in association with the CD3 and ζ signaling molecules is shown on the **left**, and a B-cell membrane IgM molecule with associated Igα and Igβ signaling molecules is shown on the **right**. The variable (V) and constant (C) Ig-like domains of the TCR α and β and IgM heavy and light chains are shown. Secreted IgM has a similar subunit structure as mIg, except for absence of transmembrane and cytoplasmic portion of the heavy chains. Secreted IgM forms pentamer. Other isotypes of Ig differ in the constant regions of the heavy chains, including the number of C domains. (Modified from Abbas, et al. *Cellular and molecular immunology*, 7th ed. Saunders/Elsevier, 2011; Figure 7.8. p. 147).

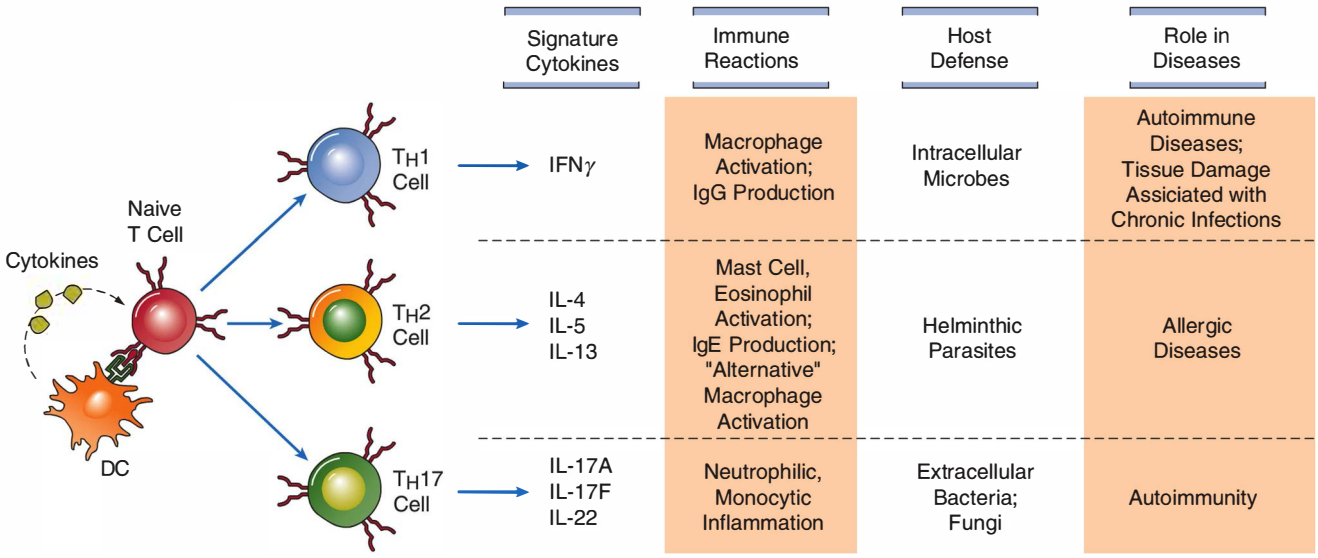

FIGURE 6.4 Helper T-cell subsets.

their functions, hence the moniker "helper T cells." T-cell help is largely provided by membrane CD154 and secreted cytokines. CD154 (CD40-ligand) is a TNF superfamily cell surface protein, which binds to the TNF receptor superfamily protein CD40 on the APC.[69] CD154 is also expressed on platelets, and CD40 is expressed on endothelium; these molecules have effects on homeostasis. Cytokines secreted by helper T cells act in a paracrine fashion by binding to cytokine receptors on the APC. The expression of CD154 and cytokines by the helper T cell requires activation by antigen, which ensures that help is selectively targeted to the appropriate cells, that is, those that have been infected or have phagocytosed microbes, and therefore can present microbial antigens to the helper T cells. In addition, cytokines secreted by activated helper T cells often have important effects on a wider range of neighboring cells besides the APCs, such as proinflammatory effects on endothelial cells.

The helper function of $CD4^+$ effector T cells on DCs is mainly mediated by CD154, and results in activation of the DCs to express more costimulatory molecules, IL-12, and type I interferons, which enhances their ability to simulate the differentiation of naïve T cells, especially naïve $CD8^+$ T cells into effector and memory cells. This function is sometimes called "licensing," and is one way helper T cells promote $CD8^+$ CTL responses to some infections. $CD4^+$ effector T cells help macrophages by enhancing their microbicidal or tissue repair functions, as is discussed for Th1 and Th2 cells below. The helper functions for B cells result in antibody class switching, increases in Ig affinity for antigens, and generation of memory B cells, as is discussed later.

T_H1, T_H17, and T_H2 subsets of $CD4^+$ helper T cells secrete distinct sets of cytokines and perform different roles in adaptive immunity[70,71] (FIGURE 6.4). Th1 cell secretes IFNγ, which together with CD154, enhances macrophage production of reactive oxygen species and lysosomal enzymes, and inflammatory cytokines such as TNF, IL-1, and chemokines. These effects, referred to as classical macrophage activation, enhance macrophage killing of ingested microbes, and promote inflammation leading to recruitment of more T cells and monocytes to sites of infection. Th1 cells are important for defense against microbial pathogens that can live within phagocytes, such as mycobacteria. Th17 cells secrete IL-17 and IL-22. IL-17 acts on many hematopoietic and tissue cell types to promote acute inflammation, and both IL-17 and IL-22 increase epithelial barrier function in the gut and skin.[72] T_H17 cells are required for defense against extracellular bacteria and fungal pathogens. T_H2 cells secrete IL-4, IL-5, and IL-13. IL-4 induces B cells to produce IgE, an antibody isotype that mediates antigen-induced mast cell degranulation, which promotes expulsion of helminths from the gut. IL-5 stimulates production and activation of eosinophils, which release granule contents that are toxic to helminths. IL-4 and IL-13 also simulates macrophages to secrete the anti-inflammatory cytokine IL-10 and growth factors that promote angiogenesis and collagen synthesis needed for tissue repair. These effects of IL-4 and IL-13 on macrophages are called alternative macrophage activation. In addition to their protective functions, T_H1, T_H2, and T_H17 cells are also mediators of tissue damage and disease. For example, prolonged T_H1 and T_H2 activation in the setting of persistent infections can result in granulomatous inflammation and tissue fibrosis. Self-reactive Th1 and Th17 cells are culprits in several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and psoriasis. Th2 cells specific for noninfectious environmental antigens promote IgE-dependent atopic disease.

T follicular helper cells (T_{FH}) are a subset of CD4+ helper T cells found in lymph node follicles, which have the specialized function of promoting B-cell differentiation into memory and long-lived plasma cells.[73] T_{FH} cells are characterized by expression of the follicular homing chemokine receptor CXCR5, ICOS, and abundant secretion of IL-21. T_{FH} cells may be heterogeneous with respect to secretion of other cytokines that induce Ig class switching to different isotypes.

CD8+ Cytotoxic T lymphocytes

The two main effector functions of $CD8^+$ CTLs are to directly kill infected (target) cells and to secrete proinflammatory cytokines, especially IFNγ.[74] CTL killing of infected cells requires recognition of microbial peptide-self class I MHC complexes, but costimulatory signals are not required, and therefore any infected class I MHC expressing cell can potentially activate and be killed by a CTL. $CD8^+$ CTL-mediated immunity is most important against viruses and certain bacteria whose proteins gain access to the cytosol. CTLs contain numerous lysosomal granules that contain perforin, a lipophilic protein that polymerizes in membranes, and serine proteases called granzymes, the most important of which is granzyme B. The main mechanism of target cell killing is by exocytic release of CTL granules into an immune synapse between the CTL and target cell, delivery of the granzymes into the target cell cytosol by perforin-dependent permeabilization of target cell membranes, and granzyme initiation of the caspase 3 and mitochondrial apoptosis pathways.[75,76] The DNA degradation that occurs during CTL-induced target cell apoptosis may serve to destroy microbial genomes. CTLs survive their lethal encounter with target cells, and are able to detach and then kill other cells. In addition, CTL may kill target cells by the Fas ligand:Fas (CD95) pathway, which involves caspase 8 proapoptotic signaling via CD95, and not granule CTL exocytosis. Robust CTL responses to an infection can overwhelm phagocytic clearance of apoptotic cells, resulting in proinflammatory necrotic death, and CTL secretion of IFNγ can enhance the inflammatory response. Thus, CTL responses to infection often cause tissue injury and exacerbate disease, such as in viral hepatitis. CTLs are also major contributors to allograft endothelial and parenchymal injury during acute rejection.

Memory T cells

Effector T cells generated in a response to specific microbial antigen are relatively short-lived, and most will die by apoptosis within several days after the microbe or antigen is eliminated. However, a residual population of antigen-specific memory T cells, 10- to 100-fold larger than the original population of naïve T cells specific for the antigen, will survive for months to years.[21,77] Memory T cells are responsible for more robust and specialized T-cell responses upon reexposure to a microbe. It is not known if memory T cells differentiate directly from naïve T cells, or if they arise from effector T cells. Generation of memory T cells depends on costimulation, and for $CD8^+$ T cells, on $CD4^+$ T cell help. Long-term maintenance of $CD4^+$ and $CD8^+$ memory T cells requires IL-7, and survival of memory $CD8^+$ T cells also requires IL-15. Two populations of memory T cells are described. Effector memory T cells are constitutively present in epithelial barrier tissues, and secrete abundant cytokines upon reactivation. Central memory T cells recirculate through lymphoid tissues, like naïve T cells, and undergo robust proliferation upon reactivation.

B CELLS AND ANTIBODY-MEDIATED ADAPTIVE IMMUNITY

Antibodies (immunoglobulins, Ig) are the recognition and effector molecules of the humoral arm of adaptive immunity. Ig molecules are expressed as membrane-bound receptors on the surface of B cells, and as soluble molecules in the blood and extracellular fluids in tissues. Humoral immune responses begin by antigen binding to membrane-bound antibody, which leads to the clonal expansion and differentiation of the B cell into an antibody secreting effector cell. The secreted antibodies perform various functions that protect against microbes, but are also responsible for tissue damage in many immune-mediated diseases. As in T-cell–mediated adaptive immunity, antibody-mediated immunity is characterized by tremendous diversity of antigen specificities, specialization of responses, and memory.

Antibody Structure

All antibody molecules share a basic structure composed of two identical heavy chain polypeptides disulfide-linked together, and two identical light chain polypeptides each disulfide-linked to one of the heavy chains (see FIGURE 6.3). The heavy chains are composed of two or three C-terminal constant (C) domains and a single N-terminal variable (V) Ig domain. The light chains contain one C-terminal C domain and one N-terminal V domain. These C and V domains have the prototypical Ig-domain conformation found in all Ig superfamily members. The antigen-binding site of an antibody molecule is formed by three hypervariable loops of the heavy chain V domain and three hypervariable loops of the light chain V domain, similar to the configuration of the TCR antigen-binding site. Two identical binding sites are formed by the two pairs of heavy and light chains in each Ig molecule, so that the basic structural unit of all antibodies is bivalent. All the antibody molecules expressed by a single naïve B cell have the identical antigen-binding site, but millions of different clones of naïve B cells are generated in every individual, each expressing antibodies with different antigen-binding sites. This diversity is generated by B-cell–specific somatic recombination of germline gene segments in the heavy and light chain loci, as described earlier. There are five different Ig classes or isotypes, named IgD, IgM, IgG, IgA, and IgE, distinguished by differences in the heavy chain constant regions. In addition, there are four subclasses of human IgG (IgG1, IgG2, IgG3, IgG4) and two subclasses of IgA (IgA1, IgA2). The heavy chains associated with each class are named by the Greek letter corresponding to the class name (δ, μ, γ, α, and ε). A single clone of B cells may change the class of antibody it produces, while maintaining the same antigen-binding site, by a process called isotype switch recombination, discussed later in this chapter.

The functions of antibodies depend on interactions of the heavy chain constant regions with other soluble or cell membrane proteins, and the different types of heavy chains perform different sets of functions because they interact with different sets of molecules.[78] The C-terminal heavy chain C domains are often referred to as the Fc region, and receptors on cells that bind heavy chains are called Fc receptors (FcRs). In membrane-bound antibodies of a particular class, there is a hydrophobic membrane spanning group of amino acids and a short cytoplasmic tail at the C-terminus of the heavy chain, while the secreted antibodies of the same class have shorter C-termini lacking these features but containing a secretory tail piece typical of all secreted proteins. Secreted IgA and IgM molecules form dimers and pentamers, respectively, in which the secretory tail pieces are disulfide-linked to the J chain protein. Two light chain isotypes exist, κ and λ with identical functions, and only κ or only λ light chains are produced by any one B cell.

All antibody functions depend on antigen recognition, which is mediated by several types of noncovalent interactions. The antigen-binding site formed from the six hypervariable loops of the heavy and light chain V domains is a planar surface, which can bind to large or small molecules, but small chemical antigens may also fit into clefts between hypervariable loops. Large molecules with different levels of structural organization provide various opportunities for antibody recognition. For example, an antibody specific for a protein may recognize a shape that depends on the native three-dimensional folded conformation, and another antibody specific for the same protein may recognize a short linear sequence of amino acids on an exposed segment of polypeptide. Antibodies can bind virtually any class of molecules produced by living organisms, as well as nonbiologic molecules. In typical immune responses to microbial antigens, the Kd of binding ranges from 10^{-7} to 10^{-11} M.

B-Cell Activation and the Generation of Antibody-Mediated Immune Responses

Two main types of antibody-mediated immune responses are distinguished by the nature of the stimulating antigen and the role helper T cells play. T-cell–independent responses are stimulated by polyvalent nonprotein antigens, such as polysaccharides and nucleic acids. T-cell–dependent responses are mounted against protein antigens and require interactions of $CD4^+$ helper T cells with B cells, both specific for the same antigen. In both types of responses, an initial step is the recognition of the antigen by the B-cell receptor on a naïve B cell.

The B-cell Receptor Complex and CR2/CD21 Coreceptor

The B-cell receptor complex on naïve B cells is made up of membrane IgM or IgD noncovalently associated with two invariant signaling proteins called Igα and Igβ that have ITAMs in their cytoplasmic tails and function in a similar manner to CD3 and ζ proteins in the TCR complex. Recognition of an antigen by membrane Ig leads to tyrosine phosphorylation of the ITAMs of Igα and Igβ by Src family tyrosine kinases, the recruitment of Syk, a protein tyrosine kinase homologous to Zap70, and then Syk-mediated phosphorylation of adaptor proteins and other signaling enzymes, analogous to those in T cells described earlier.[79] These signaling events lead to expression of genes that drive clonal proliferation and differentiation into antibody secreting cells. However, like naïve T cells, other signals besides those originating from the antigen receptor, are required for an effective response to develop. Complement activation provides one such signal, in the form of a C3d, a proteolytic product of C3b, which can be covalently attached to the same microbial surface recognized by membrane Ig. C3d binds to CR2, which is part of a signaling complex on B cells that includes CD19 and CD81. When a C3d-coated antigen engages both the BCR and CR2, the cytoplasmic tail of CD19, becomes phosphorylated by kinases associated with the cytoplasmic tails Igα and Igβb, which then, and amplifies BCR signaling. By this mechanism,

complement activation on a microbial surface or other antigen greatly increases the ability of the B cell to productively respond and develop an antibody response.

T-cell–dependent Antibody Responses

T-cell–dependent antibody responses are specific for protein antigens, and generate high-affinity antibodies of various isotypes, as well as long-lived plasma cells and memory B cells. These responses develop within secondary lymphoid organs by the bidirectional interactions of a B cell and $CD4^+$ helper T cell, each of which expresses an antigen receptor specific for the same protein.[80] Rare naïve B cells in a follicle may express BCRs that recognize some epitope on an intact monovalent microbial protein. The proteins will bind to the IgM receptors on those B cells, which stimulate endocytosis and processing of the protein, and presentation of a microbial peptide-class II MHC complex on the B-cell surface. $CD4^+$ T cells specific for the same microbial peptide-class II MHC are activated by DC APCs in the interfollicular zones, as described earlier, and differentiate into helper T cells. Changes in chemokine receptor expression on the activated B cells and T cells facilitate their migration toward one another at the edge of the follicles, and at this location B cells preset the microbial peptide antigens to the helper T cells. As an antigen-presenting B cell and helper T cell are engaged, the T cell delivers activating signals back to the B cell via cytokines and CD40 ligand. These signals stimulate some degree of B-cell proliferation and differentiation in antibody secreting plasma cells outside the follicle. This involves alternative heavy chain mRNA processing to exclude membrane anchoring sequences and include a secretory tail piece. The secreted antibodies include IgM and, due to isotype switching in some of the B cells, IgGs.

Isotype (class) switching involves rearrangements of DNA segments in the Ig heavy chain locus of a mature B cell that has already undergone V-D-J recombination and expresses antibodies of one isotype, usually IgM.[81] These DNA rearrangements depend on *activation-induced deaminase (AID)*, whose expression is induced by T_H CD154 binding to B cell CD40. AID catalyzes the replacement of cytosine residues in DNA with uracil, leading to generation of double-stranded breaks in specific intronic switch regions upstream of heavy chain gene locus.[82] During the repair of these breaks, various DNA repair enzymes act in concert as a switch recombinase, which deletes the intervening DNA between two DNA breaks, and moves the VDJ cassette from its original location adjacent to an upstream heavy chain gene locus (e.g., the μ locus) to a new downstream location just 5′ of another heavy chain locus (such as γ, ε, or α). The particular downstream heavy chain locus that ends up being used depends on the type of cytokines secreted by the helper T cells, which will enhance accessibility of different switch regions to the recombinase enzymes. For example, IL-4 from a Th2 cell will specifically promote ε heavy chain switching.

Some of the B cells that receive helper T-cell signals migrate back into the follicle and undergo remarkable proliferative expansion to form a germinal center. T_{FH} cells also migrate into the follicle and provide additional CD154 and cytokine signals to the newly expanded clone of B cells. These signals result in two fundamental features of the germinal center reaction, *affinity maturation* and the generation of *long-lived antibody-secreting plasma cells* and *memory cells*. Affinity maturation is the increase in the average affinity of Igs produced to a protein antigen as a T-dependent humoral response progresses. The underlying mechanism of affinity maturation is the generation of random point mutations (somatic hypermutation) in the V region gene segments of the proliferating germinal center B cells, resulting in many different subclones with varying changes in the affinity of the Igs they express. This is followed by the selection for survival of only those subclones that express the highest affinity antibodies.[83] V gene somatic mutations are generated by the same AID enzyme that mediates switch recombination, but in the case of affinity maturation, the repair of the introduced uracils is by base substitution, and not by dsDNA breaks and recombination. The selection of the high-affinity B-cell subclones depends on follicular dendritic cells (FDCs), a cell type unique to B-cell follicles that is not related to conventional DCs that activate T cells. FDCs bind and display intact protein antigens on their surfaces, while the overall concentration of antigen declines as a humoral response progresses. Only B cells that can bind these antigens tightly will receive survival signals, and other B cells will die by apoptosis. CD154 and IL-21 signals delivered by T_{FH} cells are also important for the selection of high-affinity B cells. Many of the selected B cells will differentiate into antibody secreting plasma cells, which are responsible for the rising titer of high-affinity antibodies that occurs during T-dependent responses to infections. Some of these plasma cells migrate to the bone marrow and survive there for months to years, and maintain titers of high-affinity antibody in the blood. Other selected B cells in the germinal center become resting memory cells, which reside within lymphoid tissues, survive for many years, do not actively secrete antibodies, but respond rapidly upon re-exposure to antigen by secreting isotype-switched high-affinity antibodies.

T-independent Antibody Responses

T-independent antibody responses are usually stimulated by polyvalent nonprotein antigens, such as bacterial capsular polysaccharide. This is because helper T cells only recognize peptides derived from protein antigens, so nonprotein antigens cannot elicit T-cell help. BCR signaling by monovalent antigens is usually not strong enough to induce B-cell proliferation and differentiation. However, polyvalent antigens can cause clustering of multiple BCR complexes and achieve a localized concentration of ITAMs and kinases that will generate sufficient signaling to productively activate a naïve B cell. TLR ligands and complement can enhance B-cell responses to these nonprotein antigens. Although some isotype switching to IgG and IgA occurs in response to nonprotein antigens, overall most T-independent responses are dominated by IgM secretion. There is also relatively little long-term memory or generation of long-lived plasma cells in T-independent responses. These properties become shortcomings of vaccines that do not activate T cells, but they have been addressed by the use of conjugate vaccines in which microbial polysaccharide antigens are covalently linked to protein carriers. The modified antigens are recognized by both B cells and T cells, and induce T-dependent antibody responses to the nonprotein component of the vaccine. The same mechanism underlies T-dependent IgE responses to nonprotein drugs (e.g., penicillin) or environmental chemicals (e.g., urushiols from poison ivy). In these cases, the allergens are reactive chemicals that covalently link to amino acid residues of self-proteins, including MHC molecules. In this context, they are called haptens, and can be recognized by antibodies, and by T cells when presented as part of an altered self-peptide bound to MHC.

Functions of Secreted Antibodies

The simplest protective function that antibodies perform is the binding to microbes or their products, thereby blocking their interactions with cellular receptors that promote infection or toxicity. This function, called neutralization, is due to stearic hindrance or by antibody-induced conformational changes in the bound antigens. Neutralization does not involve the constant regions of antibodies, and can be mediated by artificially prepared fragments of antibodies lacking the Fc regions (called Fab or F(ab)2 fragments). The most important neutralizing antibodies are high-affinity IgG molecules in the blood and IgA molecules in mucosal secretions, and the chronic production of neutralizing antiviral antibodies is the mechanism by which most successful viral vaccines work.

A major function of secreted IgG antibodies is opsonization of microbes to promote FcR-mediated phagocytosis and killing. There are several FcRs specific for IgG molecules expressed on different cell types, but the most important for phagocytosis is FcγR1 (CD64) that binds IgG1 and IgG3 with high affinity, when the antibodies are simultaneously binding antigens (i.e., forming immune complexes)[84]. CD64 is associated with a dimer of FcR γ chains, which contain ITAMs in their cytoplasmic tails, and transduce activating signals that enhance phagocytic, microbicidal, and inflammatory responses of the phagocytes. Thus, immune complexes binding to FcγR1 on neutrophils and macrophages induces their phagocytosis, destruction of the antigens, and increased local delivery of more phagocytes and antibodies.

There are several other isotype-specific FcR-dependent functions of antibodies. IgGs bound to surface antigens on infected or stressed host cells can simultaneously engage FcγRIIIA (CD16) on NK cells and thereby activate the NK cell to kill the antigen-bearing cell. This process, called antibody-dependent cellular cytotoxicity, may also contribute to killing of host cells targeted by therapeutic antibodies for cancer (e.g., anti-Her2/Nue) or autoimmune disease (e.g., anti-CD20). IgG and IgA antibodies specific for helminthic parasites bind to FcRs on eosinophils and can stimulate the release of granule contents that are toxic to the worms. Allergens or parasite antigens cross-link IgE that is prebound to high-affinity receptors for IgE (FcεR) on mast cells. This causes release of mast cell granule contents, including histamine, and rapid production of inflammatory cytokines and eicosanoids. The resulting immediate and late phase inflammatory responses have antihelminthic functions, but also cause allergic disease.

IgG1, IgG3, and IgM immune complexes bind C1q and activate the classical complement cascade.[85] As discussed earlier in the chapter, complement activation generates the opsonin C3b, which binds to the antigen–antibody complex, and promotes its phagocytosis by neutrophils and macrophages via the C3b receptor CR1 (CD 35). Complement activation also generates proinflammatory anaphylatoxins (C3a, C5a), and leads to lysis of *Neisseria* species of bacteria by MAC formation.

Dimeric IgA is secreted into the gut lumen where it functions as a neutralizing antibody to protect mucosal barriers from microbial invasion.[86] Secretory IgA is produced by lamina propria B cells, then transported through gut epithelial cells by attachment to the membrane bound poly I receptor, and is released into the lumen by proteolytic cleavage of the receptor. Secretory IgA is also found in airways and mother's milk, and it provides passive mucosal immunity to breast feeding babies. Maternal circulating IgG is transported across the placenta into the fetal circulation by the neonatal Fc receptor (FcRn), and provides critical passive immunity for neonates up to 6 months of age. FcRn is also expressed in endothelial cells and many other cells, where it protects endocytosed IgG from degradation and recycles the antibody back into the blood. This function of FcRn is largely responsible for the very long half-life of circulating Ig compared to other plasma proteins.

Regulation of Adaptive Immune Responses

Multiple mechanisms exist to prevent nonuseful and potentially dangerous immune responses, and to limit the magnitude and duration of useful responses to pathogens. These mechanisms are particularly critical for adaptive immunity because the random generation of antigen receptor specificities in lymphocytes carries the risk of producing self-reactive cells, and because the adaptive immune system is characterized by powerful effector mechanisms and long-lived memory cells. During lymphocyte development, many self-reactive B and T cells are eliminated by apoptotic death at an immature stage if their antigen receptors recognize self-antigens within the bone marrow or thymus.[87] Immature B cells that recognize antigens in the bone marrow may also undergo additional rounds of V-D-J recombination in their light chain loci, so that their specificities are changed; this is called receptor editing.[88] Many self-reactive B and T cells do escape negative selection during development, and enter the peripheral immune system. Additional mechanisms of peripheral tolerance are required to eliminate or suppress these cells. One such mechanism is the presentation of self-antigens to T cells by immature DCs that lack costimulatory molecules, which induces anergy or death of the T cells.[89] By the same mechanisms, costimulatory blockade is a potential therapeutic approach to induce tolerance to newly introduced proteins, such as in the setting of transplantation or gene therapy. T-cell responses are also regulated by inhibitory receptors of the CD28 family, namely, CTLA-4 (CD152), which binds to CD80 and CD86, and PD-1 (CD279), which binds to PD-L1 (C274) and PD-L2 (CD273).[90] Mouse models show the necessity of CTLA-4 to prevent lethal autoimmunity, and human clinical trials of blocking antibodies to CTLA-4 or PD-1 have demonstrated enhanced T-cell-mediated antitumor immune responses.

Regulatory T cells (Tregs) are a heterogeneous population of T lymphocytes that suppress effector T-cell responses, and thereby prevent autoimmune disease.[91,92] Natural Tregs mature in the thymus, and induced or adaptive Treg differentiate from naïve T cells in peripheral lymphoid organs. The best defined Tregs are $CD3^+$, $CD4^+$, $CD25^{hi}$, and $CD127^{lo}$ and express the transcription factor FOXP3, which is essential for their development. Mutations in FOXP3 lead to a lethal autoimmune disease called IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome). It is likely that induced Tregs contribute to the control of normal immune responses to foreign antigens. Tregs function by inhibiting the activation of naïve and effector T cells by APCs, through cell contact–dependent mechanisms and by the actions of secreted TGFβ.

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CHAPTER

7

Biostatistics and Epidemiology: The Basic Science of Clinical Research

KERRY L. LEE

In the basic sciences, there is an essential knowledge base and an array of tools and techniques that basic scientists must master in order to conduct state-of-the art laboratory and animal research in areas such as molecular and cell biology, biochemistry, and genetics, and to research the complex molecular or cellular mechanisms related to hemostasis and thrombosis. Similarly, there are vitally important concepts, scientific methods, and tools that must be understood for conducting clinical research, whether that research is in hemostasis and thrombosis, cancer, cardiovascular disease, or other areas of medicine. For example, to rigorously evaluate a promising new compound for reducing the incidence of thrombotic complications in a specific population of patients, a properly designed study with an adequate number of subjects where potential biases are minimized and the data are properly analyzed is required. The key question is how should you design, conduct, analyze, and interpret such a study. The answer to that important question can be found in the body of knowledge and the concepts, principles, and tools of biostatistics and clinical epidemiology. Indeed, the *basic science* of clinical research and the quantitative methodology required for conducting well-designed and properly analyzed clinical investigations largely exists within these disciplines. A basic understanding of biostatistical and epidemiologic methods is also essential to understand and critically evaluate the current medical literature in which the designs and results of clinical trials and observational studies are reported and discussed.

This chapter provides an overview of selected biostatistical and epidemiologic methods that are relevant for researchers who wish to design and conduct a clinical study. In addition, this overview is relevant for clinicians and medical practitioners who need to critically evaluate the published medical literature and use that information to make rational, evidence-based decisions in medical practice. An understanding of these methods is also important for properly synthesizing and summarizing information for the development of guidelines and policy decisions designed to improve health outcomes in the general public.

EPIDEMIOLOGY STUDY DESIGNS

The classical definition of epidemiology found in textbooks on the subject is that epidemiology is the study of the distribution and determinants of disease frequency in human populations.[1–4] This definition embodies two principal areas, namely (a) the study of disease frequency and variation in the occurrence of disease and (b) the search for the determinants of the distribution and frequency of disease and reasons for the variation in its occurrence. Relevant to the methods discussed in this chapter is the concept of *clinical epidemiology*, defined by Weiss[5] as "the study of variation in the *outcome* of illness and of the reasons for that variation." Numerous textbooks have been written on the subject (see for instance references[1–5]), and graduate training programs in epidemiology have been developed at many well-known colleges and universities, particularly in universities with a School of Public Health. Epidemiology, because it is based directly on observations of human subjects, has the advantage of relevance to populations of real people. However, classical epidemiology studies are commonly observational in nature and cannot take place under the controlled conditions possible in a laboratory experiment. Deriving reliable conclusions about cause-and-effect relationships from observational studies is challenging and must be done very cautiously and carefully. Observational epidemiologic studies have the disadvantage that possible confounding factors may bias conclusions about cause-and-effect relationships, and great care must be taken in designing such studies and analyzing the data in order to reduce the likelihood of spurious associations between a disease or disease outcome and a study factor of interest. In the epidemiology literature, the study factor of interest is often referred to as an *exposure variable*.[6]

There are two basic types of epidemiologic studies, which with some variations encompass most of the clinical research studies seen in practice today.[6] These include (a) *case–control* studies, and (b) *cohort* studies. In a case–control study, a group of cases or patients who have a disease of interest and a control group, or comparison series of subjects without the disease, are selected for investigation. Of primary interest is a comparison of the proportions in each group with a particular *exposure* (or factor) of interest. In contrast, in a cohort study, subjects are classified on the basis of the presence or absence of exposure to a particular factor (e.g., a particular treatment), and then followed for a specified period of time to assess the development of the outcome of interest (i.e., development of a particular disease or clinical event of importance) in each exposure group. Both case–control and many cohort studies are often criticized because of the potential for confounding and bias that is inherent in the fact that the design is observational. In a cohort study where the exposure factor of interest (e.g., use of a particular drug or treatment) is either self-selected or chosen by a health care provider, the subjects with that factor (e.g., people who use a particular treatment) may be systematically different from those without the factor (e.g., people who do not use the treatment) in ways that will affect the outcome of interest. In a case–control study, because the outcome of interest has already occurred at the time the exposure is assessed, there is potential for bias in the selection of subjects into the study and in their recall of prior events.

However, there are two principal strengths of observational epidemiologic studies. First, the evaluation of exposure factors that require a long duration before the outcome of interest occurs may be able to be studied productively with one of these designs, particularly a case–control design. Second, if the effect to be detected is moderate to large (on the order of a 50% or greater difference in the disease outcome of interest), then one of these observational designs may suffice. The strong association between smoking and lung cancer observed in the case–control studies conducted by Doll and Hill[7] in the 1950s is an example where although the amount of uncontrolled confounding may have affected the magnitude of the relative risk estimate, the relative risk of lung cancer due to smoking was sufficiently large that even with complete control of confounding factors, it is unlikely to have materially changed the conclusion that there is a strong positive association between smoking and lung cancer.[6]

RANDOMIZED CLINICAL TRIALS

In contrast to case–control and observational cohort studies where the "exposure" factor is observed rather than controlled as part of the experiment, a study design that can be viewed as an important special case of an epidemiologic cohort study is the randomized clinical trial.[6] Since the conduct and publication of the earliest randomized trials in the 1940s, randomized clinical trials have increasingly become accepted as the principal and most reliable method (the "gold standard") for acquiring information required for judging the efficacy and safety of therapeutic interventions. Variations of a "strength of evidence" hierarchy, such as that published in Green and Byar,[8] (FIGURE 7.1) always place the information derived from a well-designed randomized clinical trial as having greater value than information from nonrandomized observational studies. Randomized trials are widely accepted today as a powerful tool and the key to providing information required for rational, evidence-based medical care and prevention of disease.[6,9] Well-designed clinical trials are required by regulatory agencies prior to the registration of new drugs and devices. Clinical trials serve as the critical bridge between initial studies that have provided "proof of concept" of a new therapy and its ultimate widespread clinical application.

It is a legitimate question to ask why clinical trials are so essential. There are many reasons. First, most new interventions for use in clinical care have modest beneficial effects.[10] Relative risk reductions in key clinical outcomes as high as 25% are rarely achieved or exceeded by new interventions, and frequently the benefit is less although still clinically important. Second, few interventions are free of undesirable effects, and both the benefits and risks must be evaluated in order to adequately assess the usefulness of an intervention.[9] Given the uncertainties about the disease course and biologic variation in clinical outcomes, it is usually impossible to reliably judge the effects of a treatment or intervention on the basis of uncontrolled clinical observations. The randomized clinical trial has emerged as the principal research tool for developing evidence to inform and influence clinical practice. This is true in studies of hemostasis and thrombosis, as well as in numerous other areas of medicine.

In the past 20 years, many papers and a number of excellent textbooks have been published describing in considerable detail the methodology of randomized clinical trials. The popular text by Friedman et al.[9] provides an excellent expository overview of the fundamental concepts of clinical trials and the major principles and components of a well-designed clinical trial. Other useful books include Piantadosi's[11] excellent methodologic perspective on clinical trials, Cook and DeMets'[12] introduction to statistical methods in clinical trials, the older, but highly relevant text by Pocock,[13] and the recently published four-volume *Wiley Encyclopedia of Clinical Trials*.[14] In this chapter, selected topics that are pertinent to clinical researchers for planning and conducting a well-designed clinical trial and analyzing and interpreting the results of the study are discussed.

1. Anecdotal Case Reports
2. Case Series without Controls
3. Series with Literature Controls
4. Analyses Using Computer Databases
5. "Case-Control" Observational Studies
6. Series Based on Historical Control Groups
7. Single Randomized Controlled Clinical Trials
8. Confirmed Randomized Controlled Clinical Trials

FIGURE 7.1 Hierarchy of strength of evidence. (Reprinted from Green S, Byar D. Using observational data from registries to compare treatments: the fallacy of omnimetrics. *Stat Med* 1984;3:361–373, with permission.)

What is a Clinical Trial?

The text by Friedman et al.[9] defines a clinical trial as "a prospective study comparing the effect and value of interventions(s) against a control in human beings." The key elements of this definition are that a clinical trial (a) is prospective, rather than retrospective (i.e., subjects are followed forward in time), (b) involves one or more interventions or intervention strategies, (c) requires a control or comparison group against which the interventions are evaluated, and (d) involves human subjects.

The important prospective element of a clinical trial is in sharp contrast to the case–control epidemiologic study design mentioned earlier where participants are selected on the basis of the presence or absence of an event or condition of interest. Thus, by definition, a case–control study is not a clinical trial. Because a clinical trial involves human subjects, it introduces issues of ethics, safety, compliance, and the inherent biologic variation that exists among human subjects.

"Phase" Designation for Clinical Trials

In the medical literature, clinical trials are often characterized as being of a certain "phase." This terminology generally places clinical trials into four phases (phase I through phase IV).[9] Although preclinical data may be obtained from *in vitro* studies or animal models, phase I studies are generally small studies that represent the initial step or phase of testing a drug or biologic in humans to understand how well it can be tolerated and assess its toxicity. A typical goal of a phase I trial is to estimate how large a dose can be given before unacceptable toxicity is experienced by subjects. Although it does not meet the definition of a clinical trial (there is no control arm), this phase of development for a

new intervention is important and is commonly referred to as a phase I trial, although a "phase I study" is a more appropriate designation.

A phase II trial is one in which the objective is to assess the biologic activity or effect of a new compound, acquire further information regarding the appropriate level of dosing, determine whether there are encouraging signals (typically on surrogate endpoints [discussed later in the chapter]), and obtain data for designing a larger, more definitive (phase III) study that would be required to test the effectiveness of a new therapy in order to obtain regulatory approval. Finally, phase IV studies, generally referred to as postmarketing surveillance studies, are longer-term surveillance studies, designed to obtain additional information on long-term safety issues that may be associated with adoption of a new intervention.

Efficacy versus Effectiveness Trials

The terminology "efficacy" and "effectiveness" is often used in the context of clinical trials.[9] An *efficacy* trial is meant to distinguish clinical trials designed to assess the effects of an intervention in an ideal setting (where subjects and health care providers are highly compliant with the protocol, the best trained and most experienced center(s) are involved, and perhaps the study population and spectrum of risk of the population are narrowly defined). In contrast, an *effectiveness* trial is one in which the effects of an intervention are assessed in a study population enrolled across different types of clinical centers, taking into account the realities of less-than-perfect compliance with the study protocol, and typically involving a broader spectrum of patient risk. Most moderate to large multicenter trials would be considered "effectiveness" trials. The terms "comparative effectiveness trials" and "pragmatic trials" have recently become quite popular, but both terms relate to the general class of studies referred to as "effectiveness" trials.

Study Questions and Endpoints

The planning of a clinical trial depends on the specific objectives and the questions that the investigators wish to address. It is essential that those objectives and questions be well focused, clearly stated, and specified in advance. Closely related to the specific aims of the study and the questions of interest is the choice of endpoints to be examined in a trial. As advocated by Friedman et al.,[9] each clinical trial should have a primary endpoint. This choice is a key decision in designing a trial and is usually the major determinant of the study size. The primary endpoint should be (a) consistent with the primary study question and the hypothesized effect of the intervention, (b) clearly defined and specified in advance, (c) capable of being ascertained as completely as possible (ideally in every subject), (d) measured in the same way for all subjects, and (e) capable of unbiased assessment. For nonfatal endpoints, these last two points may require that the study have a blinded core laboratory to make the endpoint measurements or a blinded clinical events committee to review and adjudicate the clinical endpoints.

Beyond the important choice of the primary endpoint, it is reasonable in a clinical trial to consider one or more secondary endpoints in order to address important secondary or subsidiary questions. Again, prespecification and prioritization of these endpoints is important.

There are a variety of types of endpoint variables, including (a) binary (e.g., developing the disease of interest; whether the patient lived or died), (b) ordinal (e.g., worsening, no change, or improvement; number of significantly diseased arteries), (c) continuous (e.g., cholesterol level; lymphocyte count; distance walked in a 6-minute walk test), and (d) time to event (with censoring) (e.g., time to death or to significant nonfatal events [e.g., stroke] in long-term follow-up studies). Time-to-event endpoints typically arise in studies where patients are followed over a relatively long period of time.

Composite Endpoints

For many therapies under investigation, the goal is not necessarily to reduce mortality, but rather to prevent nonfatal events or improve symptoms. In such cases, a variety of negative outcomes are important (e.g., nonfatal myocardial infarction, stroke, or unstable angina). Furthermore, it is important with a new therapy to be relative certain that excess mortality is not being caused in the pursuit of reduced symptoms or nonfatal events. An approach frequently used is to specify a composite endpoint that includes more than one relevant outcome (e.g., death or stroke; death, myocardial infarction, or hospitalization for unstable angina).[9,15] This type of composite endpoint has been valuable in a number of studies and has led to acceptance of several new therapies in patients with acute coronary syndromes and heart failure. An advantage of a composite endpoint is that by including multiple possible outcomes where the occurrence of any one of them is counted, the event rate will be increased (compared for example to an endpoint consisting of mortality alone), and therefore the number of patients required for the study will be less. However, the interpretation can be problematic, particularly if some components of the composite are affected differently by the treatment being studied compared to its effect on other components. Furthermore, the common approach to analyzing such composite endpoints counts each component with equal weight, which may be somewhat counterintuitive. Some attempts at developing weighting schemes have been used, but clinicians have a difficult time agreeing on appropriate weights.[15,16] If considering a composite endpoint, a useful guideline is to (a) include only components that are expected to be affected by the intervention being studied, (b) include "harder" or more objective measures that are clinically important, (c) include mortality as one component, and (d) limit the number of components to a relatively small number.

Surrogate Endpoints

With clinical events as the primary endpoint, a trial can be quite long and expensive, and therefore consideration is often given to designing trials with a surrogate endpoint, namely a laboratory measurement or physical sign that is used as an alternative to a clinically meaningful endpoint.[9,15,17] Surrogate endpoints are of interest for their potential to reduce the size, cost, and duration of major trials. Furthermore, they may be helpful in explaining the mechanism of action of a therapy on clinical endpoints. Examples of surrogate endpoints might be such things as blood pressure, cholesterol reduction, CD-4 lymphocyte level, or suppression of ventricular arrhythmias. Lowering blood pressure in a population of hypertensive patients is an example of a surrogate for the clinical outcome of death, myocardial infarction,

or stroke. Surrogate endpoints are essential for early-phase studies to elucidate pathophysiological effects, but the pitfalls of assuming that a positive effect of an intervention on a surrogate endpoint will translate into a positive effect on a relevant clinical outcome have been well described. The Cardiac Arrhythmia Suppression Trial[18] and heart failure trials of inotropic drugs[19–22] are classic examples of the failure of surrogate endpoints.

There is a common misconception that if an outcome is correlated with the true clinical event, it can be used as a valid surrogate endpoint and serve as a replacement for the true clinical endpoint of interest. A valid surrogate endpoint must fulfill two criteria.[17,23] First, changes in the surrogate must be predictive of the relevant clinical outcome. The second important requirement, which can be difficult in practice to ascertain, is that a potential surrogate must fully (or nearly so) capture the effect of the intervention on the true clinical outcome. The considerations that underlie this determination are well described in the excellent article by Fleming and DeMets.[17]

Choice of Study Population

An important consideration in planning a clinical trial is the choice of the inclusion and exclusion criteria that define the study population.[9] The impact of these enrollment criteria on the ability to recruit patients and the ability to generalize the results must be considered. As general principles, eligibility criteria should include patients for whom (a) the intervention is likely to have an effect, (b) there is a high likelihood of detecting the hypothesized effect, (c) the possibility of adverse effects or safety concerns do not outweigh the possible benefits, (d) competing risks are minimal, and (e) adherence is not likely to be a major issue. There are tradeoffs between using broad, less restrictive enrollment criteria versus narrow, more restrictive criteria. With broad criteria, the results will be generalizable to a broader population, there will be a larger pool of potential patients, enrollment will be easier, and protocol violations may be reduced. On the other hand, broader inclusion criteria will generally lead to enrollment of some lower risk patients, which will lead to a lower event rate, and therefore require a larger trial. With a more narrowly defined patient population, the study can be restricted to higher risk patients, leading to a higher event rate and a smaller size trial. The negative aspects of narrow criteria are that the study is less generalizable, there will be a smaller pool of eligible patients, enrollment is typically more complicated, and thus protocol violations may be increased.

Sample Size and Statistical Power

One of the most important aspects of designing a clinical trial is to determine the number of patients that must be enrolled to accomplish the objectives of the study. The all-too-frequently used nonscientific approach for making this determination is to (a) assess the amount of money available for performing the study, (b) determine the amount that each patient will cost, and then divide (a) by (b). The adequacy of the sample size has been a deficiency of clinical trials in the past. A review of the statistical power of 71 published randomized trials that failed to find significant differences between groups revealed that 67 of the trials had a >10% risk of missing a true 25% therapeutic improvement, and with the same risk, 50 of the trials could have missed a 50% improvement.[24]

In the context of sample size for clinical trials, the basic concepts of statistical power are briefly reviewed. The power of a study is the probability of a positive result (a statistically significant result) if the intervention under study has a real effect on the primary outcome of the trial. Thus power is the probability of a "true positive" study; of correctly detecting true differences. The level of power depends on the magnitude of the effect of the intervention, and on the number of patients enrolled (sample size). The power of a study equals 1 minus the type II error probability, where the type II error probability is the probability of failing to detect a real difference.

With that basic information as background, the preferred (scientific) approach for determining sample size in a clinical study is as follows:

- Develop an estimate of the outcome expected in the control arm. This could be an event rate if the endpoint is a clinical outcome or a mean ± standard deviation if the outcome is a continuous measure. Typically, such estimates will be based on pilot data, previous studies, or other sources of relevant information.
- Determine the treatment effect to be detected (the effect should be clinically important and realistic).
- Select the desired level of power (90% is preferable; 80% may be acceptable for some studies).
- Specify the desired level of significance (commonly the level used in clinical studies is $\alpha = 0.05$).
- Calculate the number of patients required.

Formulas exist in numerous textbooks for calculating the required number of patients for a binary endpoint, for a continuous endpoint measure, and for comparing groups with respect to time-to-event endpoints.[9,25,26] Commonly available statistical software packages (SAS and others) contain procedures for performing sample size calculations.

For trials with a continuous response, a key parameter that must be specified for sample size and power calculations is the variability of the response (outcome) variable, which is typically characterized in terms of the standard deviation. Greater variability in the outcome variable requires more patients to achieve the same level of power. A continuous outcome also requires specifying a clinically meaningful difference between the mean outcome response in the intervention and control arms that is desired to be able to statistically detect.

For trials with a binary endpoint, the sample size depends on the event rate estimated for the control arm, and the magnitude of the hypothesized benefit from the intervention. Low event rates require a larger sample size for detecting meaningful differences.

For trials with a time-to-event outcome variable, the power is driven by the number of events rather than the total number of enrolled patients. Therefore, the sample size must be selected so that the number of events will be adequate. The sample size calculations involve first determining the number of events needed to achieve the desired level of power, and then based on the projected event rates, the number of patients required can be calculated.[27]

A type of trial design that has been growing in popularity is what is termed as an "event-driven" trial, meaning that the study will continue until the required number of events has been achieved. If the event rate proves to be less than projected, the study must either enroll more patients or extend the duration

of follow-up. The advantage of such a design is that the study is assured of achieving the required number of events. The main disadvantage is that it is more difficult to plan the exact number of patients and the length of follow-up and hence to develop the overall study budget.

For studies with more complex designs or features such that the standard sample size formulas may not apply directly, computer simulation of the proposed design features can be used to perform sample size and power analysis. This would normally require the help of an experienced statistician.

Useful points to remember in determining an appropriate sample size:

- Appropriately account for a realistic amount of patient withdrawals from the study, premature discontinuation of the randomly assigned study treatment, crossover from one arm to another, or other noncompliance with the protocol.
- Avoid overly optimistic projections of event rates and treatment benefit.
- Remember that large studies are required to *reliably* detect moderate treatment effects.

Randomization

Although not specifically mentioned as part of the definition of a clinical trial, a critical component of a well-designed trial is the randomization of study participants to the intervention and control arms of the study.[9]

The unique feature and strength of randomization is that it controls, on average, for the effects of potentially confounding factors that are unknown or unmeasured as well as those that are known. It is this feature that makes the randomized trial such a powerful strategy, especially for studying small-to-moderate effects.

Three important contributions of randomization include (a) it eliminates bias in allocating subjects to the different arms of a study, (b) it ensures that there are no systematic differences between treatment groups with respect to *known and unknown* risk factors, and (c) it provides a valid basis for statistical inference.[28] This third advantage is somewhat more subtle, but means that randomization guarantees the validity of the statistical tests of significance that are used to compare the treatments. Simply stated, this means that the process of randomization makes it possible to ascribe a probability distribution to the difference in outcome between treatment groups receiving equally effective treatments and thus to assign "significance levels" to observed differences.[28] This is the basis of the *P*-values reported in randomized clinical trials or any other randomized experiment.

Stratified randomization is often used in clinical trials and simply means that a separate randomization is performed within prespecified strata, defined by selected patient characteristics such as a prognostic factor known to be strongly related to the outcome of interest.[9,13] Stratification by clinical center is often used in multicenter clinical trials to ensure that treatments are allocated in similar proportions at each center. Stratification may improve balance among treatments with respect to prognostic factors and emphasize subgroups (or strata) of special interest. Stratification by clinical site is usually desirable, but stratifying by more than one or two key other factors is unnecessary and undesirable.[29,30,31]

Blocked randomization, also called "permuted block randomization," is commonly used in clinical trials to avoid any major imbalance or deviation from the desired proportion of patients allocated to each arm. Blocked randomization guarantees that any deviation will never be large, and at certain points the numbers assigned to each group will reflect exactly the correct proportions.[9,13] Blocked randomization is especially desirable if the mix or level of risk of patients enrolled might change over time.

Consideration may be given in a clinical trial to equal versus unequal allocation of patients to the different arms of the trial.[13] There are several points to consider in this matter. Equal allocation will maximize the statistical power of the treatment comparisons for a given number of patients. Equal allocation is more consistent with equipoise (indifference) toward which group a patient is assigned. Unequal allocation (allocating more patients to the experimental intervention) (a) allows obtaining more information and experience with a new therapy with respect to side effects or toxicities, (b) could reduce cost if the comparator (control) therapy is expensive, (c) does not substantially decrease efficiency (i.e., decrease statistical power) as long as the proportions allocated to each treatment arm are not too different (e.g., not more extreme than three to one),[13] and (d) may have the effect of causing participants to think that one arm of the study is preferred or more desirable than other arms.

The term *adaptive randomization* may be encountered in the clinical trials literature. There are various schemes that have been proposed for adaptive randomization, for example, adapting the allocation ratio or making other adaptations during the course of the trial. These schemes are discussed extensively in the textbook by Pocock.[13]

For the practical implementation of a randomization scheme, a commonly used approach, particularly in phase III trials, is to employ a centralized interactive voice response system where a telephone call center can be used to verify patient eligibility and provide the treatment assignment. Internet-based systems for randomization are also growing in use. The older, simple approach of an envelope system is still used in some smaller studies, but is subject to misuse (either intentional or unintentional) such as prematurely opening the envelope containing the randomization assignment before eligibility is fully ascertained or informed consent has been obtained. Selecting envelopes out of sequence is also a potential limitation. Although it is inexpensive, this approach to randomization is generally discouraged.

One important point in planning and conducting a clinical trial is that it is critical that the treatment intervention begin as soon as possible after randomization in order to avoid bias that can arise from events occurring between randomization and initiation of the assigned therapy. Thus whenever possible, it is desirable to delay randomization until the last practicable time before initiation of therapy so that there is not a major delay between randomization and the start of therapy.

Summary principles regarding randomization: (a) blocked randomization is desirable, especially for large trials, (b) stratifying the randomization by center is desirable in a multicenter trial, (c) stratifying by multiple other factors is unnecessary and may simply add complexity, and (d) treatment should be initiated as soon as possible after randomization.

Blinding of Treatment Assignment

Ideally, the treatment assignment in a randomized trial should be unknown both to the patients enrolled in the study and to the medical professionals administering the treatment and following

the patients. This approach, known as double-blinding, is a well-accepted standard in trials where blinding is possible. This feature of trial design is important for avoiding potential biases that can arise during patient assessments and data collection.[9] However, not all studies can be done in a blinded fashion. Trials of surgical procedures or implanted devices or lifestyle changes generally cannot be blinded. In studies where double-blinding is not possible, a single-blind approach (only the caregiver but not the patient is aware of the treatment assignment) may be possible. If this is also not possible, then other measures such as use of a blinded core laboratory for performing key measurements or a blinded events committee to classify and adjudicate relevant clinical outcomes should be incorporated into the study design to reduce potential bias.

Noninferiority Trials

Noninferiority trials are designed to determine whether a new intervention is not worse than an accepted standard intervention by a prespecified amount.[32,33] The setting in which a noninferiority design is most often used is in evaluating a new therapy when an established standard treatment already exists. A new intervention may be very worthwhile if it is as effective as an established therapy (not inferior) and is either (a) easier to administer, (b) has fewer side effects, (c) costs less, or (d) has less negative impact on quality of life. Noninferiority trial designs are relatively common in the pharmaceutical industry where a company wishes to test a new drug therapy against another therapy that has been shown to work, is already in use, and may be considered to be the present "standard." Such comparative trials are often referred to as "active-control" trials or "positive-control" trials.[34,35]

A key element of a noninferiority trial is to specify a value Δ, referred to as the noninferiority "margin." The study is then designed to provide sufficient power to demonstrate with high confidence that the new therapy is not worse than the standard by more than Δ if this is indeed true. The statistical formulation for testing the hypothesis of noninferiority is an interesting reversal of the usual hypothesis testing situation in a trial where the objective is to test for superiority. To explain, the more usual formulation where a trial is designed to detect a difference (i.e., show superiority) is based on testing the null hypothesis of no difference, and assessing whether the data are consistent with that hypothesis or whether the data support a rejection of the null hypothesis. In contrast, with a noninferiority trial, the null hypothesis is that the new therapy is worse than the standard by more than Δ, and the study is designed to assess whether or not the data are consistent with that hypothesis. If this null hypothesis is rejected, the interpretation is that the new intervention is not inferior to the standard. A more intuitive and useful way to conceptualize this situation is that if the upper limit of the confidence interval (typically the 95% confidence interval) for the difference between the new intervention and the standard falls below Δ, then the new therapy can be considered noninferior to the standard. Examples of the various outcomes that can arise in such studies and their interpretation are illustrated in FIGURE 7.2.

There are several caveats to keep in mind with noninferiority trials. The choice of the margin Δ is critical and depends on both knowledge of the effect of the control therapy and good clinical judgment. The sample size is driven by Δ. With a relatively small value of Δ, the study will require a large sample size. On the other hand, if Δ is relatively large, the assessment of noninferiority is not sufficiently stringent, and demonstration that the difference is simply less than a moderately large Δ is not persuasive that the new therapy is noninferior. Again, the choice of Δ is critical, and some guidelines for that choice are discussed in the excellent papers by Temple and Ellenberg,[34,35] and in a new textbook on noninferiority trials.[32] It should be emphasized that well-designed noninferiority trials do not necessarily require a smaller number of patients than a well-designed superiority trial. Indeed, a noninferiority study that is not well planned and rigorous in its attention to sample size, compliance, and the other standards described in this chapter will be biased in favor of noninferiority. In that case, acceptance by the clinical and scientific community is unlikely.

Factorial Study Design

Although there are many different variations and types of study designs that are nicely summarized in Friedman et al.,[9] one in particular that deserves special mention is the factorial design.[9,36,37] In its simplest form, a factorial trial is designed to evaluate two interventions compared with a control in a single experiment. The design is most easily conceptualized in the form

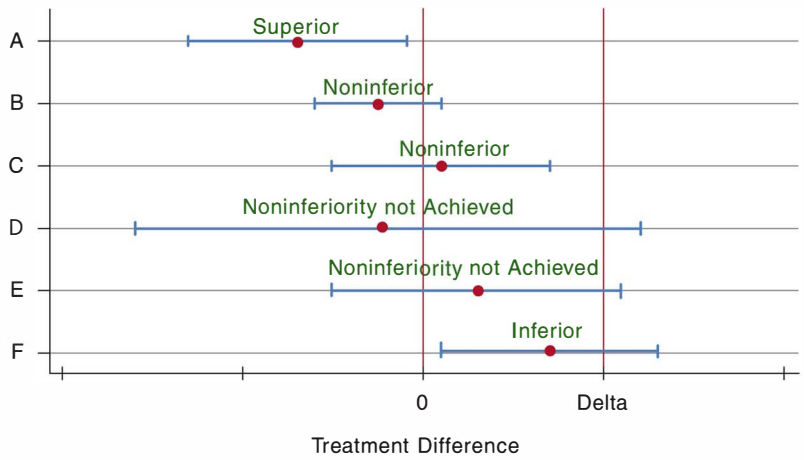

FIGURE 7.2 Illustration of the possible outcomes in a noninferiority clinical trial. The "dot" represents the observed outcome difference between a new intervention and the "standard" or comparator treatment, and the *lines* extending out from the "dot" represent the confidence interval for the true treatment difference. The *vertical line* labeled "delta" represents the noninferiority margin. For scenarios *A*, *B*, and *C* where the upper limit of the prespecified (e.g., 95%) confidence interval is less than delta, noninferiority has been achieved. In fact, for scenario *A*, the new intervention is superior to the "standard." For scenarios *D*, *E*, and *F* where the upper limit of the confidence interval extends beyond delta, noninferiority is not achieved. In scenario *F*, the new intervention is inferior to the "standard."

Study Effects of Two or More *Factors* at the Same Time.

	Drug A	Placebo
Drug B	N_{AB}	N_B
Placebo	N_A	N_O

Use All Patients in Analysis.

Effects of Drug A:

$$(N_A + N_{AB}) \text{ vs. } (N_O + N_B)$$

Effects of Drug B:

$$(N_B + N_{AB}) \text{ vs. } (N_O + N_A)$$

FIGURE 7.3 Illustration of a 2-by-2 factorial design where two different interventions (drugs in this example) are studied in a single trial. The numbers in each "cell" of the design represent the number of subjects allocated to that particular combination. N_A is the number of subjects who received drug A and the placebo for drug B, N_B is the number who received drug B and the placebo for drug A, N_{AB} is the number who received both drug A and drug B, and N_0 is the number who received the placebo for both drugs (i.e., received neither drug A nor drug B).

of a 2-by-2 table such as shown in FIGURE 7.3 and is thus referred to as a 2-by-2 factorial design. All patients are included in the analysis, and the effect of one intervention (drug A) is assessed by comparing the outcomes of all patients in the first column with the outcomes of all patients in the second column (FIGURE 7.3). The effect of the second intervention (drug B) is assessed by comparing the outcomes of all patients in the first row with all patients in the second row. Given the cost and effort in recruiting subjects and conducting clinical trials, the prospect of getting answers to two important questions (i.e., evaluating two interventions) in a single study is appealing. There are numerous examples of factorial designs, including the classic ISIS-2 study,[38] the Physicians' Health Study,[39] and the Women's Health Initiative.[40] The National Institutes of Health funded study of chelation therapy and high-dose vitamins is another example of a factorial design.[41] Some factorial studies are more complex than the 2-by-2 design, involving a third or even a fourth factor. It is also possible to leave some of the cells empty and have a partial or incomplete factorial design. Another variation of a factorial design is the large NIH-funded *Action to Control Cardiovascular Risk in Diabetes* trial, in which three different factors (intensive glycemic control, blood pressure control, and lipid control) were studied in a "double factorial design" in patients with type II diabetes.[42] All patients enrolled were in the glycemia trial, but within that structure, one 2-by-2 factorial trial addressed the blood pressure question and the other 2-by-2 factorial addressed the lipid question, hence the terminology "double factorial."[42]

As outlined in Friedman et al.,[9] the appeal of the factorial design might suggest that there really is a free lunch. However, every design has its strengths and weaknesses. A concern with the factorial design is the possibility of an interaction between the treatment factors and the impact of an interaction on the sample size requirements. In simple terms, the presence of an *interaction* in the 2-by-2 factorial example means that the effect of drug A differs depending on the presence or absence of drug B or *vice versa*. This is more likely to occur when the two interventions are expected to have related mechanisms of action.

If it is reasonable to assume that there is no interaction (which is the usual assumption in a factorial design), then with a modest increase in sample size, the two factors in a 2-by-2 factorial trial can be studied in a single trial—one that is considerably smaller than the sum of the patients that would be required for two independent trials to study the two different interventions individually. If an interaction between the two interventions is expected to exist, a factorial design is the way to rigorously make that assessment. However, since the power for statistically assessing an interaction is less than the power for assessing the "main effects" of each intervention, the sample size would need to be increased in order to provide adequate power for assessing the interaction.

Overall, the factorial design has some distinctive appeal. If there is little chance for an interaction between the treatment interventions or if it is important to determine whether there is an interaction, then such a design with the appropriate sample size can be very informative and efficient.

STATISTICAL ANALYSIS

We live today in the age of information and an era when the technical advances with computers and computer storage devices and sophisticated software make possible an astounding array of tools for processing, analyzing, displaying, and storing large amounts of data. The earlier emphasis in this chapter about avoiding bias in clinical trials applies also to the analysis of the data. Questions of what data should be collected, and how to collect that information, are important topics that are beyond the scope of this chapter. However, the development of case report forms and data collection methods and instruments deserves very careful consideration in the design and conduct of a clinical study. The amount of information and the number of measurements required to be collected on each patient in a study can have considerable impact on the quality and completeness of the information and the resources required to obtain the data.

Baseline Data

It is important for relevant baseline data (e.g., demographics, clinical history, risk factors, and presenting characteristics) to be collected in each study patient before the start of any intervention.[9] This information is important to describe the patients studied and (a) allow the scientific community to compare the study population with those of other studies, (b) allow clinicians to judge the applicability of the results to their specific patients, (c) allow an assessment of whether the study groups were comparable before the intervention was initiated, (d) allow the calculation of change scores (change from baseline to a follow-up measurement, if relevant), and (e) allow assessment of the prognostic importance of various baseline factors. In a randomized trial, ideally baseline measures should be collected prior to any knowledge of treatment assignment, but as close as possible to the time of randomization. In reporting the results of a clinical study, typically one of the first tables presented is a summary of the baseline characteristics.

Basic Analysis Issues

The distribution of continuous variables is often summarized in terms of the mean and standard deviation. These measures are most useful when the distribution of the variable is relatively symmetric (i.e., similar in shape to a symmetric, bell-shaped "normal" distribution). Often in practice, however, continuous variables may have a skewed (asymmetric) distribution, in which case the mean and standard deviation are less useful, because they are unduly influenced by extreme or outlier values. Useful descriptive statistics that are not subject to the influence of extreme values are the median (instead of the mean) and the lower and upper quartiles. The lower quartile is the 25th percentile, the median is the 50th percentile, and the upper quartile is the 75th percentile of a distribution. It is often useful to also report the full range of values, namely the smallest and largest values.

Categorical variables can be descriptively summarized by reporting the frequency and percent (the number and percent of patients) with the particular characteristic.

It is common in practice for the different arms of a study to be statistically compared with respect to the baseline characteristics of the study patients. However, if the study is a randomized clinical trial, calculating and reporting an array of *P*-values for comparing groups defined by randomization with respect to their baseline characteristics does not serve a useful purpose and is therefore unnecessary since such comparisons are not testing a useful hypothesis.[43] If there are any significant differences between groups, such differences are due to chance, since the groups are defined by randomization (unless, of course, something has gone wrong with the randomization). In observational studies where the study groups are not defined by randomization, it is wise to perform statistical comparisons of the baseline data in order to assess comparability and characterize differences between the groups.

Comparisons between two independent groups with respect to a continuous variable is commonly performed using the two-sample *t*-test.[44] It should be noted that this test is based on certain assumptions, namely that the variable has a normal distribution with similar variation in each group and that the observations are independent. Procedures that make such distributional assumptions are referred to as "parametric" tests. The independence assumption is typically satisfied as long as each data point comes from a different subject. However, as stated earlier, many clinical variables do not have a normal distribution. Although the *t*-test is fairly robust to departures from normality, there is a nonparametric analog to the *t*-test (and to numerous other parametric statistical procedures) that does not make any assumptions about the underlying distribution of the data. The nonparametric analog of the two-sample *t*-test is the Wilcoxon rank-sum test (also called the Mann-Whitney test).[45] The Wilcoxon test is almost as sensitive (powerful) as the *t*-test even when normality is satisfied, but importantly, it can be much more powerful otherwise. It represents an attractive alternative to the *t*-test.

Where there are more than two groups to be compared, the parametric generalization of the two-sample *t*-test is analysis of variance, and the nonparametric analog is referred to as Kruskal-Wallis nonparametric analysis of variance.[45] The comments above regarding the statistical power of parametric versus nonparametric procedures apply in this case also.

Comparisons between groups with respect to a categorical variable can appropriately be performed using the conventional chi-square test.[44] In the case where the frequencies in any category of the variable are small (e.g., ≤5), the use of Fisher's Exact Test[44] is recommended.

Analysis of Outcome Data

For group comparisons with respect to outcome data, the methods described above are also applicable. In the case of a binary outcome variable, it is desirable to provide additional descriptive measures of association between the treatment and outcome such as a relative risk or risk ratio. This is simply a ratio of the overall risk of the event in the intervention arm compared to that in the control arm. Another widely used measure to descriptively characterize the effect of an intervention on a binary outcome is the odds ratio, which is the ratio of the odds of the endpoint in the intervention group (the probability that the endpoint will occur divided by the probability that it will not occur) compared to the odds of the endpoint in the control group. An odds ratio of 1.0 indicates no difference between the treatment arms, an odds ratio <1 indicates a reduction of risk in favor of the intervention compared to the control arm, and an odds ratio >1 indicates a higher risk of the intervention compared to the control. A confidence interval can be calculated for the odds ratio (using, for example, the logistic regression model[46]). If the 95% confidence interval does not include 1.0, one can interpret the difference between the treatment arms with respect to the endpoint of interest as significant at the 0.05 level of significance.

If the course of a disease studied in a clinical trial is rapid (e.g., acute liver failure) or the period of follow-up is short (e.g., <30 days), a count of the number of events on each treatment (i.e., considering the endpoint as a binary outcome) is sufficient for analyzing the data. However, if there is considerable time from study entry until the outcome event occurs (as in a trial of a chronic disease) and patients are followed over an extended period of time, it is possible to achieve a more sensitive assessment of the value of each treatment by examining not only how many patients experienced the event of interest, but also how long after study entry the event occurred (i.e., by examining the pattern of when the events occurred).[30,31] Because this type of data is of such relevance, it deserves special discussion. The body of methods for analyzing such data is often referred to in textbooks as "Survival Analysis."[47–50] However, the methods are not restricted to studies where death (or survival) is the endpoint. The methods can be used for any endpoint where the time from enrollment to the time of the event, not just the fact of its occurrence, is an important consideration. These methods permit the comparison of groups with respect to their entire "survival" experience during the follow-up period.

A typical scenario for patient follow-up is shown in FIGURE 7.4. If a patient experiences an event during follow-up, the interval of time from entry into the trial until the event occurred is important. Some patients will experience the event of interest during the follow-up period and others will not. If a patient has not experienced an event prior to termination of the study, we refer to this individual's follow-up as being "censored" at the end of the trial, meaning the patient has not experienced an event, and all that is known for that patient is that their time

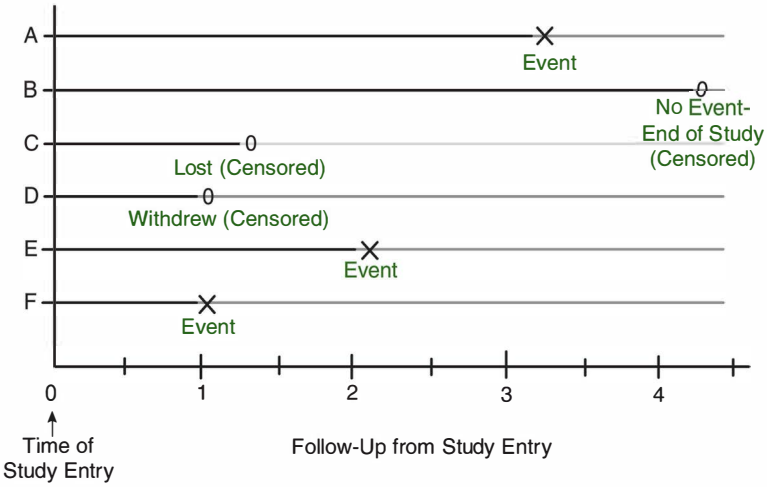

FIGURE 7.4 Typical patient follow-up scenarios in a study involving time-to-event data. Subjects *A*, *E*, and *F* all experienced the outcome of interest, but at different times during their follow-up. *B* is an example of a subject followed to the end of the study who remained event free throughout the follow-up period. *C* and *D* are examples of subjects whose follow-up was censored because they chose to withdraw from the study or became lost to further follow-up.

to an event (if it should occur in the future) is greater than their censoring time. A patient may also be censored if he or she withdraws from the study (refuses to continue to be followed) or if the patient is lost to follow-up (such losses should be kept to an absolute minimum in a well-conducted clinical trial).

With time-to-event data, a description of the results should include a calculation of event rates (or event-free rates) as a function of the length of time from study entry. These rates can be graphically presented in the form of a "survival curve" or "event rate curve" (FIGURE 7.5) and are commonly found in the clinical literature. The most common and popular approach for calculating event rates in the setting of a clinical trial and producing a "survival curve" is the method of Kaplan and Meier.[51] The calculations are straightforward, and software for performing the calculations is available in many commercial statistical software packages. The Kaplan-Meier curve is a step function, with a step at each time that an event occurs. Between adjacent event times, the curve remains constant. The Kaplan-Meier curve for each treatment arm in a clinical trial (or more generally for each defined group in a clinical study) can be viewed as an estimate of the true survival or event rate function for that group (see example in FIGURE 7.5). The precision of this estimate at any time point along the curve can be characterized with a standard error. The most popular formulation for calculating the standard error is known as Greenwood's formula.[50,52]

There are a number of statistical approaches for comparing two or more groups with respect to their pattern of "survival" (pattern of events), that is, for comparing two or more "survival curves." Several "Survival Analysis" textbooks that broadly cover the methods in this area are now available.[47–50] A popular and frequently used approach for assessing survival differences is the *log-rank* test,[49,50] also sometimes referred to as the Mantel-Haenszel

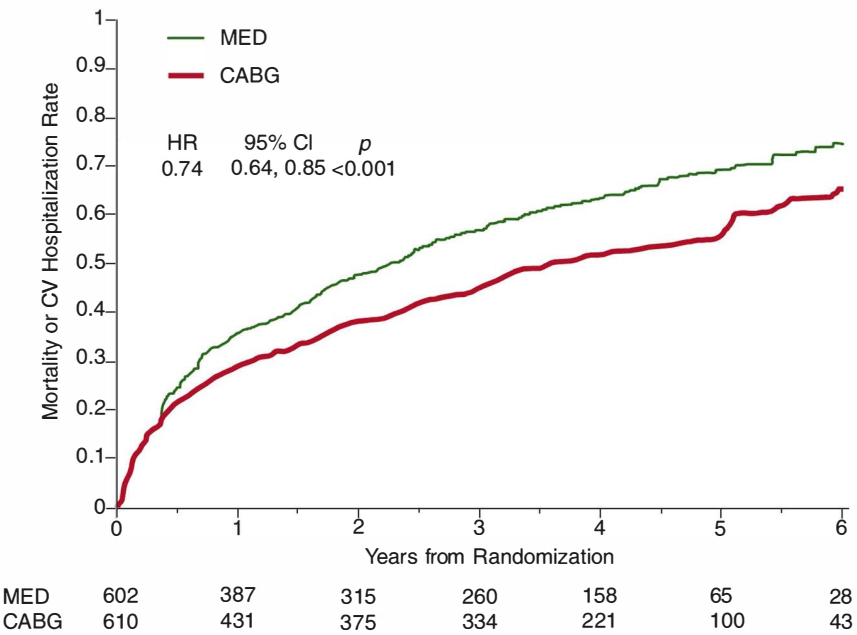

FIGURE 7.5 Illustration of Kaplan-Meier event curves from published results of the Surgical Treatment for Ischemic Heart Failure (STICH) trial comparing coronary artery bypass grafting (CABG) plus optimal medical therapy versus optimal medical therapy alone (MED) (Velazquez et al. *N Engl J Med* 2011;364:1607–1616). The endpoint is the composite of death or cardiovascular hospitalization. HR refers to the hazard ratio comparing CABG versus MED, with its associated 95% confidence interval (CI). The numbers at the bottom of the figure at annual time points are the number of patients in each treatment arm who are still in follow-up, are event free, and therefore remain at risk to experience the endpoint. Curves that depict event rates can easily be converted to event-free curves by simply subtracting the event rate at each time point from 1.0. (Velazquez et al. Coronary-artery bypass surgery in patients with left ventricular dysfunction. *N Engl J Med* 2011;364(17):1607–1616.)

test for survival data. Without providing all the mathematical details, this test simply involves (a) ranking the event and censoring times in ascending order for the study overall, (b) determining at each time point where one or more events occurred, the total number of participants in each treatment group who were at risk just before that time, and the number of events that occurred in each group at that time, (c) with this information forming a series of tables (2-by-2 tables in the case of a two-arm trial), one for each time at which an event occurs. The observed numbers of subjects in each arm who experienced an event and the numbers who did not experience an event comprise the cells of the table. The expected number of events for each arm (under the null hypothesis of no difference) can be calculated from the row and column totals for that group, the variance of the observed number can be calculated, and then the information from the individual tables is combined into an overall test statistic (a chi-square statistic with degrees of freedom equal to the number of treatment arms minus 1) for which a *P*-value and hence the degree of significance can be assessed. Log-rank statistics are seen frequently in the medical literature in connection with reports of major clinical trials.

For comparing treatment groups with respect to "survival" data, there are useful measures of association analogous to the odds ratio for a binary endpoint. In this case, the descriptive characterization of the treatment effect is given by a "hazard ratio," which can be viewed as a measure of relative risk. If the hazard ratio for the intervention arm compared to the control arm is 1.0, this implies that there is no difference in the survival pattern of the intervention group compared to control. A hazard ratio <1.0 is in the direction of some reduction in risk with the intervention arm, and a hazard ratio >1.0 is in the direction of excess risk in the intervention arm. Confidence intervals can be calculated for the hazard ratio (using for example the Cox regression model[53] [discussed later in the chapter]). If the 95% confidence interval does not include 1.0, one can interpret the difference between treatment arms as significant at the 0.05 level of significance. A single hazard ratio to characterize the overall treatment differences assumes that the hazard (risk) difference between the treatment arms is proportional over time. If this assumption is clearly violated, then different hazard ratios over different periods of the follow-up or other methods may be needed to adequately characterize the treatment differences.

"Intention-to-Treat" Analysis

A key question in the comparison of patient groups in a randomized clinical trial is which patients and which groups should be analyzed. The literature on this subject emphasizes that excluding randomized participants or observed outcomes from the analysis can lead to biased results. Analyzing clinical trial data according to the principle of "intention to treat" (ITT) has been strongly advocated in the clinical trials literature and is widely accepted by clinical trialists.[9,37] The ITT approach means that the primary comparison should include all subjects in the trial, with subjects analyzed and endpoints attributed to the treatment arms assigned by the randomization. This comparison is always of interest.

In some studies, it may be appropriate to compare the groups as randomized, but only include patients who actually receive the treatment, which in a drug study is typically defined as having received at least one dose of study drug. The rationale for this choice is that including only treated patients may provide a more sensitive appraisal of the treatment effects. However, this approach is only acceptable in a double-blind trial where knowledge of treatment assignment could not have influenced whether the patient actually received the treatment.

An "as treated" analysis may be performed, where groups are compared according to the treatment actually received. Sometimes in large trials, there are patients who either do not receive the randomly assigned treatment or incorrectly receive the treatment designated for a different arm. Such comparisons must be very cautiously interpreted, but may be useful for comparisons with respect to safety endpoints.

Finally, investigators may choose to do what is termed a "per protocol" analysis where only patients are included who fully satisfied all enrollment criteria and protocol requirements. Such comparisons must be interpreted cautiously. The full benefits of randomization are reduced or even lost when some patients are selectively excluded.

Subgroup Analysis

Regardless of the overall trial outcome, some subgroup analyses are typically performed. If the trial is positive, subgroup analysis may be undertaken to characterize patients who benefit most from the therapy versus those who may benefit less (or derive no benefit at all). If the overall trial is negative, investigators may undertake subgroup analysis in an attempt to identify at least some patients who appear to benefit from the intervention and thereby salvage something positive from the study. Some investigators feel that dividing a trial's results into smaller subgroups may offer clinicians insight into treating individual patients. However, the pitfalls of subgroup analysis have been clearly described by multiple authors in the clinical trials and medical literature.[9,15,31,38,54]

One pitfall that occurs in comparing treatments within selected subgroups in an overall positive trial is finding subgroups where the *P*-value for the treatment comparison within those subgroups does not achieve conventional significance and concluding that there is no treatment benefit for those patients. That is an improper interpretation. A study is never designed with sufficient patients in all major subgroups of interest to provide adequate statistical power for producing statistically significant treatment effects when treatments are compared within individual subgroups. Another pitfall is that the evaluation of multiple subgroups can lead to nominally significant results by chance alone. Even more complex to interpret are results where the subgroups are defined by post-randomization factors (referred to as improper subgroups).[54]

Cautious interpretation of subgroup analysis is clearly very important. It is more relevant to examine the direction of treatment differences and the general consistency of treatment effects across subgroups rather than focusing on the degree of significance in each subgroup. The process of assessing consistency can be aided quantitatively by testing whether interactions exist between the treatment and specific patient strata (i.e., whether there is statistical evidence that the relative benefit of a treatment varies across specific groups of patients). To the extent possible, subgroup analyses should be prespecified. Even then, it is wise to consider subgroup findings, particularly unexpected subgroup findings, as exploratory, subject to confirmation in another trial. Despite the attractiveness of subgroup analyses, the best estimate of the treatment effect to be expected from a qualifying patient treated outside the trial is still the overall estimate observed in the trial.[15,38]

Importance of Confidence Intervals in Interpreting Study Results

One of the important objectives of clinical trials or other clinical studies is to estimate the true effect of an intervention on important patient outcomes. There is often such a focus on *P*-values that the vitally important and valuable information in a confidence interval is sometimes overlooked. In fact, it could be argued that a confidence interval is more important than a *P*-value. In reality both are important. However, the confidence interval for the treatment effect provides valuable insight into the range of effects that are plausible based on the data. The narrower the confidence interval, the more certain we can be as to the true effect of the intervention. More studies should be designed with the objective of obtaining an estimate of the true treatment effect that is as precise as possible, rather than focusing solely on whether that effect meets the criterion for statistical significance. The case for greater use of confidence intervals in clinical trials was strongly advocated by Borenstein.[55]

Interim Analysis in Clinical Trials

The interim monitoring of safety data and often efficacy data has become a standard component in the design of major clinical trials, especially phase III trials designed to obtain regulatory approval of a new drug or device as well as many phase II studies. Such monitoring is typically performed by an independent Data and Safety Monitoring Board (DSMB) who may recommend early stopping of a clinical trial based on safety concerns, clear benefit, or other study-related information. Very cautious interpretation of interim analyses is required, and a body of statistical techniques has been developed involving various monitoring boundaries for judging statistical significance of treatment differences at interim time points.[56,57–60] Approaches have also been developed for judging "futility" (i.e., where there is a very low likelihood of obtaining a positive result if the study were to continue).[61–63] The important work of DSMBs and practical guidelines for their monitoring activity are well described in the clinical trials literature, including the excellent textbook on Data Monitoring Committees by Ellenberg et al.[64]

Number Needed to Treat

Another way of presenting the results of a clinical trial to patients, clinicians, the public, and policymakers is in terms of the number needed to treat (NNT). This quantity is the inverse of the absolute risk reduction and can be calculated with the formula $1/(p_c - p_E)$, where p_c is the rate of events in the control arm and p_E is the rate of events in the experimental intervention group. A confidence interval can be calculated for NNT using a straightforward formula.[65] The NNT, like the absolute risk reduction, provides an estimate of the absolute benefit of a therapy, in contrast to other commonly used measures of treatment effect such as an odds ratio and relative risk described previously. An example from Laupacis and Sackett[65] is illustrative of different ways of characterizing the results of a trial. Suppose patients with atrial fibrillation have a risk of stroke of 4.5% per year, which can be reduced to 1.4% per year with warfarin therapy. The relative risk reduction in this example is $(0.045 - 0.014)/0.045 = 0.69$. The absolute risk reduction is $(0.045 - 0.014) = 0.031$. The NNT is $1/0.031 = 32$. The results from this trial can thus be presented in a number of ways: (a) warfarin decreased the annual chance of having a stroke by 69%, (b) the absolute risk of stroke during 1 year is decreased by 3.1% with warfarin, and (c) 32 patients would need to be treated with warfarin for 1 year in order to avoid one additional stroke. The enthusiasm of patients, clinicians, and policymakers may be markedly affected by how the results are presented. Considering study results based on different summary measures provides a healthy perspective of the data and is helpful in developing an overall interpretation of a clinical trial.

MULTIVARIABLE ANALYSIS

In many clinical investigations, the questions of interest involve multiple variables (i.e., multiple factors or multiple measures) that are relevant to the investigation. Of considerable interest is how these factors interrelate and jointly influence the response (outcome) that is being studied. The traditional approach used in epidemiologic studies in the past has been to produce a cross-classification of the multiple factors and assess relationships with the outcome of interest by considering the individual "cells" created by this cross-classification and examining variations in the outcome across the spectrum of those cells. This approach quickly becomes impracticable as the number of variables to be investigated increases. For example, with five variables, each having two levels, the cross-classification of those variables produces thirty-two different cells. With 10 such variables, there are 1,024 cells. If the 10 variables each had three levels, this amounts to 59,049 cells. Without appropriate methods to deal with this type of data, it is impossible to identify and visualize meaningful relationships and patterns with this amount of data. Therefore, methods are required to facilitate the simultaneous assessment of the effects of multiple characteristics on the clinical outcome of interest. Fortunately, such methods do exist and are generally referred to as *multivariable analysis*, although the terms *multivariable statistical models* or *multivariable regression analysis* are also used frequently in this context.

The objective of multivariable analysis is to study the relationship between a single response or outcome variable (typically designated by the letter Y) and a set of multiple explanatory factors or characteristics (designated by $X_1, X_2, \ldots, X_p$). Y could be a continuous response variable (e.g., the change in a hematologic variable or cell count following a particular intervention), and the X variables could be factors such as age, sex, race, blood pressure, a treatment factor, and other variables of interest. The outcome variable Y could also be a discrete variable, such as the development of a thrombotic complication (yes or no), and again the X variables could be demographics or other clinical or treatment characteristics of importance. Y could also be length of patient survival or length of survival free from clinical events such as stroke or myocardial infarction. Such data could arise in either a clinical trial or an observational study. Again the X variables could be a treatment of interest and other demographic or clinical factors that may have an important prognostic relationship with the outcome.

The basic approach to this type of analysis is to study relationships using a model of the form

$$Y = F(X_1, X_2, \ldots, X_p) + \varepsilon$$

where $F(.)$ is a mathematical function, and the quantity ε designates an error term, since no model will perfectly fit all of

the data points. Sometimes the model may need to be slightly more complicated and take the form

$$G(Y) = F(X_1, X_2, \ldots, X_p) + \varepsilon$$

where G and F are different mathematical functions. The X variables are referred to as independent variables or predictor variables or explanatory variables, and Y is referred to as the dependent variable or response variable or outcome variable.

Linear Regression Analysis (Continuous Outcome Variable)

The most basic and simplest type of regression model is one in which Y is a continuous response, and there is a single predictor variable X. This model is referred to as *simple linear regression*. The model is expressed as $Y = \beta_0 + \beta_1 X + \varepsilon$, with parameters β_0 (which can be interpreted as an intercept) and β_1 (interpreted as a slope) which must be estimated from the data. If β_1 is positive, the value of Y tends to increase with increasing values of X. If β_1 is negative, the value of Y tends to decrease with increasing values of X. The most common approach for estimating β_0 and β_1 is the method of "least squares."[66] In this simple case, β_1 is a scaled version of the usual linear correlation coefficient. Once the parameters are estimated, the resulting regression line can be used to estimate the average value of Y corresponding to each value of the independent variable X. Thus, for two variables (Y and a single X variable), which could be visually conceptualized as a scatter plot, the regression line serves the role that an average does for a single variable. That is, the regression line is an estimate of the average value of Y for each value of X.

When there is more than one predictor variable (more than one X variable), the model generalizes to

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p + \varepsilon$$

where again Y is a continuous response, and the X's may be either continuous (such as age or blood pressure), discrete (such as race), or dichotomous (such as gender or whether a particular treatment was administered). The parameters β_1, β_2, …, β_p are regression coefficients that must be estimated (computed) from the data. As in the case of a single X variable, the parameter estimates are typically obtained using the method of "least squares." The parameter β_i can be interpreted as the amount by which Y changes (on average) when X_i changes by one unit and all other X's remain constant. As a simple example, suppose we postulated the model $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \varepsilon$, where Y represents systolic blood pressure (SBP), X_1 represents AGE (a continuous variable), and X_2 represents whether or not the subject is a current smoker (SMK) (a dichotomous variable). Suppose the fitted model is SBP = 48.1 + 1.7AGE + 10.3SMK. Interpreted, this means that SBP increases by 1.7 mm Hg (on average) when age increases by 1 year (and smoking status remains constant), and also SBP is increased by 10.3 mm Hg for smokers compared to nonsmokers (when age remains constant). This simple model thus enables an assessment of the relationship of age with blood pressure, controlling for smoking status, and also the relationship of smoking status with blood pressure, controlling for age.

Relationships are assessed with these types of models by testing hypotheses about the regression coefficients. The basic idea is that if a predictor variable has little or no association with the response variable, its regression coefficient will be zero, or at least near zero. Thus, hypothesis tests of whether the regression coefficients are significantly different from zero are helpful in interpreting the statistical significance of these multivariable relationships.[67]

Logistic Regression (Binary Endpoint)

Frequently in medical research, the response variable Y is a binary outcome (e.g., presence or absence of a particular disease, development of a thrombotic complication [or not], alive or dead following an acute procedure, etc.), and the questions of interest involve how a set of predictor variables may relate to whether study subjects experience the outcome of interest. Such a binary response variable is typically coded as 0 or 1, where 1 indicates that the event of interest occurred and 0 indicates that the event did not occur. The regression model commonly used in this situation is referred to as logistic multiple regression, or simply the logistic regression model. The formulation of the model is expressed in terms of the probability that the event will occur, that is, the probability that $Y = 1$. Denoting this probability by the notation $P = Pr[Y = 1]$, the logistic model can be expressed as

$$P = Pr[Y = 1] = 1/\exp[-(\beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p)].^{46,68,69}$$

The model is also often expressed in terms of the log odds of the event that $Y = 1$, namely $\log(P/(1-P))$, which is also referred to as the logit of P. In this form, the model can be expressed as

$$\text{logit}(P) = \log_e(P/(1-P)) = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p$$

where $\log_e$ refers to the natural logarithm. In this form, the model more closely resembles the classical multiple regression model for a continuous response variable.

The logistic model was popularized in the 1960s as a result of work by the statistician, Jerome Cornfield[70] in analyzing Framingham data and examining the relationships of various risk factors with the development of coronary heart disease. Cornfield used a different approach for estimating the regression coefficients than the "maximum likelihood" approach that is commonly used today.[68]

The interpretation of the regression coefficients in the logistic model is similar to ordinary multiple linear regression, except in terms of the log odds of the outcome event. The coefficient β_i is the amount by which the logit of risk (the log odds) changes when X_i changes by one unit and all other X's remain constant. A positive coefficient β_i means that the risk (or the likelihood) that $Y = 1$ (i.e., that the event will occur) increases with increasing values of X_i, whereas a negative coefficient means that the risk decreases with increasing values of X_i. As in conventional multiple linear regression (where Y is a continuous variable), relationships of various predictor variables with a binary outcome can be assessed with the logistic model through hypothesis testing of the regression coefficients.

The formulation of the logistic model lends itself nicely to calculating odds ratios and their associated confidence intervals to enhance the descriptive summarization of the relationships. For example, if the variable X_i is an indicator for treatment, that is, $X_i = 1$ for subjects who received a particular treatment and $X_i = 0$ for subjects in the control arm, then $\exp[\beta_i]$ is the odds ratio for comparing the treatment arm to the control arm, controlling

for all other variables in the model, where exp[.] represents the exponential function.[46]

Cox Proportional Hazards Regression Model (Time-to-Event Outcome)

The final multivariable model discussed in this section is one that was developed specifically for the analysis of "time-to-event" or "survival" data. The most widely used model for this type of data is the Cox proportional hazards model.[53] The Cox model, like the Kaplan-Meier method and the log-rank test described earlier, takes into account the overall structure of survival curves and accommodates varying lengths of patient follow-up and censored observations. It uses information on the time from study entry to the event of interest (the order in which the events occur) rather than simply enumerating the number of events. With this model, relationships can be studied between a "time-to-event" outcome variable and multiple patient baseline characteristics, which may be either continuous or discrete variables. A mathematical expression for the model is given by the equation

$$\log_e(\lambda(t)/\lambda_0(t)) = \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p$$

where $\lambda(t)$ is the hazard function at time t, $\lambda_0(t)$ is an arbitrary (unspecified) underlying hazard function, $X_1, X_2, \ldots X_p$ are patient characteristics, and $\beta_1, \beta_2, \ldots \beta_p$ are regression coefficients estimated from the data. The hazard function is similar to an event rate (e.g., death rate). Mathematically, it is the probability that a subject will have an event within an arbitrarily small interval of time $(t, t + \Delta t)$ given that the subject is event free prior to time t. An assumption of the Cox model is that the hazard functions for any two subjects are proportional over time. The regression coefficients are estimated by forming a (partial) likelihood function developed from the expression above, and then maximizing that likelihood function. To assess whether a particular characteristic is a significant independent predictor of "survival," a test of significance is performed on the corresponding regression coefficient to determine if it is significantly different from zero.[48,53]

The formulation of the Cox model nicely lends itself to calculating hazard ratios and their associated confidence intervals to enhance the descriptive summarization of the relationships. For example, if the variable X_i in the Cox model is an indicator for treatment, and $X_i = 1$ for subjects who received a particular treatment and $X_i = 0$ for subjects who did not, then $\exp[\beta_i]$ is the hazard ratio for subjects receiving the treatment compared to those who did not, controlling for all other variables in the model. Again, exp[.] represents the exponential function. The hazard ratio is often used as a measure of relative risk in comparing two different treatment groups in a clinical study. Confidence intervals for the hazard ratio can also be readily calculated with the Cox model. The log-rank test mentioned in an earlier section is a special case of treatment comparisons that can be performed within the framework of the Cox model.[48,50,53]

A final point about the various different models and approaches to multivariable analysis is the need to carefully check the assumptions of these models, including the linearity and additivity of the relationships.[71] Methods for assessing the fit of these models in a particular dataset, along with helpful examples, can be found in currently available textbooks.[47,50,72,73,74] The excellent book by Harrell[74] is especially noteworthy in its presentation of state-of-the-art regression modeling strategies for the analysis of medical data. A useful expository discussion of the Cox model with examples is provided in the paper titled "A Plain Man's Guide to the Proportional Hazards Function."[75]

META-ANALYSIS OF MULTIPLE STUDIES

Often referred to as a systematic overview, meta-analysis is a formal process for summarizing and combining data from multiple studies in a single analysis.[76] The objectives are to (a) compile and compare all of the available evidence for a particular treatment or treatment class, (b) obtain a more precise estimate of the true treatment effect, (c) strengthen the power to judge small but clinically important treatment effects, (d) assess the homogeneity of effects across multiple studies, (e) address questions of treatment effect in specific subgroups that may not be possible in a single trial, and (f) evaluate the generalizability of the results across trials, populations, and specific interventions.

There are important steps in a meta-analysis just as there are important steps in designing and analyzing a single clinical trial. Key among those steps is to prespecify the process for identifying the studies that will be included, abstracting the data from those studies, and then analyzing the data. Much has been written about meta-analysis, including textbooks on the subject that outline the requirements for a rigorous meta-analysis.[76–78] As far as the analytic methods are concerned, two contrasting approaches have been used, namely combining study results using so-called "fixed effects" models and alternatively using "random effects" models.[76] Fixed effects models assume that every study included in the analysis has estimated exactly the same parameters, and various approaches have been proposed for combining this information across the different studies. In contrast, random effects models allow for random variation in the effects between studies, and again various approaches have been developed for combining the information across studies. Some statisticians prefer the fixed effects approach while others prefer the random effects approach. Often in practice, the two approaches will lead to a similar interpretation of the data.

CONCLUDING SUMMARY

In this chapter, we have introduced concepts and methods that constitute the important *basic science* of clinical research. There are many aspects of planning and conducting a well-designed clinical research study that require careful consideration of the concepts and methods that have been presented. The remarkable advances of computer technology in recent years and the availability of a broad array of well-developed commercial software packages for performing statistical analyses make it possible to apply the methods outlined in this chapter with relative ease. This does not mean, however, that sound clinical research and the appropriate application of state-of-the-art biostatistical methods have simply become "push-button research" where computer software can easily perform a set of calculations, but little thought given to the appropriateness of the computer-generated results. Medical researchers today can shift their focus from concerns about computation to strengthening their understanding of appropriate methods for study design and statistical analysis and how to properly interpret the data resulting from their research. Readers of medical journals, now more

than ever, need to understand these methods in order to critically evaluate studies that deal with important issues regarding the natural history of disease, new treatments for a specific disease, and the public health benefit of implementing new treatment innovations.

There are additional details of the methods covered in this chapter that could not all be addressed due to space limitations. There are also other specialized methods (such as the analysis of longitudinal data) that were not covered. However, references are provided to a number of textbooks that provide greater depth of the methods that are covered and information on other topics. In addition, it would be of great value to serious researchers to establish a collaborative and collegial relationship with an experienced statistician.

We conclude by emphasizing that although some useful information can be derived from observational epidemiologic studies, a key take-home message from this chapter is that randomized clinical trials of adequate size that are carefully designed, conducted, and analyzed provide the strongest and most direct evidence on which to make a judgment about a cause-and-effect relationship. It is the evidence derived from randomized clinical trials that will have the greatest impact in our understanding of the effects of innovations in hemostasis and thrombosis as well as in many other areas of medicine.

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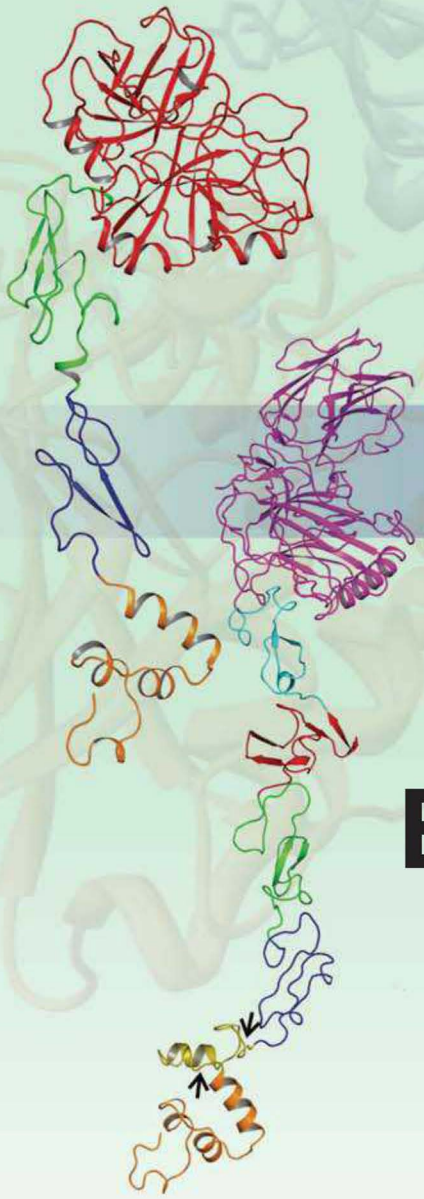

PART II

Basic Principles of Hemostasis and Thrombosis

CHAPTER 8

Overview of Basic Coagulation and Fibrinolysis

GILBERT C. WHITE, II, VICTOR J. MARDER, SAM SCHULMAN, WILLIAM C. AIRD, AND JOEL S. BENNETT

The development of a fibrin clot occurs after a series of enzymatic reactions that lead to the generation of thrombin. These reactions were originally described as an enzyme cascade[1] or waterfall,[2] a model that remains conceptually useful today (see FIGURE 8.1A). Notions of this process have evolved over the past 45 years as proteins have been purified, their function characterized, and additional factors identified. For example, factors VIII and V, which were originally depicted as enzymes in the waterfall and cascade hypotheses, were identified as nonenzymatic proteins that function as catalysts to increase the rate of activation of factor X and prothrombin, respectively. More importantly for the clinician, the idea evolved that clotting was initiated by either contact activation via a so-called *intrinsic pathway* (termed because all of the components were contained in blood) or activation by tissue factor via an *extrinsic pathway* (termed because the initiator, tissue factor, was considered extrinsic to blood). This separation into two pathways, measured by different *in vitro* clotting assays, was useful for the diagnosis of bleeding disorders and helped tie the biology closer with diagnostic testing (FIGURE 8.1B).

Over the past decade or two, concepts regarding the initiation of coagulation have continued to develop, leading to further evolution of the models for coagulation. Tissue factor and factor VIIa are now considered to be the major initiators of coagulation *in vivo*,[3,4,5] not the contact activation system, which is thought to be a key link between coagulation and inflammation. Accordingly, humoral blood coagulation is believed to initiate *in vivo* through a cell-based system (FIGURE 8.1C) that occurs when tissue factor, a membrane-associated lipoprotein that is present in most cells, is released upon cell damage or is secreted by cells such as monocytes and platelets.[6,7,8] Tissue factor forms a complex with and activates factor VII, which in turns activates factor X leading to the generation of small amounts of thrombin. This is called the "initiation phase" of coagulation. The next phase is an "amplification phase" in which the thrombin that is generated is then able to activate factor IX which activates more factor X, leading to more thrombin. The various proteins involved in coagulation, their genes, their function, and their enzymology are described in the chapters that comprise this section of the text. In this introduction, we highlight some of the features of the coagulation process. The reader is also referred to Chapters 3, 4, and 5 in the initial section of the text for background reading.

EVOLUTION OF COAGULATION PROTEINS

Just as this text is written at a point in time, the coagulation system as we describe it in this book is at a point in time. The human coagulation system has evolved over hundreds of millions of years to become the complex system it is today.[9] In lower-order organisms, thrombosis was not an issue—hemostasis was key; breeches in the vascular system had to be plugged as quickly as possible or exsanguination occurred. As reviewed in Chapter 2 of this text, the first coagulation systems were very simple, consisting of a fibrinogen, a thrombin molecule, and an activator. As organisms grew in complexity, the coagulation system evolved through domain duplication and insertion and through protein duplication. Factor VIII is a good example, a protein that has evolved over the past 450 million years to become what it is today (see FIGURE 8.2). Factor VIII is composed of three A domain repeats, a B domain, and two C domain repeats. The A domains are involved in protein–protein interactions, the B domain is removed during activation and may play a role in protein synthesis, and the C domains are involved in phospholipid binding. The A domains have their origin in blue-green algae. Over millennia, the A domain in algae duplicated and then triplicated, each A domain repeat evolving independently, to eventually form a ceruloplasmin precursor protein containing three A domain repeats, each with about 30% identity to each other. At the same time, a primordial C domain that was present in *dictyostelium* duplicated, and each C domain evolved independently. Somewhere in evolution, the ceruloplasmin precursor protein with its triplicated A domains and the duplicated C domains came together to form an A3-C2 precursor protein. Different evolutionarily unrelated B domains were inserted into the A3-C2 protein in the same position, between the A2 and the A3 domains, one B domain for factor VIII, and one B domain for factor V. The FVIII protein evolved to interact very specifically with factors IX and X in the factor X activation or "tenase" complex. The FV protein evolved to interact very specifically with factors X and II in the prothrombin-activating or "prothrombinase" complex. Presumably, these coagulation factors continue to evolve. The structure and even the function of factors VIII and V hundreds of thousands of years in the future is unknown, but evolutionary pressure will drive the process!

In a similar manner, factors VII, IX, X, and protein C, the vitamin K–dependent coagulation factors, characterized now by their serine-histidine-aspartic acid charge relay catalytic domain, the duplicated epidermal growth factor domains which are involved in protein–protein interactions, and the membrane-binding, amino-terminal γ-carboxyglutamic acid-containing domain, evolved through domain duplication and structural evolution. Prothrombin differs from the other vitamin K–dependent proteins by having two kringle domains in place of the EGF (epidermal growth factor) domains; protein Z differs by lacking the histidine and serine residues necessary

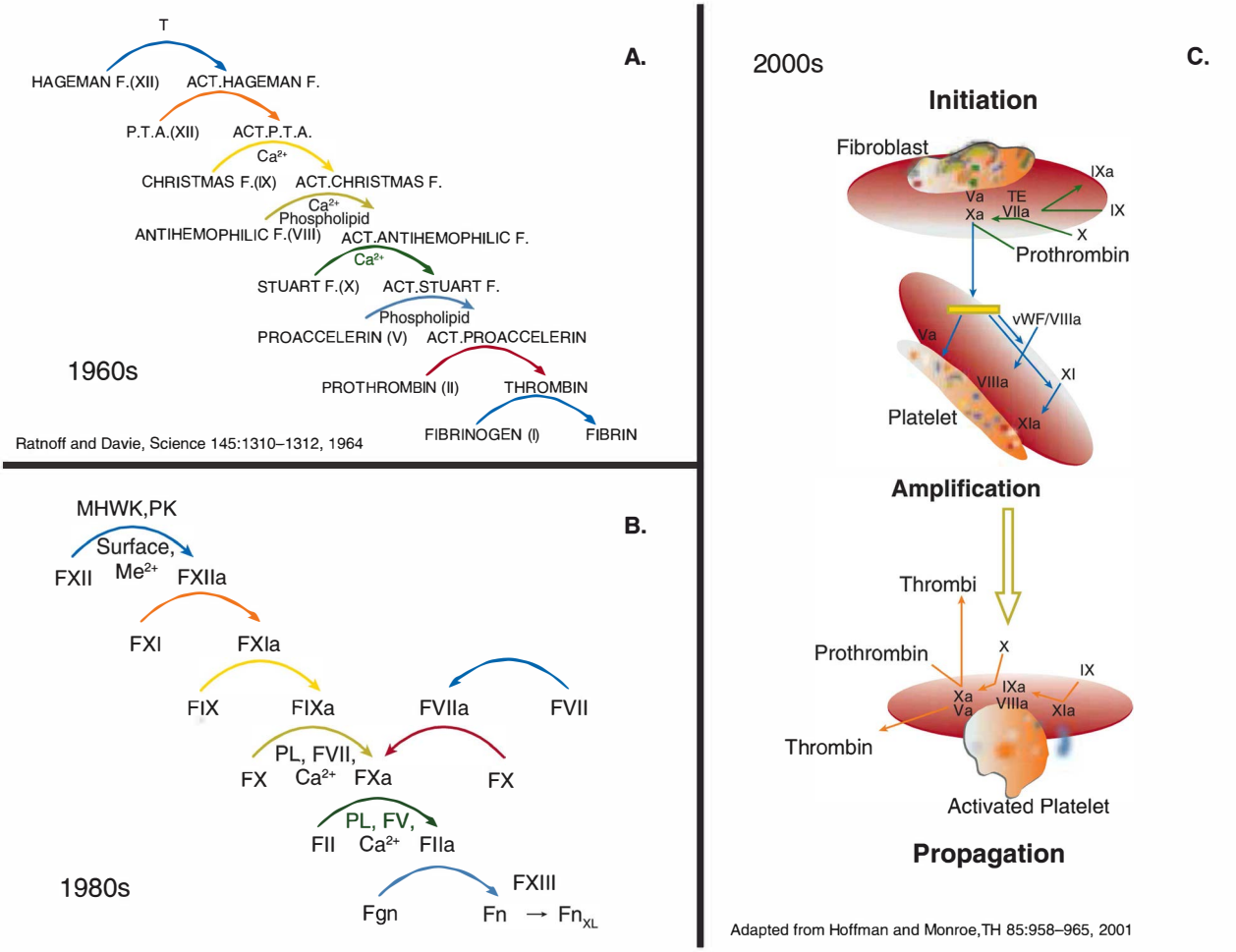

FIGURE 8.1 The human coagulation cascade through the years. **A:** The original coagulation cascade or waterfall postulated by MacFarlane[1] and by Ratnoff and Davie.[2] The development of a fibrin clot is seen as a series of sequential enzymatic reactions, starting with factor XII and ending with the conversion of fibrinogen to fibrin. **B:** Modified coagulation cascade used in the 1970s through the 1990s. This modified cascade began to separate the coagulation process into an intrinsic and extrinsic system, each assessed separately by a PT and a PTT. It also reflected the understanding that factors VIII and V were enzymatic cofactors not proteolytically active enzymes. **C:** Current concepts of coagulation, which indicate the cell-based nature of coagulation.

for catalysis in the catalytic domain. Because some of these genes are on separate chromosomes, there is speculation[9] that the duplication of the vitamin K–dependent factors came about through two rounds of total genome duplication that occurred in early vertebrate evolution some 500 million years ago.[10] Each vitamin K–dependent protein now plays a unique role in the coagulation process. Their evolution as separate clotting factors with separate functions adds to the complexity of the system, its control, and its specificity.

COAGULATION TESTING

Early on, clotting assays that were sensitive to components of the coagulation system were developed and were used to define the enzymatic pathways leading to clot formation. The two most important of these, the prothrombin time (PT) developed by Quick[11] and the partial thromboplastin time (PTT) developed by Langdell et al.[12] and later modified by Rapaport,[13] continue to be used by coagulation laboratories throughout the world, The PT measures the factors in the extrinsic pathway (FIGURE 8.1B) and is sensitive to plasma levels of factors VII, X, V, prothrombin, and fibrinogen. The PTT, which measures the intrinsic pathway (FIGURE 8.1B), is sensitive to plasma levels of the contact factors, factors IX, VIII, X, V, prothrombin, and fibrinogen. The factors common to both pathways, factors X, V, prothrombin, and fibrinogen, constitute what is called the final common pathway. It is important to emphasize that this arbitrary division into extrinsic, intrinsic, and final common pathways does not represent what occurs *in vivo* and is used simply to understand and interpret the clotting assays that are used to measure coagulation (see Chapter 50). These divisions into arbitrary pathways also do not reflect important connections that exist between the various pathways. An example is the ability of tissue factor–factor VIIa complex to activate both factor

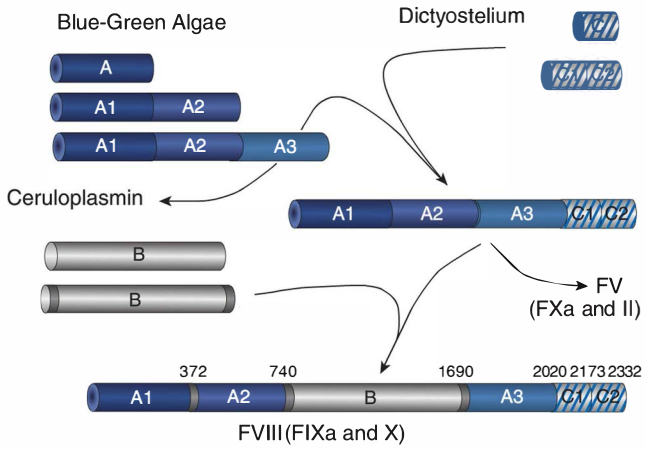

FIGURE 8.2 Evolutionary aspects of coagulation. The figure shows the evolution of coagulation factors V and VIII over an estimated 450 million years. Modern factor VIII, at the bottom, (and V, not shown) consist of triplicated A domain repeats and duplicated C domain repeats with divergent B domains interposed between the A2 and A3 domains. The A domains are derived from sequences in blue-green algae which duplicated and then triplicated. The C domains are derived from *Dictyostelium* and duplicated over time. The insertion of the B domain into factor VIII is a relatively recent event. Factor VIII has evolved (and continues to evolve) to be a cofactor in the activation of factor X by factor IXa, while factor V has evolved to be a cofactor in the activation of prothrombin by factor Xa. In similar manner, the vitamin K–dependent coagulation factors have evolved through domain building and sequence divergence. Indeed, not only have individual proteins developed by domain duplication and divergence, but the whole coagulation cascade has evolved through protein duplication and divergence. (copyright G. C. White).

IX in the intrinsic pathway as well as factor X in the common pathway. Nevertheless, the tests are useful in screening for various deficiencies in the coagulation system and as a basis for assays for the functional measurement of clotting factor activities. Tests that measure fibrinolytic activity are also available. The euglobulin clot lysis time is sensitive to proteins leading to and regulating clot lysis.

THE ROLE OF SURFACES IN COAGULATION

Functionally, the relationship between clotting and surfaces is striking.[14,15,16,17] For a process that is typically termed humoral, most of the coagulation reactions take place on biologic surfaces (see FIGURE 8.3). For example, the assembly of the prothrombinase complex, where factors Xa and thrombin-activated factor V form a complex with prothrombin, occurs on acidic phospholipids on the surface of cells. The presence of a phospholipid surface increases the rate of activation of prothrombin by several orders of magnitude (see Chapter 4). It also localizes the reaction to the site of injury and may protect the reaction from inhibitors. Likewise, the assembly of the tenase complex, where factors IXa and thrombin-activated factor VIII form a complex with factor X, occurs on acidic phospholipids. These reactions on acidic phospholipids are mediated in part by γ-carboxyglutamic acid (Gla) residues present in the amino-terminal Gla domain of the vitamin K–dependent coagulation factors.[18,19] The γ-carboxylation of glutamic acid occurs posttranslationally and confers an extra negative charge on glutamic acid that is thought to mediate calcium binding, which in turn induces a change in conformation that enables the protein to interact with acidic phospholipids on cellular surfaces.[20,21] FIGURE 8.4 shows the insertion of a Gla domain into a phospholipid surface.[21] The activation of protein C by thrombin is another reaction that occurs on a cellular surface, in this case the surface of the endothelial cell, by virtue of thrombomodulin, an endothelial-specific integral membrane protein that functions as a cofactor in the reaction, along with protein S.[22,23,24] Both protein C and protein S are vitamin K–dependent and thus interact with the membrane in the same way as factors II, VII, IX, and X. Activated protein C (APC) can thereafter interact with the endothelial protein C receptor (EPCR) to mediate the cellular effects of APC, another reaction that occurs on the cell surface by virtue of the membrane integration of EPCR.[25,26] The contact activation complex in which prekallikrein and high molecular weight kininogen form a complex with factor XII is also assembled on a surface, in this case, negatively charged surfaces like glass and kaolin, materials used to initiate coagulation *in vitro* via the intrinsic pathway.[27–30] *In vivo*, these negatively charged surfaces might be bacteria or other inflammatory generated surfaces. Thus, the assembly of cofactor, enzyme, and substrate on surfaces is a recurring theme in blood coagulation, resulting in optimal efficiency and rate of the molecular reactions, especially as a phospholipid or cell membrane provides the surface for efficient positioning of interacting enzyme complexes with proenzyme substrates.

Coagulation inhibitors such as antithrombin and heparin cofactor II are also more efficient when the reactions occur on surfaces (see Chapter 18). The typical surface in these reactions is glycosaminoglycans like heparin, heparan sulfate, and dermatan sulfate.[31,32] At least in part, heparin and the other glycosaminoglycans act as a template for the formation of a ternary complex that brings antithrombin together with its target protease, facilitating the interaction of the two reactants and thereby increasing the rate of this interaction by up to several 1,000-fold through a combination of bridging mechanisms and heparin-dependent conformational changes. A unique sequence-specific pentasaccharide in the heparin molecule contains the binding sites for both antithrombin and protease.[33] Heparin cofactor II is another serpin (serine protease inhibitor) that selectively inactivates thrombin (not factor Xa) in the presence of a heparin or dermatan sulfate template.[34] Finally, fibrin itself is an important coagulant surface. Once thrombin is bound to fibrin, it is resistant to antithrombin and even more so to antithrombin–heparin complexes.[35–37]

AMPLIFICATION AND REGULATION OF COAGULATION

An important feature of the coagulation system is the presence of amplification and regulatory influences. These are key to getting the coagulation reaction started, generating the large burst of thrombin that is needed to form a stable clot but also turning the process off so that excessive clotting does not occur. Thus, the initial formation of tissue factor–VIIa complexes can generate small amounts of thrombin that, in turn, can feed back to activate factors XI, IX, and X, as well as factors V and VIII

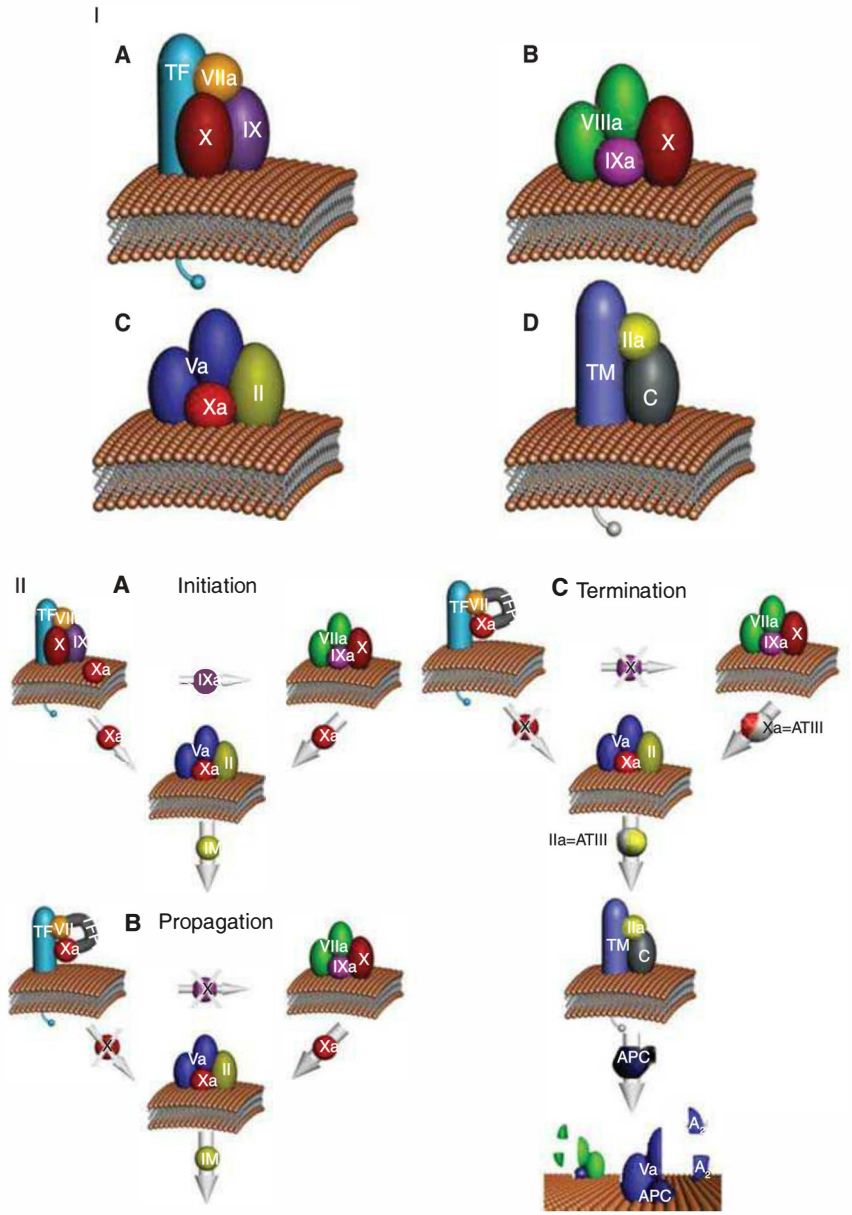

FIGURE 8.3 Surface reactions in coagulation. Coagulation reactions occur on surfaces that localize the factors and bring them together. **Part I: A:** The *tissue factor–factor VIIa complex* composed of tissue factor, which is an integral membrane protein; factor VIIa, which interacts with acidic phospholipids through its γ-carboxyglutamic acid (Gla) domain; and factors IX and X, which interact with acidic phospholipids through their Gla domains; **B:** The *tenase complex* composed of factor IXa, which interacts with acidic phospholipid surfaces through its Gla domain; factor VIII, which interacts with phospholipids through the C2 domain; and factor X, which interacts with acidic phospholipids through its Gla domain; **C:** The *prothrombinase complex* composed of factor Xa, which interacts with acidic phospholipids through the Gla domain; factor V, which interacts with phospholipids through the C2 domain; and prothrombin, which interacts with acidic phospholipids through its Gla domain; and **D:** The *protein C activation complex* composed of thrombomodulin, an endothelial integral membrane protein; protein C, which interacts with acidic phospholipids through its Gla domain; and thrombin. Other coagulation reactions such as the contact system interact with other biologic membranes, but the function remains assembly of complexes to improve enzymatic efficiency. **Part II:** The various surface reactions in the coagulation process, which are shown as isolated reactions but are in reality occurring all on the same membranes and next to each other. **A:** During *initiation*, the tissue factor–VIIa complex initiates coagulation, leading to formation of small amounts of factors IXa and Xa, which generate small amounts of thrombin. **B:** In the *propagation* phase, increased thrombin generation comes about through primarily feedback activation from factor IXa and the tenase complex. **C:** At the same time, in what is termed the *termination* phase, thrombin that is generated begins to exert an anticoagulant effect by activating protein C, which cleaves and inactivates factors V and VIII. (Figure provided by K. Mann.)

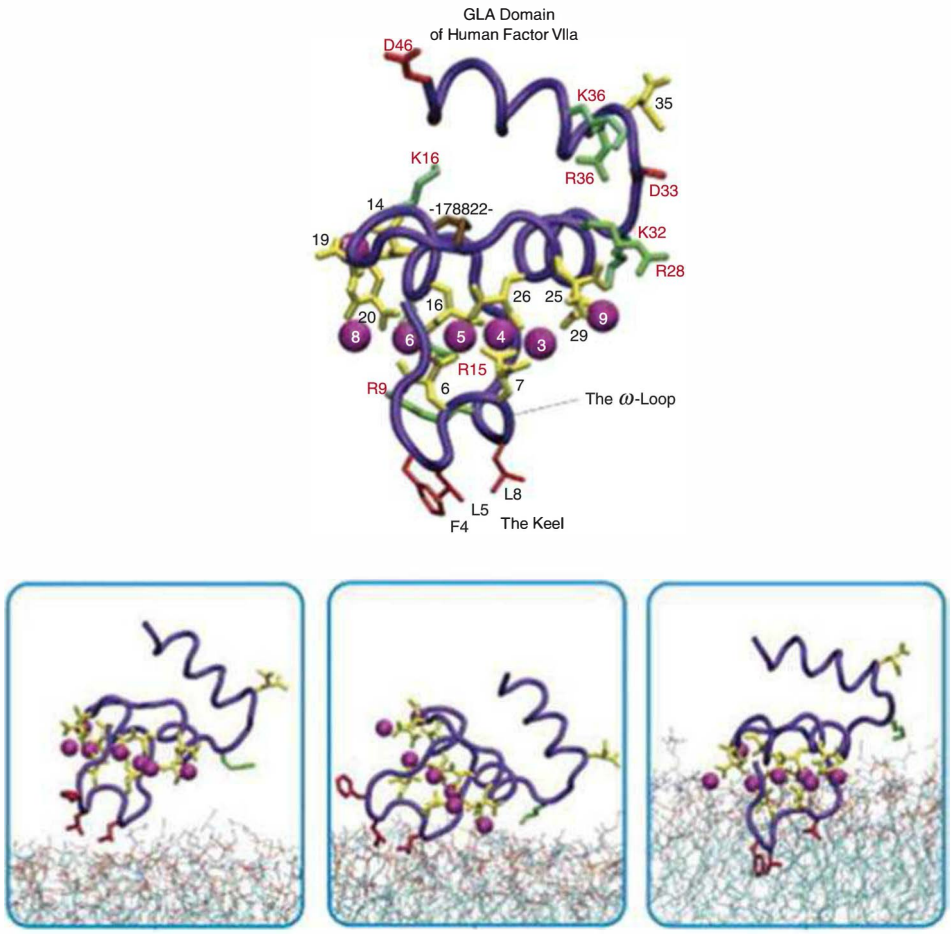

FIGURE 8.4 Insertion of a Gla domain into a phospholipid membrane. The Gla domain of human factor VIIa is depicted in the top of the figure. The backbone is shown in *purple* as a tube. Calcium ions are shown as *purple spheres* with their PDB numbering. The side chains of the hydrophobic residues of the "keel" are shown in *red*. The bottom part of the figure shows the insertion of the Gla domain into an anionic membranous bilayer of DOPS (1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine]). (Adapted from Figs 1 and 5 in Ohkubo YZ, Tajkhorshid E. Distinct structural and adhesive roles of Ca2+ in membrane binding of blood coagulation factors. *Structure* 2008;16:72–81.)

and platelets, amplifying the process and leading to generation of progressively larger amounts of thrombin. The activation of factor IX by the tissue factor–VIIa complex also serves to further activate factor X through the intrinsic pathway, further contributing to thrombin generation. There is evidence from *in vitro* work that the "burst" of thrombin is an important driver of hemostasis.[38] The greater the concentration of thrombin generated, the stronger the clot. At the same time, the generation of thrombin is strongly regulated to prevent more generalized clot formation. One mechanism of regulation relates to how thrombin is generated. For example, the generation of thrombin tends to be localized, in part, by where the tissue damage occurs and, in part, by the occurrence of clotting on cellular surfaces. Because activated platelets and inflammatory cells localize to the site of vascular damage, the generation of thrombin on the surface of these cells is also localized to the site of vascular damage. This localization promotes hemostasis without leading to more generalized thrombosis in distant parts of the circulation, although, with massive trauma and more extensive tissue damage, thrombin generation may be less localized, regulatory pathways may be overwhelmed, and disseminated intravascular coagulation may occur. Another mechanism for controlling thrombin generation is through negative feedback reactions in the coagulation system. For example, thrombin activates protein C to APC, which leads to the destruction of factors Va and VIIIa, reducing their ability to catalyze the activation of factor X and prothrombin and thereby limiting the amount of thrombin generated. Thrombin also activates plasminogen, converting it to plasmin which degrades fibrin(ogen). Plasmin, itself, also exerts positive feedback by cleavage of an activation peptide from plasminogen (connecting Glu-plasminogen to Lys-plasminogen), rendering it more susceptible to surface binding and subsequent activation by plasminogen activators. Finally, inhibitors of activated coagulation factors, such as antithrombin and tissue factor pathway inhibitor (TFPI), work to dampen thrombin generation by inhibition of activated coagulation factors or coagulation factor complexes.

The finely tuned nature of the coagulation system is underscored by the ability of even subtle genetic alterations to perturb the balance. A prevalent mutation in factor V at a site of cleavage by APC, called factor V Leiden (R506Q), results in a sevenfold increase risk of venous thrombosis in the heterozygous state and an 80-fold increase in the homozygous state.[39] This is the result of an inability of APC to induce "feedback inhibition" of factor Va in the coagulation process, leading to increased thrombin formation. Similarly, a polymorphism (G20210A) in the 3'-untranslated region of the prothrombin gene induces a mild but reproducible hypercoagulable state by virtue of increasing the transcription of prothrombin and therefore the plasma concentration of prothrombin.[40] This 25% to 35% increase in prothrombin concentration is enough to change the hemostatic–thrombotic balance. More severe alterations, including deficiency or abnormalities of antithrombin, have more prevalent effects on thrombosis. These hypercoagulable conditions are discussed in Part IV, Chapter 81 of this text.

CENTRAL ROLE OF THROMBIN AND ITS ROLE AS PROCOAGULANT AND ANTICOAGULANT

In many regards, thrombin is the central enzyme in the coagulation cascade.[41,42] Cofactors V and VIII (which are both activated and inactivated by thrombin), prothrombin (activated), factor XI (activated), factor IX (activated), factor XIII (activated), and fibrinogen ("activated") are all cleaved by thrombin. At the same time, thrombin activates the anticoagulant pathway through its effects on protein C (activated) and protein S (inactivated). As described above, thrombin is active in the fibrinolytic pathway, generating plasmin, and also activating thrombin-activated fibrinolysis inhibitor. In addition, thrombin is a strong activator of platelets, stimulating shape change, secretion, and aggregation. This effect on platelets depends on the interaction of thrombin with protease-activated receptors (PARs) on the surface of platelets. During thrombin-induced platelet activation, changes occur in the platelet membrane with the exposure of acidic phospholipids on the outer membrane leaflet. These promote coagulation by providing a procoagulant surface.[43]

Thrombin also affects the anticoagulant and procoagulant properties of the vessel wall.[44,45,46,47] Normally, the endothelial cell presents a thromboresistant surface to blood, one that is unreceptive to coagulation factors and only weakly reactive with platelets and other vascular cells. These weak interactions with platelets and leukocytes are mediated by integrins and promote rolling along the surface, but these are transient interactions and essentially serve to help localize platelets and leukocytes to the area where they might be needed in case of vascular injury. This thromboresistance is related to various properties of endothelium, from charge to heparin-like glycosaminoglycans that coat the endothelium to inhibitory molecules such as prostacyclin (PGI_2), thrombomodulin, TFPI, nitric oxide, plasminogen activators, and CD39 (ecto-NTP diphosphohydrolase). However, with the generation of thrombin, the relationship between the endothelium and blood changes. Thrombin activates endothelial cells through PARs to effect a variety of biologic responses, including contraction, relaxation of interendothelial junctions leading to increased vascular permeability, reduction in thrombomodulin expression, secretion of von Willebrand factor, downregulation of CD39, upregulation of transforming growth factor β, and secretion of endothelin which contributes to vasoconstriction; thrombin also increases the generation of nitric oxide and prostacyclin. Thus, although generating a somewhat mixed response, the overall effect of thrombin on the vessel wall would be predicted to reduce the natural thromboresistance of the vessel wall and increase the potential to participate in thrombus formation. Thrombin also plays a role in atherogenesis, partly through its ability, along with platelet-derived growth factor and fibroblast growth factor, to stimulate smooth muscle cell proliferation.

More recently, thrombin has been shown to play an important role outside its traditional role in coagulation. For example, thrombin has been linked to cancer metastasis and tumor angiogenesis.[48,49] Thrombin also is important in normal angiogenesis. Thrombin plays a key role linking coagulation and inflammation.[50,51] Thrombin upregulates proinflammatory molecules that increase endothelial permeability, in part leading to leukocyte diapedesis and transmigration of cytokines. Thrombin also affects the immune system, stimulating the release of inflammatory cytokines such as IL-6 from cells such as monocytes and acting directly on cells of the innate immune system. This capacity of thrombin to bring both the coagulation and inflammatory pathways together is a key to host defense mechanisms in man.

All of these underscore the fundamental importance of thrombin in a variety of biologic processes, from hemostasis and thrombosis to processes not initially thought to be related to coagulation such as cancer, inflammation, and the immune response.

In conclusion, the focus in this section is on the humoral coagulation system, the proteins and reactions leading to both clot formation and clot dissolution and the proteins that control or regulate those reactions. The topics are the structure of the proteins, their genetics, the role of tissue factor and its inhibitor, the cofactors V and VIII and their role in coagulation, the biology of von Willebrand factor, the vitamin K–dependent factors, the contact factors, fibrinogen, factor XIII, the inhibitors antithrombin, protein C and protein S, and the fibrinolysis system. The reader is also referred to the chapters in the initial section of the book, which describe some of the basic processes involved in thrombosis and hemostasis, including the principles of molecular biology, enzymology, cell biology, and immunology. These basic chapters are intended to provide companion reading for each of the initial Sections of the book. For example, Chapter 3 by Everett, et al., entitled Molecular Biology, is relevant to all of the Chapters in this section. Molecular biology has been an important key to understanding the biology of the coagulation system, starting with the initial cloning of the cDNAs for each protein. For most, this provided the first insight into structure and the initial correlation with function. Structure has been essential for understanding the enzymatic specificity of the proteins, their interactions with membranes, the formation of enzymatic complexes, and the interaction with inhibitory proteins. Mouse models, crystal structures, and gene transfer vectors all derive from the molecular biology of the proteins. Structure will be critically important to understand how antibodies form in response to treatment as well as for inhibiting the immune response after gene transfer. The genomic structure is important for understanding the control of protein production and the evolution of the coagulation system. Chapter 4 by Krishnaswamy provides important enzymology background for this section. The coagulation system is largely an enzymatic cascade and understanding the basic principles of enzyme biochemistry and kinetics is key to understanding the control of coagulation. Finally, Kaufman and Popolo describe the cell biology of protein production in Chapter 5. Knowing how proteins are synthesized

and the nature of their posttranslational modifications, such as γ-carboxylation of the vitamin K–dependent proteins, is important to understanding how they function. In turn, the chapters in this section provide important background for later sections on disorders of the blood coagulation system.

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CHAPTER

9

The Blood Coagulation Factors and Inhibitors: Their Primary Structure, Complementary DNAs, Genes, and Expression

DOMINIC W. CHUNG, WENFENG XU, AND EARL W. DAVIE

A BRIEF INTRODUCTION TO THE BLOOD COAGULATION CASCADE

When tissue damage occurs, platelets become activated, aggregate, and adhere to the site of vascular injury where they form a thrombus. During this process, the blood coagulation cascade is initiated when the tissue factor (TF) comes in contact with factor VII (or factor VIIa) in the flowing blood and forms a potent enzyme complex at the site of injury (FIGURE 9.1). This complex initiates a series of reactions involving coagulation proteins circulating in blood in a precursor or inactive form.[1] The complex of factor VIIa and TF catalyzes the conversion of factor X to factor Xa. Factor Xa, in turn, converts prothrombin to thrombin in the presence of factor Va, phospholipid, and calcium. Thrombin then converts fibrinogen to fibrin, leading to an insoluble clot of activated platelets and fibrin polymers. Thrombin also activates the intrinsic pathway of blood coagulation by converting factor XI to factor XIa by a mechanism that is poorly understood. Factor XIa, in turn, converts factor IX to factor IXa in a reaction requiring calcium. Factor IXa then converts factor X to factor Xa in the presence of factor VIIIa, phospholipid, and calcium. This reaction provides a second mechanism for the activation of factor X in blood. Thrombin plays a critical role in blood coagulation in that it converts fibrinogen to fibrin, factor V and factor VIII to active cofactors, factor XI and protein C to serine proteases, factor XIII to a transglutaminase, and finally, it activates platelets (FIGURE 9.1). All these activation reactions involve minor proteolysis at arginine peptide bonds. The phospholipid participating in several of these steps is provided by the activated platelets and cell membranes. Divalent calcium is also required in several steps of the coagulation cascade, and the normal level ranges from about 3.5 to 5 mM in blood.

FACTOR VII

Factor VII is highly homologous in its amino acid sequence and gene organization with the other vitamin K–dependent proteins, including factor IX, factor X, and protein C (Table 9.1). In the presence of TF, factor VII initiates the extrinsic pathway. Factor VII is a single-chain glycoprotein (Mr 50,000) that is synthesized in the liver and secreted into the blood as a zymogen composed of 406 amino acids (FIGURE 9.2).[2–5] It contains 10 γ-carboxyglutamic acid residues that are localized in the Gla domain of the protein (amino acids 1 to 35). The Gla region is followed by two epidermal growth factor (EGF)-like domains and a serine protease domain (FIGURE 9.2). The γ-carboxyglutamic residues require vitamin K for their biosynthesis.[6] Factor VII also contains a residue of β-hydroxyaspartic acid (amino acid 63), located in the first of the two EGF-like domains.[7] The first EGF-like domain stabilizes the factor VIIa–TF complex and participates in calcium binding.[8–10] The β-hydroxylation of the Asn63 is not essential for the coagulant activity of factor VIIa since recombinant factor VII is not β-hydroxylated, and functions as well as the plasma-derived counterpart.[11] The first EGF-like domain contains novel *O*-linked carbohydrates at residues Ser52 and Ser60.[12–14] The Ser52 is *O*-glycosylated, being linked to either a disaccharide (Xyl-Glc) or a trisaccharide (Xyl2-Glc) in approximately equal amounts. The Ser60 contains one residue that is conjugated to fucose by *O*-glycosylation. Recombinant factor VII/VIIa missing either one or both *O*-linked Ser residues exhibits decreased TF binding but has similar calcium binding relative to the wild-type.[9] Factor VIIa has another potential carbohydrate attachment site located at Asn322. Factors VII and VIIa contain two high-affinity calcium-binding sites located in the protease domain and the first EGF1 domain as well as six to seven low-affinity calcium-binding sites in the Gla domain.[15]

Factor VII is converted to a serine protease (factor VIIa) by minor proteolysis. Factor VIIa, however, has little if any physiologic activity until it combines with TF. The factor VIIa–TF complex then converts factor X to factor Xa in the presence of phospholipids and calcium ions.[1] Factor VIIa also converts factor IX to factor IXa in the presence of TF and calcium ions.[9] The physiological importance of the latter pathway is unclear. *In vitro* experiments evaluating the initiation phase of blood coagulation with ultrasensitive fluorescent markers indicate that factor Xa is generated almost exclusively by the factor VIIa–TF complex during the initiation phase of coagulation.[16] The conversion of human factor VII to factor VIIa is catalyzed by thrombin and factor Xa as well as factor IXa, and factor XIIa.[3,5,17–20]

The activation of factor VII is due to the cleavage of a single peptide bond (Arg152-Ile) (FIGURE 9.2). This leads to the formation of a salt bridge between the α-ammonium group of Ile153 and the carboxylate group of Asp343 and a reregistration of a β-strand in the protein,[21] resulting in a conformational change and formation of an active charge-relay system. Factor VIIa may also be formed by an autocatalytic mechanism,[22,23] but this pathway is very slow and may have

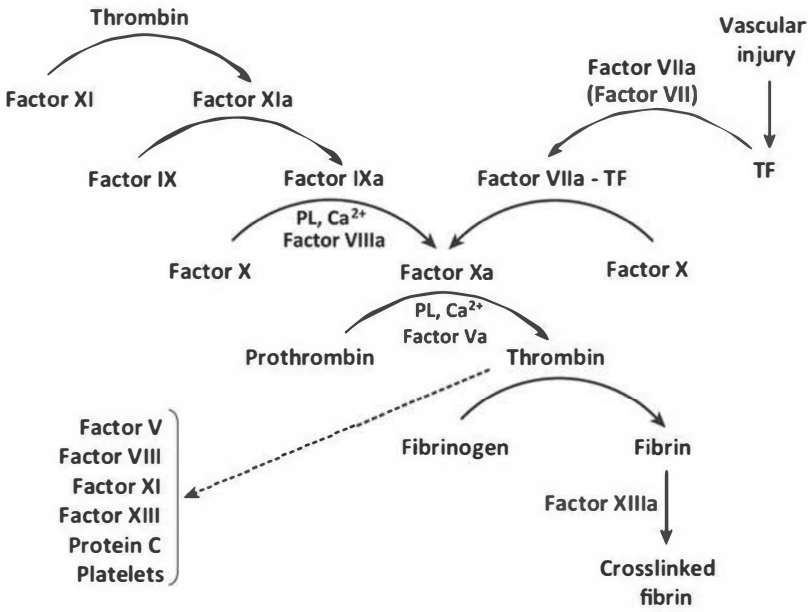

FIGURE 9.1 An abbreviated blood coagulation cascade. (Modified from Davie EW, Fujikawa K, Kisiel W. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* 1991;30:10363.)

little physiological importance.[24] Factor VIIa is a serine protease composed of a light chain (152 amino acids) and a heavy chain (254 amino acids) held together by a single disulfide bond between Cys135 and Cys262. The light chain contains the Gla domain followed by the two EGF domains, whereas the heavy chain contains the catalytic domain with the active site residues of His193, Asp242, and Ser344. The active site Ser344 is located in the same sequence of Gly-Asp-Ser-Gly-Gly-Pro that is present in all the other serine protease clotting factors, including thrombin, factor IX, factor X, factor XI, and activated protein C (APC).

The crystal structure of human factor VIIa lacking the *N*-terminal γ-carboxyglutamic acid domain is shown in FIGURE 9.3.[25] Factor VIIa has an elongated shape similar to that of a tulip, in which the Gla and EGF-like domains form the stem while the catalytic domain forms the blossom.

The cDNA and gene for factor VII have been isolated, and their sequence determined.[26,27] The sequence of the mRNA coding for human factor VII contains approximately 2,450 nucleotides (nts) that code for a prepro leader sequence of 38 amino acids and 406 amino acids present in the mature protein circulating in blood (FIGURE 9.2). A noncoding region of 1,026 nts plus a poly(A) tail follows the stop codon of TAG. The noncoding region also contains the polyadenylation recognition sequence of AATAAA, which are located 40 nts upstream from the poly(A) tail.[28]

A second clone has been identified for human factor VII, containing a prepro leader sequence of 60 amino acids.[26] This leader sequence contains an additional 22 amino acids inserted between Val at –17 and Ala at –18 in the 38–amino acid prepro leader sequence encoded by an additional exon in intron A, suggesting that the two mRNA species result from alternative splicing.[27] The removal of the prepro leader sequence requires signal peptidase in addition to a second processing protease that cleaves the peptide bond following the arginine at position –1.[29]

The gene for factor VII (F7) spans approximately 12.8 kb and consists of eight exons interrupted by seven introns. The positions of the introns with respect to the amino acid sequence and the types of intron–exon boundaries are almost exactly the same as the genes coding for the other vitamin K–dependent proteins (FIGURE 9.1 and Table 9.1). The gene coding for factor VII also contains five regions of tandem repetitive sequences, and more than a quarter of the intron sequences consist of minisatellite DNA sequences, which vary in the number of copies among individuals.[30]

The absence of a CCAAT box in the factor VII promoter contrasts with the promoter structures of factor IX, which has a functional CCAAT box, and factor X, which contains a putative CCAAT box that binds the ubiquitous transcription factor NF-Y.[31–33] The major transcription start site for factor VII is located approximately 50-bp upstream from the first initiation Met and is close to the binding sites for a Sp1-like transcription factor and HNF-4.[34,35] The G-C rich Sp1-like site is located at –100 to –94, whereas the HNF-4 site is located at –63 to –58. The factor VII HNF-4 recognition sequence, ACTTTG, is also present in the promoters of factor X and factor IX. A naturally occurring mutation in the factor IX HNF-4 site causes the hemophilia B Leiden phenotype, whereas a similar mutation in the factor VII promoter causes lifelong bleeding and virtually no detectable factor VII.[33,36]

Genetic defects leading to an abnormal factor VII include a replacement of Arg304 or Arg353 with Gln, resulting in reduced clotting activity,[37–39] presumably by a conformational change near the catalytic site. A substitution of Phe38 to Ser (factor VII central)[40] and factor VII variant Gln100 to Arg[41] result in reduced TF binding and impaired activation of factors IX and X.[40] A polymorphism originating from a decanucleotide (CCTATATCCT) inserted at position –323 relative to the first Met-initiating codon[42] and a substitution of G for T at nt –81 disrupts an HNF-4–binding site in the factor VII promoter,[36] resulting in a modest reduction in factor VII levels.[42]

Pharmaceutical preparations of recombinant factor VIIa have been very helpful in the treatment of patients with factor VIII deficiency and inhibitors as well as in patients with other coagulopathies.[43,44]

Table 9.1

Comparison of intron location and splice junction type and size for human factors VII, IX, X, protein C, and prothrombin

Intron	Protein	Location (Amino Acid)	Splice Type	Size (bp)
A	Prothrombin	–17	1	386
	Factor VII	–17	1	2,574
	Factor IX	–17	1	6,206
	Factor X	–17	1	6,542
	Protein C	–17	1	1,263
B	Prothrombin	37/38	0	659
	Factor VII	38/39	0	1,919
	Factor IX	38/39	0	188
	Factor X	37/38	0	8,836
	Protein C	37/38	0	1,462
C	Prothrombin	46	1	242
	Factor VII	46	1	68
	Factor IX	47	1	3,689
	Factor X	46	1	874
	Protein C	46	1	92
D	Factor VII	84	1	1,908
	Factor IX	85	1	7,163
	Factor X	84	1	1,447
	Protein C	92	1	102
E	Factor VII	131	1	971
	Factor IX	128	1	2,565
	Factor X	128	1	2,798
	Protein C	137	1	2,668
F	Factor VII	*15/16*[a]	0	595
	Factor IX	*15/16*	0	9,473
	Factor X	*15/16*	0	3,224
	Protein C	*15/16*	0	873
G	Factor VII	*57*	1	816
	Factor IX	*54*	1	668
	Factor X	*55*	1	1,418
	Protein C	*55*	1	1,129

bp, base pair

[a]Numbering of amino acids prior to intron F begins with residue 1 of the heavy chain of the active molecules.

Data from O'Hara PJ, Grant FJ, Haldeman BA, et al. Nucleotide sequence of the gene coding for human factor VII, a vitamin K–dependent protein participating in blood coagulation. *Proc Natl Acad Sci US A* 1987;84:5158; Leytus SP, Foster DC, Kurachi K, et al. Gene for human factor X: a blood coagulation factor whose gene organization is essentially identical with that of factor IX and protein C. *Biochemistry* 1986;25:5098; Degen SJ, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 1987;26:6165; Foster DC, Yoshitake S, Davie EW. The nucleotide sequence of the gene for human protein C. *Proc Natl Acad Sci U S A* 1985;82:4673; Yoshitake S, Schach BG, Foster DC, et al. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 1985;24:3736.

TISSUE FACTOR

Human TF (Mr 44,000) is a transmembrane glycoprotein synthesized in adventitial fibroblasts. When blood comes into contact with the subendothelium following vascular injury,[45–48] factor VII binds to TF to form a bimolecular complex in the presence of calcium ions,[1,49–54] initiating coagulation. TF is a single-chain protein containing 263 amino acids and is synthesized with a signal peptide of 32 amino acids (FIGURE 9.4).[55,56,57] The extracellular or cellular surface domain of TF is 219 residues and contains three repeating sequences of Trp-Lys-Ser. It also contains two disulfide bonds linking Cys49 with Cys57, and Cys186 with Cys209.[58] The membrane-spanning region of TF is 23 amino acids (residues 220 to 242), whereas the cytoplasmic portion of the protein at the carboxyl end of the molecule is 21 residues in length. The cytoplasmic portion also contains a half-Cys residue (Cys245) that is acylated by palmitic or stearic acid.[58] Three potential glycosylation sites with a sequence of Asn-X-Thr/Ser (Asn11, Asn124 and Asn137) are also present in the molecule.[55,56,57]

The crystal structure of TF,[59] and factor VIIa–TF complex has been determined in several different laboratories.[8,60] The extracellular region of TF (TF-219) consists of two immunoglobulin-like domains that are formed by two antiparallel β-sheets (FIGURE 9.5). The crystal structure of factor VIIa in a complex with a mutant of bovine pancreatic trypsin inhibitor (BPTI) lacks about two-thirds of the amino-terminal Gla domain of factor VIIa (FIGURE 9.6, **left**). The complex of TF (TF-219) and the factor VIIa-BPTI mutant (FIGURE 9.6, **right**),[60] enhanced by calcium about 150,000 fold,[15] is >100 Å long and 40 to 60 Å wide. In the absence of the inhibitor, the catalytic domain of factor VIIa, bound to the BPTI mutant at the top of the complex, is readily available to bind factor X and convert it to factor Xa.

The mRNA for human TF is 2.3 kb,[55,56,57] including a 5′ noncoding region of 75 bp, 885 bp of coding sequence, a stop codon, and a 3′ noncoding region of 1,141 bp, followed by a poly(A) tail. The gene for human TF (F3) spans 12.4 kb on chromosome 1,[56,61] contains six exons separated by five introns (A to E, Fig. 9.4), three full Alu sequences, one partial Alu sequence, and a typical TATA promoter element present 26-bp upstream from the cap site. TF in vascular endothelium and monocytes can be induced with several agents, such as phytohemagglutinin and endotoxin, interleukin 1, TNF, phorbol esters, and thrombin.[62–68] Functional studies indicate that basal TF transcription is controlled by the transcription factor Sp1. A distal enhancer (–227 to –172 bp) containing two AP1 sites and an NFκB site mediates the induction of the TF transcription in monocytic and endothelial cells.[69]

FACTOR X

Factor X (Mr 58,800) is a vitamin K–dependent glycoprotein (15% carbohydrate) that is synthesized in the liver and secreted into the plasma. Human factor X is composed of a light chain of 191 amino acids (Mr 16,200) and a heavy chain of 254 amino acids (Mr 42,000) held together by a single disulfide bond (FIGURE 9.7).[70,71,72,73] The light chain of human factor X contains 11 residues of γ-carboxyglutamic acid, one residue of β-hydroxyaspartic acid (residue 63),[71] and two EGF-like domains,[73–75] the first of which contains one high-affinity calcium-binding site.[76]

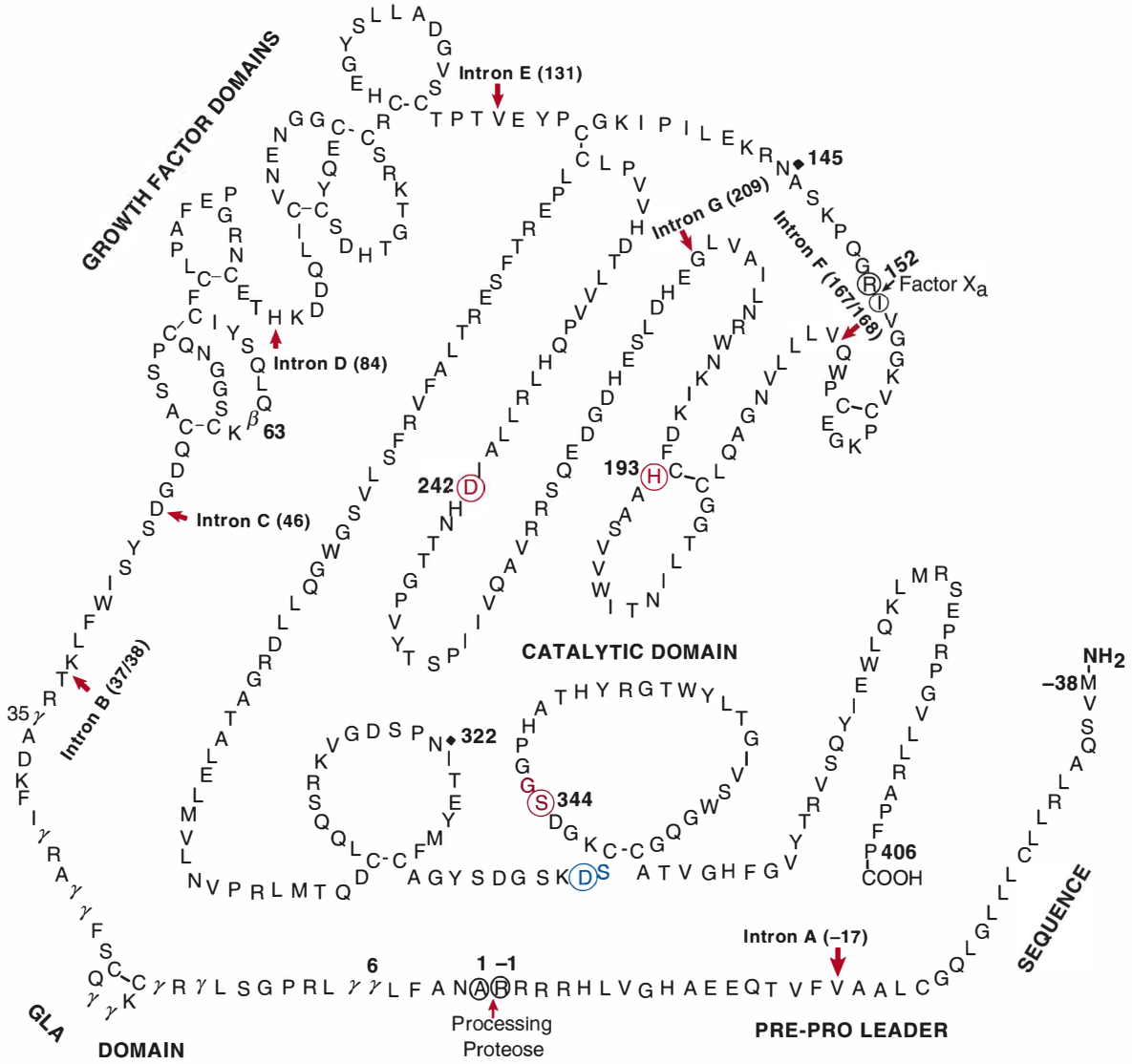

FIGURE 9.2 Amino acid sequence for human prepro factor VII. The prepro leader sequence (–38 to –1) is removed during biosynthesis by signal peptidase and a processing protease that hydrolyze the Arg(–1)-Ala bond. The single cleavage site by factor Xa is shown by a *solid arrow*. The Gla domain and potential growth factor domains are located within residues 1 to 152, which constitute the light chain of factor VIIa. The heavy chain or catalytic domain contains 254 residues, including the three principal amino acids participating in catalysis. The amino acids (His193, Asp242, and Ser344) are *circled*. Two potential carbohydrate attachment sites are shown by *solid diamonds*. The proposed disulfide bonds have been placed by analogy to those established in bovine prothrombin and epidermal growth factor. The single-letter code for amino acids is as follows: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; Q, Gln; E, Glu; G, Gly; H, His; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val; γ, γ-carboxyglutamic acid; β, β-hydroxyaspartic acid.

The heavy chain of factor X contains the activation peptide and the catalytic domain.[77] The activation peptide also contains two potential *N*-linked carbohydrate-binding sites, including an Asn39-Gln-Thr sequence and an Asn49-Leu-Ser sequence. In bovine factor X, there is an *N*-linked (Asn36) as well as an *O*-linked (Thr300) carbohydrate chain.[77]

Factor X is synthesized with a prepro leader sequence that requires two processing steps for its removal (FIGURE 9.8). These reactions are catalyzed by a signal peptidase as well as by a furin-like enzyme that cleaves the Arg residue on the carboxyl end of the propiece. Additional processing in the single-chain precursor occurs between Arg139 and Ser143, which is the *N*-terminal residue in the activation peptide. This protease activity results in the removal of a basic tripeptide of Arg-Lys-Arg and the formation of the two-chain circulating molecule that is held together by a single disulfide bond (FIGURE 9.7).

The crystal structure of factor Xa lacking the Gla domain is very similar to that of factor VIIa and factor IXa (FIGURE 9.9), an elongated molecule of 100 Å in length.[78] In the intact molecule,

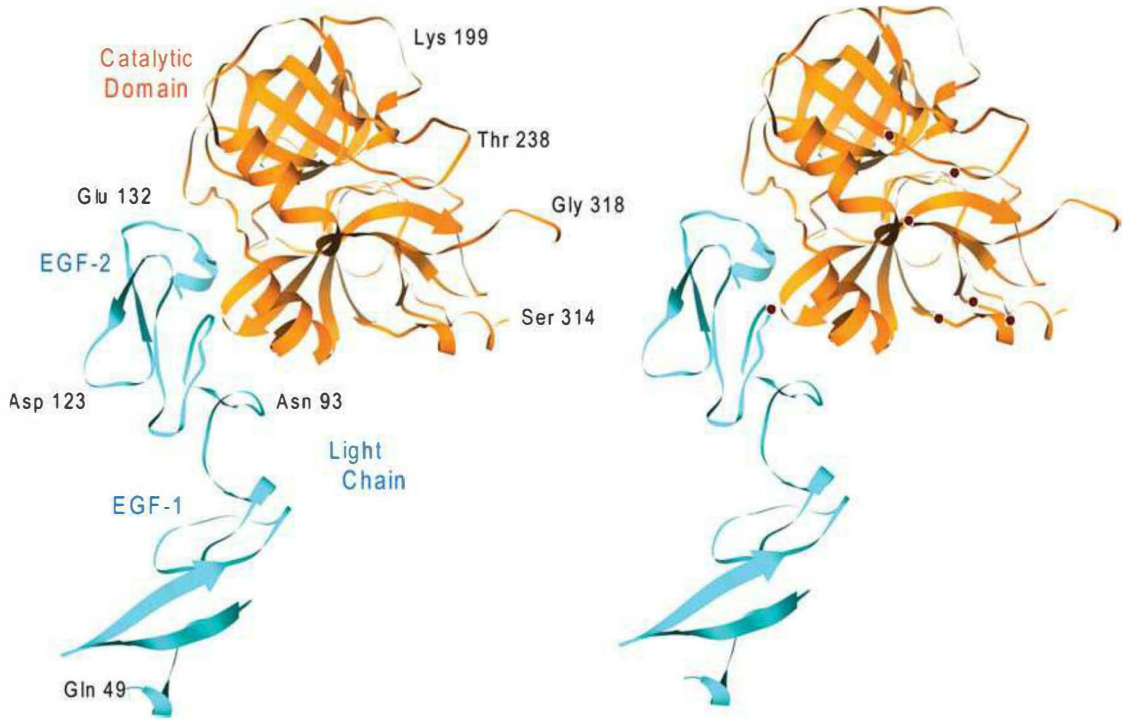

FIGURE 9.3 Ribbon structure of Gla domainless factor VIIa starting with Gln 49. The protein takes the shape of a tulip with the EGF1 and EGF2 domains (*colored cyan*) forming the stem, while the catalytic domain (*colored orange*) is the flower. The structure on the right shows the location of seven known mutations (*black dots*) in the human protein. (Modified from Pike AC, Brzozowski AM, Roberts SM, et al. Structure of human factor VIIa and its implications for the triggering of blood coagulation. *Proc Natl Acad Sci U S A* 1999;96:8925.)

the Gla region of factor X is partially buried in the phospholipid surface, which is about 61 to 69 Å from the active site.[79]

Activation of factor X involves the cleavage of an Arg-Ile1 peptide bond in the amino-terminal end of the heavy chain, liberating a small activation peptide of 52 amino acids (FIGURE 9.7).[70] During the activation reaction, the new amino-terminal Ile1 residue turns and flips into the interior of the catalytic domain. The new α-ammonium group of Ile1 then forms a salt bridge with the carboxylate group of Asp184 (*circled in green*, FIGURE 9.9),[80] generating a serine protease with an active catalytic site involving His42, Asp88, and Ser185 (*circled in blue*, FIGURE 9.9).

The gene for human factor X (F10) contains eight exons and seven introns on chromosome 13 at q32-qter in approximately 27 kb of DNA,[72,81] only 2.8 kb downstream from the gene coding for factor VII.[32] The introns are located between amino acid residues –17 (intron A), 37, and 38 (B), at residue 46 (C), and at residue 84 (D) between the two potential growth factor domains (FIGURE 9.7 and Table 9.1). Intron E is located at residue 128 following the second growth factor domain and just before the disulfide bond connecting the light and heavy chains. The last two introns (F and G) are located in the heavy chain or catalytic domain of the molecule and are present between residues 15 and 16 and at residue 55. The rest of the catalytic chain is free of introns.

The first patient identified with factor X deficiency[82–84] had a single-nucleotide change of G to A (GTG to ATG) resulting in a Val148Met change in the catalytic domain of the protein (*red arrow*, FIGURE 9.8) (Asakai, Roberts, Davie, unpublished data). Other molecular aberrations include partial deletions of exons VII and VIII, and amino acid substitutions of Arg for Gly(–20), Lys for Gla14, Ser for Pro343, and Cys for Arg366.[85–90] Amino acid changes that result in a loss of biological activity have been found throughout the catalytic domain as well as the EGF domains (FIGURE 9.10, *right panel*).

The mRNA for human factor X includes 1,475 nts that code for a prepro leader sequence of 40 amino acids, a light chain of 139 amino acids, a connecting tripeptide, and 303 amino acids that constitute the heavy chain.[73,75] The processing or polyadenylation sequence of ATTAAA is unusual in that it is located in the coding sequence and precedes the stop codon by one nucleotide.[28]

Two transcription start sites, a major one at –16-bp and a minor one at –10-bp upstream from the initiation Met codon, are present in the human factor X 5′-flanking region.[91] Full liver-specific promoter activity is contained within a 457-bp region upstream from the translation start site.[32] An apparent CCAAT sequence, present at –120 to –116 bp, binds the ubiquitous transcription factor NF-Y.[34] An HNF-4 functional element has been localized between –63 and –42 bp, and HNF-4 binding is required for liver-specific expression. Two additional positive regulatory regions have been identified at –215 to –149 bp and at –457 to –351 bp.[32]

FACTOR V

Factor V (Mr 330,000) is a glycoprotein that is synthesized as a single-chain molecule of 2,196 amino acids in liver and megakaryocytes and circulates in blood as an inactive cofactor

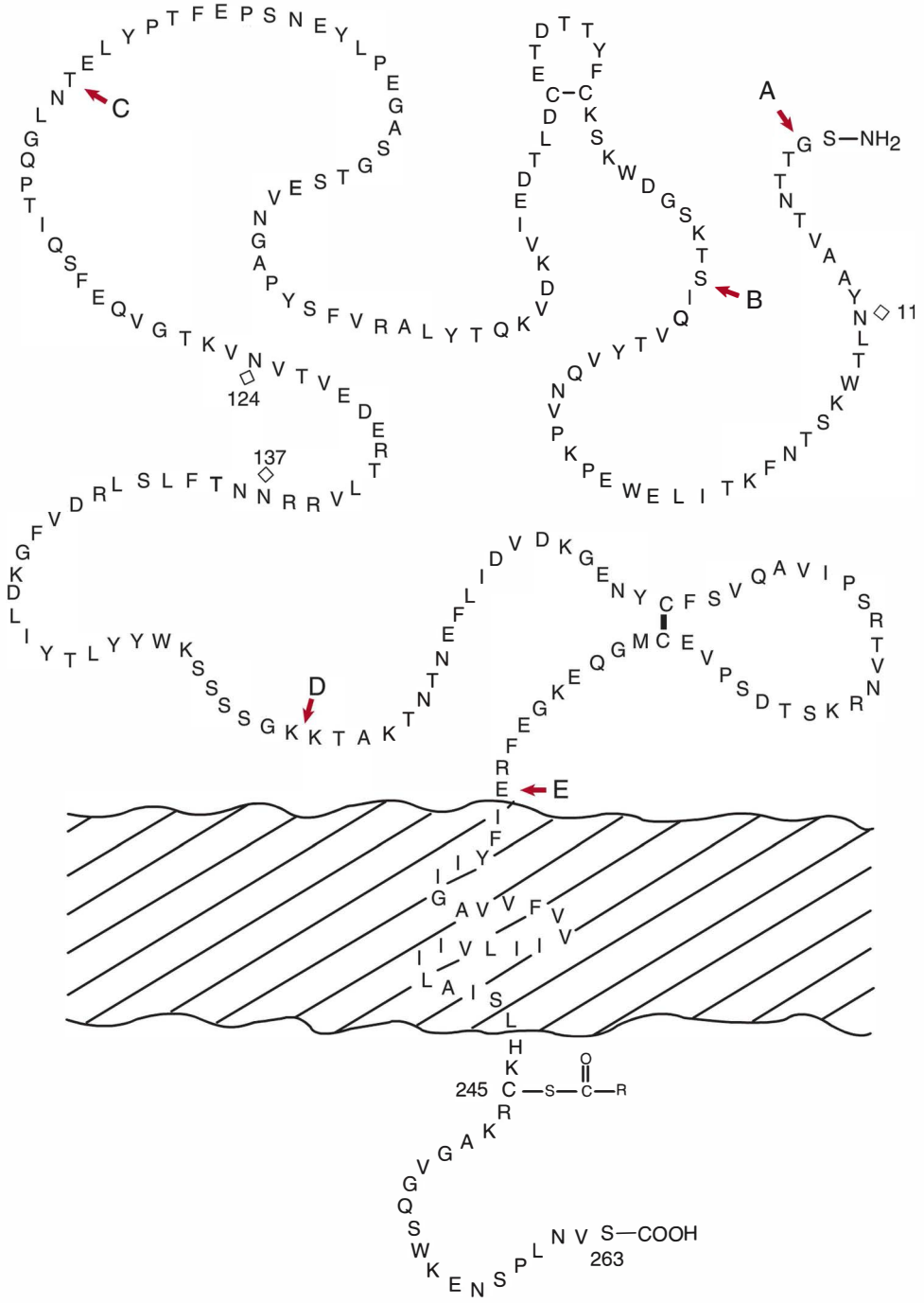

FIGURE 9.4 Amino acid sequence of human TF. The *solid arrows* indicate the position of the five introns (A to E). The three potential carbohydrate-binding sites are indicated by *open diamonds*. Cys 245 is acylated by palmitic or stearic acid.

at 7 μg/mL.[92] Approximately 20% of the factor V in blood is present in the α-granules of platelets.[93] Factor V is comprised of six domains (A1-A2-B-A3-C1-C2) that are highly homologous to those in factor VIII and ceruloplasmin (FIGURE 9.11).[94–97] The second and third A domains of factor V and factor VIII are separated by a large connecting B domain. In factor V, this domain is 836 amino acids in length, is located between amino acids 710 and 1,545, and has two tandem repeats of 17 amino acids and 31 tandem repeats of nine amino acids with a consensus sequence of [TNP]LSPDLSQT (FIGURE 9.12). The B domain in factor V shows no similarity in amino acid sequence to that present in factor VIII. Interestingly, chimeric cDNA expression constructs in which the connecting regions of factor V and factor VIII were exchanged show that sequences within the factor V connecting region increase the expression of the factor VIII chimera and its corresponding mRNA by twofold in COS1 cells.[98] In another

FIGURE 9.5 Ribbon structure of the extracellular domain (219 *N*-terminal residues) of TF. The two immunoglobulin-like domains TF-1 and TF-2 (*magenta*) are formed by two antiparallel β-sheets. The *N*-terminal Ser is located in TF-1. (Modified from Harlos K, Martin DM, O'Brien DP, et al. Crystal structure of the extracellular region of human tissue factor. *Nature* 1994;370:662.)

study, the connecting region or B domain of factor V did not require the chaperone proteins calnexin or calreticulin for efficient secretion, whereas the connecting region or B domain of factor VIII was required for both chaperone protein binding and proper secretion of factor VIII in a Chinese hamster ovary (CHO) cell line.[99] The third A domain in factor V, as well as factor VIII, is followed by two C domains. Each of the C domains is approximately 150 amino acids in length, with sequence identity of 35% to 50%.

The membrane-binding C2 domain of factor Va contains a β-barrel motif composed of eight antiparallel strands (FIGURE 9.13),[100] which form two tightly packed β-sheets of five and three strands. The bottom portion of the β-barrel is rich in basic amino acids and consists of three β-hairpin loops that form the calcium-independent membrane-binding site of factor Va.

Before participation in the coagulation cascade, the single-chain factor V undergoes minor proteolysis[95] by thrombin at Arg709, Arg1018, and Arg1545. Factor V can be activated by factor Xa, but the physiological importance of this activation pathway has not been established.[101]

Factor Va is composed of a heavy chain (A1-A2 domains, Mr 110,000) derived from the amino-terminus of the protein and a light chain (A3-C1-C2 domains, Mr 78,000) derived from the carboxyl-terminus of the molecule (FIGURE 9.11). The fragments corresponding to the central connecting B region are released from factor V during its conversion to factor Va by thrombin. The newly generated heavy and light chains associate and are held together by calcium that is bound to a high-affinity site

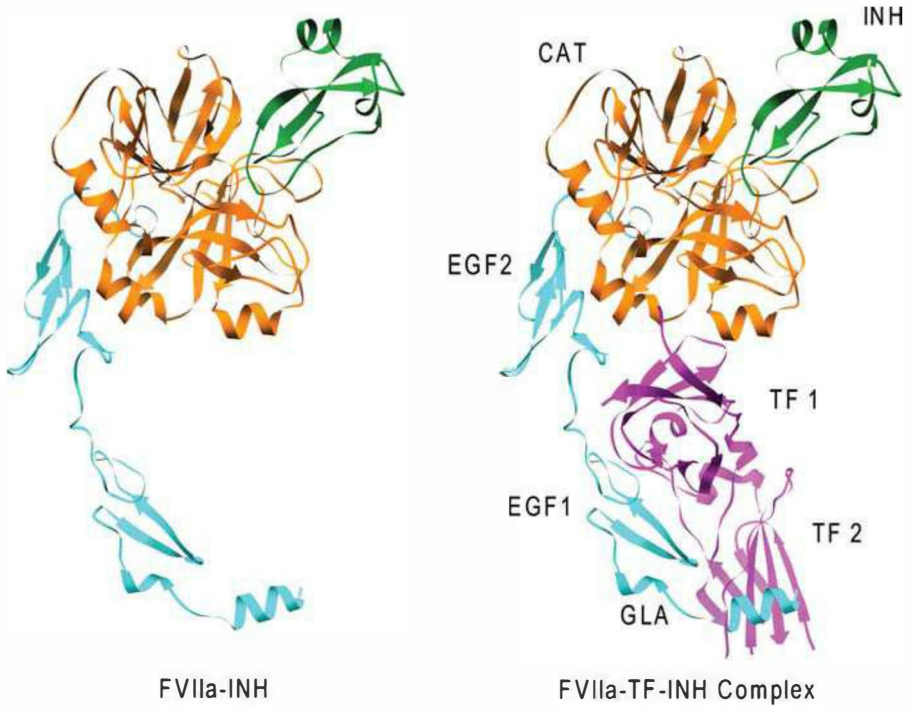

FIGURE 9.6 Ribbon structure of factor VIIa–inhibitor and factor VIIa–inhibitor–TF complex (Zhang E, St Charles R, Tulinsky A. Structure of extracellular tissue factor complexed with factor VIIa inhibited with a BPTI mutant. *J Mol Biol* 1999;285:2089). Factor VIIa (*cyan* and *orange*) complexed with pancreatic trypsin inhibitor (*green*) is shown in the left panel. The factor VIIa–inhibitor complex bound to TF (*magenta*) is shown in the panel on the right.

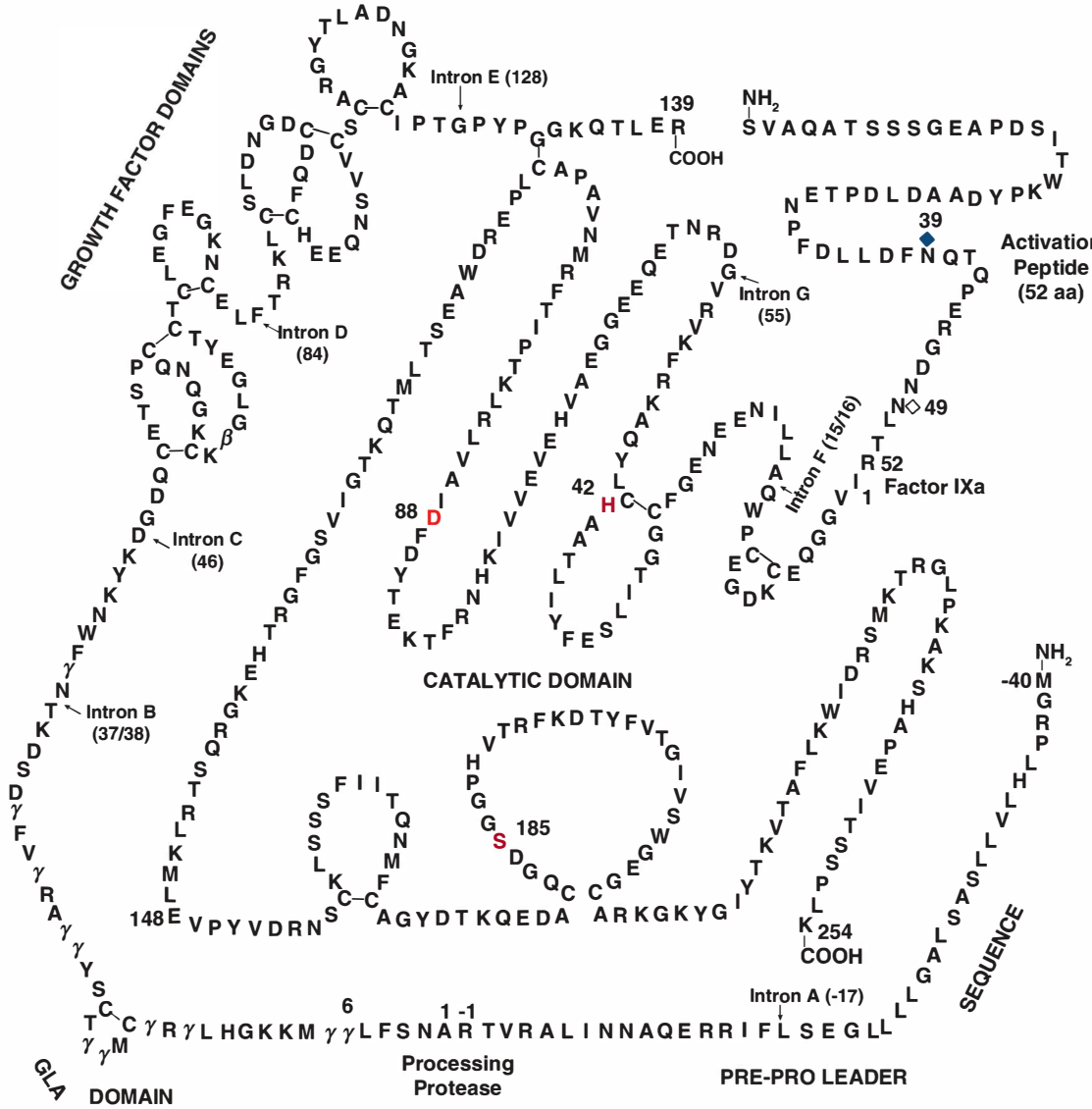

FIGURE 9.7 Amino acid sequence of human prepro factor X and the location of the seven introns (A to G). The tripeptide of Arg-Lys-Arg that connects the light chain to the heavy chain is not shown. The prepro leader sequence (–40 to –1) is removed during biosynthesis by signal peptidase and a processing protease that cleaves the Arg(–1)-Ala bond. The Gla domain and potential growth factor domains are located in the light chain within residues 1 to 139. The activation peptide of 52 amino acids is released from factor X during its conversion to factor Xa. The serine protease or catalytic domain of factor X contains 254 residues, including the catalytic triad of His42, Asp88, and Ser185, which are *circled*. Two *N*-linked carbohydrate attachment sites in the activation peptide are shown by *solid diamonds*. The proposed disulfide bonds in factor IX have been placed by analogy to those in bovine prothrombin and epidermal growth factor. The single-letter code for amino acids is given in FIGURE 9.2.

formed when the two chains combine. During the activation of prothrombin by factor Xa, the C1 and C2 domains of the light chain of factor Va are bound to phosphatidylserine in the membranes.[102] Important amino acid residues in the C2 domain of factor Va involved in the phospholipid binding include Trp2063 and Trp2064.[103] Modeling experiments and peptide inhibition studies suggest that the factor Xa binding to factor Va occurs via the A2 domain.

The cDNA for factor V is approximately 7 kb in size and codes for a leader peptide of 28 amino acids and a mature protein of 2,196 amino acids. The 3′ noncoding sequence also contains the typical sequence of AATAAA that functions as a polyadenylation signal.[28]

The gene coding for human factor V (F5) is located on chromosome 1q21-35 within 300 kb of the genes for the selectin family of leukocyte adhesive molecules.[104,105] The factor V gene spans more than 80 kb of DNA and consists of 25 exons[106] (72 to 2,820 bp) and 24 introns (400 bp to >11 kb) of DNA.

The organization of the gene for human factor V shows remarkable similarity to that of human factor VIII. The factor VIII gene, however, contains one additional exon (i.e., 26 rather

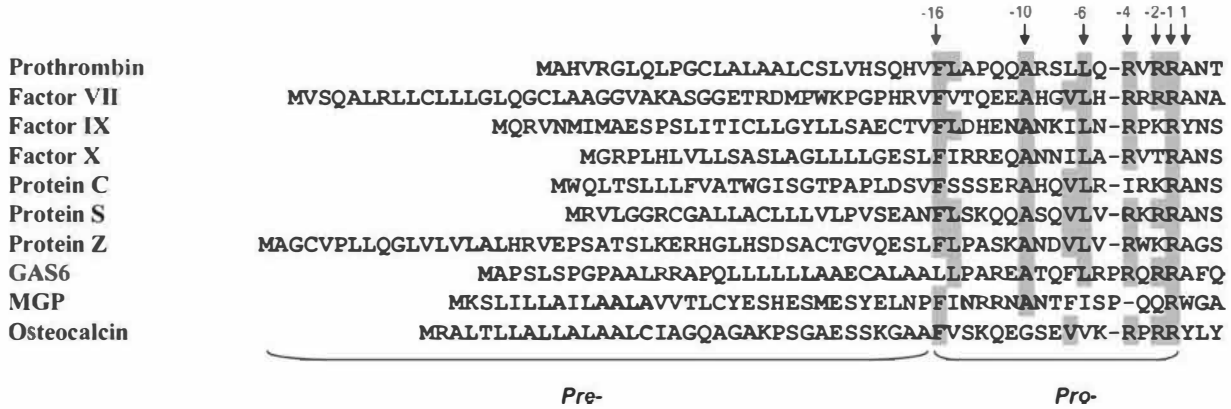

FIGURE 9.8 Prepro leader sequence of the vitamin K–dependent human plasma proteins. The putative hydrophobic core of each signal sequence is *shaded*. Identical and conserved amino acid residues within the propeptide region are also *shaded*. Numbering is relative to the mature amino-termini of the proteins.

than 25). The gene for factor VIII is also much larger than the gene for factor V, being approximately 180 kb in size. A comparison of the genomic DNA sequences for factor V and factor VIII indicated that 21 of the intron–exon boundaries occur at exactly the same location in the amino acid sequences coded by the two genes.[106] Of particular interest, the connecting B region factors V and VIII are both coded by a single very large exon. In the factor V gene, this exon is located between the 12th and 13th introns, whereas in factor VIII, it is between the 13th and 14th introns.

Recently, a homozygous factor V Leiden mutation (A for G at nt 1,691) has been shown to cause APC resistance and is the major known cause of hereditary thrombophilia.[107,108] This mutation results in the replacement of Gln for Arg at ammo acid residue 506, and this change disrupts the cleavage site for the inactivation of factor Va by APC,[12] and confers a lifelong risk of thrombosis.[109]

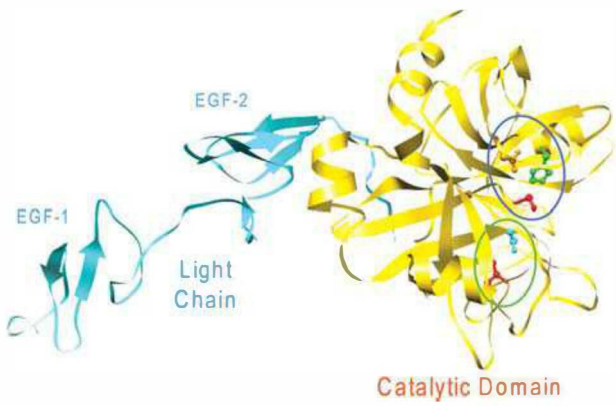

FIGURE 9.9 Ribbon structure of Gla domainless factor Xa starting with Asp 46. The catalytic triad of His 42, Asp88, and Ser185 is *circled* in blue, while the salt bridge formed between the carboxylate group of Asp184 and the α-ammonium group of Ile1 is *circled* in *green*. The EGF1 and EGF2 domains are *colored cyan*, while the catalytic domain is *yellow*. (Modified from Padmanabhan K, Padmanabhan KP, Tulinsky A, et al. Structure of human des(1-45) factor Xa at 2.2 A resolution. *J Mol Biol* 1993;232:947.)

PROTHROMBIN

Prothrombin (Mr 71,600) is a glycoprotein containing 8.2% carbohydrate that is synthesized in the liver and secreted into the blood, where it circulates as a precursor to a serine protease at a plasma concentration of 100 μg/mL.[1] The amino acid sequence of human prothrombin is 579 amino acid residues (FIGURE 9.14).[110,111,112,113–116] The sequence of human prothrombin[117,118] is identical with about 46% within the heavy chain and has a similarity of about 75% with that of other vertebrates. Prothrombin contains an amino-terminal Gla domain of about 40 amino acids followed by two kringle domains, each containing approximately 80 amino acids and a carboxyl-terminal region with a typical serine protease domain homologous to pancreatic trypsin (FIGURE 9.14).[114,119,120]

During biosynthesis in the rough endoplasmic reticulum, prothrombin undergoes removal of a signal peptide sequence by signal peptidase, carboxylation of amino-terminal glutamic acid residues, cleavage of the propiece by a furin-like proprotein convertase, and the addition of three carbohydrate chains (FIGURE 9.14). The carboxylation of 10 glutamic acid residues located within the first 40 amino-terminal residues in prothrombin is catalyzed by γ-glutamyl carboxylase, which recognizes the propeptide sequence and is responsible for the vitamin K–dependent conversion of the amino-terminal glutamic acid residues to γ-carboxyglutamate. The γ-carboxyglutamyl residues coordinate Ca^{2+} ions, leading to the binding of the Gla region to an anionic phospholipid surface. The addition of the three *N*-linked carbohydrate chains occurs at Asn78 and Asn100 in the first kringle domain while the third carbohydrate chain is added to the serine protease domain at Asn53 in the catalytic chain.[111]

During the final stages of blood coagulation, prothrombin is converted to thrombin. Thrombin is a serine protease composed of a light chain of 49 amino acids (Thr272 to Arg320) and a catalytic heavy chain of 259 residues (Ile1 to Glu259)

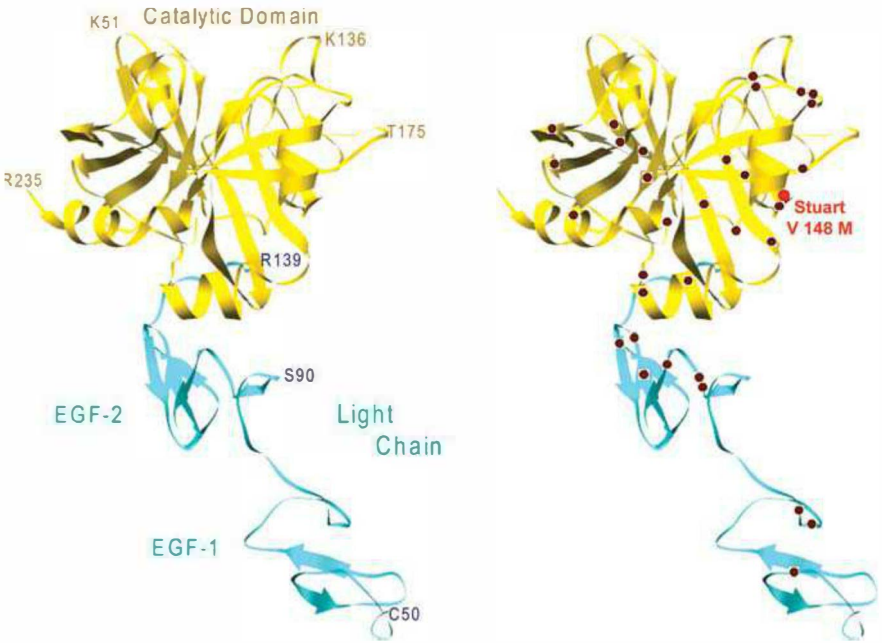

FIGURE 9.10 Ribbon structure of Gla domainless factor Xa starting with Asp46 and the Gla domainless factor Xa showing the location of missense mutations in 34 patients with factor X deficiency. The protein has a shape of a tulip with the EGF1 and EGF2 domains (*colored cyan*) forming the stem while the catalytic domain (*colored yellow*) contributes the flower. (Modified from Padmanabhan K, Padmanabhan KP, Tulinsky A, et al. Structure of human des(1-45) factor Xa at 2.2 A resolution. *J Mol Biol* 1993;232:947; Mutations are mainly from Peyvandi F, Menegatti M, Santagostino E, et al. Gene mutations and three-dimensional structural analysis in 13 families with severe factor deficiency. *Br J Haematol* 2002;117:685.)

(FIGURE 9.14), held together by a single disulfide bond. Human thrombin undergoes some additional autolysis at the Arg13Thr bond in the light chain, resulting in the removal of a 13–amino acid fragment, reducing the light chain of α-thrombin to 36 residues.

Thrombin generation is due to the cleavage of two internal peptide bonds in prothrombin catalyzed by factor Xa present in the prothrombinase complex.[121] The first cleavage at the Arg320-Ile bond generates a protease called meizothrombin (FIGURE 9.15), followed by cleavage of the Arg 271-Thr bond forming thrombin and fragment 1.2, a polypeptide containing the Gla domain and two tandem kringle domains. The replacement of the Arg271 by Cys results in a defective molecule (prothrombin Barcelona), demonstrating the importance of this cleavage site.[122]

In the absence of factor Va, the first cleavage of prothrombin by factor Xa occurs at the Arg271-Thr peptide bond generating fragment 1.2 and prethrombin-2. In a second step, prethrombin-2 is cleaved at Arg320. The catalytic efficiency of the prothrombin-2 pathway, however, is slow and insufficient for physiological clot formation.[121]

Thus far, there is no crystal structure available for prothrombin. In 1989, Bode et al.[123,124] published the first detailed crystallographic structures of thrombin and compared its structure with pancreatic trypsin, with which it shares considerable structural similarity. The amino acid sequence of thrombin including the catalytic residues of His43, Asp99, and Ser205 is shown in FIGURE 9.16. These residues correspond to His57, Asp102, and Ser195 as described in the charge-relay system present in chymotrypsinogen.[125] These three important amino acids (His43, Asp99, and Ser205) are enclosed by a red-dashed oval in thrombin (FIGURE 9.16A). The His, Asp, and Ser in the catalytic site of thrombin are also located in the middle of an equatorial cleft that separates the adjacent upper and lower β-barrels or hemispheres of a roughly spherical thrombin molecule (FIGURE 9.16, A,B).[123] The upper lip of the substrate-binding cleft contains the 60 loop

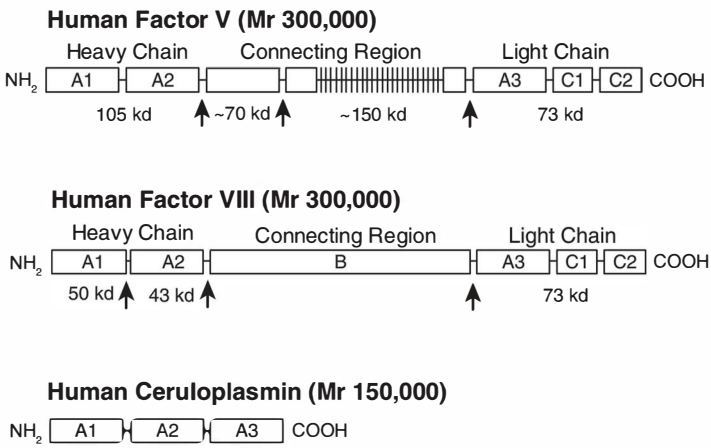

FIGURE 9.11 Structural domains in factor V, factor VIII, and ceruloplasmin. Thrombin cleavage sites are indicated by *solid arrows*. The identity of the domains is indicated by letters A, B, and C inside *boxes*. The 31 tandem repeats in the connecting region of factor V are indicated by *vertical bars*. The A domains in factor V correspond approximately to amino acids 1 through 331, 337 through 711, and 1,649 through 2,196. The C domains in factor VIII correspond approximately to amino acids 2,020 through 2,172 and 2,173 through 2,332. The A domains in ceruloplasmin correspond approximately to amino acids 1 through 338, 348 through 699, and 711 through 1,047.

	1139	P	T	D	I	S	Q	M	S	P
	1148	S	S	E	H	E	V	W	Q	T
1	1157	V	I	S	P	D	L	S	Q	V
2	1166	T	L	S	P	E	L	S	Q	T
3	1175	N	L	S	P	D	L	S	H	T
4	1184	T	L	S	P	E	L	I	Q	R
5	1193	N	L	S	P	A	L	G	Q	M
6	1202	P	I	S	P	D	L	S	H	T
7	1211	T	L	S	P	D	L	S	H	T
8	1220	T	L	S	L	D	L	S	Q	T
9	1229	N	L	S	P	E	L	S	Q	T
10	1238	N	L	S	P	A	L	G	Q	M
11	1247	P	L	S	P	D	L	S	H	T
12	1256	T	I	S	L	D	F	S	Q	T
13	1265	N	L	S	P	E	L	S	H	M
14	1274	T	L	S	P	E	L	S	Q	T
15	1283	N	L	S	P	A	L	G	Q	M
16	1292	P	I	S	P	D	L	S	H	T
17	1301	T	L	S	L	D	F	S	Q	T
18	1310	N	L	S	P	E	L	S	Q	T
19	1319	N	L	S	P	A	L	G	Q	M
20	1328	P	L	S	P	D	P	S	H	T
21	1337	T	L	S	L	D	L	S	Q	T
22	1346	N	L	S	P	E	L	S	Q	T
23	1355	N	L	S	P	D	L	S	E	M
24	1364	P	L	F	A	D	L	S	Q	I
25	1373	P	L	T	P	D	L	D	Q	M
26	1382	T	L	S	P	D	L	G	E	T
27	1391	D	L	S	P	N	F	G	Q	M
28	1400	S	L	S	P	D	L	S	Q	V
29	1409	T	L	S	P	D	I	S	D	T
30	1418	T	L	L	P	D	L	S	Q	I
31	1427	S	P	P	P	D	L	D	Q	I
	1436	F	Y	P	S	E	S	S	Q	S
	1445	L	L	L	Q	E	F	N	E	S
	1454	F	P	Y	P	D	L	G	Q	M
	1463	P	S	P	S	S	P	T	L	N
Consensus		T N P	L	S	P	D	L	S	Q	T

FIGURE 9.12 Thirty-one tandem repeats in the connecting region of factor V. The consensus sequence for the nine amino acid repeats is shown at the bottom. Residues identical to the consensus sequence are shaded.

(*magenta shading*, FIGURE 9.16), which is primarily hydrophobic and makes contact with hydrophobic residues present amino-terminal to the scissile bond of the substrate. The lower lip or γ-loop in the substrate-binding cleft (*green shading*, FIGURE 9.16) is more hydrophilic than the 60 loop and plays a role in determining the substrate specificity at the carboxyl side of the scissile bond in the substrate.

Thrombin cleaves peptide bonds immediately following Arg, a specificity that is due to a conserved Asp201 present six amino acids prior to Ser205 and located in the bottom of the

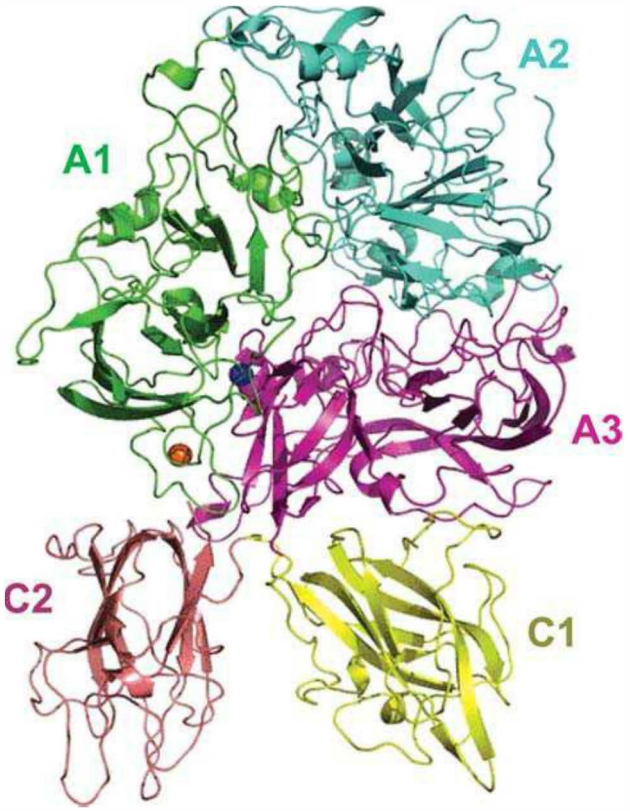

FIGURE 9.13 Model of human factor Va obtained by molecular dynamics simulations (Macedo-Ribeiro S, Bode W, Huber R, et al. Crystal structures of the membrane-binding C2 domain of human coagulation factor V. *Nature* 1999;402:434). The A1 domain is represented in *green*, A2 in *cyan*, A3 in *magenta*, C1 in *yellow*, C2 in *pink*. Ca^{2+} and Cu^{2+} are in *orange* and *blue*, respectively.

S1 primary specificity pocket. This specificity of thrombin is particularly evident for macromolecular substrates and inhibitors and is largely due to two surface regions that are distinct from the catalytic triad of Ser, His, and Asp.[126] These two surface regions are located on opposite ends of the substrate-binding groove in thrombin and have been called the anion-binding exosite I and exosite II (FIGURE 9.16C–E). Exosite I contains mainly basic amino acids including Lys21, 106, and 107 and Arg62, 68, 70, and 73 (FIGURE 9.16C–E).

Exosite II (heparin-binding site) consists mainly of the basic amino acids Arg89, 98, and 245 and Lys248 and 252. The sulfated glycosoaminoglycans present in heparin bind to thrombin by an electrostatic interaction and accelerates the rate of thrombin inhibition by antithrombin III.

The cleavage of the Arg320-Ile peptide bond in prothrombin catalyzed by factor Xa results in a new free amino-terminal Ile1 in the catalytic chain of the activated enzyme. The newly generated Ile1 turns and flips into the activation pocket of thrombin where its α-ammonium group forms a salt bridge with the carboxylate group of Asp204 (*dashed blue oval*, FIGURE 9.16A). This Asp is located just prior to the catalytic Ser205. The salt bridge induces a conformational rearrangement in the protein, leading to an active charge-relay system in the serine protease (*dashed red oval*, FIGURE 9.16A), essentially identical to that occurring in

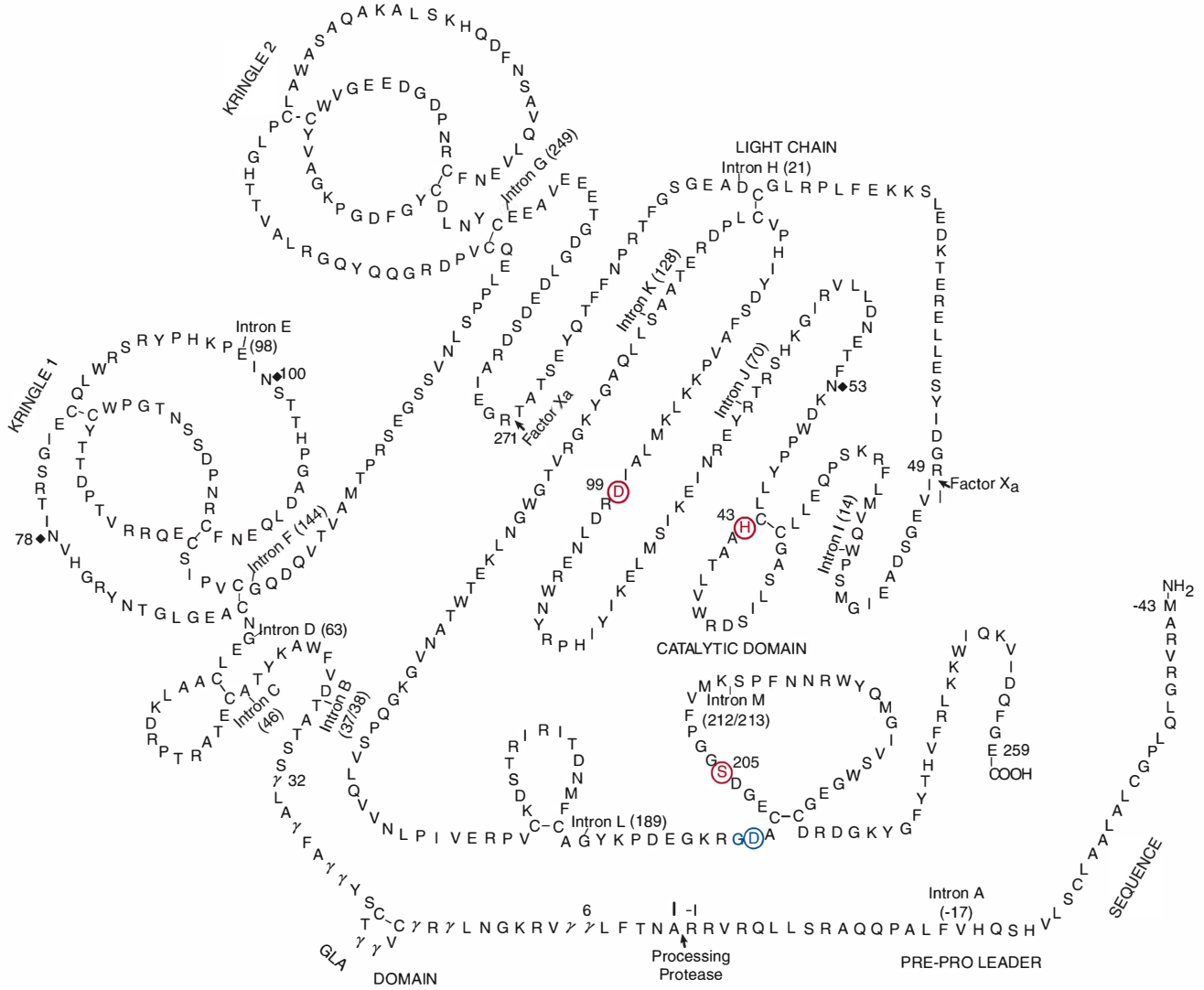

FIGURE 9.14 Amino acid sequence for human prepro prothrombin and the location of the 14 introns (A to M) (Degen SJ, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 1987;26:6165). The prepro leader sequence (−43 to −1) is removed during biosynthesis by signal peptidase and a processing protease that hydrolyzes the Arg(−1)-Ala bond. The Gla domain and the kringle domains are located within residues 1 through 271, which constitute fragment 1. This fragment is released from prothrombin during its conversion to thrombin by factor Xa. The light chain in thrombin is generated by the cleavage of the Arg319-Ile bond by factor Xa, and this chain is attached to the catalytic domain by a single disulfide bond. The serine protease or catalytic domain of thrombin contains 259 residues, including the three principal amino acids participating in catalysis. These three amino acids (His363, Asp419, and Ser525) are *circled*. Three potential carbohydrate attachment sites are shown by *solid diamonds*. The proposed disulfide bonds in human prothrombin have been placed by analogy to those in the bovine molecule.

the activation of pancreatic trypsin and all the other coagulation factors that form serine proteases.

The gene for human prothrombin (F2) is present in approximately 21 kb of DNA,[111,112,127,128] and it contains 13 introns (A through M) (FIGURE 9.14) (84 to 9,447 bp) and 14 exons (25 to 315 bp) located in the coding and 3′ noncoding portions of the gene (Table 9.2). The first intron (A) is in the prepro leader sequence at residue −17 while the second intron (B) follows the Gla domain between residues 37 and 38 in the mature protein. The third intron (C) is nine residues later at residue 46. These three introns are located in positions analogous to the first three introns in the coding regions of the genes for factor VII, factor IX, factor X, and protein C (Table 9.1).[27,72,129,130,131] The fourth intron (D) is just before the first kringle, whereas the next intron (E) is present within the first kringle. The fifth intron (F) is located immediately following kringle 1 (residue 144) and the sixth (G) immediately after kringle 2 (residue 249). The seventh intron (H) occurs in the region of prothrombin that becomes the light chain and the remaining five introns (I through M) are located in the catalytic domain at positions 14, 70, 128,

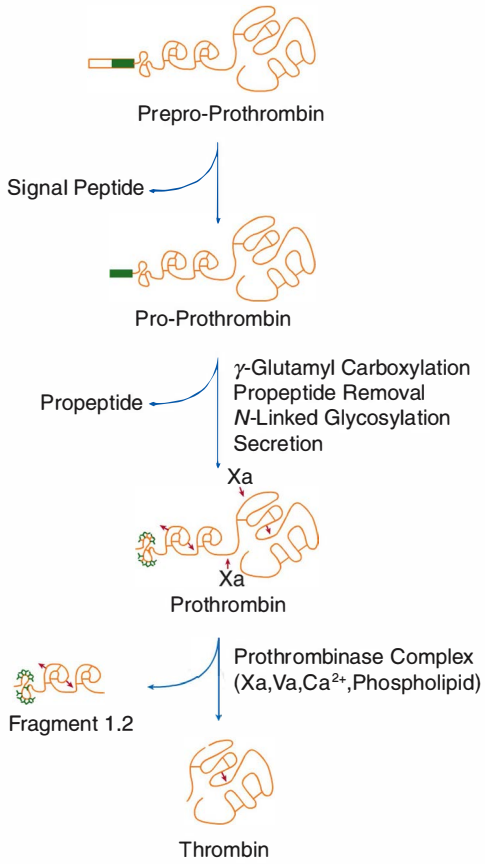

FIGURE 9.15 The processing and biosynthesis of prothrombin and the generation of thrombin. (Reprinted from Davie EW, Kulman JD. An overview of the structure and function of thrombin. *Semin Thromb hemost* 2006;32(Suppl 1):3, with permission.)

189, and between residues 212 and 213. The sequences at the splice junctions agree with the GT-AG rule of Breathnach and Chambon[132,133] and the consensus sequence of Mount,[133] except for one splice site at the 5′ end of intervening sequence L.[112]

The gene for prothrombin also contains 30 copies of Alu repetitive sequences, which make up 39% of the gene.[112] This family of DNA sequences is composed of approximately 300 nts, and the human haploid genome contains roughly 350,000 copies of Alu repetitive sequences.[134] In the prothrombin gene, many Alu sequences are tightly clustered and include five sets of tandem repeats. Intervening sequence L (9.5 kb) contains 20 Alu repeats, five of which occur in head-to-tail orientation with no additional DNA between them. The prothrombin gene also contains two copies of partial KpnI repeats (170 bp and 326 bp) located in intervening sequence L.

The mRNA for human prothrombin includes 1,866 nts that code for a prepro leader sequence of 43 amino acids and a mature polypeptide chain of 579 amino acids (FIGURE 9.14).[111,112] The prepro leader sequence includes a hydrophobic stretch of amino acids (residues –37 to –26) and ends with an arginine residue just before the amino-terminal alanine that is present in the circulating protein. The Arg-Ala peptide bond, however, is not cleaved by signal peptidase.[135] This enzyme cleaves the prepro polypeptide chain near the middle of the prepro leader sequence at one of the small amino acid residues such as alanine cysteine, or serine, leaving a propiece (18 to 24 amino acids) still attached to the polypeptide; the propiece is then cleaved by a second processing protease with a substrate preference for a basic residue at –4, –2, and –1.[29]

A prepro leader sequence, typical of the vitamin K–dependent coagulation factors (FIGURE 9.8), plays a role in the carboxylation reaction that occurs on the lumen side of the rough endoplasmic reticulum.[136,137] The conserved Phe and Ala residues at positions –16 and –10 appear to play an important role in the recognition sequence for this carboxylase.[138,139]

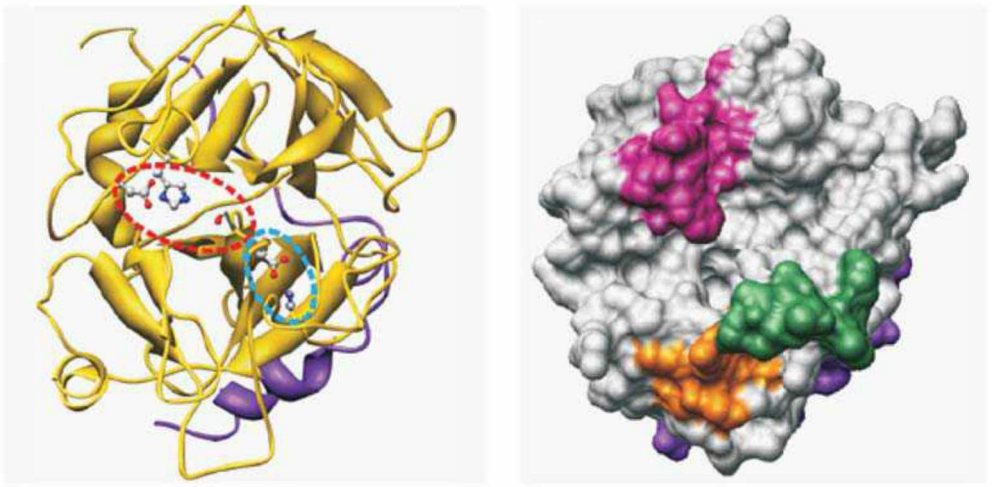

FIGURE 9.16 The catalytic and anion-binding exosites I and II in thrombin. The ribbon structure of thrombin **(A)** shows the catalytic triad of His43, Asp99, and Ser205 (enclosed in a *red-dashed circle*) and the salt bridge formed between Ile1 and Asp204 (enclosed in a *blue dashed circle*). The surface representations of thrombin are shown in the left **(C)**, standard **(B** and **D)**, and right **(E)** orientations. The substrate-binding cleft in thrombin runs from left to right **(B,D)** and is bordered by an upper lip or the 60 loop (*magenta shading*) and a lower lip or γ-loop (*green shading*). Residues constituting exosite I (*dark blue*) and exosite II (*light blue*) are enclosed by *dashed ovals* in

(Continued on page 123)

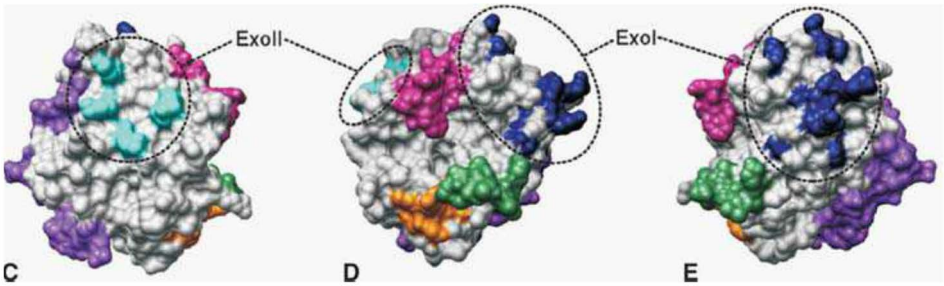

FIGURE 9.16 *(Continued)* **(C), (D)**, and **(E)**, while the Na-binding loop is shown in *orange*. (From Davie EW, Kulman JD. An overview of the structure and function of thrombin. *Semin Thromb Hemost* 2006;32(Suppl 1):3.)

Like the other vitamin K–dependent coagulation factors, the liver-specific expression of prothrombin is transcriptionally regulated. The immediate 5′-flanking sequence does not contain TATA or CCAAT boxes, and the two major transcription start sites are located at –36- and –23-bp upstream of the initiator codon.[140] Full tissue-specific promoter activity is located within 1,000 bp of the transcription start sites.[140–142] A weak positive element lies in the region 400-bp upstream of the mRNA coding sequence, accounting for approximately 5% of the total promoter activity in HepG2 cells. A liver-specific enhancer element is located in the region between –940 and –860 bp. DNA protein–binding studies and functional reporter gene analysis with mutant promoter constructs have demonstrated that this 80-bp enhancer contains an HNF-1–binding site flanked by a G-C–rich motif that binds a ubiquitous Sp1-like transacting factor.[140,141,143] All together, six different transcription factors bind to the prothrombin enhancer region and at least three (HNF-4-alpha, HNF-3-beta, and Sp1/Sp3) are important in the regulation of prothrombin expression.[143]

A common G to A substitution at nucleotide position 20,210 in the 3′-untranslated end of the prothrombin gene results in an elevated level of circulating prothrombin and a 2.8-fold increased risk for venous thrombosis.[144,145]

Table 9.2

Location and size of exons and introns in the human prothrombin gene (F2)

Exon	Nucleotide Positions	Length (bp)	Amino Acids	Intron	Type[a]	Nucleotide Positions	Length (bp)	Number of Alu Repeats
I	+1–79	79+[b]	–43 to –17	A	1	80–465	386	—
II	466–626	161	–17 to 37	B	0	627–1,285	659	—
III	1,286–1,310	25	38–46	C	1	1,311–1,552	242	—
IV	1,553–1,603	51	46–63	D	1	1,604–3,929	2,326	4
V	3,930–4,035	106	63–98	E	2	4,036–4,131	96	—
VI	4,132–4,268	137	98–144	F	1	4,269–6,606	2,338	3
VII	6,607–6,921	315	144–249	G	1	6,922–7,245	324	—
VIII	7,246–7,374	129	249–292	H	1	7,375–7,458	84	—
IX	7,459–7,585	127	292–334	I	2	7,586–8,742	1,157	2
X	8,743–8,910	168	334–390	J	2	8,911–9,407	497	—
XI	9,408–9,581	174	390–448	K	2	9,582–10,123	542	1
XII	10,124–10,305	182	448–509	L	1	10,306–19,752	9,447	20[c]
XIII	19,753–19,823	71	509–532	M	0	19,824–19,969	146	—
XIV	19,970–20,210	241	533—poly(A)	—	—	—	—	—

bp, base pair.
[a]Intron placement as discussed by Mount SM. A catalogue of splice junction sequences. *Nucleic Acids Res* 1982;10:459, in which a type 0 indicates placement between two codons, a type 1 interrupts a codon between the first and second bases, and a type 2 occurs between the second and third bases of the codon.
[b]The length of the 5′ noncoding region of the messenger RNA for human prothrombin is unknown; therefore, the length of exon I is measured from the initiator methionine.
[c]This intron also has two copies of partial Kpn repeats.
Reprinted from Degen SJ, Davie EW. Nucleotide sequence of the gene for human prothrombin. *Biochemistry* 1987;26:6165, with permission.

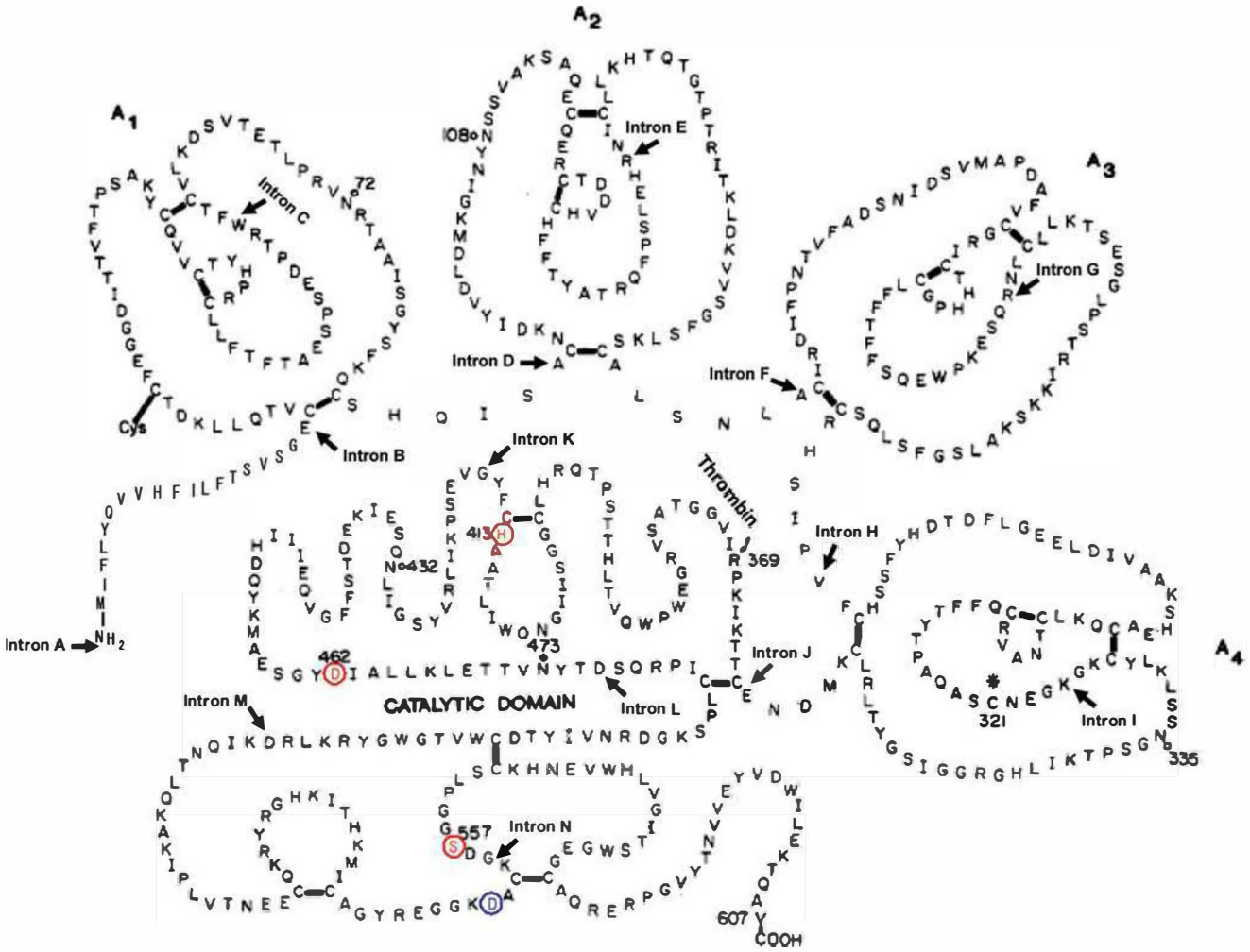

FIGURE 9.17 Amino acid sequence of human prepro factor XI. The signal sequence (−18 to −1) is removed during biosynthesis by signal peptidase by cleavage of the Gly(−1)-Glu bond. Factor XI circulates in plasma as a homodimer connected by a single disulfide bond linking Cys321 in the fourth apple domains of both subunits. The location of the 14 introns (A to N) is shown by *solid arrows*. The four apple domains (of 90 or 91 amino acids) are labeled A1, A2, A3, and A4. The site of cleavage (Arg369-Ile) catalyzed by thrombin during the conversion of factor XI to factor XIa is shown with a *small arrow*. The three members of the catalytic triad (His413, Asp462, and Ser557) are *circled*. The four *N*-linked carbohydrate attachment sites (Asn72, 108, 432, and 473) are shown by *solid diamonds*.

FACTOR XI

Factor XI (Mr 143,000) is a plasma glycoprotein (5% carbohydrate) composed of 1,214 amino acids synthesized in the liver and secreted into the plasma as a zymogen that circulates as a complex with high molecular weight kininogen (HMWK).[146] Factor XI is an unusual zymogen to a serine protease in that it contains two identical polypeptide chains, each with a catalytic site,[147] linked by a single disulfide bond in the fourth apple domain (Cys321) (FIGURE 9.17).[148,149] Each of these chains contains four tandem repeats of 90 (or 91) amino acids ("apple" domains) that range in identity from 23% to 34% (FIGURE 9.17)[150] and are linked by disulfide bonds between the first and sixth, second and fifth, and third and fourth half-Cys.[148] An extra half-Cys (Cys11) present in apple 1 forms a disulfide bond with another half-Cys residue, whereas Cys321 in each fourth apple domain links the two identical polypeptide chains of the protein together by a disulfide bond. The four apple domains in factor XI are also highly homologous to the four tandem repeats present in plasma prekallikrein but have not been identified in any other protein.[151,152] Present evidence indicates that factor XI is bound to HMWK through apple 1, whereas apple 2 is involved in the interaction of factor XI with factor IX.[153–156] The two catalytic domains in factor XI contain amino acid sequences typical of the pancreatic trypsin family of serine proteases and are located in the carboxyl end of the protein (FIGURE 9.17). The four apple domains and the catalytic domains in the dimer of factor XI are readily observed in the crystal structure of the protein (FIGURE 9.18A,B).[157] Each apple domain (60 × 60 × 20 Å) consists of a single α-helix surrounded by seven antiparallel β-strands. The four tandem apple domains form a shape that resembles a flat saucer. In the dimer, the two saucers are located between the two catalytic domains where they form an inverted V shape.

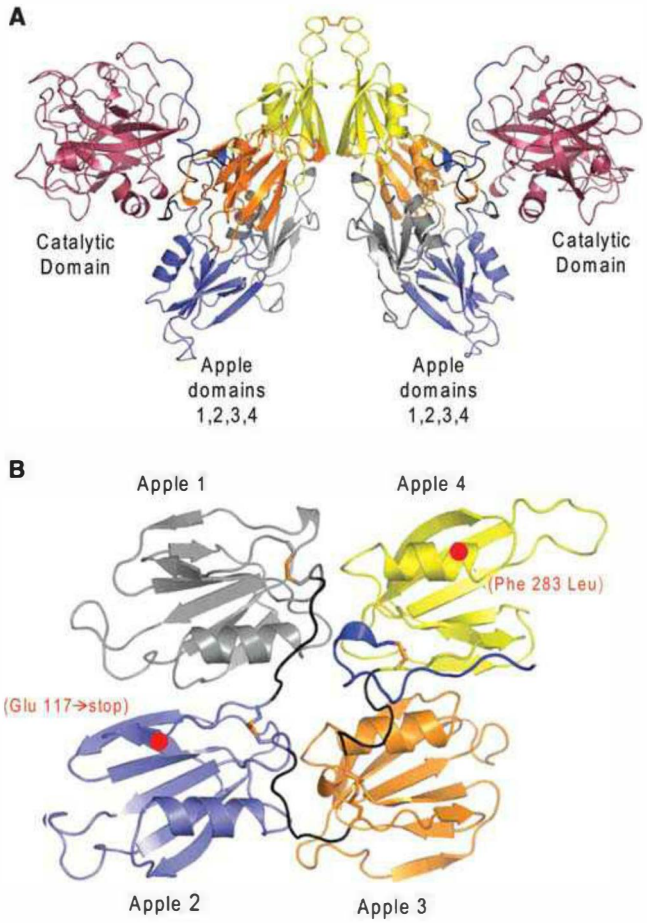

FIGURE 9.18 Ribbon structures of the factor XI dimer **(A)** and the four apple domains of factor IX **(B)**. The two catalytic sites are on the outside of the catalytic domains (colored *magenta*), while the two sets of four apple domains are in the middle where they are linked by a single disulfide bond between Cys321 from each of the apple four domains **(A)**. Apple 1 is colored *gray*, apple 2 is *blue*, apple 3 is *orange*, and apple 4 is *yellow*. (Reprinted from Papagrigoriou E, McEwan PA, Walsh PN, et al. Crystal structure of the factor XI zymogen reveals a pathway for transactivation. *Nat Struct Mol Biol* 2006;13:557, with permission.)

Factor XIa is a glycoprotein with five potential *N*-glycosylation sites in each chain. There are four carbohydrate chains, including two on the heavy chain (Asn72 and Asn108) and two on the catalytic or light chain (Asn432 and Asn473), but none on Asn335 of the heavy chain.

The conversion of factor XI to factor XIa catalyzed by thrombin readily occurs in the presence of a negatively charged material such as dextran sulfate, sulfatide, or heparin.[158,159] Factor XI can also be activated *in vitro* by factor XIIa in the presence of HMWK and a negatively charged surface.[160] Both of these activation reactions are due to the cleavage of an internal Arg369-Ile peptide bond in each of the two polypeptide chains,[150] resulting in the formation of factor XIa, a serine protease composed of two heavy chains (each 369 amino acids) and two light chains (each 238 amino acids) held together by three disulfide bonds. Each of the two light chains contains a serine protease domain that starts with Ile370. During the activation of factor XI, the newly generated Ile370 turns and flips into the activation pocket where its α-ammonium group forms a salt bridge with the carboxylate group of Asp556. This generates an active serine protease that includes the catalytic triad of His413, Asp462, and Ser557 in the light chain of each factor XIa subunit.

The cDNA for human factor XI has been isolated from a λgt11 expression library prepared from human liver. This mRNA was approximately 2,100 nts long.[150] These data also indicated that factor XI is synthesized as a single polypeptide chain with a typical leader sequence of 18 amino acids (FIGURE 9.17). Each of the two chains present in the mature molecule contains 607 amino acids. Also, the cDNA for factor XI contains a potential polyadenylation or processing sequence of AACAAA rather than the typical AATAAA.[28] This sequence is located 21 nts upstream from the poly(A) tail and is present in the 166 nts that constitute the 3′ noncoding sequence of the mRNA.

The gene for human factor XI (F11) is approximately 23 kb, located on the distal end of the long arm of chromosome 4 (4q35)[161,162] and contains 15 exons interrupted by 14 introns (FIGURE 9.17). The first exon codes for the 5′-untranslated region, whereas exon II codes for the signal peptide. The four apple domains are coded by the next eight exons. Each apple domain is coded by two exons interrupted by a single intron, and these introns are located in essentially the same position within each of the four apple domains. The carboxyl-terminal region of factor XIa containing the catalytic chain is coded by five exons, four of which are located in the same positions as those in the genes for human tissue plasminogen activator and human urokinase.

Factor XI deficiency is an unusually mild bleeding tendency that occurs in either sex.[163] Factor XI deficiency is found primarily in the Ashkenazi Jewish population,[164] and mutations occurring almost entirely in two regions of the gene coding for apple 2 and apple 4. The first mutation (type II) results in a stop codon at residue 117 in apple 2, where GAA coding for Gln117 is replaced by a stop codon of TAA (FIGURE 9.18B), leading to the synthesis of a truncated polypeptide and loss of biological activity. The second principal mutation (type III) results in an amino acid substitution at Phe283 in the fourth apple domain by Leu,[165] resulting in reduced dimerization of the molecule and a lowered secretion.[166] The third, far less common mutation (type I), disrupts normal mRNA splicing and changes a nucleotide sequence of GTAAC to ATAAC at the last intron–exon boundary.[165]

In a study of 43 patients with severe factor XI deficiency, 49% were due to the type II mutation and 47% to type III.[167,168] More than 152 different mutations have been published that occur in all four apple domains as well as in the catalytic domain.[169]

FACTOR IX

The gene for factor IX (F9), like factor VIII, is located in the distal region of the long arm of the X chromosome. No link, however, exists between the genes for these two proteins.[170,171,172] The factor IX gene is in region Xq27 and is closely linked to the fragile X site.[173]

Factor IX (Mr 57,000) is a single-chain glycoprotein (17% carbohydrate) composed of 415 amino acids (FIGURE 9.19).[170,171,172] Like prothrombin and factor VII, it is a vitamin K–dependent protein and contains 12 residues of γ-carboxyglutamic acid

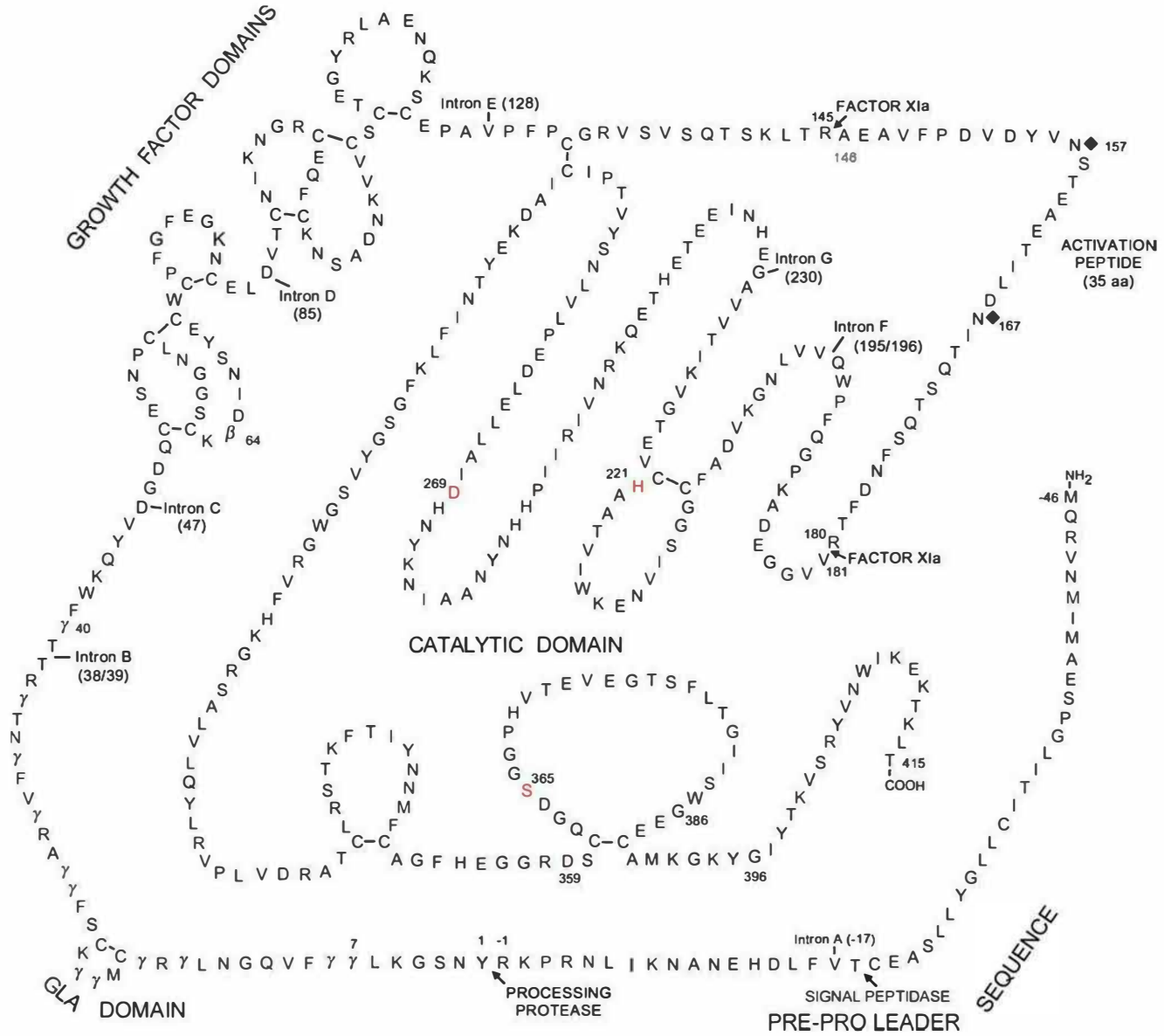

FIGURE 9.19 Amino acid sequence of human prepro factor IX and the location of the seven introns (A to G). The prepro leader sequence (–46 to –1) is removed during biosynthesis by signal peptidase and a processing protease that hydrolyzes the Arg(–1)-Tyr bond. The Gla domain and epidermal growth factor domains are located within residues 1 through 145, which constitute the light chain of factor IXa. The activation peptide of 35 residues is released from factor IX during its conversion to factor IXa by factor XIa. The amino acids at which this cleavage occurs (Arg145-Ala and Arg180-Val) are *circled*. The serine protease or catalytic domain of factor IX contains 235 residues, including the three principal amino acids participating in catalysis. These three amino acids (His221, Asp269, and Ser365) are also *circled*. Two potential carbohydrate attachment sites in the activation peptide are shown by *solid diamonds*. The proposed disulfide bond in factor IX have been placed by analogy to those in bovine prothrombin and epidermal growth factor.

(Gla domain) located in the amino-terminal region of the protein. The Gla domain is followed by two EGF-like domains, which show considerable homology with the corresponding regions of factor VII, factor X, protein C, and protein S.[73–75,174] The two EGF domains in factor IX are followed by an activation glycopeptide and a catalytic domain of 235 amino acids (FIGURE 9.19). The individual domains are readily identified in tulip-shaped crystal structure (FIGURE 9.20),[175] which has a stem composed of the amino-terminal Gla domain and two EGF domains attached to the catalytic domain that forms the blossom (FIGURE 9.20).

Factor IX is cleaved at two internal arginine peptide bonds (Arg145-A1a and Arg180-Val, FIGURE 9.19) by factor XIa or the factor VIIa–TF complex, generating factor IXa, a serine protease composed of a light chain and a heavy chain held together by a disulfide bond.[1,176] During the activation reaction, a glycopeptide of 35 amino acids is released, generating

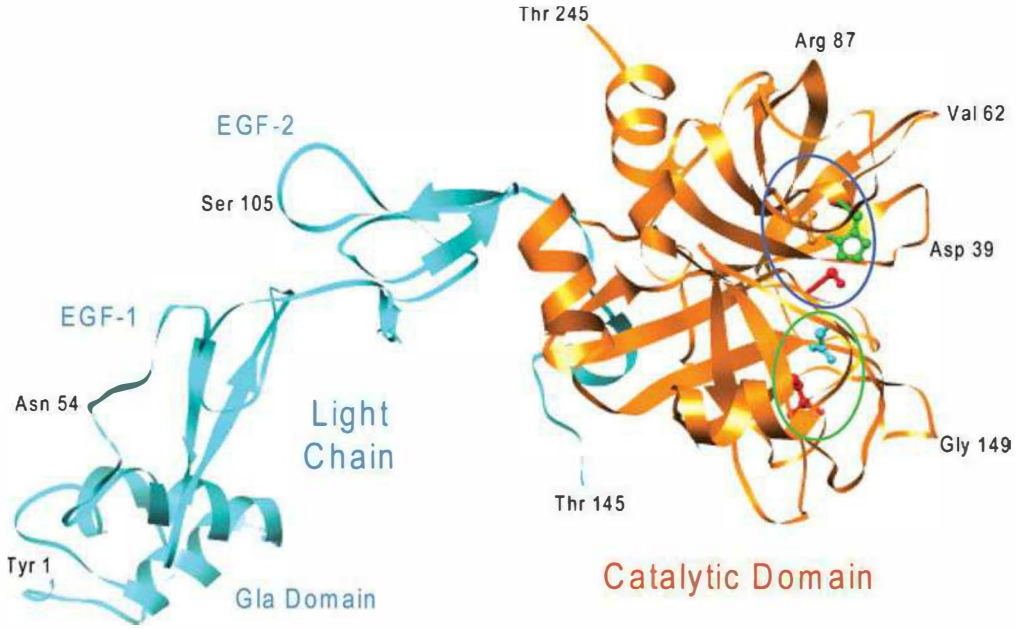

FIGURE 9.20 A ribbon structure of human factor IXa. The salt bridge formed between Val181 and Asp364 during the activation of factor IX is *encircled* in *green*, while the catalytic triad of His221, Asp269, and Ser365 are *encircled* in *blue*. The light chain of factor IXa, including the Gla domain and the two epidermal growth factor domains are *colored cyan*, while the catalytic domain is *colored orange*. (Modified from Davie EW, Kulman JD. An overview of the structure and function of thrombin. *Semin Thromb hemost* 2006;32(Suppl 1):3.)

a new amino-terminal Val181 in the heavy chain. The Val181 is then inserted into the activation pocket of the enzyme where its α-ammonium group forms a salt bridge with the carboxylate group of Asp364, a residue located next to the active site Ser365 (*encircled in green*, FIGURE 9.21), generating a serine protease with an active catalytic triad of His221, Asp269, and Ser365 (*encircled in blue*, FIGURE 9.20).

A model of the 1:1 factor IXa complex with factor VIIIa in the presence of phospholipid and calcium based on the lowest energy structure is shown in FIGURE 9.21.[177] The A2 and A3

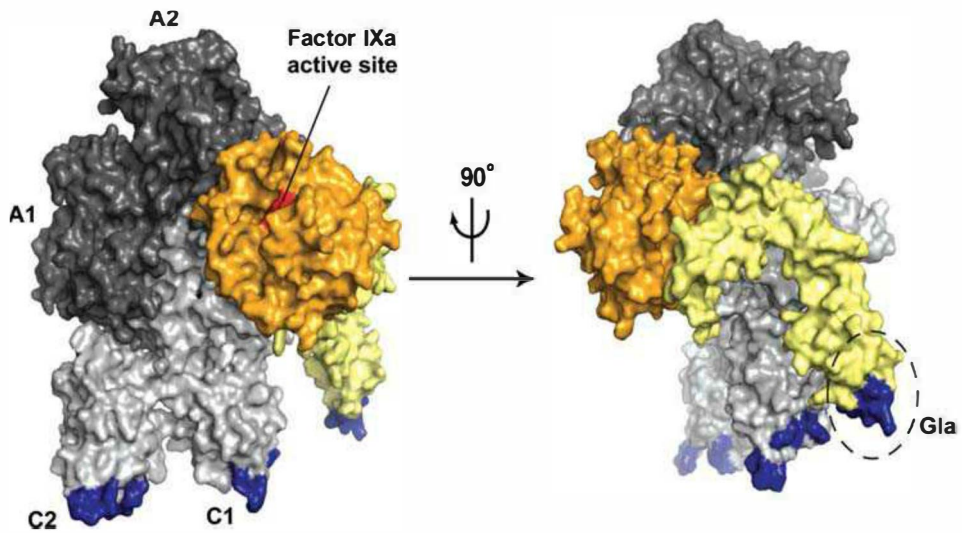

FIGURE 9.21 Space-filling model of the factor IXa–factor VIIIa complex with a front **(left)** and side **(right)** view (Shen BW, Spiegel PC, Chang CH, et al. The tertiary structure and domain organization of coagulation factor VIII. *Blood* 2000;96:979). The factor VIIIa A1, A2, and A3 domains are colored *dark gray*, while the C1 and C2 domains of factor VIIIa are colored *light gray*. The factor IXa light chain is colored *yellow*, while the catalytic domain is colored *orange*. Phospholipid-binding regions are colored *blue*. (From Ngo JC, Huang M, Roth DA, et al. Crystal structure of human factor VIII: implications for the formation of the factor IXa–factor VIIIa complex. *Structure* 2008;16:597.)

Table 9.3

Location and size of exons and introns in the human factor IX gene (F9)

Exon	Nucleotide Positions[a]	Length (bp)	Amino Acids[b]	Intron	Nucleotide Positions	Length (bp)
I	1–117	117	–46 to –17	A	118–6,325	6,206[c]
II	6,326–6,489	164	–17 to 37	B	6,490–6,677	188
III	6,678–6,702	25	38–47	C	6,703–10,391	3,689
IV	10,392–10,505	114	47–85	D	10,506–17,668	7,163
V	17,669–17,797	129	85–128	E	17,798–20,362	2,565
VI	20,363–20,565	203	128–195	F	20,566–30,038	9,473
VII	30,039–30,153	115	196–234	G	30,154–30,821	668
VIII	30,882–32,757	1,935	234–415	—	—	—

bp, base pair.
[a]Includes 30 nts of the 5′ end and 1,390 nts of the 3′ noncoding sequences.
[b]Amino acids encoded in each exon; negative numbers refer to amino acids in the prepro leader sequence.
[c]Includes the 50 extra nucleotides in the polymorphic DNA.
Reprinted from Yoshitake S, Schach BG, Foster DC, et al. Nucleotide sequence of the gene for human factor IX (antihemophilic factor B). *Biochemistry* 1985;24:3736, with permission.

domains of factor VIIIa interact with multiple domains in factor IXa to form the complex.[178,179] The C1 and C2 domains in factor VIIIa and the Gla domain on factor IXa (*colored dark blue*) are at the bottom of the complex bound to the membrane surface. The catalytic site in factor IXa is at the top of the complex about 75 to 80 Å above the membrane surface and is readily available to bind and cleave factor X.

The gene for human factor IX (F9)[129,131] contains approximately 34 kb of DNA, including eight exons (I to VIII) and seven introns (A to G) located within the coding and 3′ noncoding region of the gene (FIGURE 9.19). The eight exons range in size from 25 bp (exon III) to 1,935 bp (exon VIII). Exons I and II code for a prepro leader sequence and a Gla domain, whereas exons IV and V each codes for EGF domains. The last three exons code for the activation peptide and the catalytic domain. The seven introns range in size from 188 nts (intron B) to 9,473 nts (Intron F) (Table 9.3). The introns are located in positions essentially identical to those in factor VII, factor X, and protein C (Table 9.1).

Originally, it was suggested that the initiation site for mRNA synthesis starts at an A 29 nt upstream from the initiation methionine, but other studies indicate three major transcription sites, including +1, +4, and +30 as well as a minor site at +8.[180] Therefore, there may be considerable heterogeneity of transcription initiation, reflecting the absence of a well-defined or typical TATA-like promoter element in the 5′ end of the gene. The 5′ end of the gene also contains three Met codons (at –46, –41, and –39) for polypeptide initiation, but the Met(–39) is the only one conserved in the human, monkey, dog, and murine factor IX genes.[181,182] This suggests that the Met(–39) is the most probable initiation site for human factor IX biosynthesis under physiologic conditions.

The transcriptional regulation of the factor IX gene has been the most extensively studied of all the vitamin K–dependent coagulation factors. This is due to the Leiden phenotype, a rare form of hemophilia B that is characterized by spontaneous correction of severe childhood hemophilia B with the onset of puberty.[183] The genetic defects that confer the factor IX Leiden phenotype have been found to be single-nucleotide substitutions within a 54-bp region of the factor IX transcriptional promoter.[33,184–188] The developmental timing of the expression of the factor IX gene characterized by these mutations suggests that the transcription of the factor IX gene is, in part, hormonally mediated. A consensus androgen response element (ARE) has been identified between nts –36 and –17 of the factor IX promoter.[189,190] This sequence overlaps a functional HNF-4–binding site. Interestingly, a substitution of C for G at –26 bp causes lifelong hemophilia B, presumably by disruption of the ARE, whereas substitutions at –21 and –20 bp (A, G, or C for T in the sequence ACTTTG) result in disruption of the HNF-4–binding site and the Leiden phenotype.[33,187,190] These data suggest that androgen binding can partially overcome the negative effect of impaired HNF-4 binding to the promoter. Other Leiden mutations include nucleotide substitutions at positions –6, –5, + 8, and +13 relative to the transcription start site.[191] These mutations correspond to a disruption in the proximal C/EBP-binding site (+1 to +18 bp) and a disruption in the binding site of an uncharacterized transactivating regulatory factor (–15 to –1 bp).[184,192] Experiments investigating the molecular mechanisms explaining Leiden-specific mutations outside the putative ARE have identified interactions between C/EBP and the hormonally regulated transcription factors D-site–binding protein and hepatic leukemia factor.[193–196] Accordingly, the hormonal sensitivity of the factor IX promoter may be mediated by both direct androgen receptor binding to the promoter and the indirect regulatory effects of androgen receptor on the expression of developmentally regulated transcription factors. The factor IX promoter is complex and includes a negative regulatory or silencer activity in the region spanning –1,700 to –1,400 bp.[197] This region contains the short sequence ATCCTCTCC, which has been associated

with the silencer activity in several genes expressed primarily in the liver.[198]

Kurachi et al.[199,200] have found that the factor IX gene contains two age-related stability elements (ASE): a short sequence from –797 to –776 bp with an enhancer-like ASE-5′-element and the other ASE element, which confers an increased mRNA stability, in the middle of the 3′-untranslated region in an extensive stretch of dinucleotide repeating structures.

The gene for factor IX contains four Alu sequences.[131] The first is located in intron A, with the next three in intron F. A fifth Alu sequence is present in the 3′-flanking region of the gene, located approximately 230 nts downstream from the poly(A) adenylation site. Each of the five Alu sequences contains short direct flanking repeats ranging from 7 to 14 nts long. The gene for factor IX contains other repetitive sequences, including two KpnI repetitive elements, in the 5′ end of the gene and within intron D.

A major polymorphic site involving a deletion of 50 nts is present within intron A[201]; a second is a TaqI site after the fourth exon in the gene; and[173,202], a third site is in exon VI that codes for the activation peptide[203] results in the conversion of Ala3 (GCT) to Thr (ACT). The threonine appears to be more prevalent in the general population, with a frequency perhaps as high as 0.8.A fourth polymorphism in intron C, in which a G has been changed to a C, results in a loss of a recognition site for endonuclease XmnI.[201] These polymorphic sites have made it possible to establish methods for carrier detection of factor IX deficiency.

At present, hundreds of factor IX abnormalities have been defined at the protein and gene level. Factor IX Chapel Hill is due to a change of a G (in CGT) to an A (in CAT) in exon VI, causing a substitution of a His for Arg145.[204] Accordingly, the cleavage of a critical Arg-Ala bond by factor XIa during the coagulation process is blocked, reducing the activity of factor IX Chapel Hill to approximately 8% of normal.

One of the most interesting cases of hemophilia also known as the "royal disease" was associated with Queen Victoria of England (1819 to 1901), who was a carrier of hemophilia (factor VIII or factor IX deficiency). One of her granddaughters was Empress Alexandra (1872 to 1918),[205] the mother of Crown Prince Alexis (1904 to 1918), who inherited severe hemophilia from his mother (FIGURE 9.22A).[206] In July 2007, a grave was discovered near Ekaterinburg, which contained bone fragments identified by DNA sequencing to belong to Prince Alexis and his sister Anastasia, who were the two youngest children of Nicholas II and Alexandra.[207–209] Sequence analysis of DNA from members of the Tsar's family showed no missense or small insertion or deletion mutations in the coding regions of genes for factor VIII or factor IX, but an A to G mutation (nt 10,389) was found in intron C located three base pairs upstream of exon IV in the splice acceptor site of the factor IX gene.[210,211] This resulted in a change of the wildtype sequence of AAAG/ATGGA to

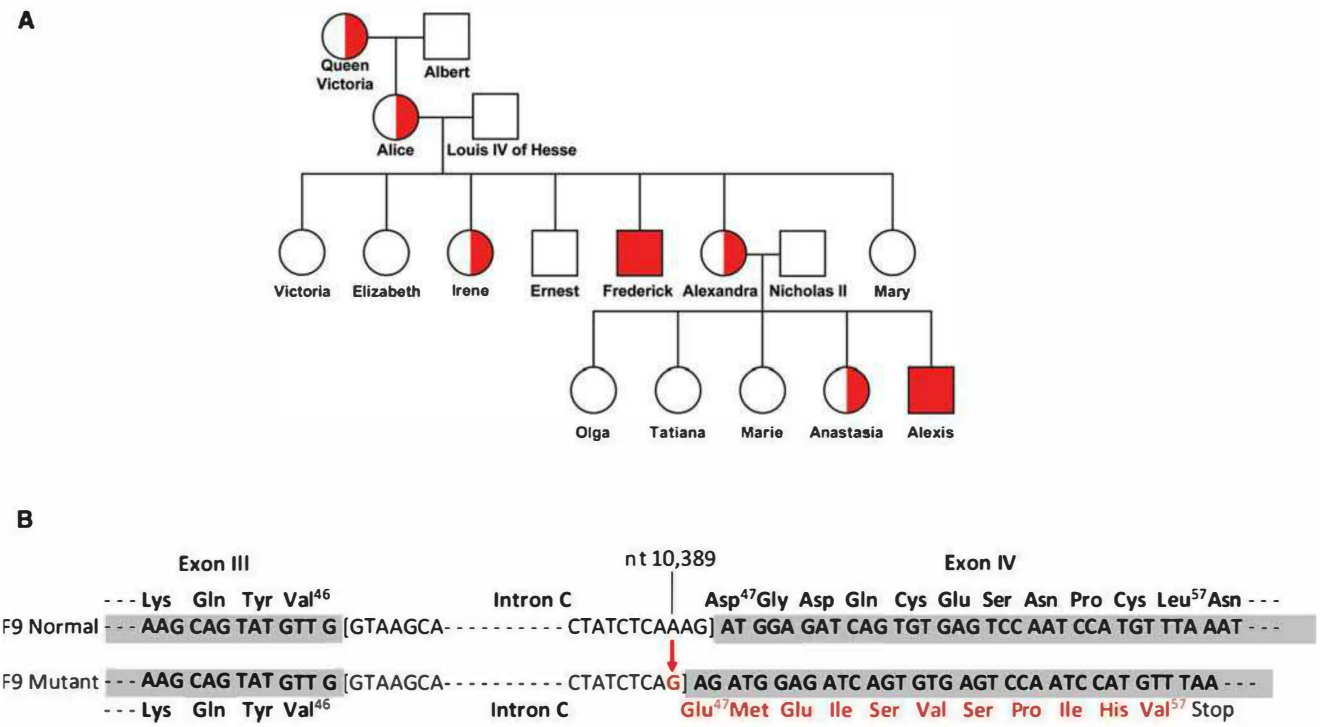

FIGURE 9.22 **A:** A partial family tree of Empress Alexandra. The female carriers of factor IX deficiency are shown with half-red circles, while males with factor IX deficiency are shown with red squares. **B:** The DNA sequence in intron C of the normal and mutated factor IX gene. The A to G mutation (nt 10,389 in the factor IX gene) occurred three base pairs upstream of exon IV in the splice acceptor site. (Modified from Lannoy N, Hermans C. The 'royal disease'—haemophilia A or B? A haematological mystery is finally solved. *Haemophilia* 2010;16:843 and Rogaev EI, Grigorenko AP, Faskhutdinova G, et al. Genotype analysis identifies the cause of the "royal disease". *Science* 2009;326:817.)

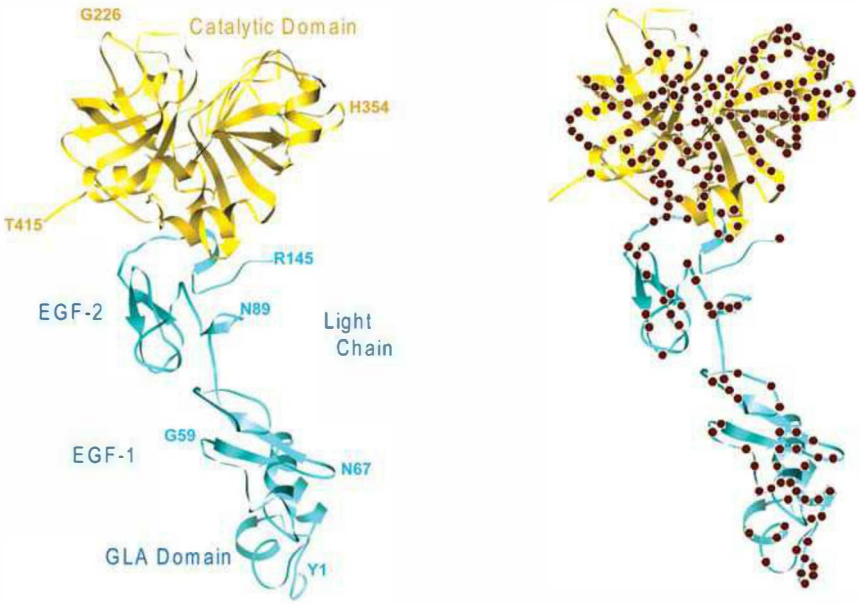

FIGURE 9.23 Ribbon structure of human factor IXa (Shen BW, Spiegel PC, Chang CH, et al. The tertiary structure and domain organization of coagulation factor VIII. *Blood* 2000;96:979). The left panel shows the light chain colored *cyan* and the catalytic chain *yellow*. The right panel shows the location of missense mutations (*black dots*) in 92 unrelated factor IX patients residing in the Pacific Northwest region of the United States. (Reprinted from Xu W, Thompson AR, Davie EW. with permission.)

AG/AGATGGA and a different nucleotide sequence in the mRNA coding for factor IX. In addition, a premature downstream stop codon is formed and the amino acid sequence of exon IV ends with an abnormal 11–amino acid sequence (FIGURE 9.22B). As expected, an analysis of the DNA from Alexandra, a heterozygous carrier, indicated the presence of both wild and mutant alleles, while the DNA from Alexis had only the mutant allele. One of Alexis's sisters presumed to be Anastasia was also a heterozygous carrier of the mutation (FIGURE 9.22A). This splice junction mutation in the factor IX gene has been found in three unrelated patients with severe hemophilia B.[212–214]

Another factor IX abnormality is due to a point mutation that changes an essential G in a splice junction site to a T[215] at the 5′ end of intron F, changing the exon–intron junction from GAG/GTACT to GAG/TTACT. A similar mutation in the GT donor splice sequence in the β-globin gene results in β-thalassemia.[216] Gene deletions have been reported in four out of five patients with antibodies to factor IX.[217] Two patients had deletions of more than 18 kb of DNA coding for factor IX, a third patient had a deletion of more than 9 kb, and in a fourth patient, who had <0.2% circulating factor IX antigen (factor IX Seattle 2), a deletion of a single adenine in exon V resulted in a frameshift that converted Asp85 to a Val, a stop codon at position 86, and a polypeptide of only 85 amino acids that terminated after the first EGF domain.[218] The location of the amino acid changes in factor IX from patients from 91 unrelated families with factor IX deficiency due to missense mutations is shown in FIGURE 9.23 (Xu W, Thompson AR, Davie EW. Unpublished data). The mRNA for human factor IX is approximately 2,800 nts long and includes 138 nts that code for a prepro leader sequence of 46 amino acids and 1,245 nts that code for 415 amino acids present in the circulating protein (FIGURE 9.20).[129,131,172] The prepro leader sequence of 46 amino acids includes two additional methionines that could also generate a prepro leader sequence of 41 or 39 amino acids. It is unknown which of these start sites is used under physiologic conditions. The stop codon of TAA in the mRNA is followed by a rather long 3′ noncoding sequence of 1,387 nts and a poly(A) tail. The polyadenylation sequence of AATAAA is located 16 nts upstream from the poly(A) tail.

The isolation of a cDNA coding for human factor IX has made it possible to express this protein in rat hepatoma cells, chick embryo fibroblasts, and BHK cells.[219–221] The full-length cDNA for human factor IX is coexpressed with the processing enzyme furin in CHO cells.[222] Recombinant factor IX has procoagulant function that is indistinguishable from plasma-derived factor IX[223] and is produced for therapeutic use in patients with hemophilia B.

Although most gene therapy delivery systems target the liver, other targets include myoblasts, primary fibroblasts, keratinocytes, capillary endothelial cells, and hematopoietic stem cells have been employed,[224–228] using viral vectors derived from retroviruses, adenoviruses, and herpes simplex viruses, as well as nonviral vectors such as naked DNA, DNA protein complexes, and liposome-encapsulated DNA.[229] One successful preclinical study of gene transfer for hemophilia B was accomplished by infusion of an amphotropic retroviral vector encoding canine factor IX cDNA into the Chapel Hill inbred strain of hemophilia B dogs after partial hepatectomy.[230,231]

FACTOR VIII

Factor VIII (Mr 330,000) is a large glycoprotein that is synthesized as a single-chain multidomain protein of 2,332 amino acids with a domain organization of A1-A2-B-A3-C1-C2. An internal homology (30%) of the A domain consists of a triplicated sequence at residues 1 to 329, 380 to 711, and 1,649 to 2,019 (FIGURES 9.11 and 9.24). A second duplication (40% homology) of 150 amino acids (C1 and C2 domains) is located in the carboxyl terminus of the molecule (residues 2,020 to 2,174 and 2,175 to 2,332). Copper is bound to the processed and secreted factor VIII,[232,233] but it is unclear if this ligand binding stabilizes or is necessary for the interaction between the heavy and light chains.

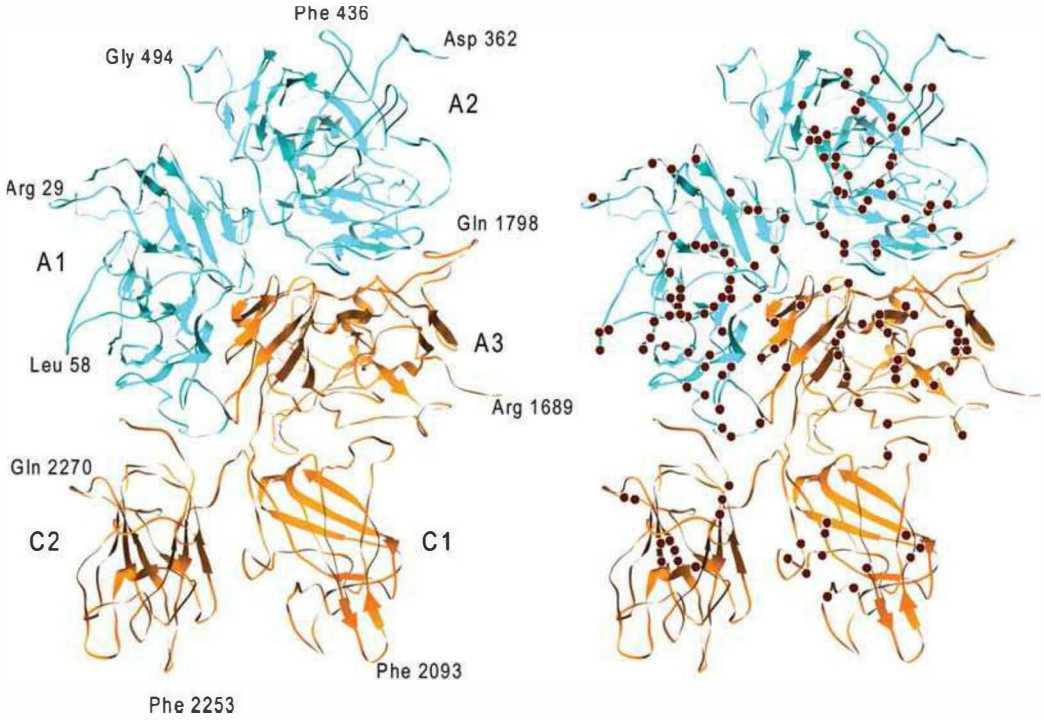

FIGURE 9.24 The ribbon structure of human factor VIII with a deleted B domain. The two structures shown in the left and right panels have their A1 and A2 domains colored *magenta*, while the A2, C1, and C2 domains are colored *orange*. The location of missense mutations resulting in severe hemophilia are shown in black dots in the A1, A2, A3, C1, and C2 domains in the right panel. (Modified from Liu ML, Shen BW, Nakaya S, et al. Hemophilic factor VIII C1- and C2-domain missense mutations and their modeling to the 1.5-angstrom human C2-domain crystal structure. *Blood* 2000;96:979.)

The heavily glycosylated B domain (connecting region) has little if any role in factor VIII coagulant activity. Factor VIII is synthesized primarily in the liver as a single-chain protein (2,332 residues) and processed into heterodimeric light and heavy chains that circulate in complex with von Willebrand factor (vWF).[234] The amino-terminal heavy chain (Mr 90,000 to 200,000) contains the A1-A2 domains and portions of the B domain, while the light chain (Mr 80,000) contains the carboxyl terminal A3-C1-C2 domains, after cleavage at Arg1648 in the B-A3 junction and other B domain sites. The binding of factor VIII to vWF as well as to membrane phosphatidylserine involves the C1 and C2 domains.[235]

Unprocessed factor VIII contains 25 potential Asn-linked carbohydrate attachment sites, located mainly in the connecting B region of the molecule between the heavy and light chains that are generated by thrombin (FIGURE 9.11). Six tyrosine sulfation sites are predicted in factor VIII, including Tyr346 in the 50-kDa fragment of the light chain; Tyr718, 719, and 723 in the 43-kDa fragment of the light chain; and Tyr1664 and 1680 in the heavy chain. Tyrosine residues in the light chain are partially sulfated in recombinant factor VIII expressed in CHO cells and fully sulfated in COS1 cells; sulfation of these residues does not influence coagulant activity but is essential for vWF binding.[236–238] Proteolytic cleavage by thrombin occurs primarily at Arg740 and Arg372 in the heavy chain and Arg1689 in the light chain. The three chains now contain the A1, the A2, and the A3-C1-C2 domains, held together by calcium, with the connecting B domain (amino acids 741 to 1,648) being split out by these proteolytic cleavages. Precisely which cleavage(s) is responsible for the activation and inactivation of factor VIII by thrombin or other proteases is unclear at present.

All five domains including the A1, A2, A3, C1, and C2 are readily identified in crystal structures[177,239,240] (FIGURE 9.24). One calcium is present in the A1 domain coordinated by Glu110 and Asp116, Asp125, and Asp126. The second calcium in the A2 domain is coordinated by Asp538 and Asp542. One copper ion was coordinated by two His and a single Cys residue in the A1 domain, while a second copper ion was coordinated by two His residues and a single Cys residue in the A3 domain. Glycosylation sites occur at Asn42 and Asn239 in the A1 domain as well as at Asn1810 in the A3 domain and Asn2118 in the C1 domain. The C1 and C2 domains are important for binding to platelets,[240] while the C2 is the binding site to vWF.[239,240]

The mRNA for human factor VIII is approximately 9,000 nts long and codes for a leader sequence of 19 amino acids and a mature protein of 2,302 amino acids.[241,242–244] The mature polypeptide chain ending in tyrosine is followed by a stop codon of TGA and 1,802 nts of noncoding sequence. A polyadenylation or processing site of AATAAA is present 19 nts upstream from the polyadenylation signal.[28]

The gene for factor VIII (F8) is located on the tip of the long arm of the X chromosome in region Xq28.[241] It contains approximately 186 kb of DNA and consists of 26 exons (69 to 3,106 nts)

and 25 introns (200 nts up to 32.4 kb).[241,242–244] Approximately 5% of the gene is present in the exons and the remaining 95% is noncoding DNA. A start site for initiation of mRNA synthesis[241] is likely at a G located 170 nts upstream from the initiating methionine.

Interestingly, intron 22 of the factor VIII gene contains an independent gene oriented in the opposite direction.[245] A transcript of 1.8 kb is present in many cells, and its cDNA isolated from a human liver library contains a long open reading frame. A comparison of the genome and cDNA sequence has revealed that this independent gene within intron 22 contains no introns.

Full promoter activity is located within the 300 bp from the initiation codon. The factor VIII regulatory region is complex in that DNA protein–binding studies show the presence of 19 binding sites distributed along the region from –1,175 to –9 bp, many of which are currently being investigated.[246–248]

The functional cis-acting elements within the fully active factor VIII promoter include HNF-1 (–64 to –35 bp), NFκB (–123 to –79 bp), C/EBPα (–203 to –149 bp), C/EBPβ (–275 to –252 bp), and HNF-4 (–312 to –279 bp).[246,247,249] Stimulation of cultured human hepatocytes with the cytokine IL6 results in a sixfold increase of factor VIII mRNA.[250] A vast number of genomic DNAs from patients with hemophilia A have been analyzed using a variety of methods.[251,252] Gross rearrangements of the gene for factor VIII, including deletions and insertions, have been found in 1% to 5% of hemophiliacs.[253,254] Accordingly, most of the defects in the gene for factor VIII are caused by point mutations. Most deletions and nonsense mutations, which create termination codons, lead to truncated molecules of factor VIII and cause severe hemophilia. Most deletions are the result of nonhomologous recombination between dissimilar sequences in which short-sequence homologies facilitate end-joining reactions.[255] Deletion breakpoints, however, are not clustered in particular regions of the factor VIII gene.[256] Deletions of the factor VIII gene are also associated with a fivefold increased risk of developing inhibitors compared with other severe hemophiliacs without gene deletion.[256]

Insertions of the transposable line 1 repetitive sequence into exon XIV have been found in two sporadic cases.[257] In each case, the inserted sequence contained most of the second open reading frame for a reverse transcriptase and poly(A) sequence; moreover, a target-site duplication at the 5′ and 3′ ends flanked the insertion.[258,259] Most cases of moderately and mildly severe hemophilia A are caused by single-point mutations that lead to impaired biosynthesis, secretion, and stability of the molecule.[260] Only a few mutations have proved to be causative for functional abnormalities of factor VIII protein, including substitutions of Arg372 and Arg1689 by Cys or His and of Tyr1680 by Phe.[261–263] The mutation at Arg372 results in a molecule that is resistant to the thrombin cleavage required for activation.[264,265] Tyr1680, as well as Arg1689, is included in a putative binding site of the light chain for vWF.[266] A number of point mutations occur at the CpG dinucleotides.[267,268] Because of the extreme heterogeneity of defects in the factor VIII molecule, intragenic DNA polymorphisms, such as the BclI site in intron 18, the XbaI site in intron 22, and a hypervariable dinucleotide repeat in intron 13, will continue to be useful in a pedigree analysis for genetic prediction.[261,269] The location of many of the amino acid changes in factor VIII due to missense mutations is shown in FIGURE 9.24.

The preparation of factor VIII by methods of recombinant DNA technology for the routine treatment of patients with classic hemophilia has been accomplished, including a recombinant factor VIII lacking the connecting B domain.[270]

FIBRINOGEN

Fibrinogen (Mr 340,000) is a dimeric plasma glycoprotein consisting of three pairs of polypeptide chains $(A\alpha,B\beta,\gamma)_2$, with apparent molecular weights of 67,600, 52,300, and 48,900, respectively, held together by disulfide bonds and folded in a trinodular structure.[271–273] The Aα chain is composed of 610 amino acids, while the Bβ chain has 461, and the γ chain has 411.[274–276]

The cDNA for the Aα chain of human fibrinogen is about 2,100 nts in length and includes a region that encodes a nascent Aα polypeptide of 644 amino acids,[277] which contains an *N*-terminal signal peptide of 19 amino acids, and a *C*-terminal extension of 15 amino acids (residues 611 to 625), which is removed from the nascent Aα chain in the hepatocyte or after secretion into the circulation. The mature Aα chain is free of carbohydrate.[278] Residues 1 to 16 of the mature Aα chain is fibrinopeptide A, which is cleaved and released by thrombin during the conversion of fibrinogen into monomeric fibrin. The remaining polypeptide chain, containing residues 17 to 610, becomes the α chain of fibrin. The Aα chain contains eight tandem repeats of approximately 13 amino acids (residues 270 to 372) of unknown function. Residues 472 to 552 of the Aα chain, least conserved among vertebrate fibrinogens, are characterized as a hypervariable region. The Aα chain contains two RGD sequences, including residues 95 to 97 in a region of the molecule that forms coiled coils with the Bβ and γ chains and a second including residues 572 to 574 that are close to the *C*-terminus of the molecule. While the RGD sequence at residues 572 to 574 does not play a significant role in platelet adhesion,[279] this sequence binds to $\alpha_v\beta_3$ and mediates adhesion to endothelial cells.[280] Three nonsynonymous polymorphic sites in the Aα chain have been identified[281]: Ile(–14)Val in the signal peptide, Thr312Ala, and Thr437Ala in the mature polypeptide. An alternatively spliced, extended variant of the Aα chain, known as the αE chain, contains 847 amino acids[282,283] and amounts to 1% of the Aα chain population. Alternative splicing of a downstream exon (sixth exon) results in the replacement of residues 611 to 625 in the prevalent Aα chain by a sequence of 236 amino acids (residues 611 to 847). Since residues 611 to 625 are proteolytically removed from the Aα chain, the overall effect of alternative splicing is to extend its length by 236 amino acids. The extended sequence is homologous to the *C*-terminal globular domains of the Bβ and γ chains: it contains two *N*-glycosylation sites,[282] binds calcium,[284] and interacts with integrins $\alpha_M\beta_2$ and $\alpha_X\beta_2$ on leukocytes.[285] Typically two αE chains are incorporated into fibrinogen, thus giving rise to a homotypic variant form of fibrinogen called fib-420 $(\alpha E,B\beta,\gamma)_2$ with a molecular weight of 420,000.[286] The level of fib-420 is threefold higher in newborns (3%) than in adults (1%). Conversion of fib-420 to fibrin-420 by thrombin is accompanied by a reduced rate of polymerization, suggesting the additional *C*-terminal globular domain in the αE chain delays lateral protofibril aggregation, and promotes a greater degree of fibril branching.[287] Posttranslational modifications also contribute to Aα chain heterogeneity. Twenty-five percent of Ser3 and Ser345

in the Aα chain are phosphorylated.[288,289] The first amino acid residue of the Aα chain, Ala1, is removed by an aminopeptidase in 10% of the nonphosphorylated chains.[290] The *C*-terminal half of the Aα chain is very sensitive to proteases, and 25% of the Aα chains have been cleaved at Lys583 by plasmin,[291] while cleavage at Asn269, Gly297, and Pro309 has been observed.[292]

The cDNA for the Bβ chain of human fibrinogen is about 1,890 nts in length and contains a sequence that encodes a nascent Bβ chain of 483 amino acids.[293] After removal of the signal peptide of 22 amino acids, the mature Bβ chain (461 amino acids) is incorporated into the six-chain fibrinogen. The first residue Gln is cyclized into pyroglutamic acid, which protects the *N*-terminus of the Bβ chain from degradation by aminopeptidases.[290] Residues 1 to 14 of the mature Bβ chain form fibrinopeptide B, which is removed by thrombin during the conversion of fibrinogen into fibrin. Pro31 of the Bβ chain is partially hydroxylated (20%),[294] and Asn364 is the site for an *N*-linked carbohydrate attachment.[295] At least three polyadenylation sites, resulting in varying lengths of 3′ noncoding regions, have been identified in the cDNAs for the Bβ chain. Two nonsynonymous polymorphisms have been identified in the Bβ chain including Pro77Ser and Arg448Lys.[281] The *C*-terminal region of the Bβ chain, residues 198 to 461, folds into a globular structure called the β-nodule, which contains the "b" polymerization site that plays a role in lateral aggregation of fibrils.[296]

The cDNA for the γ chain of human fibrinogen is about 1,590 nts in length,[297] and contains a region that encodes the nascent γ chain of 437 amino acids and additional amino-terminal 26 residues that are the signal peptides. The mature γ chain of 411 amino acids contains a single *N*-linked glycosylation site at Asn52.[298] Two nonsynonymous polymorphisms have been identified including Tyr114His and Gly165Arg.[281] The region encompassing Val143 to Val411 folds into a globular γ nodule that contains the "a" polymerization site.[299,300] The *C*-terminal 12 amino acids of the γ chain (residues 400 to 411) bind to integrin $\alpha_{IIb}\beta_3$, and this sequence is essential for platelet aggregation.[201] Alternative polyadenylation and truncation of the precursor mRNA in the ninth intron leads to truncation of the mRNA and translation read-through of the ninth exon into the ninth intron. Translation of this form of mRNA gives rise to the variant γ' chain, in which the *C*-terminal four amino acids in the γ chain (residues 408 to 411) are replaced by an alternative sequence of 20 amino acids (residues 408 to 427).[301,302] The γ' chain, which amounts to approximately 10% of the total γ chain content, is usually present in the form of a heterodimeric fibrinogen $(A\alpha B\beta)_2\gamma\gamma'$. The unique alternative *C*-terminal extended sequence of the γ' chain contains two sulfated tyrosine residues,[303] and this anionic region binds factor XIII[304] and thrombin.[305,306] The γ' variant exhibits a number of unique functional properties. It binds poorly to platelets and inhibits platelet aggregation,[307] delays lateral fibril aggregation,[308] alters the structure of fibrin,[309] and delays fibrinolysis.[310] Elevated levels of γ' chain may be associated with increased risk of cardiovascular diseases,[311–313] ischemic stroke,[314] and thrombotic microangiopathies.[315]

The genes for the three chains of human fibrinogen, designated FGA (fibrinogen alpha chain), FGB (fibrinogen beta chain), FGG (fibrinogen gamma chain), respectively, are linked and are located in chromosome 4q28[316] in a cluster approximately 53 kb in length (FIGURE 9.25). The genes are arranged in the order of FGG-FGA-FGB, and the FGB gene is oriented in a direction opposite to that of the other two genes.[317] The FGG gene is approximately 8,617 nts in length and contains 10 exons.[318] Differential polyadenylation and translation read-through from the ninth exon into the ninth intron leads to expression of the γ' isoform. The ratio of the $\gamma'$to total fibrinogen is regulated by the differential utilization of two polyadenylation signals including one in the ninth intron (pA1) giving rise to the γ' chain and one in the 10th exon (pA2) giving rise to the γ chain. A single-nucleotide polymorphism in the pA2 polyadenylation site (C10034T) increases its affinity for the polyadenylation factor CstF. As a result, this polymorphism leads to a reduced expression of the γ' chain.[319]

The FGA gene, located 14 kb downstream of the FGG gene, is approximately 7,618 nts in length[318] and contains six exons. Exons I to V of the FGA gene encode the prevalent Aα chain, while alternative splicing appended the downstream exon VI to the mRNA to encode the αE isoform. Exons V and VI of the FGA gene are unusually large, encoding residues 153 to 625 of the Aα chain and residues 611 to 847 of the αE chain, respectively.

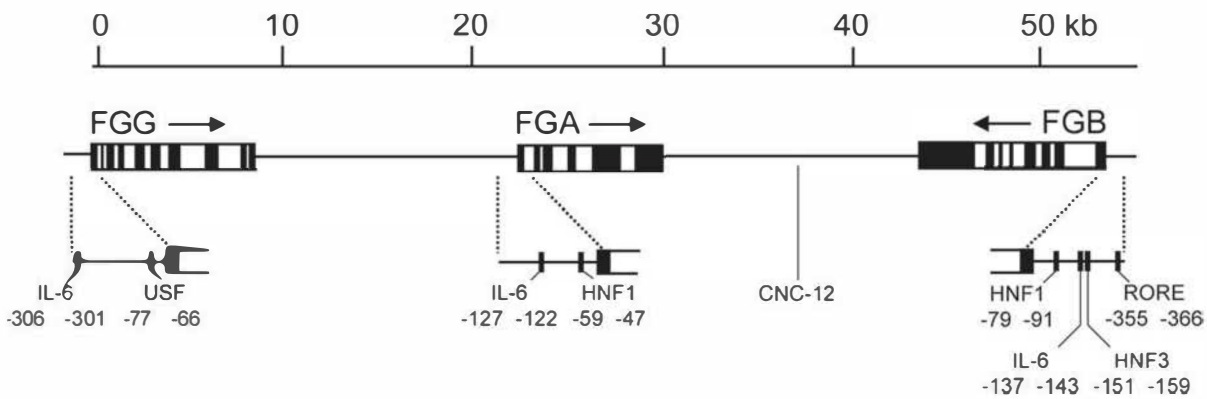

FIGURE 9.25 Arrangement of the fibrinogen genes on chromosome 4q28 and locations of regulatory elements. HNF-1, hepatocyte nuclear factor-1; HNF-3, hepatocyte nuclear factor-3; USF, upstream stimulatory factor; IL6, interleukin 6–responsive element; RORE, retinoic acid receptor-related orphan receptor alpha-binding element. CNC-12, intergenic noncoding sequence.

The FGB gene is approximately 9,784 nts in length[318] and is located 13 kb downstream of the FGA gene in an orientation opposite to that of the FGG and FGA genes. The FGB gene contains eight exons with no evidence of differential splicing. Two Alu repeat sequences including one in intron 5, and one in the 3′ flanking region, have been identified.

Fibrinogen is primarily synthesized in hepatic parenchymal cells, and liver-specific expression of the FGA and FGB genes has been attributed to a hepatocyte nuclear factor-1 (HNF-1)–dependent mechanism.[224,320] HNF-1–responsive elements are located –47 to –59 and –79 to –91 nts from the transcription initiation sites of the FGA and FGB genes, respectively (FIGURE 9.25). Liver-specific expression of the FGG gene is attributed to an upstream stimulatory factor (USF)-dependent mechanism, and a USF-responsive element is located –66 to –77 nt in the FGG gene. A 340-bp noncoding sequence named CNC-12, located in the intergenic region between the FGA and FGB genes, exhibits liver-specific enhancer activity. This activity suggests that this single conserved region within the fibrinogen gene cluster contributes to the coordinate expression of the fibrinogen genes in liver.[321]

Fibrinogen is an acute-phase protein and its level in plasma is increased four- to sevenfold during an acute-phase state induced by tissue damage, inflammation, or stress.[322] Functional IL6-responsive elements in the transcriptional regulatory regions of the three fibrinogen genes have been identified (FIGURE 9.25).[224,320,322–325] IL6 binds to IL6 receptors on hepatocytes and activates the transcription of NF-IL6, which in turn upregulates the expression of acute-phase proteins,[326] including the three chains of fibrinogen. A HNF-3–binding site in the promoter of the FGB gene (–151 to –159 nt), upstream of an IL6 element (–137 to –142 nt), is also essential for a full response to IL6 induction.[327] A binding site (RORE) for the retinoic acid receptor–related orphan receptor alpha has been identified in the promoter region (–355 to –366 nt) of the FGB gene.[328]

A single-nucleotide polymorphism in the 5′-flanking region of the FGB gene, –455G/A, has been proposed to be associated with elevated plasma fibrinogen and cardiovascular disease,[329] but this association was not confirmed.[145,330,331] Regions with small influence on fibrinogen expression may be present on chromosomes 2, 10, 17, and 20,[332] as well as the gene for HNF-1 on chromosome 12, and an unknown locus on chromosome 14.[333]

Disturbance of the circadian rhythm mediated by homozygous inactivation of the core circadian protein Bmal1 in mice is accompanied by a prothrombotic phenotype, in which circulating levels of several plasma proteins, including fibrinogen, are 40% elevated.[334] A screen of 470 microRNAs (miRNA) in HuH7 hepatoma cells shows that expression of 23 miRNAs causes significant reductions in fibrinogen synthesis, while four miRNAs causes increased synthesis.[335] Posttranslational modifications of fibrinogen may be prothrombotic or antithrombotic.[336,337]

FACTOR XIII

Factor XIII (Mr 320,000) (fibrin-stabilizing factor) is a proenzyme of plasma transglutaminase. In blood, it circulates as a heterotetramer composed of dimers of A and B subunits (A2B2), held together by noncovalent bonds. The A subunits contain the catalytic site with transglutaminase activity,[338,339] while the exact function of the B subunits remains unknown. The heterotetramer circulates in association with fibrinogen.[340]

In the presence of fibrin, thrombin converts factor XIII to factor XIIIa,[341,342] which catalyzes the formation of intermolecular epsilon-(γ-glutamyl)-lysine bonds with substrates such as fibrin monomers, α_2-plasmin inhibitor, fibronectin, and collagen.[343–345] The cross-linking in fibrin polymers occurs between Gln398 in the γchain of one fibrin molecule and Lys406 in the γ chain of an adjacent fibrin molecule. A deficiency of factor XIII can result in a lifelong bleeding tendency, delayed wound healing, and recurrent spontaneous abortion.

The A Subunit of Factor XIII

In blood, the A subunit (Mr 75,000) of factor XIII circulates as a heterotetramer with the B subunit,[346] while in various tissues and cells it exists as a dimer (A2). The exact site of the biosynthesis and mechanism of secretion of the A subunit into the blood has not been established. The concentration of the A subunit in plasma is 15 μg/mL, essentially all in complex with the B subunit,[346] but this may vary in disease states.[345,347]

The A subunit consists of 731 amino acids (FIGURE 9.26) with an amino-terminal sequence of Acetyl-Ser-Glu-Thr. There are two putative calcium-binding sites based on the homology to the EF hand.[348] The active-site Cys residue is located at position 314 within the sequence of Tyr-Gly-Gln-Cys-Trp and is identical to the active site of human and guinea pig tissue transglutaminase.[349] Eight more Cys residues are present in the A subunit, but none forms disulfide bridges. Although there are six potential Asn-linked glycosylation sites in the A subunits, little or no carbohydrate has been detected in the molecule.

In the conversion of factor XIII to factor XIIIa by thrombin, an activation peptide of 37 amino acids (Mr 4,000) is released from the amino-terminus[350,351] by cleavage between Arg37 and Gly38. In the presence of calcium ions, the modified A2B2 tetramer dissociates into an active A' dimer and two B subunits and unmasks the active sites, a process that is accelerated by fibrin.[352]

The crystal structure of the A subunit of factor XIII (FIGURE 9.27)[353] shows four distinct domains in the A subunit which follow the amino-terminal activation peptide of 37 residues. These include a beta sandwich (about 140 residues), a central core (about 330 residues), and two barrels (each about 100 residues). The central core contains Cys314 (shown by a *red star* in FIGURE 9.27) that is essential for catalytic activity of the enzyme, which may share a common ancestry with other cysteine proteases.[354]

The mRNA for the A subunit (~3.8 kb) lacks a sequence coding for a typical hydrophobic leader sequence that is usually required for protein secretion. The mRNA for the A subunit has a long 3′ noncoding region of approximately 1.5 kb.[350,355,356] The gene for the A subunit of human factor XIII (F13A1) is located on chromosome 6p24-25,[357,358] spanning more than 160 kb and including 15 exons interrupted by 14 introns.[356] The activation peptide is encoded by the second exon, the first putative calcium-binding site by the sixth, the active-site Cys by the seventh, the second putative calcium-binding site by the 11th, and a thrombin inactivation site by the 12th exons (FIGURE 9.26).

The homology of the A subunit of factor XIII to tissue keratinocyte transglutaminase and erythrocyte band 4.2 protein is 41%, 45%, and 27%, respectively.[349,359–361] However the active-site Cys residue in the band 4.2 protein is replaced by an Ala residue so that it lacks transglutaminase activity. A homologous transglutaminase has also been purified from hemocytes of horseshoe crab,[362] and

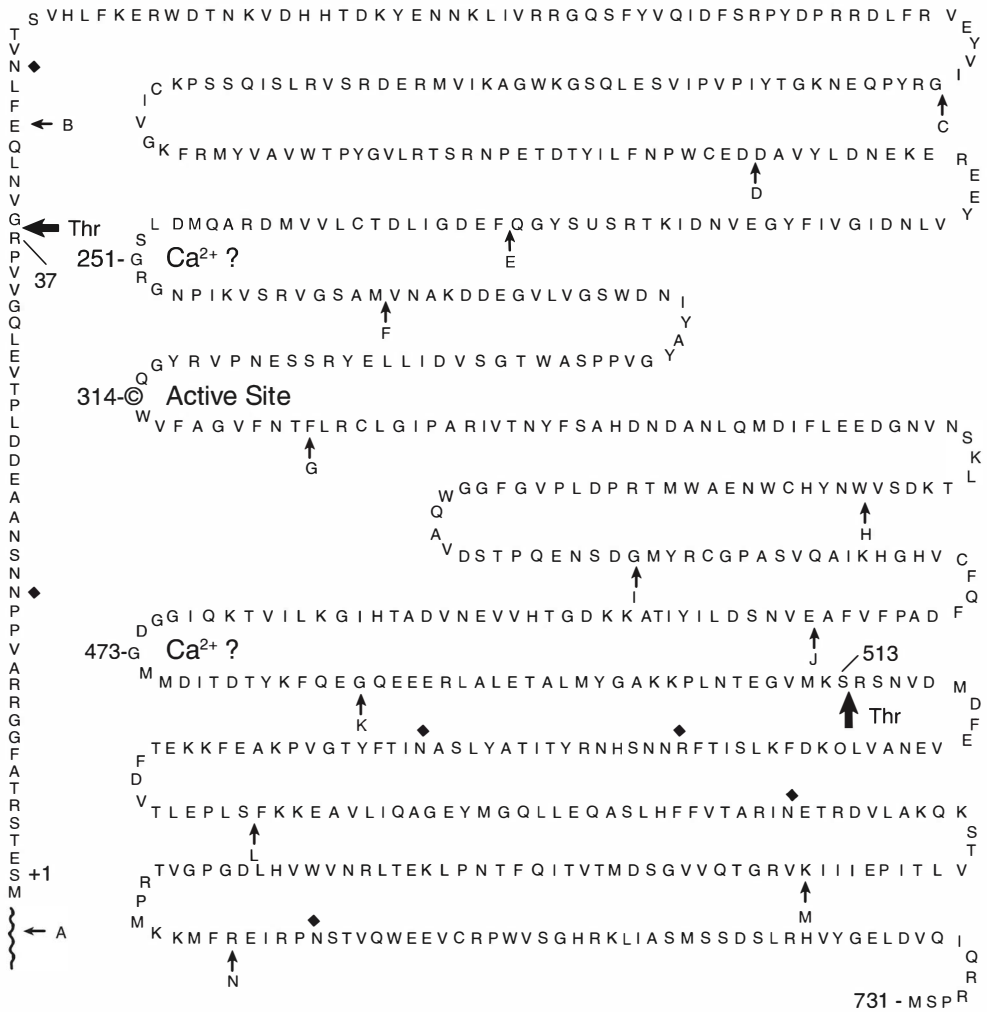

FIGURE 9.26 Amino acid sequence for the A subunit of human factor XIII and the location of the 14 introns (A to N). The thrombin cleavage site at Arg37-Gly, leading to the activation of the molecule, is shown with a *solid arrow*. The locations of the introns are shown with *small arrows*. Potential calcium-binding sites are shown with the active site Cys314. A thrombin inactivation site at Lys513-Ser is shown with an *open arrow*. Potential *N*-glycosylation sites are identified by *open diamonds*.

the gene for band 4.2 protein has the same structure as that of the A subunit of factor XIII.[363] The gene for keratinocyte transglutaminase has been localized on chromosome 14, which is different from that for the A subunit of factor XIII.[364] Thus far, 67 mutations have been observed in the F13A1 gene (FIGURE 9.27, *right panel*).[365]

The B Subunit of Factor XIII

The B subunit (Mr 80,000) of factor XIII is a plasma glycoprotein (8.5% carbohydrate) synthesized in the liver that circulates in blood both as a complex with the A dimer (XIII-A2B2) and as a free XIII-B2 dimer at a concentration of 21 μg/mL.[366] Administration of A subunits to patients with an A subunit deficiency causes an increase in the plasma level of the B subunit as well.[367,368] Three families with a bleeding tendency secondary to B subunit deficiency have been reported.[369–371]

The mRNA for the B subunit is approximately 2.3 kb long and codes for a hydrophobic leader sequence of 20 amino acids that is involved in secretion.[372,373] The mature protein starts with the unblocked amino-terminal sequence of Glu-Glu-Lys-Pro and consists of 641 amino acids (FIGURE 9.28). Almost the entire (98%) B subunit is composed of 10 tandem repeats, called sushi structures[374] or glycoprotein-1 structures, each consisting of 60 to 65 amino acids with highly conserved Cys, Pro, Gly, Tyr, Phe, and Trp residues. Sushi structures are found in at least 26 different proteins and two cDNAs from mammalian sources.[344,374] The B subunit contains 40 Cys residues (four in each of the 10 sushi structures). The first and third, and the second and fourth Cys residues in each sushi structure of the B subunit are most likely linked by disulfide bonds analogous to those determined in β_2-glycoprotein I and C4b-binding protein.[375,376] Three potential Asn-linked glycosylation sites are present in the B subunit. An Arg-Gly-Asp sequence exists in the last sushi structure of the B subunit, but its function is unclear.

The gene for the B subunit of factor XIII (F13B) is localized on chromosome 1 at bands 1q31-q32.1,[377] and other genes coding for proteins containing multiple sushi structures are also clustered on chromosome 1 at q32.[378] The gene for the B subunit

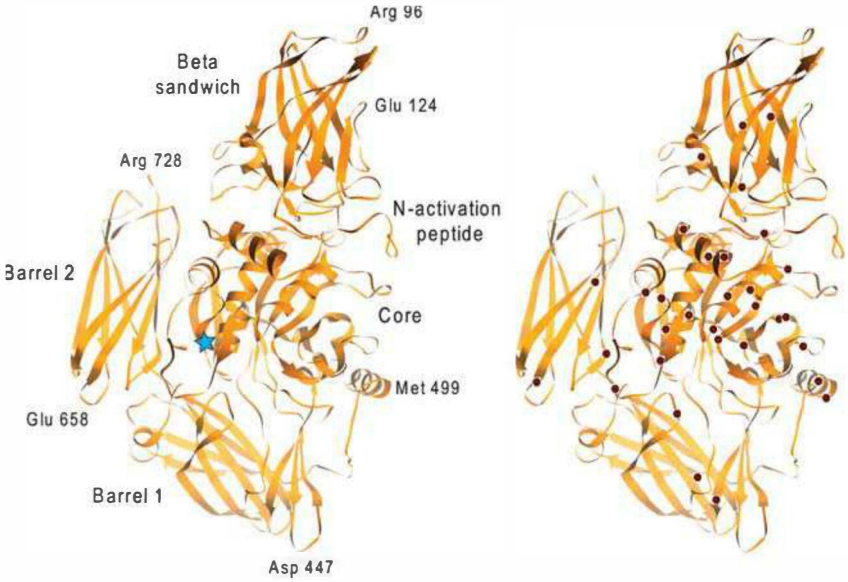

FIGURE 9.27 Ribbon structure of factor XIIIA. The protein contains our distinct domains including a beta sandwich, a central core, barrel 1, and barrel 2. Cys314 in the central core is shown by a *cyan* star and is part of the Cys-His-Asp triad of the catalytic site. Sixty-seven mutations that occur throughout the protein are shown by black dots in the structure in the right panel. (From Yee VC, Pedersen LC, Le Trong I, et al. Three-dimensional structure of a transglutaminase: human blood coagulation factor XIII. *Proc Natl Acad Sci U S A* 1994;91:7296; Hsieh L, Nugent D. Factor XIII deficiency. *Haemophilia* 2008;14:1190.)

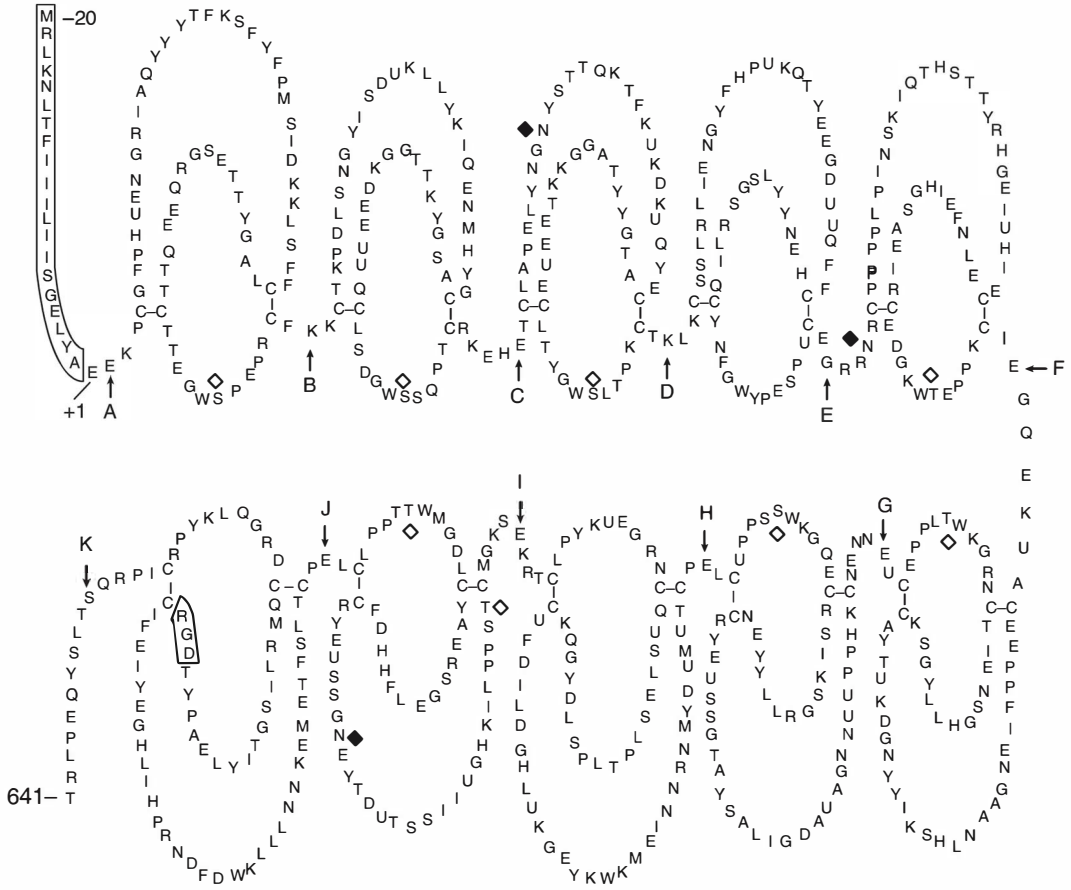

FIGURE 9.28 Amino acid sequence of the B subunit of human factor XIII and location of the 11 introns (A to K), indicated by *solid arrows*. Residues –20 to –1, enclosed in a *box*, constitute the signal sequence. The ten sushi domains are linked by disulfide bonds by analogy with those in β_2 glycoprotein and C4b-binding protein. (Naski MC, Lorand L, Shafer JA. Characterization of the kinetic pathway for fibrin promotion of alpha-thrombin-catalyzed activation of plasma factor XIII. *Biochemistry* 1991;30:934; Folk JE, Finlayson JS. The epsilon-(gamma-glutamyl)lysine crosslink and the catalytic role of transglutaminases. *Adv Protein Chem* 1977;31:1.)

spans approximately 28 kb and includes 12 exons interrupted by 11 introns.[372] All the intron–exon boundaries follow the GT-AG rule.[28] The leader sequence is encoded by the first exon, whereas the carboxyl-terminal region of the protein and the 3′ noncoding region are encoded by the 12th exon. Each of the 10 sushi structures is encoded by a single exon, and its 5′ and 3′ intron–exon boundaries are type I.[372] All sushi structures in the genes for other proteins are also encoded by single exons, with very few exceptions.[377]

Although the presence of polymorphisms in amino acid sequences has been suggested by isoelectric focusing technique, no mutations have been reported thus far. On the contrary, restriction fragment length polymorphisms are detected in the genomic DNAs.[378] Genetic defects of the complete B subunit deficiency indicate that a nucleotide deletion destroyed an intron–exon splice junction in one allele and replacement of a Cys residue with Phe diminished a disulfide bridge in another allele.[379]

PROTEIN C

Protein C (Mr 62,000) is a vitamin K–dependent glycoprotein that circulates in blood as a precursor to a serine protease.[380–383] Protein C is composed of a light chain of 155 amino acids (Mr 21,000) and a heavy chain of 262 amino acids (Mr 41,000), held together by a disulfide bond (FIGURE 9.29).[384,385] The light

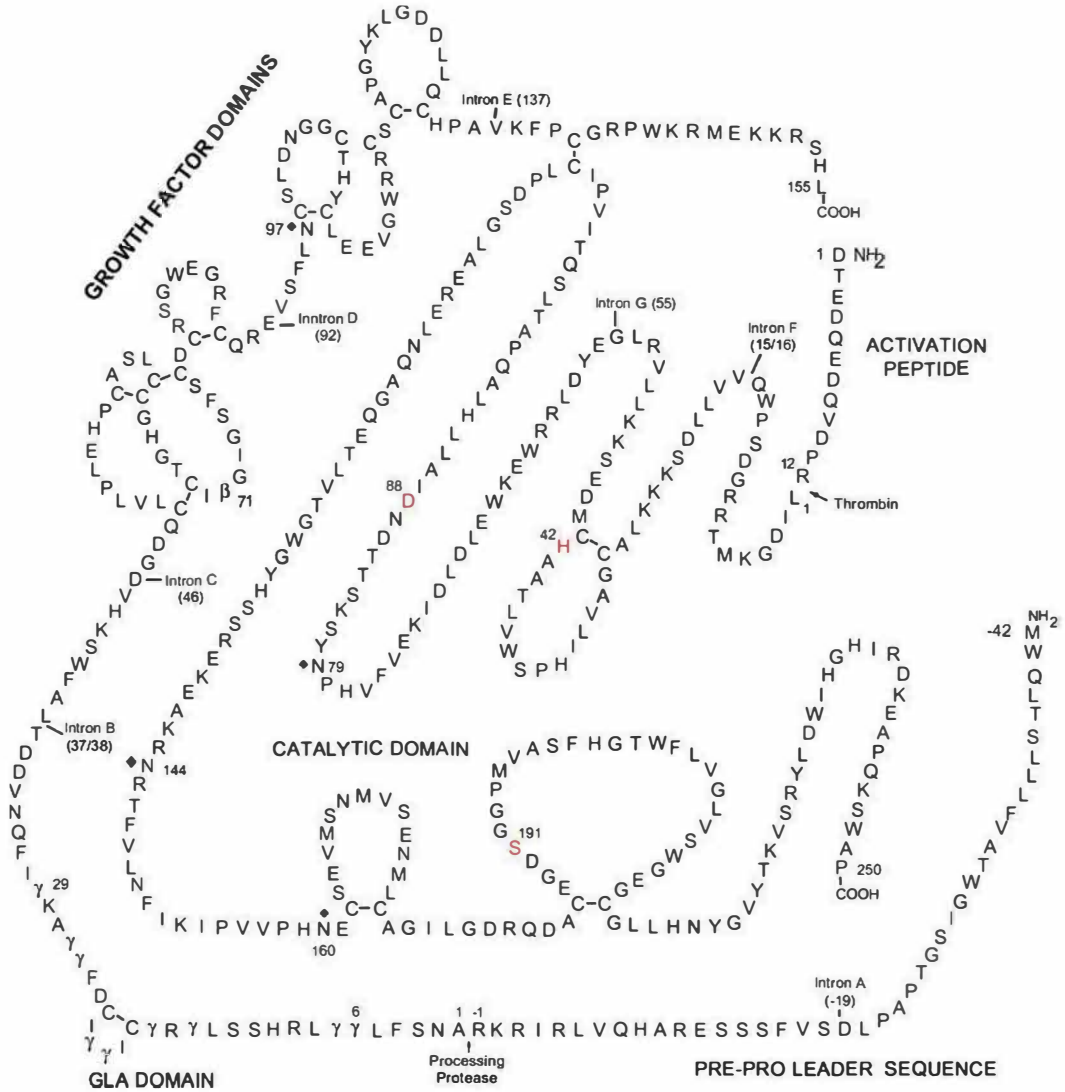

FIGURE 9.29 Amino acid sequence of human prepro protein C and location of the seven introns (A to G). The eighth intron in the 3′ noncoding sequence is not shown. The prepro leader sequence (–42 to –1) is removed during biosynthesis by signal peptidase and a processing protease that cleaves the Arg(–1)-Ala bond. The Gla domain and epidermal growth factor domains are located in the light chain within residues 1 to 155. The connecting peptide of Lys-Arg present between the light and heavy chains is not shown. The activation peptide of 12 residues is released from protein C by thrombin during its conversion to APC. The peptide bond (Arg-Leu) cleaved by thrombin is indicated by an arrow. The serine protease or catalytic domain of protein C contains 250 residues, including His42, Asp88, and Ser191, which participate in catalysis. Two potential carbohydrate attachment sites in the activation peptide are shown by *solid diamonds*. The proposed disulfide bonds in protein C have been placed by analogy to those established in bovine prothrombin and epidermal growth factor.

chain contains nine γ-carboxyglutamic acid residues followed by two EGF-like domains.[130,386–389] The light chain also has one β-hydroxyaspartic acid that has been reported to be involved in Ca^{2+} binding and anticoagulant activity.[10,390]. The heavy chain of protein C contains an activation peptide followed by a catalytic or serine protease domain. The protein also has four potential attachment sites for carbohydrate bound to asparagine, including residue 97 in the light chain and residues 79, 144, and 160 in the heavy chain.

Protein C activation by thrombin is greatly accelerated by thrombomodulin.[381,391–394] APC then inactivates factors Va and VIIIa by minor proteolysis in the presence of calcium and phospholipid.[381,395–398] Protein S functions in the latter reactions as a cofactor.[398–400] The conversion of protein C to a serine protease (APC) by thrombin is due to the cleavage of an Arg-Leu peptide bond at the amino-terminal region of the heavy chain, generating an activation peptide of 12 amino acids and a new amino-terminal Leu1 (FIGURE 9.29). The newly formed α-ammonium of Leu1 flips back into the catalytic domain where it forms a salt bridge with the carboxyl group of Asp190 and a serine protease with an active charge relay system involving His42, Asp88, and Ser191 (circled *in red*, FIGURE 9.29).

The mRNA for human protein C is approximately 1,700 nts in length and codes for a prepro leader sequence, a single-chain polypeptide of 419 amino acids, and a stop codon.[387] The 419 amino acids include a dipeptide located between the light chain and the heavy chain. The 3′ noncoding sequence consists of 68 nts and includes a processing or polyadenylation sequence of ATTAAA, which is located 16 nts upstream from the poly(A) tail. The prepro leader sequence in protein C is 42 amino acids in length (FIGURE 9.29). It is likely that the signal peptidase cleaves the prepro leader sequence following the serine residue located at –18 and removal of the propepetide by a furin-like protease that cleaves at Arg(–1). The light and heavy chains of circulating protein C are initially connected by a Lys-Arg dipeptide that is removed during processing.

The gene for human protein C (PROC) is present on chromosome 2 at q14-21, and in approximately 11 kb of DNA.[130,389,401] The gene contains nine exons and eight introns (FIGURE 9.29). The first intron is located in the 5′ noncoding region of the gene while the other seven are present in the coding region of the gene (FIGURE 9.29).[389] The introns range in size from 92 to 2,668 nts, and the exons range in size from 25 to 885 nts. A1l the intron–exon boundaries follow the GT-AG rule.[132] The 5′ untranslated region of the protein C gene contains an additional short noncoding sequence corresponding to an untranslated exon. This exon is separated from the translation start codon by a 1,463-bp intronic sequence.[402] Intron A is located in the prepro leader sequence; intron B follows the Gla domain; intron C is located just before the first potential growth factor domain; intron D separates the two potential growth factor domains; intron E follows the second potential growth factor domain; and introns F and G are located in the heavy chain that contains the catalytic domain.

High-level expression of the gene for protein C in HepG2 cells is regulated by a 1,500-bp 5′ flanking region, which includes 66 bp of the noncoding exon.[402] The major transcription start site is located 65-bp upstream from the first intron–exon boundary or 1,515-bp upstream from the initiation Met codon.[402] Two minor transcription start sites are located at –7 and +13 bp relative to the major transcription start site. Cis elements include HNF-1 (–10 to +9 bp), HNF-3 (–25 to –11 bp), a unique liver-specific binding site (12 to +30 bp), and an Spl site in the 5′ noncoding exon (+58 to +65 bp). A strong silencer region is located between –162 and –82 bp, and two possible liver-specific enhancer regions, which interact coordinately with promoter elements, are located between –1,462 and –162 bp.

Several genetic defects of protein C have been found in patients with hereditary thrombophilia, including a nonsense mutation of Arg306, a replacement of Arg169 with Trp at the activation cleavage site, the conversion of Trp402 to Cys,[403–405] promoter mutations of +3 C to T and –2 T to C that disrupt the HNF-1 site, and –15 T to A, –20 A to G mutations that disrupt the HNF-3–binding site,[406,407] and –2 mutations that disrupt HNF-1 binding.[408]

PROTEIN S

Human protein S (Mr 69,000) is a single-chain plasma glycoprotein (7.8% carbohydrate) that contains 10 γ-carboxyglutamic acid (Gla) residues[41] and requires vitamin K for hepatic biosynthesis.[410] Protein S functions as a cofactor of APC in the inactivation of factors Va and VIIIa,[389–400,411] and its deficiency leads to a predisposition for venous thrombosis.[412–415] The concentration of protein S in human plasma is approximately 25 μg/mL,[416,417] 40% of which exists free and 60% in stoichiometric complex with C4b-binding protein.[416] Complex formation does not lead to inactivation of C4b, although protein S no longer functions as a cofactor.[418,419] In the bovine system, another plasma protein binds to protein S.[420]

The amino acid sequence of human protein S has been determined by cDNA cloning.[421,422] The size of its mRNA is approximately 3.5 to 4.0 kb, including two polyadenylation signals at the 3′ end. Protein S is synthesized with a hydrophobic signal sequence and prepropeptide of 41 amino acids (FIGURE 9.30). The mature protein consists of 635 amino adds and starts with a sequence of Ala-Asn-Ser. Starting from the amino-terminus, there is a Gla domain, a thrombin-sensitive region, a short stretch of aromatic residues, four EGF domains, and a sequence homologous to steroid hormone–binding globulin (SHBG, FIGURE 9.30).[423] The cleavage by thrombin of two Arg-Ser/Ala bonds in the second disulfide loop results in the release of a peptide containing residues 53 to 70 and the loss of protein S cofactor activity.[423–426]

The first EGF domain of bovine protein S contains one β-hydroxyaspartic acid (residue 95), while each of the remaining three EGF domains contains one β-hydroxyasparagine (residues 136, 178, and 217).[427] One Asp and three Asn residues at the same positions in human protein S are also very likely hydroxylated, but these residues are not required for anticoagulant cofactor activity or for binding to C4b-binding protein.[428] The carboxyl-terminal portion of the SHBG region is important for the binding of protein S to C4b-binding protein.[429] Potential Asn-linked glycosylation sites are clustered in the middle of the SHBG region. Cloning of human cDNA for protein S has made it possible to express a functional recombinant protein S in mammalian cells.[430,431]

The gene for human protein S (PROS1) has been localized to chromosome 3q11,[432,433] spanning more than 80 kb and containing 15 exons interrupted by 14 introns.[434,435,436] All nucleotide sequences around the intron–exon boundaries follow the AG/GT rule and the consensus sequence. There are six Alu repeats located throughout the gene. The signal sequence is encoded by the first exon, the propeptide and Gla domain by the second exon, the short stretch of aromatic residues by the third exon, and the

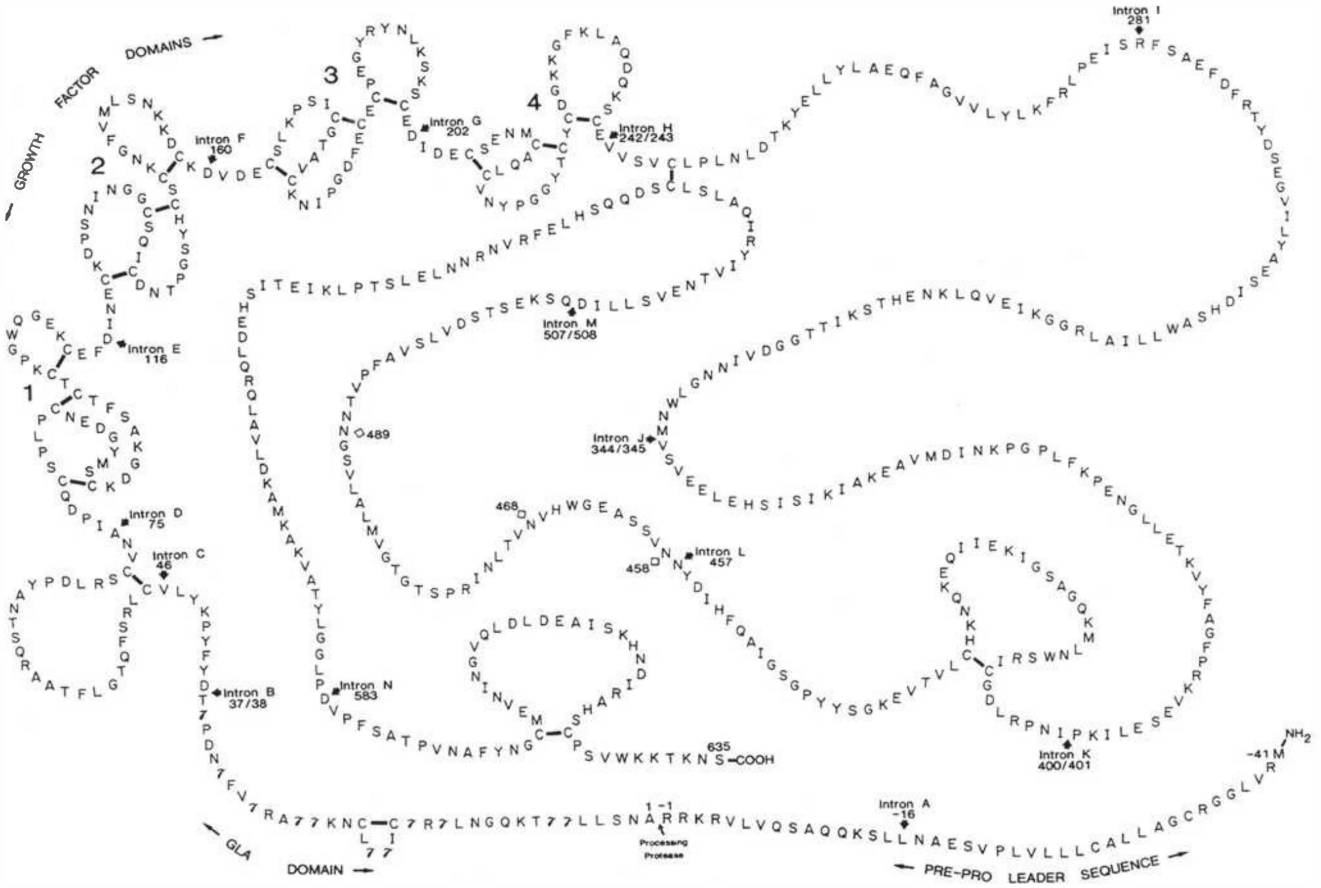

FIGURE 9.30 Amino acid sequence of human prepro protein S and location of the 14 introns (A to N), indicated by *solid arrows*. The prepro leader sequence includes residues –41 to –1. *Open diamonds* indicate potential carbohydrate attachment sites of the Asn-X-Ser/Thr type. Large numbers denote orders of four epidermal growth factor domains, which are followed by a region homologous to SHBGs.

thrombin-sensitive region by the fourth exon. Each of the four EGF domains is encoded by a single exon, while the SHBG region is encoded by seven exons as in the human SHBG gene.[437] Three potential glycosylation sites are encoded within a single exon.

Two copies of the gene for human protein S have been identified, and the second gene is very likely a pseudogene.[438,439] The latter spans over 55 kb and shows the absence of exon I, as well as many nucleotide substitutions, including a stop codon, insertions, and deletions resulting in frameshifts and the consequent in-frame stop codons. Overall homology between the exons of the gene for protein S and those of the pseudogene is 97%, indicating that these two genes diverged recently. A deletion of the middle portion of the gene coding for protein S has been identified in the DNA of patients with protein S deficiency,[439] and dimorphism at position 460 (Ser to Pro) in one of three potential Asn-linked glycosylation sites has been detected.[440]

Antithrombin III

Antithrombin III (Mr 58,200) is a major serine protease inhibitor synthesized in the liver and secreted into the plasma, where it circulates at about 150 μg/mL. Antithrombin III is a single-chain glycoprotein (9% carbohydrate) containing 432 amino acids (FIGURE 9.31) and four glycosylation sites located at Asn96, Asn135, Asn155, and Asn192 (α form). About 10% of the plasma protein is glycosylated at only three positions and lacks glycosylation at Asn135 (β form).[441,442] Antithrombin III is a member of the SERPIN family of suicide protease inhibitors (Table 9.4), 34 of which have been found on 10 different human chromosomes.[443] These proteins have an amino acid sequence homology of 25% to 35% and share a conserved tertiary core domain of about 350 residues that includes eight or nine α-helices and three β-sheets (FIGURE 9.31).

Antithrombin III is a potent inhibitor of thrombin, factor IXa, factor Xa, and factor XIa, a process that is accelerated 2,000-fold or more by heparin, which induces a conformational change and accelerates binding to thrombin.[444–447] The substrate recognition sequence containing the reactive center loop (RCL) of about 20 residues and its reactive Arg393 are located at the top of the inhibitor and are partially obscured in the absence of heparin. In the presence of heparin, an extension (*shown in blue*, FIGURE 9.32) forms at the top of helix D (*shown in yellow*) and a shift to the right occurs in the RCL (*red with black and green spheres*). This makes the reactive Arg393 in the RCL of the inhibitor more readily available to interact with target serine proteases.

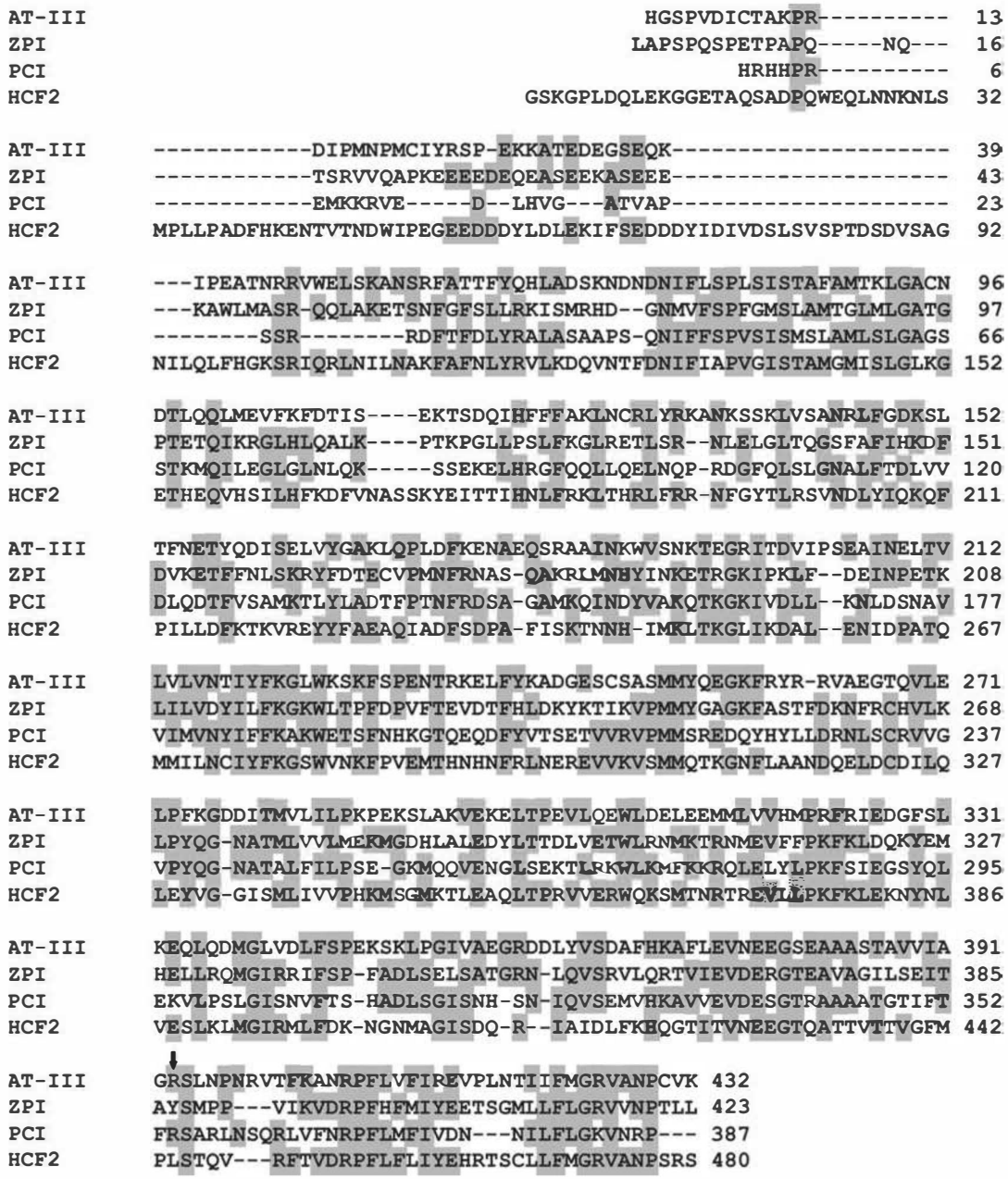

FIGURE 9.31 Alignment of the amino acid sequences of four SERPIN inhibitors of blood coagulation. Identical amino acids in the four proteins are shaded. AT-III, antithrombin III; ZPI, protein Z inhibitor; PCI, protein C inhibitor; HCF2, heparin cofactor 2.

The cDNA coding for antithrombin III was prepared from human liver RNA and predicted a 32–amino acid signal sequence followed by a 432–amino acid polypeptide chain.[441] At the 5′ end of the cDNA clone, a single transcription start site was identified 72 bp prior to the ATG initiation codon. At the 3′ end of the cDNA clone, a poly(A) sequence of AATAAA was observed 49 bp following the termination codon. Typical control regions in the gene for antithrombin III (SERPINC1), such as a TATA box, a CCAAT box, or GC elements, were not identified in the 5′-untranslated region. The gene for antithrombin III is located at chromosome 1q23-25 and contains about 13.4 kb of DNA.[448] It contains seven exons (including exons IIIA and IIIB) and six introns.[448] The gene also has nine full-length Alu repeat elements and one partial length Alu sequence, an unusually high number of Alu repeat sequences for a human gene. More than 50 mutations in the gene coding for antithrombin III have been identified, often located in regions of the gene coding for amino acids located in or near the reactive site loop or the heparin-binding site in antithrombin III.[449]

Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a serine protease inhibitor that occurs in multiple isoforms called TFPIα, TFPIβ, and TFPIδ.[450–453] It was originally called extrinsic pathway inhibitor

Table 9.4

Protein inhibitors of blood coagulation

Protein	Type	Specificity	Coinhibitor
APC	Serine protease	Factor Va Factor VIIIa	Protein S PL, Ca^{2+}
Tissue factor pathway inhibitor	Kunitz	Factor VIIa–TF	
Antithrombin III	SERPIN	Thrombin, factor IXa Factor Xa, factor XIa	Heparin Heparan sulfate
PZ–dependent protease inhibitor	SERPIN	Factor Xa, factor XIa	PZ PL, Ca^{2+}
Protein C inhibitor	SERPIN	APC Thrombin–TM	
Heparin cofactor 2	SERPIN	Thrombin	Dermatan sulfate

TF, tissue factor; TM, thrombomodulin; PL, phospholipid.

and lipoprotein-associated coagulation inhibitor. TFPIα (Mr 43,000) is a single-chain glycoprotein protease inhibitor containing 276 amino acids and three potential *N*-linked glycosylation sites and consists of an acidic *N*-terminal region, three tandem Kunitz-type protease inhibitor domains, and a basic *C*-terminal region involved in membrane anchoring (FIGURE 9.33). TFPIα is the major isoform produced in human tissue.[452]

TFPIβ (222 amino acids) and TFPIδ (162 amino acids) are shorter molecules formed by alternative mRNA splicing.[454] TFPIβ contains the first and second Kunitz domains, but lacks

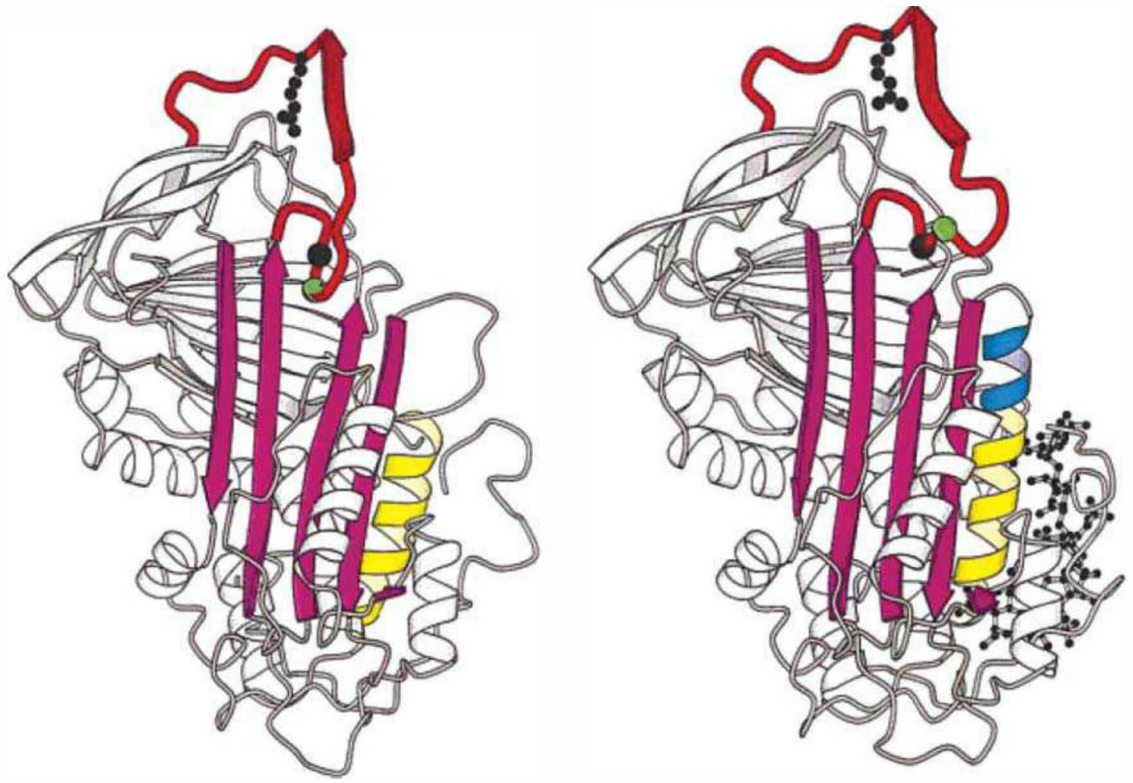

FIGURE 9.32 Ribbon structures of antithrombin III and antithrombin III–heparin (pentasaccharide) complex.[446] The conformational change in antithrombin III induced by heparin is readily seen by comparing the structure on the left (no heparin) and the one on the right with heparin which is bound to the lower right-hand side of the molecule.

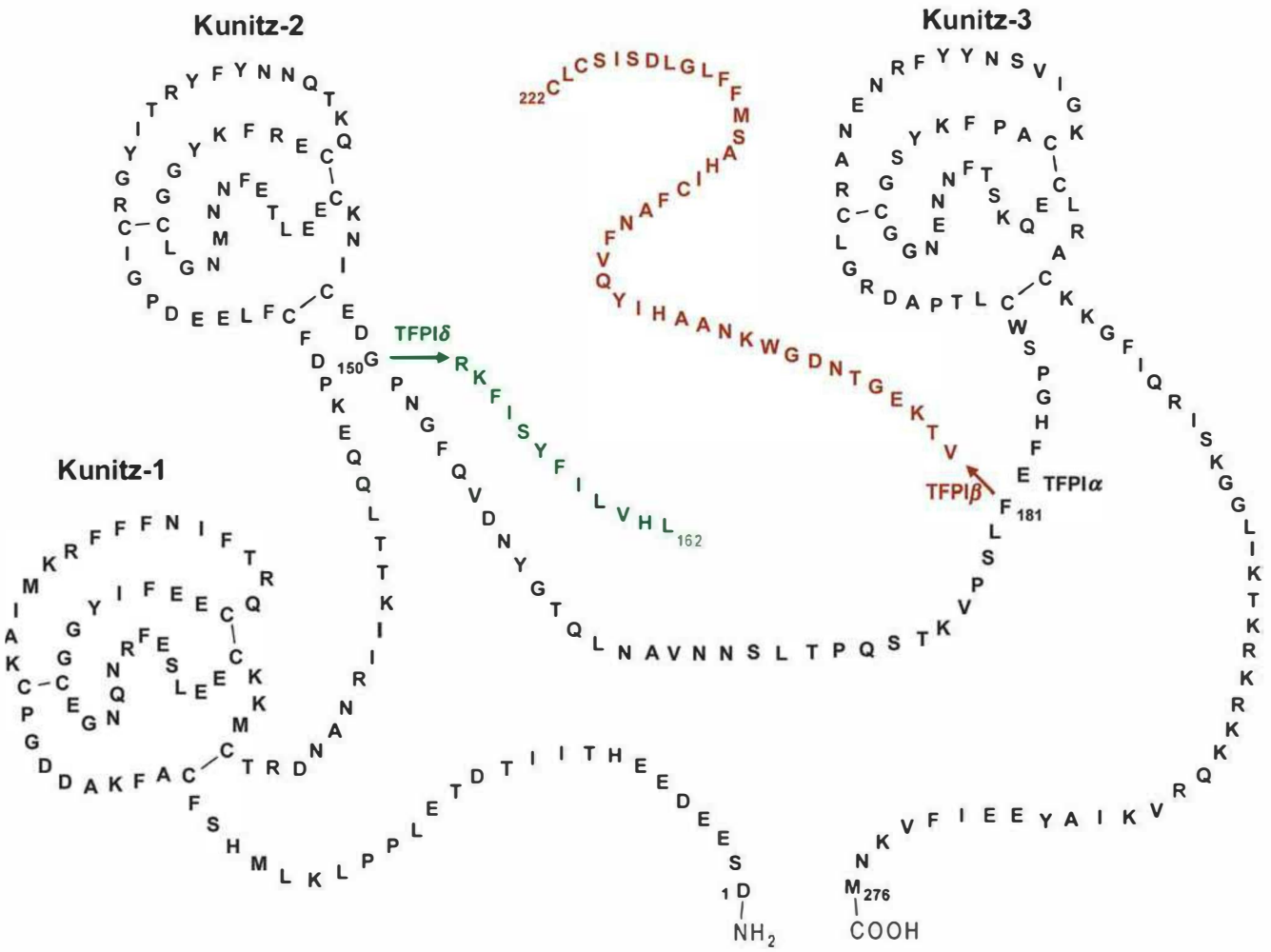

FIGURE 9.33 Amino acid sequence of the α, β, and δ isoforms of TFPI. The acidic *N*-terminal region of TFPIα is followed by three tandem Kunitz-type protease inhibitor domains and a basic *C*-terminal region. The shorter TFPIβ is spliced at Phe181 to Val shown in the red peptide. This reduces the length of the β isoform to 222 amino acids. In the shortest isoform, TFBIδ, the Gly150 is spliced to Arg present in the green peptide reducing the length of the δ isoform to 162 amino acids. (Modified from Maroney SA, Ellery PE, Mast AE. Alternatively spliced isoforms of tissue factor pathway inhibitor. *Thromb Res* 2010;125(Suppl 1):S52.)

the third Kunitz-type inhibitor and the *C*-terminal end. In contrast, the two Kunitz-type protease inhibitor domains in TFPIβ are followed by a completely different *C*-terminal region encoding a glycosyl-phosphatidylinositol anchor attachment sequence that is unrelated to the basic C-terminal region present in TFPIα. TFPIδ (162 amino acids) also has the first two Kunitz domains, followed by still another *C*-terminal sequence of 12 residues (FIGURE 9.33). The physiological role of this isoform is not known.

Factor Xa is inactivated when it binds to the second Kunitz domain of TFPI, while the factor VIIa–TF complex binds to the first Kunitz domain, leading to the inactivation of the factor VIIa–TF complex as well as factor Xa. These reactions may block the continuation of factor Xa generation by the extrinsic pathway of blood coagulation.[455] When major vascular damage occurs, it appears likely that the concentration of TFPI is insufficient to completely inhibit the factor Xa and factor VIIa–TF complex activity.

The cDNA coding for TFPI of placental and fetal liver cDNA libraries[456] contains 1,431 nts that include a 5′ noncoding sequence of 132 nts followed by an open reading frame of 912 nts and a 3′ noncoding region of 387 nts. The open reading frame codes for a signal peptide of 28 residues and a protein of 276 amino acids. A larger mRNA of 4.4 kb was also observed.[457]

The gene for TFPI is on chromosome 2 at 2q31-2q32.1 and spans about 70 kb[458,459] and contains nine exons and eight introns. The signal sequence and *N*-terminal region are coded by exon III, while exons IV, VI, and VIII code for the three Kunitz domains. The *C*-terminal region of the inhibitor is coded by exon IX. Thus far, no TATA box or CCAAT sequences have been observed in the 5′ end of the gene.

Protein Z and Protein Z–Dependent Protease Inhibitor

Protein Z (PZ) is a vitamin K–dependent plasma glycoprotein,[460] consisting of 396 amino acids including 13 Gla residues, one β-hydroxyaspartic acid, and four carbohydrate chains.[461–463] Plasma PZ concentration is 2.9 μg/mL,[464] in complex with protein Z–dependent protease inhibitor (PZPI).[465] PZ is composed

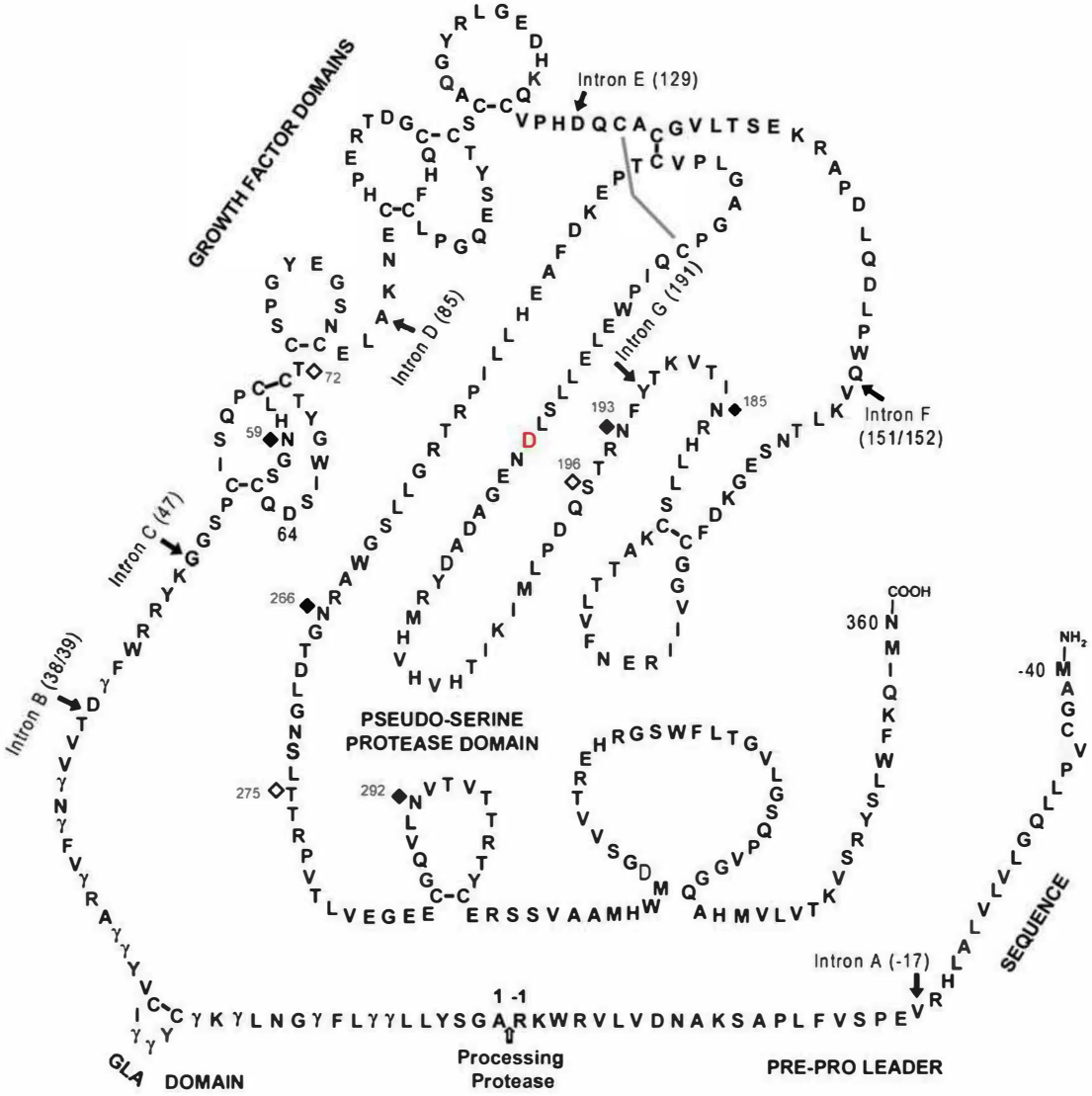

FIGURE 9.34 Amino acid sequence of human prepro PZ and location of introns A to G. The prepro leader sequence (–40 to –1) is removed during biosynthesis by signal peptidase and a processing protease that cleaves the Arg(–1)-Ala bond. Potential *N*-linked carbohydrate attachment sites Asn59, 185, 193, 266, and 292 are shown by *solid diamonds*, while potential *O*-linked carbohydrate attachment sites Thr72, 196, and 275 are shown by *open diamonds*. The single residual amino acid of the catalytic triad Asp221 in the pseudo-serine protease domain is shown in *red*. (From Huang X, Dementiev A, Olson ST, et al. Basis for the specificity and activation of the serpin protein Z–dependent proteinase inhibitor (PZPI) as an inhibitor of membrane-associated factor Xa. *J Biol Chem* 2010;285:20399.)

of 360 amino acids (FIGURE 9.34), including 13 Gla residues and eight potential carbohydrate chains bound to Asn-X-(Ser/Thr) at positions 59, 185, 193, 266, and 292 and three potential *O*-linked carbohydrate attachment sites (Ser/Thr)-X-X-Pro at residues 72, 196, and 275. It seems probable that Asp64 in human PZ is β-hydroxy-Asp, since this residue was identified as β-hydroxy-Asp in bovine PZ.[461] The Gla region of PZ is followed by two EGF-like domains and a serine protease-like domain (FIGURE 9.34). However, the protein is not a zymogen of a serine protease.

The cDNA for PZ is approximately 1.6 kb in length and codes for a prepro leader peptide of 40 amino acids and the mature circulating protein (FIGURE 9.34).[463] The prepro leader contains a typical secretion signal rich in hydrophobic residues, followed by a propeptide sequence that is essential for the carboxylation of 13 amino-terminal Glu residues. The *C*-terminus of the prepropeptide contains an Arg-Trp-Lys-Arg sequence that is recognized by a furin-like protease that cleaves between the last Arg and the *N*-terminal Ala (FIGURE 9.8).

The gene for human PZ (PROZ) is about 14 kb in length and contains nine exons including one alternative exon. The exon/intron boundaries for the eight regular exons are typical consensus sequences of GT-AG. The gene for PZ is localized to chromosome 13 at band q34.[466]

Inhibition of factor Xa by PZPI is accelerated about 2,000-fold in the presence of PZ, phospholipid, and calcium.[467,468,469] PZPI also inhibits factor XIa but does not require PZ for this reaction and does not inhibit other coagulation factors.

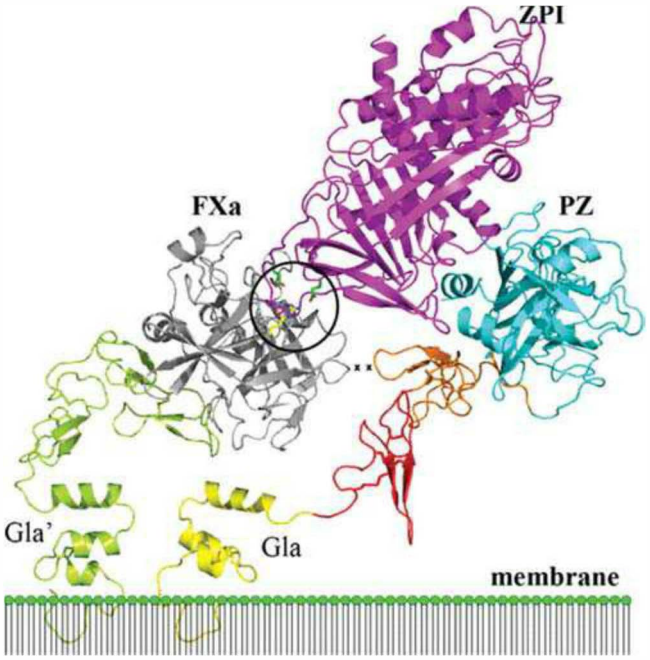

FIGURE 9.35 A model of the ribbon structure of the complex of PZ–PZ inhibitor–factor Xa on a phospholipid surface. PZ pseudo-serine protease domain is colored *cyan*, PZ EGF1 and EGF2 are colored *orange*, and PZ Gla domain is colored *yellow*; protein Z protease inhibitor (ZPI) is colored *magenta*, factor Xa catalytic domain (FXa) is colored *gray*, and factor Xa EGF1, EGF2, and Gla domain are colored *green*. The active site of factor Xa bound to the reactive center of PZ inhibitor is enclosed in a *black* circle. (Huang X, Dementiev A, Olson ST, et al. Basis for the specificity and activation of the serpin protein Z–dependent proteinase inhibitor (ZPI) as an inhibitor of membrane-associated factor Xa. *J Biol Chem* 2010;285:20399.)

PZPI (Mr 72,000) is a single-chain molecule composed of 423 amino acids (FIGURE 9.31), homologous to other members of the SERPIN family of protease inhibitors. Tyr387 was predicted to be at the P1-reactive center of PZPI in contrast to Arg, which is the P1 residue in antithrombin III and protein C inhibitor. PZ circulates in complex with PZPI[470] (FIGURE 9.35). PZ is bound to PZPI through polar and ionic interactions while the Gla region of PZ is bound to calcium and the membrane surface shown at the bottom of the structure. PZPI has nine α-helices and three β-sheets, typical of the SERPIN family. In the ternary complex, the Gla region of factor Xa is also bound to the membrane surface, while its catalytic site binds to PZPI through its P1 Tyr. The phospholipid membrane plays an important role in bringing the three proteins together.

A cDNA of 2.44 kb coding for PZPI[467] contains six potential ATG start sites at the 5′ end of the clone; a sixth ATG is the likely initiation site. The open reading frame of 1,335 bp contains a 21–amino acid signal peptide and five potential *N*-linked glycosylation sites. Northern blot analysis indicated the presence of PZPI mRNA in liver but none in heart, lung, brain, spleen, testis, and kidney.

Protein C Inhibitor

Protein C inhibitor (Mr 57,000) is a single-chain SERPIN that is a potent inhibitor of APC[471] and the thrombin–thrombomodulin complex that converts protein C to APC.[472] Its concentration in plasma is 1 μg/mL.[473] The inhibitor is a glycoprotein composed of 387 amino acids (FIGURE 9.31), containing five potential carbohydrate chains including three starting at residues 230, 243, and 319 (Asn-X-Ser/Thr) and two at residues 20 and 39 (Thr/Ser-X-X-Pro).[473] The P1-reactive site in protein C inhibitor is Arg354, which is the same as in antithrombin III (FIGURE 9.31). The protein contains only one cysteine residue similar to α_1-antitrypsin and α_1-antichymotrypsin. Protein C inhibitor forms a 1:1 molar complex with APC in a reaction accelerated about 50-fold by the presence of heparin. Protein C inhibitor also inhibits thrombin, factor Xa,[474] factor XIa, plasma kallikrein,[475] urokinase,[476] prostate-specific antigen,[477] and sperm acrosin.[478]

A cDNA coding for protein C inhibitor[473] is 2,106 bp in length and codes for a signal peptide of 19 amino acids, a mature protein of 387 amino acids, and a stop codon. The 3′ noncoding region is 839 bp in length but lacked a typical polyadenylation sequence. The cleavage site for signal peptidase is Leu-His.

The gene for protein C inhibitor (SERPINA5) is 11.5 kb in length and contains five exons and four introns. The boundaries for the introns and exons all follow the typical GT-AG sequence. The gene for protein C inhibitor is located on chromosome 14q32.1.[479,480] The 5′ noncoding region of the gene lacks a typical TATA box and CCAAT sequences, but contains several cis elements including an Sp1-binding site (–302 to –294 bp) with strong promoter activity and two AP2-binding sites (–350 to –343 bp) with strong enhancer activity.[481]

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CHAPTER

10 Genetics of Coagulation

RODNEY M. CAMIRE AND PARIS MARGARITIS

The determination of the gene, complementary deoxyribonucleic acid (cDNA), and amino acid sequences of all of the proteins known to be involved in the hemostatic process has been completed. This task, begun in the early 1980s, has allowed for (a) deduction of amino acid sequences from the cloned cDNAs of several species, (b) characterization of gene structure and organization, (c) characterization of mutations responsible for inherited abnormalities, (d) characterization of polymorphisms useful for gene-tracking studies, (e) production of large quantities of recombinant protein for research and for therapeutic purposes, and (f) the development of animal models of hemostatic disease through transgenic and gene knockout technologies. It has also made possible the contemplation of novel gene-based approaches for the treatment of bleeding diatheses. This chapter focuses primarily on various genetic aspects of procoagulant, anticoagulant, and fibrinolytic factors involved in human blood coagulation, with emphasis on gene structure and mutations that predispose to bleeding or thrombosis. We have also focused on mutational mechanisms and how new genomic information will impact genetic analysis.

GENOMIC INFORMATION

The completion of the Human Genome Project in April 2003 was an enormous scientific achievement,[1,2] providing substantial advances in the understanding of gene structure and organization, genetic variation, comparative genomics, genomic medicine, and gene interaction with environmental factors. There are currently three major Web portals that serve as central hubs for genome-related resources.[3] The first is the National Center for Biotechnology Information (NCBI) run by the National Institutes of Health (NIH) (http:// www.ncbi.nlm.nih.gov/); the second is Ensembl (http://www.ensembl.org/index.html), which is a collaborative effort between the Wellcome Trust Sanger Institute and European Molecular Biology Laboratory's (EMBL's) European Bioinformatics Institute; and the third is the University of California, Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu) developed by an academic research group at the UCSC.

An immediate application of genomic sequence information is to accelerate the identification of genes that are associated with human disease. There are several strategies to accomplish this, the most common being genetic linkage analysis (genome-wide linkage scan or homozygosity mapping),[4] by which the chromosomal location of a disease-causing gene can be identified. Genome-wide linkage analysis usually requires families in which several individuals are affected with the disease of interest. Evenly spaced DNA markers are used to trace the inheritance of each copy of each chromosome. Chromosomal segments that do not influence disease segregate randomly, whereas the particular copy that, in each family, carries the disease-causing mutation will be shared among affected family members more often than would be predicted by chance.[5] The DNA markers that are typically used are termed microsatellites, which are di-, tri-, or tetranucleotide tandem repeats in DNA sequences and can be readily followed by simple polymerase chain reaction (PCR) techniques. Typically, the segment of the genome between identified microsatellite markers that influences the disease state is quite large: millions to tens of millions of base pairs in length, spanning dozens to hundreds of genes. However, the human genome project allows researchers to move rapidly from large chromosomal regions to individual candidate genes because all genes identified within any linkage region are immediately available.

The development of a haplotype map of the human genome will facilitate the identification of genes that are affected by environmental factors. A haplotype block of DNA, consisting of a group of nearby alleles or genetic markers that are inherited together, is roughly consistent among all humans, but different individuals have different versions of the blocks, allowing one to identify the relationship between haplotype blocks and diseases.[6] The International Haplotype Map Project (HapMap; http://hapmap.ncbi.nlm.nih.gov/) that aims to define the haplotype structure of human populations should greatly facilitate genetic linkage studies by allowing researchers to find genes that affect health, disease, and individual responses to medications and environmental factors.

MUTATIONS: MECHANISMS AND DATABASES

Alterations in a gene sequence can be neutral, detrimental, or favorable, and are semantically referred to as a mutation when the variation leads to a phenotypic change, a polymorphism when the genetic change is present in >5% of alleles in a population, and a rare sequence variant when the change occurs in <5% of alleles. Therefore, distinct populations show variations not only in disease prevalence but also in the frequency of polymorphic changes.

Types of Genetic Mutations

Point Mutations

A point mutation is defined as a change of a single base pair of a DNA sequence in a gene caused by the substitution of one nucleotide for another. A single codon in frame (missense mutation) may predict replacement of a single amino acid in an otherwise normal protein. When the change results in a stop codon (nonsense mutation), a truncated protein product

results. Sometimes, the mutated proteins are not properly produced, secreted into the blood, or located in the physiologic site. When they do circulate in substantial amounts, the variant protein often circulates with impaired function. When a single base change is phenotypically silent, this is commonly referred to as a single nucleotide polymorphism (SNP). Outside the actual coding region, it is even more likely that nucleotide substitutions are silent. Notable exceptions are mutations in binding sites for transcription factors that regulate gene activity, mutations that affect splicing of the immature RNA, and mutations that may alter the stability of the RNA.

Up to approximately 50% of the single nucleotide substitutions occur as transitions within cytosine–phosphoguanine (CpG) dinucleotides, the most common hot spot for mutation.[7,8] This finding is all the more remarkable when one considers that CpG dinucleotides are underrepresented in most genes, presumably because of the evolutionary loss of these dinucleotides. CpG dinucleotides are hot spots for mutation because they are sites of methylation of the cytosine residue (i.e., a CpG becomes a TpG or a CpA).[7] Methylcytosine readily deaminates to thymine spontaneously. After the C-to-T transition occurs in the reverse or "antisense" DNA strand, the sequence in its complementary coding strand is changed from G to A. Because the common (but not exclusive) codon for Arg is CGx, many CpG dinucleotide mutations are found within Arg codons, producing Arg-to-stop, Gln, His, Cys, or Trp mutations. When the same CpG transition occurs among different families, it often is possible to distinguish independent mutational events from founder effects through haplotype or pedigree analyses. The founder effect is the establishment of a new, typically isolated, population by a few original "founders" that carry only a fraction of the total genetic variation of the parental population.

Insertion/deletion

Insertion/deletion is a type of variation that is defined as a change in the DNA sequence created by the addition or subtraction of nucleotides. When the insertion or deletion is within the triplet open reading frame and the change is a number of nucleotides that is not a multiple of three, this disruption results in a frameshift mutation. During protein translation, a frameshift usually results in a premature stop codon producing a grossly abnormal protein with impaired function and viability.

Categorization of Mutations

Strictly speaking, a recessive mutation implies that heterozygotes (one copy of mutation) have a normal phenotype and that only homozygotes (two copies of the mutation) are phenotypically affected. Recessive-negative mutations display a predominantly recessive inheritance with a nearly infinite number of different mutations. Most mutations in the genes encoding the coagulation and fibrinolytic genes are recessive negative and come to clinical attention when present in homozygous (doubly or compound heterozygous) or hemizygous (for *X*-linked disorders) form.

A dominant mutation refers to a genetic change that lacks phenotypic differences between affected heterozygous and homozygous individuals. Dominant-negative mutations show a dominant inheritance with a narrow spectrum of mutations. From the perspective of family studies, this distinction is informative in that dominant mutations should be suspected after successive generations are affected; recessive mutations normally occur only within one generation. Less commonly, dominant (incomplete) inheritance is seen when there is a mutation that causes a gain of function, the best known example of which is factor V_{Leiden}. Genetically, gain-of-function mutations are clearly less heterogeneous than loss-of-function mutations, as there are infinite ways to destroy gene function, whereas only a few mutations lead to improvement of gene or protein function.

In reality, mutations are not always strictly dominant or recessive, as the penetrance of a mutant phenotype can vary dramatically, largely because of concomitant genetic changes. Therefore, recessive-negative mutations are not always entirely recessive. For example, although homozygous protein C deficiency produces severe thrombotic disease and purpura fulminans at birth,[9] heterozygotes may either experience thromboembolic disease later in life or never develop venous thrombosis.[10,11]

Normal Variants and Polymorphisms

Normal variants and polymorphisms are particularly useful in gene-tracking studies of families with a severe bleeding or thrombotic disorder. In such studies, a polymorphism is used as a marker of a defective gene, followed throughout a family when the actual gene mutation is not known. This approach has been particularly useful in genetic counseling for hemophilia A or B, and is increasingly applied as markers of risk factors for venous thrombosis and cardiovascular disease.[12] In the case of protein C, fibrinogen, factor VII, plasminogen activator inhibitor-1 (PAI-1), and the prothrombin 20210GA variant, a particular relation exists between polymorphisms and plasma levels of specific clotting factors.

Epigenetics

In addition to mutations, epigenetic changes within the genome can also have a direct effect on phenotype. Epigenetics refers to those mechanisms that control gene expression in a potentially heritable way yet do not involve changes to the underlying DNA sequence.[13] These traits exist on top of or in addition to the traditional molecular basis for inheritance. Driving these changes are covalent modifications to cytosine bases and histones (e.g., DNA methylation and acetylation of histones) and changes in positioning of nucleosomes.[14] Epigenetic changes to DNA plays an important role in the normal way cells respond to their environment and contribute to the regulation of many cellular processes. It is not surprising then that aberrant placement of epigenetic marks or defects in the epigenetic machinery lead to human disease. This is now well established in cancer and is becoming more appreciated in cardiovascular and autoimmune disease as well as neurologic disorders.[15] Thus epigenetic factors constitute a key link between genetics, disease and environment playing a decisive role in the underpinnings of human pathology. The Human Epigenome Project (HEP; http://www.epigenome.org/index.php) with the stated goal of cataloging and interpreting genome-wide DNA methylation patterns of all human genes in all major tissues, should allow researchers to make these important links.

Multifactorial Disease

Contrasting with rare monogenetic diseases are the milder and much more common multifactorial or complex diseases, such as type 2 diabetes mellitus, hypertension, coronary artery disease, and venous thrombosis. In venous thrombotic disease, thrombotic episodes typically occur relatively late in life, and in up to

50% of patients, genetic risk factors such as heterozygosity for mutations in coagulation inhibitors or cofactors are found.[16]

Databases of Mutations

Mutations in the genes for essentially all of the coagulation proteins can be found at the Human Gene Mutation Database (HGMD) at the Institute of Medical Genetics, at http://www.hgmd.cf.ac.uk/ac/index.php searching using the relevant gene symbol (Table 10.1). Additionally, a coagulation sequence and structure database (CoagBase; www.isth.org/default/index.cfm/standards/coagbase/) is maintained by the International Society of Thrombosis and Haemostasis, and ClotBase (http://www.clotbase.bicnirrh.res.in) provides a wide range of useful information including a catalog of known mutations for many coagulation factors.[17]

Table 10.1

Characteristics of human coagulation factor genes

Genes	Gene Symbol	Gene ID	Location	Exons	Gene	mRNA	cDNA
Prothrombin	F2	2147	11p11–q12	14	20.3 kb	1,997 bp	1,866 bp
Factor X	F10	2159	13q34–qter	8	26.7 kb	1,502 bp	1,467 bp
Factor VII	F7	2155	13q34–qter	8	13.1 kb	2,478 bp	1,335 bp
Factor IX	F9	2158	Xq27.1–q27.2	8	31.3 kb	2,804 bp	1,386 bp
Factor XI	F11	2160	4q34–q35	15	22.6 kb	2,217 bp	1,878 bp
Tissue factor	F3	2152	1p22–p21	6	11.6 kb	2,153 bp	888 bp
Factor VIII	F8	2157	Xq28	26	~186 kb	9,030 bp	7,056 bp
Factor V	F5	2153	1q23	25	~80 kb	6,914 bp	6,675 bp
Protein C	PROC	5624	2q13–q14	9	10.8 kb	1,843 bp	1,386 bp
Thrombomodulin	THBD	7056	20p12–cen	1	4.2 kb	4,048 bp	1,728 bp
Protein S	PROS1	5627	3p11–q11.2	15	80 kb	3,309 bp	2,031 bp
EPCR	PROCR	10544	20q11.2	4	6 kb	1,483 bp	717 bp
ATIII	SERPINC1	462	1q23–q24	7	13.5 kb	1,395 bp	1,395 bp
TFPI	TFPI	7035	2q31–q32.1	9	~85 kb	1,431 bp	915 bp
Heparin cofactor II	SERPIND1	3053	22q11.21	5	13.6 kb	2,217 bp	1,500 bp
Factor XII	F12	2161	5q33–qter	14	12 kb	2,048 bp	1,848 bp
Prekallikrein	KLKB1	3818	4q34–q35	15	31 kb	2,245 bp	1,917 bp
HMW kininogen	KNG1	3827	3q26–qter	11	27 kb	~3,200 bp	1,935 bp
tPA	PLAT	5327	8p12–p11	14	32.7 kb	~2,600 bp	1,689 bp
Plasminogen	PLG	5340	6q26–q27	19	53.5 kb	~2,900 bp	2,433 bp
Plasmin inhibitor	SERPINF2	5345	17p13	10	~16 kb	~2,300 bp	1,476 bp
PAI-1	SERPINE1	5054	7q21.3–q22	9	12.3 kb	~2,800 bp	1,209 bp
TAFI	CPB2	1361	13q14.11	11	~48 kb	~1,700 bp	1,272 bp
Fibrinogen Aα chain	FGA	2243	4q28	5	5.4 kb	2,182 bp	1,935 bp
Fibrinogen Bβ chain	FGB	2244	4q28	8	8.2 kb	1,918 bp	1,476 bp
Fibrinogen γ chain	FGG	2266	4q28	10	8.4 kb	1,559 bp	1,314 bp
Factor XIII A chain	F13A1	2162	6p25.3–p24.3	15	>160 kb	~4,000 bp	2,199 bp
Factor XIII B chain	F13B	2165	1q31–q31.2	12	~28 kb	~2,200 bp	1,986 bp
vWF	vWF	7450	12p13.3	52	~178 kb	8,923 bp	8,442 bp
γ-Glutamylcarboxylase	GGCX	2677	2p12	15	13 kb	3,245 bp	2,277 bp
Epoxide reductase I	VKORC1	79001	16p11.2	3	~5 kb	1,003 bp	492 bp

The size of the cDNAs is defined as the number of nucleotides from the start to the stop codons.
EPCR, endothelial cell protein C receptor; TFPI, tissue-factor pathway inhibitor; HMW, high-molecular-weight; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin-activatable fibrinolysis inhibitor; vWF, von Willebrand factor.
Genomic and protein information for each gene can be obtained by using the Gene ID and searching "All Databases" at http://ncbi.nlm.nih.gov/.
Mutations can be found by searching for Gene Symbol at http://www.hgmd.cf.ac.uk/ac/index.php. Some coagulation factors have their own mutation databases. These can be found within each section.

MOLECULAR GENETICS OF HEMOSTATIC PROTEINS

Hemostatic proteins can be divided into procoagulant serine proteases, procoagulant cofactors, anticoagulants, contact activators, and fibrinolytic factors, plus several other relevant proteins fibrinogen, factor XIII, and von Willebrand factor (vWF). While not considered coagulation factors *per se*, both γ-glutamyl carboxylase and vitamin K 2,3-epoxide reductase I (VKOR) play an essential role in hemostasis. These enzymes are involved in the posttranslational carboxylation of specific glutamic acid residues using vitamin K within the amino-terminal Gla domains of vitamin K–dependent proteins (prothrombin, factor VII, IX, X, protein C, S, and Z).[18,19] Warfarin inhibits VKOR, and a deficiency of either of these enzymes is classified as vitamin K–dependent coagulation factors deficiency, typically characterized by hemorrhagic manifestations.[20] Knockout of the γ-glutamyl carboxylase gene in mice results in offspring surviving to term but dying uniformly at birth of massive intra-abdominal hemorrhage.[21]

The proteins considered in this chapter and their gene symbols, chromosomal location, size of gene, mRNA, and cDNA sequences are shown in Table 10.1. Most of the hemostatic proteins have been experimentally knocked-out in mouse models, and where appropriate, these studies are summarized in Table 10.2.

Table 10.2

Genetic models of thrombosis and hemostasis

Gene Knockout	Strain	Viable	Mortality Observed (Days of Gestation)	Phenotype	References
Prothrombin	C57BL/6	No	9–11	Fatal hemorrhage/yolk sac defect	31
Factor X	Swiss	No	11–13	Partial embryonic lethal/intra-abdominal bleeding	54
Factor VII	C57BL6/J	No	At birth	Fatal perinatal bleeding	77
Factor IX	C57BL/6	Yes	—	Severe bleeding following trauma	93–95
Factor XI	C57BL/6	Yes	—	Normal/prolonged aPTT	114
Tissue factor	Several	No	9.5–12	Fatal embryonic bleeding	125–127
Factor VIII	129sv	Yes	—	Severe bleeding following trauma	137
Factor V	C57BL/6J	No	9.5–10	Fatal hemorrhage/yolk sac defect	166
Protein C	Swiss	No	18.5–19	Consumptive coagulopathy	195
Protein S	129sv	No	15.5–17.5	Consumptive coagulopathy	229,230
Thrombomodulin	129sv	No	8.5–10	Embryonic lethal/yolk sac defect	203,205
EPCR	Swiss	No	9–10	Embryonic lethal/placental thrombosis	453
ATIII	C57BL/6J	No	15–16	Consumptive coagulopathy/severe hemorrhage	454
TFPI	C57BL/6	No	10–13	Intrauterine lethality	266
Heparin cofactor II	C57BL/6	Yes	—	Normal; differences in thrombosis model	285
tPA	C57BL/6	Yes	—	Develop normally	330
Plasminogen	C57BL/6	Yes	—	Predispose to thrombosis; ligneous conjunctivitis	346–348
Plasmin inhibitor	C57BL6/J	Yes	—	Develop normally; enhanced fibrinolytic potential	367
PAI-1	C57BL/6	Yes	—	Develop normally; mild hyperfibrinolytic state	397,398
TAFI	C57BL/6	Yes	—	Normal	410
Fibrinogen A chain	C57BL/6	Yes	—	Wound healing defect/failure of pregnancy	424
Factor XIII A chain	C57BL/6	Yes	—	Develop normally; severe uterine bleeding; miscarriages	439,440
vWF	C57BL/6J	Yes	—	Defects in hemostasis and thrombosis; model type 3 vWD	451
γ-Carboxylase	C57BL/6J	No	9.5–18	Massive intra-abdominal hemorrhage	21

EPCR, endothelial cell protein C receptor; ATIII, antithrombin III; TFPI, tissue factor pathway inhibitor; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin-activatable fibrinolysis inhibitor; vWF, von Willebrand factor.

Procoagulant Serine Proteases

Prothrombin

The vitamin K–dependent serine protease zymogen prothrombin plays a central role in the coagulation pathway. Following its activation by the prothrombinase complex (i.e., factor Xa, factor Va, calcium ions, and anionic membranes), the resulting product thrombin participates in numerous biologic processes, including the cleavage of fibrinogen to fibrin, which leads to clot formation (see Chapter 8). The gene encoding prothrombin is on chromosome 11p11–q12 and is approximately 20 kb in length with 14 exons.[22] The mRNA for human prothrombin was cloned in 1983 and is approximately 2 kb in length and includes a 1,866-bp cDNA.[23] The major site of synthesis of prothrombin is in the liver,[24] but minor sites are present in the central nervous system (CNS), skeletal and smooth muscle cells, and the kidney.[25–28] Prothrombin is expressed as a single polypeptide chain of 622 amino acids. Following removal of a 43–amino acid leader sequence, it is secreted into plasma as a 72-kDa protein containing 579 amino acids at a concentration of 1.4 μM.[23,29]

Homozygosity or compound heterozygosity for loss-of-function mutations in the prothrombin gene leads to a bleeding diathesis. There are no known cases of a total deficiency of prothrombin, consistent with the studies in mice showing lethality in utero with a complete absence of the protein.[30,31] Prothrombin deficiency has a prevalence of approximately 1:1,000,000 to 1:2,000,000.[32] Greater than 60 different mutations are known, most of which lead to a variant protein,[32] a number of which have been subjected to detailed biochemical analysis. For example, thrombin Quick I (Arg to Cys at 382) has near normal activity with thrombin-specific low molecular weight (LMW) substrates, but about 100 times lower activity with fibrinogen than that observed with thrombin.[33,34]

A common polymorphism that constitutes a major genetic risk factor for thrombosis is the 20210G to A mutation.[35] Position 20210 in the prothrombin mRNA is where prothrombin pre-mRNA undergoes 3′ cleavage and polyadenylation. This polymorphism was originally found in probands with a strong personal and familial history of venous thrombosis and is associated with slightly elevated plasma levels of prothrombin (~25%).[35] Further epidemiologic data confirmed that the DNA variant confers a three- to fourfold risk factor for venous thrombosis.[36–39] The prevalence of the 20210A allele is between 1% and 2% and is dependent on the geographic origin of the subjects.[40] Homozygotes are relatively rare.[39]

Initial reports indicated that the mutation, located 20 nucleotides downstream of the poly A signal, increases the post-translational 3′ end processing efficiency, thereby leading to a higher rate of transcription.[41] Additional studies showed that the mutant allele influences mRNA stability increasing cytoplasmic half-life.[42] However, these studies have been called into question because prothrombin 20210G and 20210A mRNA levels were found at equal levels in fresh liver tissue from a 20210G/A heterozygote.[43] This study was able to demonstrate that the 20210A mutation affects the position of the 3′-cleavage/polyadenylation reaction, an event that may lead to its abnormal mRNA function. Additionally, *in vitro* studies indicate that the G or A allele at 20210 have equal stability further implicating the importance of mRNA processing and/or translation, not cytoplasmic half-life, in regulating prothrombin expression.[44]

Factor X

Factor X is a vitamin K–dependent plasma protein that plays a central role in blood coagulation. In the presence of its cofactor protein factor Va, activated factor X cleaves two peptide bonds in prothrombin to form thrombin. Factor X is synthesized primarily in the liver, but other tissues also may contribute, such as lung, heart, ovary, and small intestine.[45] The gene for factor X has been cloned and is approximately 27 kb in length, contains 8 exons, and is located on chromosome 13q34–qter, 2.8 kb from the factor VII gene.[46,47] The cDNA has an open reading frame of 1,467 nucleotides and encodes a protein of 488 amino acids (preprofactor X).[48–50] Following proteolytic processing, mature factor X (59 kDa) is a two-chain serine protease zymogen of 445 amino acids, covalently associated through a single disulfide bond, circulating at a concentration of 170 nM.[51,52]

Factor X deficiency is a rare (1:1,000,000) autosomal recessive bleeding disorder.[53] Heterozygotes are usually free of symptoms but may exhibit abnormal bleeding during surgery. Over 50 factor X–deficient families are reported and over 100 different mutations have been identified.[53] Large deletions have been identified but most defects are missense mutations often associated with detectable factor X antigen levels and mild-to-asymptomatic disease.[53] Mice rendered experimentally deficient in factor X show frequent embryonic lethality, and those that survive die shortly after birth from massive intra-abdominal bleeding.[54,55]

A collection of factor X mutations has been described that have differential responses to the intrinsic and extrinsic pathways. For example, factor X Vorarlberg is associated with a marked reduction of activity in the extrinsic system and a much more modest reduction of activity in the intrinsic system.[56,57] These types of mutations imply that factor VIIa/tissue factor (TF) and factor IXa/factor VIIIa likely have different sites of interaction with factor X, despite the fact that they cleave the same bond within the zymogen. At least eight naturally occurring factor X variants display marked differences in activity in the intrinsic and extrinsic systems.[56–64]

Factor X was originally called Stuart-Prower factor after the first individuals in whom factor X deficiency was discovered.[65,66] One of the index cases of the Stuart family is homozygous for a replacement of Val by Met at position 298.[67] A descendent of the Prower case was shown to be a compound heterozygote for an Asp282 to Asn and an Arg287 to Trp mutation.[68] An informative historic sketch on the discovery of factor X has been published.[69]

Factor VII

Factor VII is a vitamin K–dependent serine protease zymogen that circulates in plasma as a single-chain polypeptide (50 kDa) of 406 amino acids at a concentration of 10 nM.[70–72] Like most clotting factors, it is synthesized primarily in the liver, as a 444–amino acid precursor protein. Activated factor VII, in concert with its cofactor, TF, initiates blood coagulation following vascular injury by activating factors IX and X.[73] The factor VII gene is relatively small (~13 kb), contains eight exons, and is located on chromosome 13q34–qter, only 2.8 kb upstream of the factor X gene.[74,75] The full-length mRNA is almost 2.5 kb, whereas the cDNA sequence is 1,335 nucleotides long.

Factor VII deficiency is a rare autosomal disorder with a prevalence of approximately 1:500,000.[76] Individuals with factor VII antigen and activity levels <1% display a moderate-to-severe

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bleeding phenotype. Mutations associated with this phenotype include frameshift, splice site, promoter, and missense mutations, with the total number of mutations now reaching more than 110.[76] The situation is less clear for mutations associated with measurable levels of factor VII activity, as many are asymptomatic.

It is not entirely clear whether a complete absence of factor VII is incompatible with life. For example, the factor VII knockout mouse develops normally to term, but dies shortly after birth from major abdominal and intracranial hemorrhage.[77] In addition, McVey et al.[78] identified a splice site mutation in the factor VII gene that was associated with a complete absence of factor VII and that resulted in fatal intracerebral hemorrhage at the age of 14 days. In contrast, Peyvandi et al.[79] have reported a homozygous 2-bp deletion in the factor VII gene that was associated with a complete absence of factor VII and was nonlethal. Although it is likely that some compensatory mechanism is affecting the phenotype in this individual, this mechanism has not yet been identified.

Several mutations have been identified in the promoter region of the factor VII gene; one of the mutations appears to disrupt the Sp1 binding site, whereas the other two interfere with binding of the transcription factor hepatocyte nuclear factor (HNF)-4 or the binding of an unidentified protein near the transcription state site.[80–82] Additional transcription factors (early growth response [EGR]-1, cAMP response element binding [CREB] protein, and upstream stimulatory factor-1) also bind within the proximal factor VII promoter region.[83] A mutation at −39 resulting in reduced plasma levels and activity may disrupt binding of CREB.[84]

Interest in the relation between elevated factor VII levels and an increased risk of cardiovascular disease was stimulated by the findings of the Northwick Park Heart Study (NPHS I).[78,85] However, results from a second prospective study (NPHS II) did not show a clear correlation between elevated factor VII levels and myocardial infarction risk or the inverse correlation with a factor VII lowering haplotype,[86] and further case–control studies showed that this polymorphism is associated with a lower risk of peripheral arterial disease.[87]

Since cardiovascular disease is a multifactorial outcome, it is likely that the relationship between plasma factor VII levels and arterial thrombosis is influenced by additional thrombotic risk factors such as diabetes, high body mass index, and elevated cholesterol levels.

Factor IX

Factor IX is a vitamin K–dependent serine protease zymogen that plays a critical role in the middle phase of blood coagulation. Activated factor IX is the serine protease component of the intrinsic Xase enzymatic complex, which is also composed of factor VIIIa, anionic membranes, and calcium ions (see Chapters 9 and 14). Factor IX is synthesized primarily in the liver and is composed of 461 amino acids. Following proteolytic processing and removal of the preproregion, it circulates in plasma as a single-chain 415–amino acid protein (55 kDa) at a concentration of 90 nM. The factor IX gene was cloned between 1982 and 1984 and lies on the long arm of the X chromosome, band q27.1.[88–92] The full gene sequence is known and is approximately 31 kb in length and has eight exons.[92] The mRNA is approximately 2.8 kb in length, whereas the cDNA is 1,386 bp long.[89,92]

Loss-of-function mutations in the factor IX gene result in hemophilia B. The prevalence of this *X*-linked disorder is 1 in 25,000 men, whereas women are rarely affected. The fivefold difference in prevalence between hemophilia A and B is roughly equivalent to the difference in size of the coding portions of the factor VIII and IX genes. The clinical manifestation of hemophilia B is almost identical to that for hemophilia A. Several mouse models of hemophilia B have proved useful in the development of new therapies.[93–95]

The first mutation in the factor IX gene, described in 1983,[96] was a deletion that showed up in a screening using Southern blotting of genomic DNA. The first point mutation in factor IX, factor IX Chapel Hill, was discovered by protein sequencing, not by DNA analysis.[97] At present, over 375 different mutations are known in the factor IX gene, many of which can be found in an international database (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html). Some mutations occur independently in patients from diverse geographic regions, mostly mutations at CpG dinucleotides, accounting for approximately 50% of the independent missense mutations found in different families.

The numerous mutations in the 5′-untranslated region of the factor IX gene are associated with a certain form of hemophilia B (hemophilia B Leyden), arguably the most fascinating of all promoter mutations.[98,99,100] The unique feature of these variants is that individuals exhibit "recovery" from severe hemophilia with the onset of puberty. Therefore, the disease is characterized by the absence of factor IX expression in childhood (factor IX levels <2%) and a gradual rise in factor IX levels after the onset of puberty (see FIGURE 10.1). Studies using transgenic mice that robustly mimic the hemophilia B Leyden phenotype have uncovered the elusive molecular mechanism underlying the puberty-onset spontaneous amelioration of factor IX levels. This process relates to a puberty-onset gene switch (age-related stability element/age related increase element) and that growth hormone is directly responsible for the puberty-onset recovery of factor IX production.[101]

Three factor IX mutations (i.e., Ala10 to Val, Ala10 to Thr, and Asn9 to Lys) are associated with a unique type of genetic predisposition to bleeding during oral anticoagulation therapy that is attributable to increased warfarin sensitivity.[102–104] The Ala10 and Asn9 residues are within the factor IX propeptide region and are critical for interaction with γ-glutamyl carboxylase and, therefore, γ-carboxylation; mutations at these sites impair this reaction. When receiving coumarins, patients with these variants showed a disproportionate decrease of factor IX activity approaching that of severe hemophilia B. As a consequence, these patients may have bleeding at the very beginning of oral anticoagulation. After discontinuation of treatment, the factor IX levels return to normal.

While mutations in factor IX are generally not associated with thrombosis, elevated levels of factor IX have been associated with venous thrombosis.[105] A patient has been described with a missense mutation in factor IX (Arg338 to Leu; factor IX-Padua) that leads to thrombophilia.[106] The levels of the mutant protein were normal in the patient, but the coagulant activity was approximately eight times the normal level. To date, this is the only reported gain-of-function mutation resulting in a hyperfunctional factor IX protein predisposing to thrombosis.

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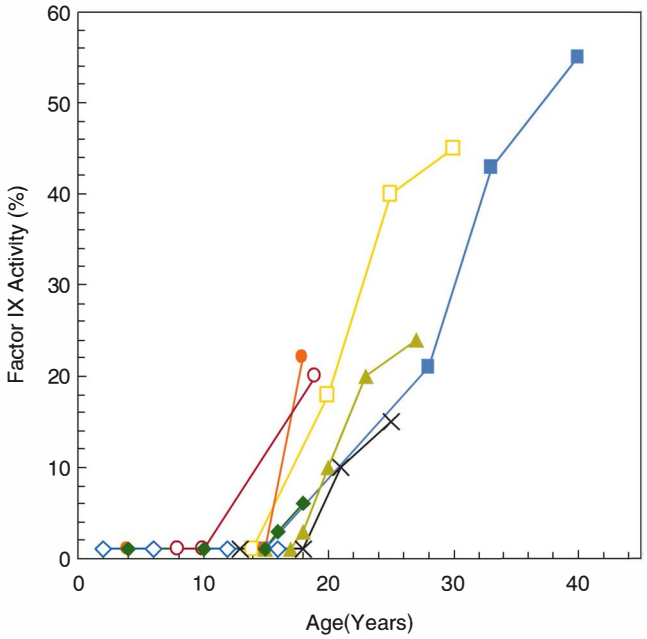

FIGURE 10.1 Factor IX levels measured over time in eight individuals with Leyden variant hemophilia B. These mutations in the promoter result in severe factor IX deficiency until puberty when factor IX levels rise gradually to approximately 30%. (Data from Briet E, Bertina RM, van Tilburg NH, et al. Hemophilia B Leyden: a sex-linked hereditary disorder that improves after puberty. (*N Engl J Med* 1982;306:788–790.)

Factor XI

Factor XI is a serine protease zymogen that contributes to hemostasis by activating factor IX (see Chapter 15A). Factor XI is converted to factor XIa through proteolytic cleavage, either by factor XIIa or by thrombin. A near full-length cDNA encoding factor XI was cloned in 1986, and the structure of the factor XI gene was determined in 1987.[107,108] The gene consists of 15 exons spread over 23 kb of DNA. The factor XI gene is on chromosome 4q34–q35, adjacent to the prekallikrein gene.[108] Factor XI is synthesized in the liver and circulates in plasma at a concentration of 30 nM.[109,110] It is a disulfide-bond–linked dimer of two 80-kDa polypeptides of 608 amino acids each.[109]

Factor XI deficiency is an autosomal disorder characterized by trauma- or surgery-induced hemorrhage and is only rarely characterized by spontaneous bleeds.[111] A mutation database specific for factor XI can be found at: http://www.factorxi.org/. It is usually rare in most ethnic groups but is highly prevalent among Ashkenazi Jews,[112] mutated factor XI genes being as high as 13%.[112,113] Homozygosity for factor XI deficiency is associated with a very mild bleeding tendency, even when factor XI levels are quite low, consistent with factor XI–deficient mice that do not show any increased tendency to bleed.[114]

The recurrence of mutations in Jews is thought to be the result of founder effects.[113] This interpretation is supported by the fact that in other ethnic groups the mutations are more diverse. Well over 150 different mutations are known in factor XI, many of which are associated with decreased antigen levels. The mutations vary from splice site defects and amino acid replacements to frameshift mutations.

Procoagulant Cofactors

Tissue Factor

TF is a cell-associated cofactor protein for activated factor VII, and the enzyme complex is considered to be the physiologic trigger of normal hemostasis and thrombotic disease (see Chapter 11).[73] The gene for TF resides on the short arm of chromosome 1 (p21–p22) and is approximately 12 kb long and contains six exons.[115,116] The size of the predominant TF mRNA in cells is approximately 2.2 kb, with the cDNA being 888 bp long.[115,117–119] TF is an integral membrane protein of 263 amino acids and is initially synthesized with a 32–amino acid signal peptide. TF is expressed constitutively by epithelial cells and adventitial fibroblasts surrounding blood vessels as well as by cardiomyocytes and astrocytes in the brain and is exposed to the blood following injury.[120,121] TF is present in the circulation, associated with hematopoietic cells and their microparticles.[122] Cardiovascular disease, sepsis, and cancer have increased "circulating TF" levels, thus potentially contributing to pathologic thrombosis.[123]

Several studies have identified binding sites for transcription factors that regulate basal and inducible TF gene expression in different cell types. Basal expression appears to be regulated by the Sp1 transcription factor, whereas inducible expression is regulated by c-Fos/c-Jun, c-Rel/p65, and EGR-1.[124] Regulation of TF expression by inflammatory mediators and angiogenic factors suggests that it may contribute to both inflammation and angiogenesis.

At present, there are no published missense mutations in the TF gene and no known TF-deficient patients, suggesting that TF deficiency may be lethal. Support for this concept comes from TF knockout mice that die *in utero* between days 8.5 and 10.5.[125–127] Promoter polymorphisms in the TF gene, however, have been identified. Six novel promoter polymorphisms have been found that are distributed over two haplotypes with equal frequencies, one of which (TF-603A) has been linked to venous thromboembolic disease, suggesting that it might protect against thrombosis by reducing the level of circulating TF, although no link with myocardial infarction has been found.[128] These polymorphisms significantly influence constitutive TF gene expression in human monocytes, but have no major effect on whole blood clotting time.[129]

Factor VIII

Factor VIII is a metal ion–dependent procofactor that circulates in plasma as a heterodimer of a heavy chain and light chain (see Chapter 12). Thrombin-activated factor VIII is the cofactor protein for factor IXa and plays a key role in the activation of factor X.[130] The essential role of factor VIII in blood coagulation is evidenced by the severe bleeding diathesis associated with its deficiency (hemophilia A). The factor VIII gene was originally cloned in 1984 and is approximately 186 kb in length and has 26 exons and lies on the X chromosome (q28).[131–133] The gene encodes a mature mRNA of approximately 9 kb, and the cDNA is 7,056 nucleotides long.[134,135] Preprofactor VIII has 2,351 amino acids, whereas mature factor VIII has 2,332 amino acids and circulates in plasma at a concentration of approximately 0.7 nM.[134–136] A mouse model of hemophilia A was described in 1995.[137] Accounts describing the discovery of the factor VIII protein and gene have been published.[138,139]

The first description of mutations in the factor VIII gene dates to 1985, in which two nonsense mutations in the coding

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sequence and two partial deletions of the factor VIII gene were described.[140] The finding of four distinctly different mutations in the factor VIII gene of otherwise similar patients was the first hint that a large variety of mutations exist in the factor VIII gene in patients with hemophilia.

There is also an unusually common inversion in the factor VIII gene that is highly prevalent among patients with severe hemophilia A.[141,142] This common rearrangement in the factor VIII gene was discovered in 1993 and is present in approximately 40% to 50% of all severe hemophilia A cases.[141] Because it is so prevalent and relatively easy to detect, the discovery of this rearrangement has revolutionized genetic counseling for hemophilia A. It results from an unequal crossover event between a sequence in intron 22 of the factor VIII gene and one of two extragenic copies of this sequence that lie distal on the X chromosome. Inversion of the factor VIII gene by homologous recombination leads to complete disruption of the factor VIII coding sequence and consequently to a severe form of hemophilia A (FIGURE 10.2). Another factor VIII inversion occurs in intron 1 and breaks the factor VIII gene, resulting in production of two chimeric mRNAs[143,144]; the prevalence of this inversion in patients with severe hemophilia A is approximately 4% to 5%.[144]

The molecular pathology of the factor VIII gene is updated and reviewed in an electronic version of the hemophilia A database: http://hadb.org.uk/. As of 2010, there were more than 1,400 unique mutations in factor VIII reported, including some 900 different point mutations. Missense mutations in FVIII span the entire molecule with a relatively equal distribution, except for the central B-domain (FIGURE 10.3), consistent with data indicating that this region is not needed for biologic activity.[145] The effect of these mutations on gene function is diverse. Severe dysfunction is produced by point mutations that introduce premature stop codons and by mutations that destroy splice junctions between introns and exons. On the other hand, these types of missense mutations that predict replacement of the normal amino acid by another amino acid also contain most, if not all, of the mild and moderately severe forms of hemophilia A.

Gene deletions of <100 bp up to several hundred kilobases have been found in many severely affected patients, and gene deletions may be present in up to 5% of such patients.[146] Insertions are relatively uncommon in the factor VIII gene. As with deletions, it is important to distinguish in-frame from frameshift insertions. In the group of large insertions are the so-called LINE (long inserted element) retrotransposons.[147,148] These DNA elements comprise approximately 5% of the human genome. Intact LINEs encode a protein with reverse transcriptase-like activity and have the capacity to spread through the genome through an RNA intermediate. The LINE first identified in patients with hemophilia A was derived from an element on chromosome 22. Subsequent to the original description in hemophilia A, insertion of LINEs has been found to disrupt various other genes.

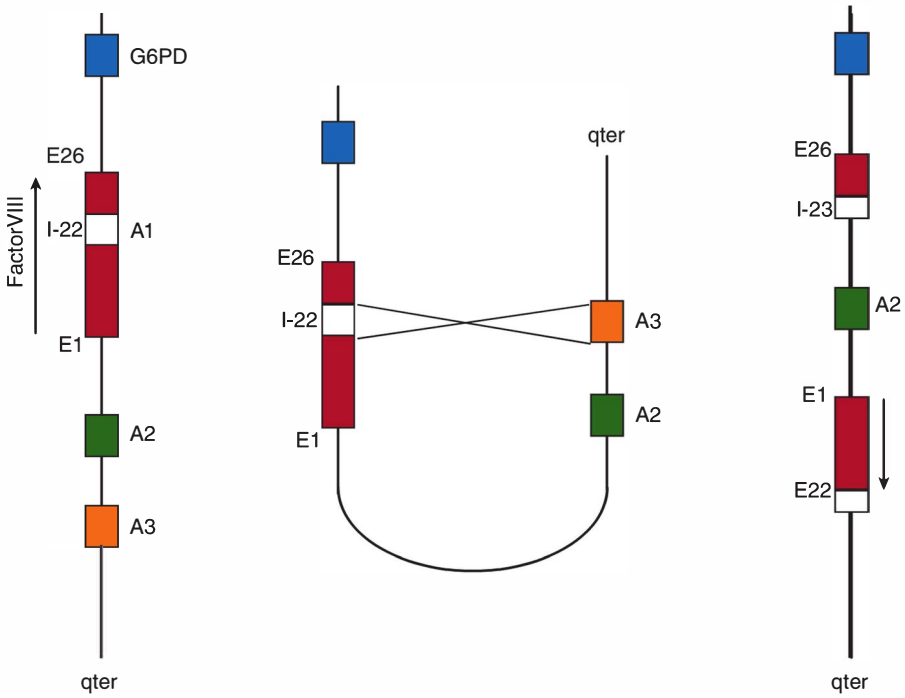

FIGURE 10.2 Factor VIII intron 22 inversion. The **left panel** depicts the normal alignment of the factor VIII gene on the X chromosome. Intron 22 (*white box*) of the factor VIII gene harbors a copy of the so-called A1 gene, whereas highly homologous A2 and A3 genes are located toward the tip of the X chromosome (indicated as qter). The **middle panel** shows alignment of the A1 gene with the A3 gene as it may occur during male spermatogenesis. When this alignment of A1 with A2 (or alternatively A3) results in a nonhomologous recombination, a rearrangement results as depicted at the **right panel**. In this rearranged X chromosome, the integrity of the factor VIII gene is completely disrupted because it is cut in two parts that are now aligned in opposite directions.

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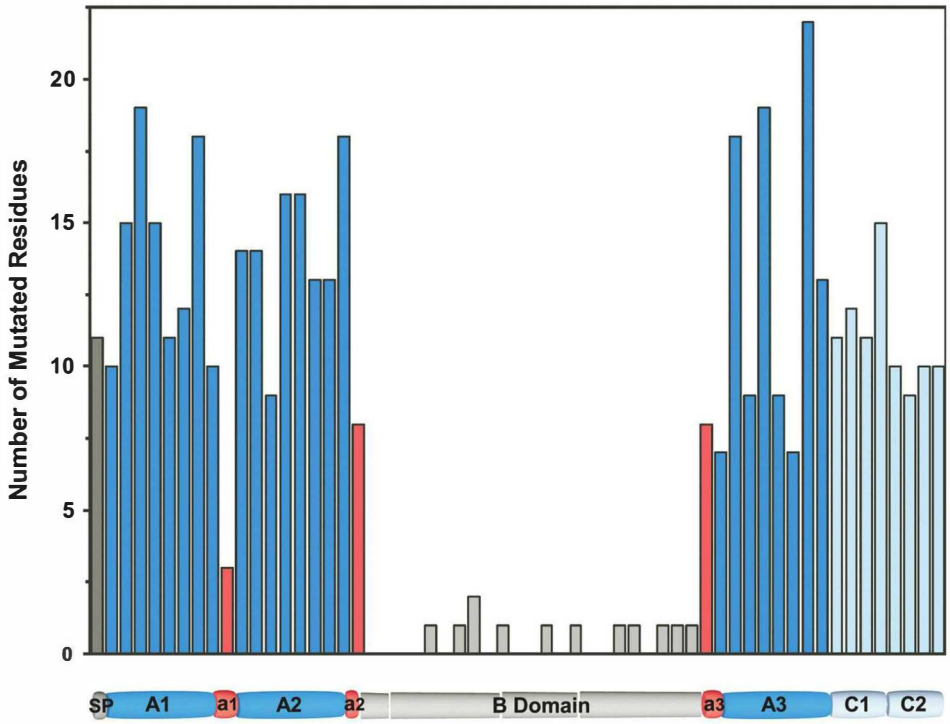

FIGURE 10.3 Graphic representation of missense mutation within factor VIII. Mutations in factor VIII leading to hemophilia A are presented in 40 residue blocks spanning the molecule. Mutations were derived from the hemophilia A mutation database (http://hadb.org.uk) with nonsense mutations excluded. (*J. Coagul Disord* 2010;2:19-27, with permission.)

Factor V

Activated factor V is the cofactor protein for the serine protease factor Xa in the prothrombinase complex. The precursor of factor Va is factor V, a large heavily glycosylated, single-chain protein that is homologous to factor VIII (see Chapter 12).[149] The factor V gene is located on the long arm of chromosome 1q23, not far from the antithrombin gene and is approximately 80 kb in length and contains 25 exons.[150] The first factor V cDNA sequences were published in 1986 to 1987.[151–153] The mRNA is approximately 7 kb, whereas the cDNA sequence is 6,675 nucleotides long. Factor V is synthesized in the liver as a 2,240–amino acid protein and is secreted after preproprocessing as a 2,196 single-chain protein and circulates in plasma at a concentration of 20 nM.[154–156] In addition to plasma factor V, approximately 20% of the total factor V in whole blood is contained in the α-granules of platelets.[156] Although it was originally thought that megakaryocytes synthesize factor V,[157,158] studies in humans indicate that most of the platelet-derived factor V originates from plasma through an endocytotic mechanism.[159–162]

Factor V deficiency is a rare recessively inherited disorder associated with a mild-to-severe bleeding tendency caused by loss-of-function mutations in the factor V gene.[163] It was first described in the 1940s by Owren[164] in Norway. Its incidence is about 1:1,000,000 and there have been more than 200 cases described so far. Because factor V deficiency is so rare and the gene is large, relatively few patients have been characterized genetically, with over 50 mutations reported, including missense mutations, insertions, deletions, and splice-site mutations.

The factor V phenotype is quite variable and correlates poorly with plasma FV levels.[165] Patients with undetectable levels of FV (<1%) due to homozygous nonsense, frameshift, or missense mutations, exhibit the gamut of phenotypes from asymptomatic to severe bleeding. These observations are difficult to reconcile, as FV knockout mice display a severe phenotype where approximately 50% of the embryos do not survive past mid-gestation and the remaining die within hours after birth from excessive hemorrhage.[166] Two recent studies have provided some insight into the possible mechanism that may blunt the phenotype in FV deficiency. These studies show that these patients consistently have low levels of Tissue factor pathway inhibitor (TFPI) and small but detectable amounts of platelet FV which, when released from activated platelets, contributes to thrombin generation.[167,168]

In some cases, factor V deficiency is coinherited with factor VIII deficiency, the molecular basis for which is now clear.[169] Combined factor V and VIII deficiency is a distinct autosomal recessive disorder, and the affected patients demonstrate a moderate bleeding tendency. Approximately 50% of the families described to date are of Mediterranean origin, and the disorder is particularly prevalent among Jews of Middle Eastern and Sephardic origin.[170] Elegant positional cloning studies into combined factor V and VIII deficiency initially identified a candidate gene called *ERGIC-53* (now called *LMAN1*).[169,171,172] *LMAN1* is a mannose-binding type 1 transmembrane protein localized to the endoplasmic reticulum (ER)–Golgi intermediate compartment that likely functions as a molecular chaperone of a specific subset of secreted proteins, including coagulation factors V and VIII. DNA sequence analysis has identified over 30 different mutations in *LMAN1* spread across the entire gene. However, in a subset of patients, no mutation in *LMAN1* could be found, suggesting that mutations in another gene may also

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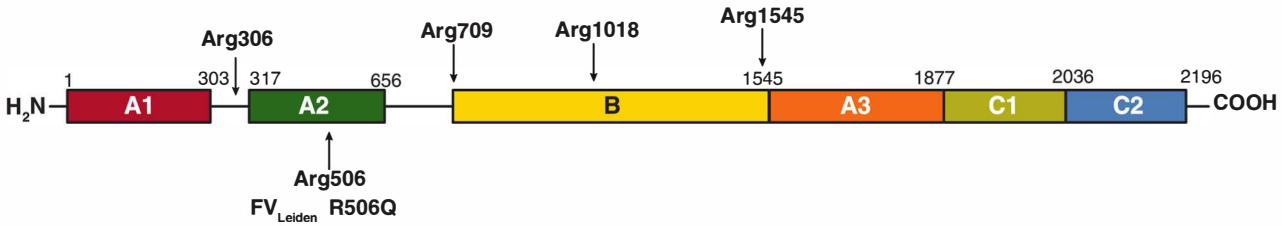

FIGURE 10.4 Illustration of the molecular defect called factor V_{Leiden}. Central in the figure is a schematic illustration of the domain structure of mature factor V as it circulates in plasma (A1-A2-B-A3-C1-C2). Factor V is activated by thrombin or factor Xa. The major cleavage sites for these activators are Arg709, Arg1018, and Arg1545. Activated factor V can be inactivated by (APC). Inactivation occurs by cleavage of a site (Arg306) between domains A1 and A2, at Arg506 within the A2 domain, and at Arg679 (not shown). In factor $V_{Leiden,}$ the codon for Arg506 at the A2 cleavage site is changed to Gln. This amino acid replacement results in loss of the A2 inactivation site and therefore in a factor Va molecule relatively resistant to APC. Mutations have also been identified at the Arg306 cleavage site: factor $V_{Cambridge}$ and factor $V_{Hong\ Kong}$.

be associated with combined factors V/VIII deficiency. This second gene and corresponding mutations causing factor V/VIII deficiency is termed *MCFD2* (multiple coagulation factor deficiency 2).[173] *MCFD2* is localized to the ER–Golgi intermediate compartment through a direct interaction with *LMAN1*. The phenotypes associated with mutations in *MCFD2* and *LMAN1* are indistinguishable and associated only with deficiency of factors V and VIII, although a selective delay in secretion of other proteins has been noted.[169]

The most common genetic defect described in factor V is a gain-of-function mutation. In 1993 Dahlbäck et al.[174] observed that plasma from individuals who experienced venous thrombotic episodes had a reduced response to activated protein C (APC), a condition that was termed *APC resistance*. The genetic basis of this abnormality is a G1691 to A mutation in the factor V gene, which results in a change of Arg506 to Gln at a critical APC inactivation cleavage site in factor Va (FIGURE 10.4).[175,176,177] The mutation was termed *factor* V_{Leiden} and is associated not with a bleeding diathesis but with an increased thrombotic risk. Factor Va_{Leiden} is less readily inactivated by APC, thereby enhancing the procoagulant capacities of factor V.[178–180] This genetic abnormality is prevalent in approximately 3% of whites, representing the most common genetic risk factor in venous thrombosis.[181] An historic sketch on the discovery of APC resistance has recently been published by Dahlbäck.[182]

Most individuals are heterozygous for factor V_{Leiden}, but homozygosity is also seen, and there also exists the so-called pseudohomozygosity for factor V_{Leiden},[183,184] in which one factor V allele carries the 506 mutation and the other harbors a loss-of-function mutation. Mutations at Arg306, another critical APC cleavage site, are also associated with APC resistance and thrombotic disease.[185,186]

Inhibitors of Coagulation

Protein C

Protein C is a vitamin K–dependent plasma serine protease zymogen that plays a central role in regulating blood coagulation as an anticoagulant (see Chapter 19). Thrombin in complex with thrombomodulin on endothelial cells is the major activator of protein C. APC exerts its anticoagulant function by inactivating, via limited proteolysis, factors Va and VIIIa.[182] Human protein C is encoded by a gene on chromosome 2q13–q14 and spans approximately 11 kb and contains nine exons.[187–189] The protein C gene encodes mRNA transcripts of 1.8 and 1.6 kb; the difference appears to result from alternative polyadenylation signals present in the transcript.[190] The cDNA is 1,386 nucleotide long and encodes a mature protein of 461 amino acids. Prior to secretion, a 42–amino acid preprosequence is removed as well as internal proteolytic cleavage, resulting in the secretion of a 419–amino acid two-chain protein (62 kDa) that circulates in plasma at a concentration of 60 nM.[190,191]

Loss-of-function mutations in the protein C gene lead to protein C deficiency, first described in 1981.[192] After this original report, many others confirmed that heterozygosity for protein C deficiency in a significant number of families with venous thrombosis, a disease expected to be related to low levels of protein C. In homozygotes, life-threatening thrombotic symptoms often start immediately after birth and take the form of purpura fulminans.[193,194] Inactivation of the protein C gene in mice leads to lethal perinatal consumptive coagulopathy and is inconsistent with even short-term survival.[195]

More than 300 different mutations have been described for the protein C gene. Each of these mutations is assumed (or has been proved) to lead to absent or defective protein C. Mutations in the protein C gene can be found at: http://www.itb.cnr.it/procmd/. The mutations range from missense mutations (some leading to variant proteins that have proven useful for structure–function analysis of plasma purified protein), promoter mutations, and RNA processing mutations to frameshift mutations.

The protein C database of mutations contains many multiple entries for certain mutations. For instance, the Arg230 to Cys mutation has been documented in 28 apparently unrelated families. Two factors are important when considering these multiple occurrences of mutations. The first is that they may result from a founder effect. In one example, the common origin of several families was traced back to the 18th century.[196] The second explanation for the recurrence of mutations is that they have arisen independently. On the basis of geographic considerations or haplotyping, this has been confirmed for several mutations.[196,197] These recurrent mutations typically occur at CpG dinucleotides. When one plots the relation between specific mutations and the geographic dispersal, the observed curve closely matches the curve that is expected on the basis of the likelihood of a given mutation to occur.[198] This analysis indicates that most multiple reports of mutations reflect recurrent mutation rather than founder effect.

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Thrombomodulin

Thrombomodulin is an endothelial cell surface glycoprotein (GP) receptor for thrombin. The thrombin–thrombomodulin complex initiates the protein C anticoagulant pathway by activating the zymogen protein C (see Chapter 19). Several groups have cloned and sequenced the thrombomodulin cDNA and gene.[199–201] The gene is 4.2 kb in length and is located on chromosome 20p12–cen and is unusual in that it does not contain any introns and has a high GC content. The mRNA is approximately 4 kb in length, whereas the cDNA is 1,728 bp long and encodes a protein of 575 amino acids. The mature protein is 559 amino acids in length following removal of the signal sequence. An informative historic sketch on the discovery of thrombomodulin has been recently published.[202]

Given its function in the protein C pathway, a hereditary deficiency of thrombomodulin is a strong candidate for pathophysiologic involvement in venous thrombosis. Mice genetically modified to inactivate or alter (missense mutation) the thrombomodulin gene show increased fibrin deposition and a prothrombotic state.[203–205] Some evidence from clinical studies supports a role for thrombomodulin in thrombotic disease. For example, in the Atherosclerosis Risk in Communities study, soluble thrombomodulin shows a strong, graded, inverse association with and is a good predictor of incident coronary artery disease.[206] Mutations in the thrombomodulin gene occur in patients with thromboembolic disease[207,208] or atypical hemolytic uremic syndrome,[209] several of which have been recently biochemically characterized.[210] The Ala455 to Val polymorphism in whites is not a risk factor for venous thrombosis[211,212]; however, it may be associated with cardiovascular risk in certain populations and may also be associated with varicose veins through linkage with promoter mutations.[213,214]

Protein S

Protein S is a vitamin K–dependent GP with anticoagulant effect in the coagulation pathway (see Chapter 19). Protein S circulates in plasma in two forms: free (~40%) and bound to C4b binding protein, a component of the complement system. Only free protein S has anticoagulant properties. Protein S acts as a cofactor for APC in the inactivation of the procoagulant factors Va and VIIIa, possibly by potentiating the binding of protein C to procoagulant phospholipid surfaces; however, the precise mechanism is not known, and the effects of protein S in modulating APC function *in vitro* tend to be minor.

The human genome contains two protein S genes, one of which is active (*PROS1*), and the other a pseudogene (*PROS2*).[215–217] The *PROS1* gene is approximately 80 kb in length and contains 15 exons and is located on chromosome 3 (3p11.1–q11.2).[218–235,236] The major mRNA species from *PROS1* is approximately 4 kb long, whereas the cDNA is 2,031 nucleotides in length.[218,219] The protein S precursor protein is 676 amino acids long, whereas mature protein S is a single-chain GP of 635 amino acids. The *PROS2* gene is a highly homologous pseudogene of *PROS1* that contains a variety of mutations, such as stop codons and deletions that prohibit proper translation. Moreover, no transcription product of the *PROS2* gene is identified.[220]

Mutations in the protein S gene lead to protein S deficiency, an autosomal dominant trait affecting up to 5% of families with congenital thrombophilia.[221] After the first cases of heterozygous protein S deficiency were described in 1984, numerous reports have appeared.[222–224] The prevalence in the general population is not known with great certainty. The clinical symptoms in heterozygous protein S deficiency are comparable to protein C deficiency and are relatively mild. Only a few cases of homozygous deficiency have been described, and these were associated with severe thrombotic disease and purpura fulminans immediately after birth.[225–228] Mice deficient in protein S (PROS1$^{-/-}$) die in utero from a fulminant coagulopathy and associated hemorrhages.[229,230]

The mutations of protein S deficiency are collated in the HGMD database, currently describing the findings in almost 200 patients. The spectrum of mutations is similar to that for hemophilia B and protein C deficiency, including 154 different point mutations and a translocation.[231] Only 14 of the mutations occur in CpG dinucleotides and are compatible with methylation-mediated deamination.[7] This differs from what was found in hemophilia B or protein C deficiency, in which approximately 30% of the point mutations occur in CpG dinucleotides.

Endothelial Cell Protein C receptor

The endothelial cell protein C receptor (EPCR), a cell-specific type 1 transmembrane protein that binds both protein C and APC, was identified in 1994.[232,233] The protein functions by augmenting protein C activation by the thrombin–thrombomodulin complex, primarily by reducing the apparent K_m for protein C.[234] A soluble form of EPCR (43 kDa) circulates at a concentration of approximately 2.5 nM.[235] The gene for EPCR spans about 6 kb of genomic DNA, contains four exons, and is localized to chromosome 20 (20q11.2).[236,237] The mRNA is approximately 1.4 kb, whereas the cDNA is 717 nucleotides in length and encodes a protein of 238 amino acids.[232] In adults, EPCR is localized primarily on endothelial cells of large blood vessels, but during development it is expressed at high levels on trophoblast giant cells.[238,239]

In view of the role EPCR plays in the protein C pathway, deficiencies of the protein might be expected to be thrombogenic. Although homozygous EPCR deficiency has not been described in humans, the EPCR knockout mouse exhibits placental thrombosis and an early embryonic (E10.5) lethal phenotype.

In 2001, the first mutation in the *EPCR* gene was reported, a 23-bp insertion in the *EPCR* gene in survivors of myocardial infarction and deep vein thrombosis.[240] Its role in thrombosis is difficult to assess because the allelic frequency is low.[241–244] Point mutations have also been described in the promoter region of the gene in four thrombophilic patients and also in the coding region (Arg96 to Cys), but the involvement of these mutations in gene regulation or functional activity could not be clearly demonstrated.[245,246] Elevated levels of soluble EPCR associated with the A3 haplotype (characterized by 13 polymorphisms) are associated with an increased risk of venous thrombosis,[247] but this is controversial, given the wide range of soluble EPCR in plasma of A3 carriers, with only the high levels being associated with venous thrombosis.[247–249] An additional SNP in the A3 haplotype has been associated with reduced risk of premature myocardial infarction, partly by increased levels of soluble EPCR.[250] Further studies are needed in order to evaluate the actual importance of the *EPCR* gene variations in thrombotic diseases.

Antithrombin III

Antithrombin is the major serpin family regulator of blood-clotting proteases in plasma (see Chapter 18). This anticoagulant protein downregulates blood clotting by inhibiting thrombin, factor Xa, and factor IXa, thereby forming a stable equimolar

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complex with the enzymes by an unusual mechanism that is shared by other serpin family protease inhibitors.[251] Inhibition of protease activity is greatly accelerated by the participation of the glycosaminoglycans heparin and heparin sulfate that induce conformational changes that enhance the serpin's reactivity with the enzymes.

The relative abundance (approximately 2.5 μM; 150 μg/mL) of antithrombin and its ease of purification from plasma facilitated the deduction of most of its primary structure by Edman degradation in 1979.[252] The first cDNA sequence was cloned between 1982 and 1983,[253–255] and 10 years later the full gene sequence was published.[256] The 13.5-kb gene is localized on chromosome 1q23–24 and consists of seven exons.[257–259] The mRNA is approximately 1.4 kb long, whereas the cDNA is 1,395 bp long. The deduced primary structure of the protein indicates a 32–amino acid signal sequence and a mature protein of 432 amino acids.

Because of its essential role as an anticoagulant, individuals with deficiencies of antithrombin are at increased risk for thrombosis. The prevalence of this condition in the general population is approximately 1:500.[260] The prevalence in patients with a history of thromboembolic disease is approximately 5%.[261] The first report of antithrombin deficiency was published in 1965,[262] and the first mutation was characterized in 1983.[263] The mutations (>250) are collated on a regular basis in a database of antithrombin mutations: (http://www1.imperial.ac.uk/departmentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/).

Tissue Factor Pathway Inhibitor

TFPI is a trivalent 34-kDa-proteinase inhibitor. The inhibitor, a Kunitz-type protein, plays an essential role in regulating coagulation through the inhibition of a quaternary complex of TFPI, factor VIIa, TF, and factor Xa. The first Kunitz domain (K1) of the TFPI protein is an *N*-terminal acidic region that inhibits factor VIIa complexed to TF. The second Kunitz domain (K2) inhibits factor Xa, and the third Kunitz domain (K3) contains the *C*-terminal basic region. An account of the discovery and isolation of TFPI has been published recently by Broze.[264]

The gene for TFPI is approximately 85 kb, located on chromosome 2q31–q32.1, and contains nine exons. Unlike deficiencies of other coagulation inhibitors (i.e., antithrombin, protein C, and protein S), severe deficiencies of TFPI have yet to be described. However, there may be a decreased threshold for thrombosis as suggested by TFPI resistance, which is defined as poor anticoagulant response to TFPI. This resistance was observed in 4.7% of controls and in 11.0% of patients with venous thrombosis.[265] Heterozygous TFPI knockout mice are phenotypically normal, despite lower levels of TFPI in a factor VIIa/TF inhibition assay.[266] In homozygous TFPI knockout mice, embryonic lethality with signs of yolk sac hemorrhage was seen in 60% of the litter between E9.5 and E11.5.[266] Past E11.5, CNS and tail hemorrhages were present, and none of the knockout mice survived to the neonatal period.

To date, five polymorphisms have been described in the TFPI gene, two in the promoter, two in the coding region, and one in intron 7.[267–270] Several of these have been assessed for their effect on the risk of venous thrombosis, with conflicting results. The Pro15 to Leu polymorphism was first reported to be associated with venous thrombosis,[267,271] but this was not confirmed by subsequent studies.[272,273] There was no association found between the risk of venous thrombosis or coronary heart disease with either the Val264 to Met or the C-399T and T-287C promoter polymorphisms.[267,274] The T-33C polymorphism in intron 7 appears to be an independent risk factor for venous thrombosis but larger independent studies are needed for verification.[275]

Heparin Cofactor II

Heparin cofactor II (HCII), a plasma serine protein inhibitor (serpin), specifically binds and inactivates thrombin in the presence of heparin, heparan sulfate, and dermatan sulfate (see Chapter 18), inhibiting the rate of thrombin activity by 1,000-fold.[276] HCII is highly homologous with antithrombin III, with the notable exception that the equivalent of Arg393 in antithrombin III is a Leu. Its stimulation by dermatan sulfate is unique among the serpins. A full-length cDNA for HCII was isolated and sequenced in 1988,[277] and the complete sequence of the gene, which has five exons and is nearly 13.6 kb in length has been localized to chromosome 22q11.21.[278]

HCII is synthesized in the liver and circulates at a concentration of approximately 1 μM.[279] During the third trimester, HCII levels increase to 150%[280] and thrombin-HCII levels increase fourfold,[281] whereas a 50% decrease in the HCII levels occurs in women with preeclampsia.[282]

Inherited deficiency of HCII has been documented in at least 15 families.[283] Heterozygous deficiency may predispose patients to both arterial and venous thromboembolic disorders, but solid data to support these claims still are lacking.[284] Several mutations in the HCII gene have been described and characterized, three of which are missense mutations and two are frameshifts, one an insertion and the other a deletion. Mice deficient in HCII are viable and fertile without any gross abnormalities, and are born at the expected Mendelian frequency.[285] However, a photochemical model of induced endothelial cell injury suggests that HCII may inhibit arterial thrombosis.

Contact Activation Factors

Factor XII

Factor XII (Hageman factor) is an activator both of the coagulation system and the kinin system. It is synthesized in the liver and circulates as an inactive zymogen. During contact activation, factor XII is proteolytically cleaved at several sites by plasma kallikrein to yield enzymatically active factor XIIa. Factor XIIa is capable of activating prekallikrein and factor XI and is therefore the first component of the contact activation system,[286] but it does not undergo autoactivation.

The amino acid sequence of factor XII was determined between 1983 and 1985,[287,288] and factor XII cDNA was cloned in 1985.[289] The mRNA is 2,048 nucleotides long and encodes a protein of 615 amino acids, and the gene is 12 kb long with 14 exons and is located on chromosome 5q33–qter.[290,291] The promoter of factor XII is TATA-less and contains a functional estrogen response element modulated by HNF-4 in an estrogen-dependent manner.[292]

Factor XII deficiency was first described by Ratnoff and Colopy in 1955 in a patient named John Hageman, who had a prolonged clotting time but no hemorrhagic symptoms.[286] The markedly prolonged activated partial thromboplastin time (aPTT) (>150 seconds) corrects to normal with a 1:1 mix with normal plasma. There is no recognized bleeding tendency or

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clinical consequences,[293–296] and in fact, Mr. Hageman died of pulmonary embolism.

To date, there are over 200 reported cases of factor XII deficiency,[297] with over 20 mutations in the factor XII gene, most of which are missense. The first patient in whom a specific point mutation was identified had a Cys571 to Ser change,[293] affecting the disulfide bond that stabilizes the active center Ser in serine proteases and likely responsible for the moderately severe decrease of factor XII activity (3% level). In one large study of 31 unrelated patients from Austria, Germany, and Switzerland, several novel mutations were found, including missense mutations that directly or indirectly affected the catalytic triad of factor XII, two of which caused shifts in the reading frame of the gene.[295]

In the factor XII 5′ untranslated region, a common C-to-T transition associated with lower levels of factor XII has been proposed to play a protective role against coronary disease as well as a role in responsiveness to therapy.[298,299] In some, but not all, studies reported, low levels of factor XII coagulant activity have been associated with recurrent abortion in up to 14% of women.[300]

Prekallikrein

Plasma prekallikrein (KLKB1, previously known as KLK3) is the zymogen of a serine protease and is activated by factor XIIa. The active enzyme, kallikrein, shows proteolytic activity on a number of substrates, of which, factor XII, high molecular weight (HMW) kininogen, and urokinase are probably of physiologic importance.[301,302] Prekallikrein circulates as a complex with HMW kininogen (HK) at a concentration of about 40 μg/mL. Prolylcarboxypeptidase is a physiologic endothelial cell activator of plasma prekallikrein.[303]

Both amino acid sequence analysis and cDNA cloning have been used to determine the primary structure of prekallikrein.[304] The mRNA is approximately 2.2 kb long and encodes a protein of 638 amino acids.[304] The human prekallikrein gene is approximately 31 kb long, contains 15 exons, and is located on chromosome 4q34–35, not far from the factor XI gene.[305]

Several kindred with plasma prekallikrein deficiency (Fletcher factor deficiency) have been reported[297,306–309] with no detectable prekallikrein activity. Despite this, affected individuals have no bleeding tendency or any other clinical problems. In 2003, the first reports on the genetic basis for prekallikrein deficiency were reported in two separate families[310,311] and to date six missense mutations have been reported.

High Molecular Weight Kininogen

The plasma kininogens are the substrate for kallikrein. HK and LMW kininogen (LK) are two related plasma proteins that derive from the same gene on chromosome 3q26–qter through alternative splicing.[312–315] Normal, mature, secreted, full-length HK is a 626–amino acid protein.

Deficiency of HK is a rare disorder lacking a bleeding or thrombotic phenotype despite a prolonged aPTT >150 seconds. There are approximately 35 reported cases of HK deficiency.[297] One of the originally described patients who was deficient in kininogen with the associated Williams trait was homozygous for a C-to-T transition at nucleotide 586, resulting in an Arg-to-stop mutation in the fifth exon.[316] Fitzgerald trait has a nonsense mutation at amino acid 502 with no functional HK activity and 40% LK antigen.[317]

In a 6-year-old boy who had extensive left vertebral basilar artery thrombosis and a left vertebral artery dissection subsequent to a traumatic injury, a frameshift mutation of a single base pair deletion at amino acid 480 of the mature protein was associated with absent plasma HK because of degeneration into a stop codon downstream.[318]

Fibrinolytic Factors

Tissue-type Plasminogen Activator

Once a clot has been formed, the fibrinolytic system comes into play to dissolve the clot in a regulated fashion. Like the coagulation system, the fibrinolytic system is a network of reactions in which zymogens are proteolytically cleaved to form active enzymes. The final step in the cascade of reactions is the activation of plasminogen to plasmin, which is the principal degrading enzyme of insoluble fibrin.

Tissue-type plasminogen activator (tPA) is a plasma serine protease that mediates plasmin generation and clot lysis by specifically cleaving and activating clot-bound plasminogen.[319] Endothelial-derived tPA is the main endothelial cell–derived blood activator of the fibrinolytic system, modulates cerebrovascular tone, and may play a part in the hemodynamic changes occurring in brain trauma and ischemia.[320–322] The tPA gene is relatively large (i.e., 32.7 kb), contains 14 exons, and is located on chromosome 8 (p12–p11).[323–326] The *tPA* mRNA was isolated in 1983 using a human Bowes melanoma cell line[327] encoding a mature protein of 527 amino acids with a molecular mass of 68 kDa. Endothelial cells secrete tPA primarily as a single-chain GP, but it is converted to the two-chain form by plasmin-mediated cleavage at Arg275.[319] In normal plasma, the antigen concentration of tPA is approximately 0.005 μg/mL (i.e., 70 pM), and most of it in complex with PAI-1.[328,329] A tPA knockout mouse developed normally and did not exhibit any macroscopic abnormality,[330] although microscopic examination revealed mild glomerulonephritis. An informative historic sketch on discovery of tPA has recently been published.[331]

Of the numerous nucleotide sequence differences that have been identified within the *tPA* gene, the most studied is a 311-bp Alu insertion/deletion in intron 8.[332] In one study, the presence of one insertion was associated with an increased risk of myocardial infarction, whereas homozygous carriers had a twofold adjusted risk.[333] These findings, however, could not be confirmed in other investigations, calling into question the role of this variation in arterial disease.[334] Moreover, the risk of acute myocardial infarction and the Alu repeat appears to be dependent on the population studied,[333,335,336] suggesting that additional genetic factors may influence such risk.

Evidence exists that tPA levels can predict cardiovascular disease in apparently healthy individuals.[337] In a search for genetic variations that may effect tPA protein production, eight polymorphisms were identified.[338] One of these polymorphisms located at position –7,351 within the enhancer region of the *tPA* gene was identified and was strongly correlated with endothelial tPA release rates.[338] This polymorphism is within a Sp1 binding site, and the T allele inhibits Sp1 binding and was associated with <50% of the tPA release observed compared to the wild-type C allele. This polymorphism has a strong association with first myocardial infarction and lacunar stroke.[339]

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Plasminogen

The terminal event in the activation of the fibrinolytic system is the generation of the serine protease plasmin, which has a variety of functional properties, the most notable being dissolution of fibrin clots.[319] The precursor of plasmin is plasminogen, a single chain GP of 92 kDa that circulates in plasma at a concentration of 200 μg/mL (i.e., 2 μM). The primary amino acid sequence of plasminogen was reported in 1978.[340] The plasminogen gene encodes an mRNA transcript of approximately 2.9 kb, whereas the cDNA is 2,433 bp in length and encodes a 791–amino acid mature protein with a 19–amino acid signal sequence.[341,342] The gene for plasminogen is localized on chromosome 6q26–q27 in proximity to two genes for apolipoprotein A.[343,344] The gene spans 53.5 kb and contains 19 exons, making it the largest gene among the constituents of the fibrinolytic system.[345] Mice deficient in plasminogen, in addition to developing thrombotic complications, also develop fibrin-rich conjunctival lesions that appear indistinguishable from human ligneous conjunctivitis.[346–348]

Plasminogen deficiency is classified into two types: type I or hypoplasminogenemia (i.e., antigen and activity reduced) and type II or dysplasminogenemia (i.e., normal antigen, dysfunctional protein). The first case of hereditary heterozygous deficiency of plasminogen (type II) linked to thrombophilia was reported by Aoki et al.[349] in 1978, and it is estimated that plasminogen deficiency occurs with a prevalence of approximately 0.5% in the general population and 1% to 3% in thrombosis patients.[350–352] Despite this frequency, there does not appear to be a clear association between plasminogen deficiency and an individual thrombotic risk.[353] Although initial reports suggested that type II plasminogen deficiency in combination with factor V_{Leiden} is a risk of thrombosis, further studies failed to confirm that.[353]

Severe deficiency of plasminogen leads to occlusive hydrocephalus and congenital ligneous conjunctivitis, a rare hereditary disorder characterized by the formation of proliferative pseudomembranes on the conjunctiva and other mucosal membranes.[354–356] The abnormalities in these patients reflect the important role of plasminogen in wound healing and are due to homozygous loss-of-function mutations in the plasminogen gene, such as Arg216 to His or Trp597 to Stop. None of the homozygous or heterozygous individuals developed spontaneous thrombosis, but one of the children had repeated thrombotic occlusions of an implanted catheter.

Plasmin Inhibitor

Within the circulation, rapid inhibition of free plasmin occurs with plasmin inhibitor (PI), which is a serine protease inhibitor (serpin) and which was formally called α_2-plasmin *inhibitor* or α_2-antiplasmin.[357] PI is the primary inhibitor of plasmin and is a single-chain GP with an M_r of 70,000 and circulates in plasma at a concentration of 70 μg/mL (1 μM).[358] Partial and full-length cDNA clones of PI were isolated between 1986 and 1987. The full-length mRNA is approximately 2.3 kb long, encoding a mature inhibitor protein of 464 amino acids (Met-form).[359–361] There is a second form of the inhibitor in plasma that is 12 amino acid shorter (Asn-form),[362] a result of cleavage by anti*p*lasmin *c*leaving *e*nzyme (APCE).[363] The PI gene contains 10 exons and is distributed over approximately 16 kb of DNA and is localized on chromosome 17p13.[364,365]

PI deficiency is a rare autosomal recessive disorder and is characterized by a bleeding tendency, with homozygous patients being more affected.[366] Type I deficiency is defined as the absence or decreased concentrations of the protein, where type II is characterized by a dysfunctional protein. The first deficiency states were reported in 1978, and since then more than 40 cases have been reported.[366] In contrast to patients with a severe deficiency of PI who have mild-to-severe hemorrhagic tendencies, mice that are deficient in PI have a transient enhanced endogenous fibrinolytic activity and do not have an overt bleeding tendency.[367]

There are nine published reports detailing the molecular basis of PI deficiency: (a) a trinucleotide insertion responsible for an additional Ala within the 353 to 357 fragment,[395] (b) a frameshift mutation in exon 10 that is responsible for elongation of the protein,[368] (c) a trinucleotide deletion responsible for a Glu137 deletion,[369] (d) a G-to-A transition responsible for a Val384 to Met substitution,[370] (e) a splicing donor site suppression in intron 2,[371] (f) a single T deletion at nucleotide position 332 in exon 5 that causes a frameshift and shortening of the protein,[372] (g) a splicing donor site mutation in intron 6 that leads to exon 6 skipping,[373] (h) a deletion in codon 71,[374] and (i) cytosine deletion at codon 399.[375] In addition, a polymorphism (Arg6 to Trp) appears to be responsible for enhancing fibrinolysis.[376]

Plasminogen Activator Inhibitor 1

PAI-1, the principal inhibitor of fibrinolysis, is a 50-kDa serpin (serine protease inhibitor) that interacts with and inhibits both tPA and urokinase plasminogen activator. The source of plasma PAI-1 is unclear since endothelial cells, adipocytes, hepatocytes, and megakaryocytes can all secrete it into the circulation.[377] Cofactors for PAI-1 activity include heparin, the presence of which increases PAI-1 activity toward thrombin, and vitronectin, a stabilizing factor. PAI-1 levels show diurnal variations, with a peak level in the early morning, a finding that appears to be associated with the 4G/5G polymorphism.[378]

The *PAI-1* gene is located on chromosome 7q21.3–q22, covers approximately 12.3 kb, and consists of nine exons and eight introns.[379–384] The *PAI-1* gene encodes two distinct species of mRNA approximately 2.3 and 3.4 kb in size. The cDNA of PAI-1 has been cloned by seven different groups and is 1,209 bp in length.[385–389] The promoter of PAI-1 contains a TATA box, lacks a CAAT box, and contains numerous regulatory sequences including two enhancer-like glucocorticoid response elements and nucleotides.[390] Studies have shown a correlation with a functional 4G or 5G genotype in the PAI-1 promoter. Both the 4G and 5G alleles GACACGTG($G_{4\ or\ 5}$) AGT (4G/5G) bind a transcriptional activator at –675 in the PAI-1 5′ untranslated region. However, the 5G allele also binds a repressor protein in an overlapping binding site, resulting in lower levels in the 5G than in the 4G allele. Numerous correlations have shown a significantly higher prevalence of particular cardiovascular disease states with the 4G allele.[391–394]. PAI-1 deficiency has also been documented by a frameshift mutation leading to a premature stop codon[395] or, more recently, in a compound heterozygote patient with a Ala15 to Thr mutation in the signal peptide, resulting in 10% normal activity.[396]

PAI-$1^{-/-}$ knockout mice are viable and show no apparent histologic or hemostatic abnormalities. Challenge with endotoxins in the knockout mice induces a greater resistance to venous thrombosis and a mild hyperfibrinolytic state.[397,398] Other physical challenges in this knockout mouse show tumor growth, angiogenesis, and increased responses to injury.

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Thrombin-activatable Fibrinolysis Inhibitor

Thrombin-activatable fibrinolysis inhibitor (TAFI), also known as procarboxypeptidase B and procarboxypeptidase U, is a zymogen found in plasma that may play an important role in hemostasis by linking both coagulation and fibrinolysis.[399] Activated TAFI, when exposed to a fibrin clot, catalyzes the removal of *C*-terminal lysines, thereby diminishing the cofactor activity for plasminogen activation. This in turn reduces plasmin production on fibrin, slowing clot lysis or prolonging fibrinolysis.[399] This inhibitor has been characterized by three independent laboratories, and was cloned in 1991.[400–402] The TAFI mRNA is approximately 1.7 kb in length, whereas the cDNA is 1,272 nucleotides long. The human *TAFI* gene was characterized in 1999, is located on chromosome 13q14.11, and consists of 11 exons spanning approximately 48 kb of DNA.[403] TAFI is synthesized in the liver as a prepropeptide of 423 amino acids, which includes a 22–amino acid signal sequence. It has a molecular mass of 60 kDa and circulates in plasma at a concentration of approximately 70 nM.[404]

Making use of a large population-based case–control study on venous thrombosis, the Leiden Thrombophilia Study found that an elevated TAFI antigen level was only a mild risk factor for venous thrombosis.[405] In addition, an elevated TAFI level was a mild risk factor for coronary artery disease and ischemic stroke.[406,407] In contrast, however, two studies have reported that high TAFI antigen levels are associated with a lower risk of coronary events.[408,409] Although no reports have yet described individuals with TAFI deficiency, the phenotype is likely to be mild because TAFI knockout mice do not exhibit any abnormal phenotypes, even in arterial and venous injury models.[410]

In addition to several polymorphisms in the promoter and coding sequence, another SNP is associated with reduced TAFI levels, a C to T change at nucleotide position 1,040 resulting in a Thr325 to Ile substitution.[411,412] However, biochemical characterization revealed that this particular mutation increases the thermal stability of the protein, resulting in enhanced activity of activated TAFI and consequently in an increased antifibrinolytic potential.[412,413] At present, it is unclear whether this polymorphism influences thrombotic disease, although it is a contributing factor in hereditary thrombophilia.[414]

Other Hemostatic Proteins

Fibrinogen

The final reaction in the coagulation cascade is the formation of an insoluble fibrin network from fibrinogen. Fibrinogen is a complex molecule, a 340-kDa GP that consists of two disulfide-linked monomers (see Chapter 16). Each of the monomers consists of three polypeptide chains encoded by three distinct genes: Aα, Bβ, and γ.[415] The fibrinogen to fibrin transformation is initiated after thrombin proteolytically removes two acidic peptides, fibrinopeptide A and fibrinopeptide B, from the *N*-terminus of the Aα and Bβ chains, respectively. Removal of these peptides exposes sites (often denoted A and B knobs) on the fibrinogen molecule that favor intermolecular reactions between fibrin dimers and therefore allows polymerization and formation of a fibrin network.[415]

The cDNAs encoding the Aα, Bβ, and γ chains were cloned in 1993.[416–419] The mRNA for the Aα chain is approximately 2.2 kb nucleotides long and encodes a 625–amino acid long mature protein.[416,419] The Aα gene also has been cloned and is approximately 5.4 kb and contains five exons,[419] and there is also a variant Aα form in plasma (<2%) that is extended by 236 amino acids and that is encoded by a sixth exon. The Bβ mRNA counts 1,918 nucleotides and encodes a protein of 461 amino acids. The Bβ gene is approximately 8 kb and contains eight exons.[418] The fibrinogen γ gene is approximately 8.4 kb long and contains 10 exons. The gene encodes two slightly different forms of the γ chain: γA and γB.[416] The most abundant form is γA, which is produced from mRNA and spliced at nucleotide 7,774 in exon 9. The γB mRNA is not spliced after exon 9, leading to read-through at the donor splice site and the addition of 20 codons before a stop codon is reached. Exon 10 is not used, and polyadenylation occurs at a different site. The genes encoding the fibrinogen chains cover approximately 50 kb and are located on chromosome 4q28.[420–422] The genes in the fibrinogen locus are linked in the order $\gamma \rightarrow$ A$\alpha \leftarrow$ Bβ.[444] The direction of transcription of the Bβ gene is therefore reversed compared to the direction of transcription of the other two genes. Transcription of the three genes occurs in a coordinated fashion, in which transcription from the Bβ gene is the rate-limiting step.[423]

A knockout model shows that homozygous mice deficient in fibrinogen (Aα chain–deficient) are born at the expected Mendelian frequency,[424] but, approximately 30% manifest overt bleeding in the peritoneal cavity, skin, and soft tissues around joints in the neonatal period. Long-term survival in the mice deficient in fibrinogen varies and is highly dependent on genetic background; in pregnancy, mice show fatal uterine bleeding around the 10th day of gestation.

Afibrinogenemia, first described in 1920, is a rare autosomal recessive disorder associated with spontaneous, postsurgical, and postpartum bleeding of variable severity.[76] It is characterized by a severe deficiency of fibrinogen both in the plasma and the platelet. More than 30 afibrinogenemia-causing mutations have been reported, most in the Aα with just a few in the Bβ gene.

Dysfibrinogenemia is characterized by a variant form of fibrinogen, mostly because of a missense mutation that replaces an amino acid.[425,426] Most patients of dysfibrinogenemia present themselves in heterozygous form, with abnormal fibrinogen subunits covalently linked to normal ones, leading to an overall abnormal fibrinogen and fibrin. A large number of different mutations have been documented (>500) in dysfibrinogenemia, and they are collected and presented on an electronic database: http://site.geht.org/site/Pratiques-Professionnelles/Base-de-donnees-Fibrinogene/Database-English-Version/Fibrinogen-variants-Database-_79_.html.[427]

Homozygotes for dysfibrinogenemia may have severe bleeding, but this is not always the case. Most bleeding episodes are mild to moderate and may occur postoperatively, postpartum, antepartum, and as a result of delayed wound healing. Thrombotic problems include deep venous thrombosis, thrombophlebitis, pulmonary embolism, arterial thrombosis, and joint arterial and venous thrombosis. In patients with a history of venous thrombosis, the prevalence of dysfibrinogenemia is approximately 0.8%.[428] Patients who experience a thrombotic event may also have a history of bleeding (27%).

Elevated fibrinogen levels are an important risk factor for cardiovascular disease.[429–433,434] The best studied genetic determinants are two promoter polymorphisms in the fibrinogen Bβ gene, a G/A site at nucleotide −455 and a C/T variant site at −148. The two variant sites are in complete linkage equilibrium.[429] Individuals homozygous for the −455A allele have 0.28 g/L higher fibrinogen levels than GG homozygotes.

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Additional SNPs that are associated with circulating fibrinogen levels have been identified by genome-wide association studies that reside outside the fibrinogen genes.[430] Extrapolating from several studies on polymorphic fibrinogen gene variations, it is suggested that qualitative and quantitative modifications may be equally or more important in determining the risk of venous thromboembolism and cardiovascular disease, respectively.[431]

Factor XIII

Activated factor XIII is the final component of the blood clotting cascade and is responsible for cross-linking fibrin (see Chapter 17). Plasma factor XIII is a tetrameric molecule composed of two A subunits (83 kDa) and two B subunits (80 kDa), and platelet factor XIII consists only of two A subunits.[432] The factor XIII-A subunits belong to the transglutaminase family of proteins. Thrombin cleaves a peptide bond within the A chain, forming factor XIIIa, which stabilizes the fibrin clot. This is accomplished by a transglutaminase reaction forming covalent bonds between Lys and Gln side chains near the carboxyl-termini of the $\gamma\gamma$ chains (within strands) and between $\gamma\alpha$ chains (within the mesh). The factor XIII-A subunit gene has been localized to chromosome 6p24–25 (F13A) and is approximately 160 kb and contains 15 exons.[433,434] The mature A subunit has 731 amino acids. The factor XIII B subunit gene (F13B) is approximately 28 kb in length, has 12 exons, and is located on chromosome 1q31–32.1.[435,436] The mature protein has 641 amino acids and contains 10 short consensus repeats known as *sushi* domains.[437]

Factor XIII deficiency is a rare autosomal disorder that is associated with a severe bleeding diathesis when factor XIII levels are <1%. It is subdivided in type I and II for FXIII B or A deficiency, respectively.[438] Women with inherited factor XIII deficiency have recurrent abortions and difficulty carrying a pregnancy to term. The inheritance of this disorder is not completely recessive because increased bleeding has been reported for some heterozygous individuals, but most heterozygotes are asymptomatic.

The role of factor XIII during pregnancy and placental/uterine hemostasis was demonstrated with factor XIII A knockout mice.[439,440] Despite being able to become pregnant, knockout mice with factor XIII A frequently died of excessive vaginal bleeding with massive placental hemorrhage and subsequent necrosis at gestational day 10. This phenotype correlates with spontaneous miscarriage in humans with factor XIII deficiency.

More than 70 mutations have been identified in factor XIII deficiency, most of which affect the A gene, most of which being missense or nonsense. A mutation database can be found at: http://www.f13-database.de/. Polymorphisms of both the A and B subunit genes should prove useful in distinguishing A chain from B chain defects in family studies.[432] A common mutation in the factor XIII A subunit has been implicated in some, but not all, studies as a risk factor for vascular disease. This Val34 to Leu dimorphism, which is three amino acids from the activation site of factor XIII, is protective against myocardial infarction and predisposes to intracerebral hemorrhage,[441,442] especially in homozygote form.[443] The contribution of this mutation in coronary heart disease is controversial.[438]

von Willebrand Factor

vWF is a large multimeric adhesive GP that is involved in adhesion of platelets to the subendothelium at sites of vascular damage (see Chapter 13). vWF also plays a critical role in stabilizing the procoagulant cofactor, factor VIII. In response to numerous stimuli, vWF is released from platelet α-granules and from Weibel-Palade bodies of endothelial cells, and is constitutively secreted by endothelial cells and possibly produced in placental syncytiotrophoblasts.[444] In plasma, vWF circulates at concentrations of approximately 10 mg/mL. The vWF protein (monomer, 250 kDa) exists as a series of multimers that are linked by disulfide bonds located near the *C*-terminal end of each subunit (see FIGURE 10.5).

The *vWF* gene, located on the short arm of chromosome 12 (12p13.3), is approximately 178 kb in length and contains 52 exons.[445,446] A partial, highly homologous pseudogene is present on chromosome 22q11–13 and is approximately 21 to 29 kb in length.[446] This pseudogene has no known function, but its presence complicates genetic analysis of von Willebrand disease (vWD). Several groups originally cloned the cDNA for vWF

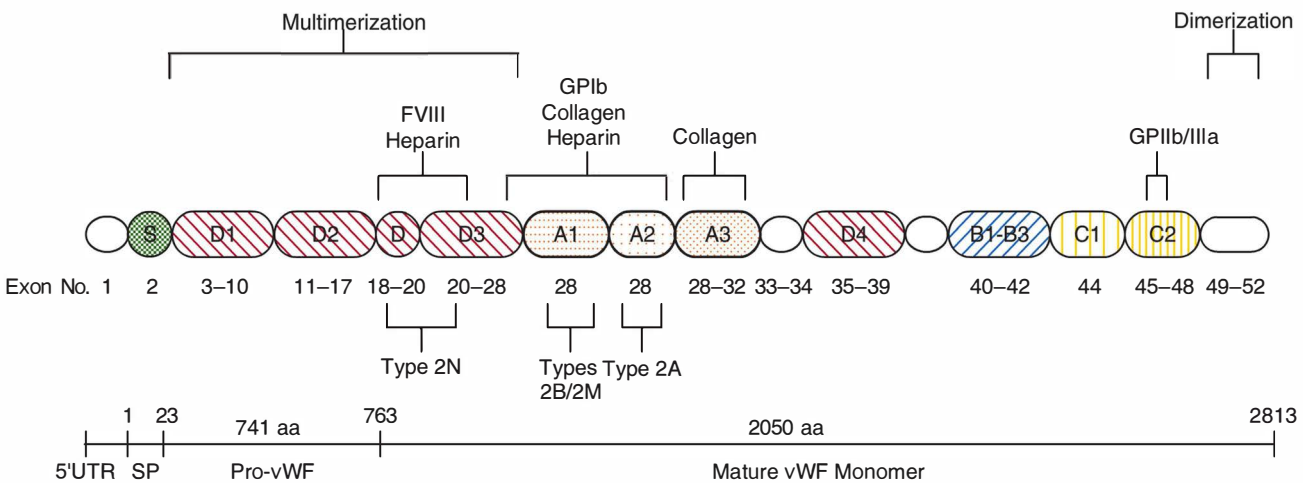

FIGURE 10.5 Gene organization of vWF. The 52 exons of the *vWF* gene are shown along with the indicated protein domain regions. Potential functional binding sites with other proteins are also shown. Type II mutation hot spots between exons 18 and 28 are indicated. GP, glycoprotein. See also Chapter 13.

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in 1985 to 1986,[447–450] known to be almost 9,000 bp long and encoding a protein of 2,813 amino acids.

vWD is a congenital bleeding disorder caused by defects at the vWF locus. This autosomally inherited mucocutaneous bleeding disorder was first reported by Erik von Willebrand in 1926 in a large family from the Aland Islands off the coast of Finland. On the basis of phenotypic and genetic characteristics, an updated classification of vWD has been adopted.[20] Type 1 deficiency includes patients with a partial quantitative deficiency of vWF levels in plasma. Type 2 deficiency refers to a qualitative deficiency and there are four subtypes (2A, 2B, 2M, and 2N). Type 3 vWD comprises patients with extremely low or undetectable levels of vWF. Symptomatology of vWF deficiency includes easy bruising, nosebleeds, and hematomas. Excessive menstrual bleeding and heavy bleeding following oral cavity, tooth extraction, tonsillectomy, and adenoidectomy are also common. A mouse model of vWF has been described, and these mice very closely mimic severe (type 3) human vWD.[451] Mutations and polymorphisms identified in the *vWF* gene can be found at: http://www.shef.ac.uk/vwf/index.html.

The inheritance of vWD varies with the type of deficiency. Type 2 deficiency accounts for approximately 75% of all vWD.[452] Heritable variances are modest and not solely linked to the vWF locus. Notably, variation in vWF is highly linked to an individual's ABO blood type. In non–type 3 disease, vWF levels increase during pregnancy and during stress, such as at the time of drawing blood. Individuals with blood type O have vWF levels 25% to 35% lower than individuals of blood types A, B, or AB. Variations are influenced by polymorphisms including a −1793G > C SNP. vWD is viewed as the most frequent bleeding disorder, with an estimated prevalence of 1% in the general population. The frequency of vWD is highly dependent on the criteria used in establishing the diagnosis. Experts differ on the definition of vWF type 1 deficiency from <15% levels to levels below 50%.[452]

Type 1 and types 2A, 2B, and 2M variants are most commonly dominant. Type 2A is the most common subclass and refers to qualitative variants with decreased platelet-dependent function associated with absence of HMW forms of vWF multimers; type 2B mutations increase the affinity of vWF for platelet GP Ib; type 2M refers to variants that share decreased platelet-dependent function that is not the result of the absence of HMW forms; and finally, type 2N refers to those vWF variants with a decreased affinity for factor VIII (FIGURE 10.5).

Type 3 deficiency is the most severe form of vWD and inherits as a recessive trait. This form is caused by homozygosity for complete loss-of-function mutations in the *vWF* gene. As is the case in hemophilia A and B, an almost infinite number of possible mutations lead to this phenotype. This fact, combined with the large size of the *vWF* gene, underlines the difficulty in routinely identifying mutations in type 3 deficiency.

SUMMARY

The completion of the Human Genome Project in 2003 has made gene and cDNA sequences immediately available, thereby providing a powerful means of discovering hereditary factors in multifactorial/multigenic diseases such as thrombosis. However, it is not genes alone but the interplay of genetic and environmental factors that determines phenotype. A challenge for the future will be to determine which combinations of genetic changes directly or indirectly influence complex disease states, and genome sequences from other animals may also prove useful in defining informative genetic changes. These sequences should also prove useful in furthering our understanding of the evolution of blood coagulation. The availability of genetic information for the hemostatic proteins has also made possible the safe and efficient production of pharmacologically relevant proteins for therapeutic purposes and provides new methods for the treatment of diseases using gene- and cell-based therapy approaches.

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CHAPTER

11 Tissue Factor and the Initiation and Regulation (TFPI) of Coagulation

JAMES H. MORRISSEY AND GEORGE J. BROZE, JR.

Blood circulates as a liquid, but once it escapes from an injured blood vessel, it must rapidly gel, or clot, to form a hemostatic plug in order to limit further blood loss. On the other hand, pathologic activation of the blood clotting system within the lumen of blood vessels is a major cause of human death and disability. This chapter focuses on the mechanism of initiation of the blood clotting system, both in normal hemostasis and in many thrombotic diseases. Two main pathways for triggering the blood clotting cascade were initially described: the intrinsic pathway and the extrinsic, or tissue factor (TF), pathway. The intrinsic pathway is so called because it appeared to be an intrinsic property of plasma; that is, when blood or plasma is placed in a glass tube, it will clot spontaneously. This pathway is more accurately called the *contact pathway* because it is activated when plasma contacts artificial surfaces like glass or clay. The contact pathway *per se* is not thought to be of primary importance in normal hemostasis because individuals in whom it is inactive (owing to a severe congenital deficiency in factor XII) exhibit no bleeding tendency. The contact pathway is considered in detail in Chapter 15B.

The TF pathway of blood clotting is triggered when plasma comes into contact with cells that express the integral membrane protein, TF, on their surfaces. TF is abundant on a variety of cell types found outside the vasculature but is present only in tiny amounts within the vasculature. After vascular injury, blood is exposed to cells bearing TF, whereupon TF binds factor VII—one of the soluble clotting factors—with high affinity. It is the complex of TF and factor VII (or, more accurately, the activated form known as *factor VIIa*) that initiates the blood clotting cascade in normal hemostasis. In addition, TF triggers the clotting cascade in a variety of thrombotic disorders.

OVERVIEW OF THE INITIATION OF BLOOD COAGULATION BY TISSUE FACTOR AND FACTOR VII

The clotting cascade comprises a series of reactions involving the activation of zymogens (inert precursors of enzymes) by limited proteolysis. With the exception of thrombin, the newly generated proteases have low enzymatic activity until they bind to specific protein cofactors on suitable phospholipid (PL) surfaces. Such binding events potentiate the activity of clotting proteases by as much as several 100- to 1,000-fold.

The first enzyme in the clotting cascade consists of two subunits: a serine protease, factor VIIa (the catalytic subunit), and a protein cofactor, TF (the positively acting regulatory subunit). Because TF is an integral membrane protein, the complex of TF and factor VIIa (TF:VIIa) is tethered to the cell surface. Free factor VIIa is a weak enzyme, but TF:VIIa is the most potent known activator of the blood clotting cascade.

All the serine proteases of the blood clotting cascade, including factor VII, circulate in the plasma as inert zymogens. Furthermore, most protein cofactors of the clotting cascade circulate as inert procofactors. (TF is a notable exception; it does not require proteolysis for activity.) A nagging question for many years has been: How can the clotting cascade be initiated through the assembly of a collection of inert precursors? The answer appears to come from the fact that trace amounts of active factor VIIa are present in the circulation at all times.

The active forms of most serine proteases of the clotting system have extremely short plasma half-lives (measured in seconds) because plasma contains high concentrations of protease inhibitors. Free factor VIIa, however, is not reactive with plasma protease inhibitors.[1] When injected into animals or humans, factor VIIa has a long circulating half-life of approximately 2 hours, similar to the approximately 5-hour half-life of zymogen factor VII.[2] Therefore, any factor VIIa that is generated *in vivo* can circulate for considerable periods.

TF binds factor VII or VIIa with high affinity, resulting in a 1:1 complex of the two proteins on the cell surface. As depicted in FIGURE 11.1, once bound to TF, factor VII is rapidly converted to factor VIIa by limited proteolysis.[3] So, there are two ways to form the TF:VIIa complex: through direct capture of circulating factor VIIa by TF or through capture of factor VII followed by conversion to factor VIIa.

Once formed, the TF:VIIa complex triggers clotting in two ways. TF:VIIa can activate factor IX via limited proteolysis (reaction 1 in FIGURE 11.2). The newly generated factor IXa then binds with a protein cofactor on a PL surface to form the IXa:VIIIa complex, which catalyzes the conversion of factor X to Xa. Alternatively, TF:VIIa can directly activate factor X (see reaction 2 in FIGURE 11.2). This latter mechanism predominates in most *in vitro* conditions, in which factor X is the preferred substrate for TF:VIIa.

Where does the first burst of serine protease activity come from if all the enzymes and their essential cofactors circulate as inactive precursors? It turns out that trace levels of factor VIIa are present in the circulation of all normal individuals, making up approximately 1% of the total factor VII concentration (but with considerable individual variation).[4] Exposure of TF to plasma therefore results in assembly of both TF:VII and TF:VIIa complexes, and only the latter are enzymatically active. However, these low initial levels of TF:VIIa are thought to trigger enough of the clotting cascade to result in the back-activation of the rest of the bound factor VII by downstream proteases. A variety of coagulation proteases activate factor VII to VIIa *in vitro*, including factors IXa, Xa, and XIIa; thrombin; plasmin; and factor

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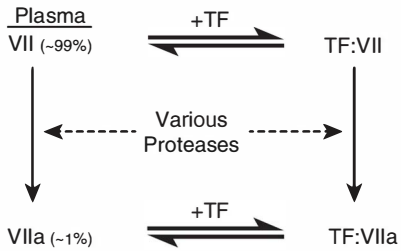

FIGURE 11.1 Two pathways for the formation of the TF and factor VIIa complex (TF:VIIa). Plasma contains a mixture of approximately 1% active factor VIIa and 99% zymogen factor VII. Both factors, VII and VIIa, bind reversibly to TF with similarly high affinities to generate TF:VII and TF:VIIa complexes, respectively. Factor VII can be converted to factor VIIa by a variety of plasma proteases (see text), and factor VII bound to TF is an especially good substrate for conversion to factor VIIa.

VII–activating protease.[3,5–11] In addition, the TF:VIIa complex catalyzes the autoactivation of factor VII bound to TF.[12,13] It is currently unclear which of these proteases plays the major role in activating TF-bound factor VII during clotting, although many consider factor Xa the most likely candidate.

It is also presently unclear where or how circulating factor VIIa is generated. One important clue comes from the discovery that patients with hemophilia B (who lack factor IX) have plasma factor VIIa levels that are only 10% of normal.[14] Thus, factor IX (presumably as factor IXa) participates in formation of much of the circulating factor VIIa found in plasma. Another clue is that factor VIIa levels rise postprandially, especially after high-fat meals.[15,16] This rise occurs in individuals who are deficient in factor XI or XII but not in factor IX,[17] suggesting that activation of factor VII occurs on lipoprotein particles, apparently involving factor IXa. Consistent with this notion, plasma factor VIIa levels are very low in patients with familial abetalipoproteinemia.[18] And finally, factor VIIa levels are higher in lymph than in plasma,[19] suggesting that at least some of the factor VIIa may be generated extravascularly and returned to the circulation by the lymph.

TISSUE FACTOR PROTEIN STRUCTURE

TF is also known as *thromboplastin, CD142*, and *coagulation factor III*. It is a glycosylated membrane protein consisting of a single-polypeptide chain of 261 or 263 amino acids (the two forms are nearly equal in abundance), with variability in length owing to microheterogeneity at the amino-terminus.[20–22] The calculated molecular weight of TF's polypeptide chain is 29,447 Da or 29,593 Da, whereas the mobility of the fully glycosylated protein on sodium dodecyl sulfate (SDS) gels suggests a molecular weight of approximately 45,000 Da.[23] TF belongs to the class 2 cytokine receptor superfamily.[24] Topographically, TF is a type I integral membrane protein, with its amino-terminus located outside the cell and its carboxyterminus inside the cell. The overall domain structure of TF is given in FIGURE 11.3. The extracellular portion is composed of two fibronectin type III domains, which are a variation on the immunoglobulin fold.

The extracellular domain of TF has three *N*-linked carbohydrate chains,[25] which were found to be completely dispensable

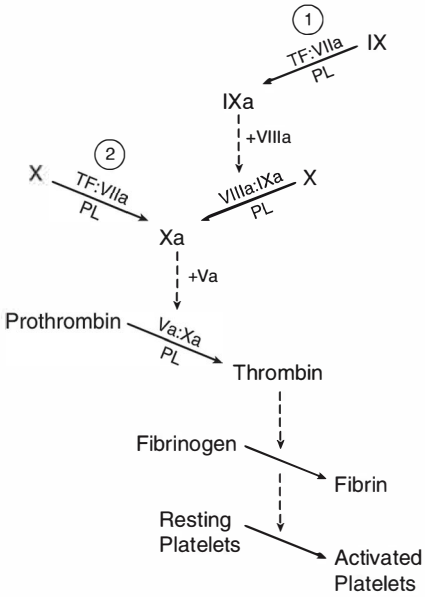

FIGURE 11.2 Simplified map of the blood clotting cascade initiated by the complex of TF and factor VIIa (TF:VIIa). The TF:VIIa complex can initiate clotting by activating either factor IX (*reaction 1*) or factor X (*reaction 2*). Regardless of the mechanism for triggering the clotting system, all clotting pathways converge at the formation of factor Xa, which assembles on a PL surface with its protein cofactor (factor Va) to catalyze the conversion of prothrombin to thrombin. Thrombin is responsible for converting fibrinogen to fibrin (again, by limited proteolysis), which spontaneously polymerizes to form a gel, or fibrin clot. In addition, thrombin is a potent activator of platelets (see Chapter 33). In this simplified clotting cascade, a number of back-activation reactions have been omitted. For example, factors Va and VIIIa circulate as inert precursors (factors V and VIII, respectively) and are activated following limited proteolysis by thrombin or factor Xa. The reactions involving hemostatically relevant factors XI and XIII, as well as all of the inhibitors of the clotting system, have also been omitted.

for clotting activity since the recombinant protein produced in bacteria was fully functional.[26] Recently, this conclusion was challenged by a study reporting that TF without carbohydrates had substantially reduced cofactor activity toward factor VIIa,[27] although a following study by others reported no detectable role for the carbohydrate chains of TF in any of its functions, including coagulant activity, intracellular trafficking, or cellular signaling.[28]

The extracellular domain of TF has two disulfide bonds,[29] at least one of which (the Cys186–Cys209 bond) is reported to be essential for coagulant activity,[30] although this conclusion has been disputed.[31] The Cys186–Cys209 disulfide bond has been proposed to play a role in TF encryption/decryption,[32] although this conclusion has also been disputed[31,33] (see section, *Modulation of Factor VIIa Activity by Tissue Factor*).

TF has a single membrane-spanning domain that anchors the protein to the cell surface. Membrane anchoring of TF is essential for full procoagulant activity, although the exact nature of the membrane anchor does not appear to be important for its clotting activity.[34]

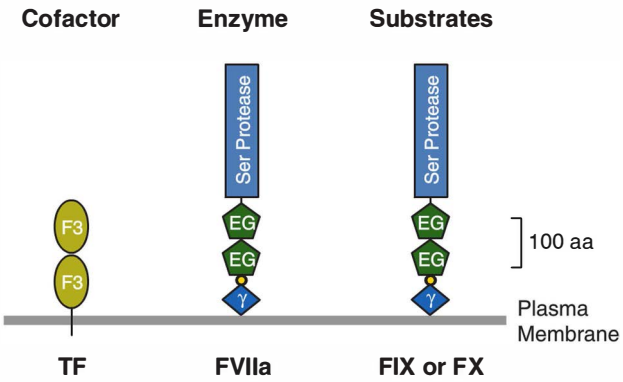

FIGURE 11.3 Schematic representation of the domain structure of TF, factor VII/VIIa (FVIIa), and factors IX and X (FIX and FX). The two fibronectin type III domains of TF are indicated as *F3*. The Gla domains of factors VII, IX, and X are indicated by *γ*, whereas the EGF-like domains are indicated by *EG* and the serine protease domains by *Ser Protease*. The aromatic stack is indicated by a small circle between the Gla and EGF domains. aa, amino acids.

The short (23–amino acid long) cytoplasmic domain of TF contains a cysteine residue to which palmitate or stearate is attached by a thioester linkage.[29] In addition, the cytoplasmic domain can be phosphorylated on serine.[35] Deletion of the cytoplasmic domain has no discernible effect on TF procoagulant activity,[34] making its role in blood clotting unclear, but there is increasing evidence that the cytoplasmic domain of TF may be important in some of TF's nonhemostatic roles (see section, *Nonhemostatic Functions for Tissue Factor and Factor VII*).

TF has been purified from human brain and placenta using factor VII affinity chromatography[23,36] and immunoaffinity chromatography.[37,38] Larger quantities can be obtained as the recombinant protein expressed in eukaryotic or prokaryotic cells.[26] Purified TF can readily be reconstituted into PL vesicles by using a variety of techniques.[39–41] A soluble form of TF consisting of the isolated extracellular domain[42] has proved useful in structure/function studies because it is easy to produce and handle.

Several groups have solved x-ray crystal structures of human and rabbit soluble TF.[43–47] Soluble TF is an elongated and apparently rather rigid molecule in which the two fibronectin type III domains are joined at an angle of approximately 120 degrees. In addition, the x-ray crystal structures of the complex of soluble TF and factor VIIa have also been determined (discussed in the section, *Structure of the Tissue Factor and Factor VIIa Complex*). Further reference to crystal structures of TF and factor VII may be found at http://www.tf7.org/xray.htm.

SITES OF TISSUE FACTOR EXPRESSION: NORMAL AND PATHOLOGIC

TF, being an integral membrane protein, is mainly found on the surface of cells in which it is synthesized. TF is abundant in a variety of cell types distributed throughout the body, including adventitial cells surrounding all blood vessels larger than capillaries; differentiating keratinocytes in the skin; and a number of epithelial cell types, including those present in mucous membranes and many organ capsules.[48,49,50] The distribution of TF can be rationalized by the requirement that it be present throughout the body, ready to trigger the clotting cascade at any time and place following vascular injury. This pattern of expression has been described as constituting a protective "hemostatic envelope" surrounding the vasculature, organ structures, and the entire organism.[48] In addition, TF is abundant at anatomic sites in which the risk—or the deleterious consequences—of bleeding is high, such as the renal glomerulus and throughout the brain.[48,50]

Spontaneous bleeding in patients with hemophilia occurs most commonly in anatomic locations such as skeletal muscle and joints, which contain especially low levels of TF.[48] Patients with hemophilia may bleed at these sites because direct activation of factor X by low levels of TF:VIIa is insufficient for hemostasis, and they lack the amplification step provided when TF:VIIa activates factor IX. Conversely, patients with hemophilia do not tend to bleed excessively from superficial cuts, which may be explained by the abundance of TF in the skin—exposure of large amounts of TF should allow direct activation of factor X in quantities sufficient for hemostasis.

In normal blood vessels, TF is readily detectable only in the adventitial cells within the vessel wall; TF antigen or messenger ribonucleic acid (mRNA) is usually undetectable in vascular smooth muscle and endothelial cells.[48,49,50] Atherosclerotic plaques, however, contain significant levels of TF mRNA and antigen, associated with monocytes and smooth muscle cells within the plaque.[49,51–54] TF antigen is also present in the acellular core of atheromas, most likely derived from cells that have undergone necrosis. TF in plaques can bind factor VIIa and is fully functional.[53,54] In healthy arteries, TF is separated from the lumen by both the intima (endothelium and underlying basement membrane) and the media (multiple layers of vascular smooth muscle cells). In atherosclerosis, however, blood is separated from TF by only a thin monolayer of endothelial cells—a much more dangerous situation. It is generally accepted that myocardial infarction is triggered by rupture or fissure of atherosclerotic plaques in coronary arteries,[55] exposing TF to blood within the lumen of the artery and triggering the formation of a thrombus that may occlude the vessel. Similar mechanisms likely precipitate other forms of arterial thrombosis, such as ischemic stroke.

TF is expressed in a variety of malignancies, where it can lead to thrombotic disease (e.g., in Trousseau syndrome). This can be caused by the expression of TF by the tumor cells themselves, by infiltrating monocytes, or by stromal cells. The association of TF with malignancy and its role in tumor pathology is reviewed in detail[56,57] and in Chapter 125.

Experiments using specific inhibitors of TF:VIIa have demonstrated that coagulopathies associated with sepsis and septic shock are mediated by TF, and furthermore that TF:VIIa contributes directly to mortality in sepsis.[58,59] Mice engineered to express very low levels of TF were protected against coagulopathy and mortality following challenge with bacterial endotoxin, and this was true even when low TF expression was limited to hematopoietic cells.[60] These studies underscore the importance of monocyte TF in driving the life-threatening coagulopathy of sepsis.

TISSUE FACTOR GENE: STRUCTURE AND REGULATION

Located on chromosome 1 at p21–22,[22,61] the TF gene (locus abbreviation, *F3*) spans 12.4 kb and contains six exons interrupted by five introns.[62] The first exon contains the

5′ noncoding sequence of the mRNA and also encodes the signal peptide, which directs the protein to the membrane and which is removed during biosynthesis. The first exon is located within a CpG island, a GC-rich deoxyribonucleic acid (DNA) region approximately 1 kb long that contains an unusually high density of CpG codons. The next four exons encode the extracellular domain, whereas the sixth exon encodes the transmembrane and cytoplasmic domains. The sixth exon also contains the relatively long 3′ noncoding sequence, which is unusual because it includes an Alu-family repeat sequence that persists in the mature mRNA. The mRNA for TF is approximately 2.3 kb long, although in some cell types such as monocytes, minor amounts of a larger TF mRNA species (~3.1 kb) are also observed.[22] The longer mRNA retains the first intron, and it is thought not to encode an active protein.[63,64]

Another alternatively spliced form of TF mRNA, initially discovered in certain leukemia cells, lacks the 5th exon.[65] When translated, it would encode a TF protein lacking a portion of the extracellular domain (normally encoded by exon 5) that is replaced by a novel amino acid sequence derived from reading exon 6 out of frame. Bogdanov et al.[66] have identified this alternatively spliced TF mRNA in a variety of normal cell types and have also discovered that the protein product of this alternatively spliced TF mRNA can be detected at low levels in plasma as an apparently soluble protein (discussed in the section, *Blood-Borne Tissue Factor*).

The TF gene is inducible in a variety of cultured cells. Quiescent fibroblasts in the dermis have no detectable TF antigen,[48] and serum-starved fibroblasts in culture also do not express TF. When cultured quiescent fibroblasts are stimulated with serum or with various mitogenic cytokines, transcription of the TF gene is rapidly induced.[67,68]

Expression of TF on activated monocytes and macrophages is an important effector function of these cells in inflammation. Monocytes isolated from peripheral blood usually have undetectable levels of TF but express this protein strongly after stimulation with various inflammatory mediators.[69,70] This directly parallels the observation that TF is expressed in circulating monocytes following intravenous administration of endotoxin or bacteria.[71] Furthermore, leukocytes (in particular, monocytes) have been shown to mediate the Schwartzman reaction in endotoxin-stimulated animals.[72] As with fibroblasts, the primary level of regulation of TF gene expression in monocytes is transcriptional.[73] In addition, endotoxin treatment of monocytes prolongs the half-life of TF mRNA.[63]

In cell culture, vascular endothelial cells can be induced to express TF robustly in response to a variety of inducers, including interleukin-1, tumor necrosis factor-α, and bacterial endotoxin.[69,70] *In vivo*, however, TF is expressed during severe sepsis by endothelial cells in only a few, highly restricted areas, such as in the splenic microvasculature.[74] This suggests that additional factors, which are not necessarily replicated *in vivo* during sepsis, may govern TF expression in cultured endothelial cells. Endothelial expression of TF has been observed *in vivo* in other conditions, however, including placental villitis[75] and graft rejection.[76,77]

In addition to studies with fibroblasts, monocytes, and endothelial cells, induction of TF gene expression has been studied in other cell types, including vascular smooth muscle cells and keratinocytes.[69,70] A variety of agents that are capable of inducing TF expression have been identified.[78] Transcriptional control of TF gene expression has been studied in several cell types, including monocytic cells, fibroblasts, and vascular endothelial and smooth muscle cells. These studies have identified transcription factor-binding sites flanking the promoter of the TF gene: Sp1 sites are important in basal transcription of the TF gene, whereas Egr-1 sites, AP-1 sites (binding c-Fos/c-Jun heterodimers), and a nuclear factor kappa B site (binding c-Rel/p65 heterodimers) are important in inducible expression of TF in response to bacterial endotoxin, serum, or phorbol ester, depending upon the cell type.[79] TF gene expression is also induced by hypoxia in several cell types.[80]

FACTOR VII PROTEIN STRUCTURE

Factor VII, a glycosylated plasma protein, consists of a single-polypeptide chain of 406 amino acids with an overall molecular weight of approximately 50,000 Da.[7,81,82] Synthesized by the liver, it circulates in plasma at a concentration of about 500 ng/mL (10 nM).[83] The domain structure of factor VII is depicted in FIGURE 11.3. Starting with the amino-terminus of the protein, it consists of the γ-carboxyglutamic acid–rich domain (Gla domain), the hydrophobic or aromatic stack, two epidermal growth factor (EGF)-like domains, and the serine protease domain. Molecular cloning revealed that factor VII has the same domain structure as factors IX, X, and protein C; shares considerable sequence homology with these coagulation serine proteases; and has a similar gene organization of introns and exons[84,85] (see Chapter 9).

Typical of vitamin K–dependent coagulation proteins, factor VII is synthesized with a signal peptide on its amino-terminus that directs the protein to the endoplasmic reticulum for secretion, followed by a propeptide that directs the cell to modify glutamic acid residues in the Gla domain. The signal and propeptides are removed before secretion. Two types of complementary DNA clones for human factor VII have been identified, one of which has an additional 22 amino acids in the propeptide.[84] Products of alternative splicing, these two forms of factor VII mRNA encode identical mature protein and appear to be functionally equivalent.

Factor VII contains 10 γ-carboxyglutamic acid (Gla) residues in its Gla domain, resulting from vitamin K–dependent modification of glutamic acid residues during biosynthesis. As with other vitamin K–dependent coagulation proteins, these Gla acid residues are essential for activity. Specifically, the Gla domain confers on factor VII the ability to bind, in a reversible and Ca^{2+}-dependent manner, to membranes containing negatively charged PLs. In the crystal structure of the TF:VIIa complex, the Gla domain contains seven bound Ca^{2+},[86] although three of these Ca^{2+}-binding sites are probably actually occupied by Mg^{2+} *in vivo*.[87]

The aromatic stack of factor VII is a short amino acid sequence containing a small cluster of aromatic residues. It is adjacent to the Gla domain and, although encoded by a separate exon, is classified by some investigators as a part of the Gla domain. The two EGF domains have a typical growth factor-fold and disulfide-bonding pattern. The first EGF domain binds a single Ca^{2+} with relatively high affinity,[86,88] whereas the second EGF domain does not bind Ca^{2+}. The first EGF domain of bovine factor VII contains the modified amino acid, β-hydroxyaspartic acid, whereas this modification is absent in human factor VII.[89]

The serine protease domain of factor VII is homologous to the digestive enzymes trypsin and chymotrypsin and exhibits

trypsin-like substrate specificity (i.e., cleaving peptide bonds after arginine or lysine). It has a typical serine protease catalytic triad composed of His193, Asp242, and Ser344. As with many other coagulation serine proteases, the protease domain of factor VII binds a single Ca^{2+}.[86,90] The protease domain also binds zinc ions, which inhibit factor VIIa enzymatic activity.[87,91,92]

Zymogen factor VII is converted to factor VIIa, the enzymatically competent form, by proteolysis of a single peptide bond between Arg152 and Ile153. The result, factor VIIa, is composed of two disulfide-linked polypeptide chains. The light chain of factor VIIa, consisting of the Gla domain, aromatic stack, and both EGF domains, has 152 amino acids and a molecular weight of approximately 20,000 Da. The heavy chain, consisting of the serine protease domain, has 254 amino acids and a molecular weight of approximately 30,000. Unlike many other coagulation zymogens, factor VII does not release an activation peptide when converted to factor VIIa.

Factor VII contains two *N*-linked carbohydrate chains attached to residues Asn145 and Asn322.[89] It also contains two short, *O*-linked carbohydrate chains attached to the first EGF domain: glucose, glucose–xylose, or glucose–$(xylose)_2$ attached to Ser52; and a single fucose attached to Ser60.[93]

Factor VII can be purified from plasma using conventional chromatography[7,81,82] or immunoaffinity chromatography.[94] Recombinant human factor VII has been produced in cultured mammalian cells.[89] Truncated forms of factor VII have been produced in which the Gla domain has been removed (Gla-domainless factor VII) by limited proteolysis[95,96] or by expression of the truncated recombinant protein.[97] Gla-domainless factor VII may or may not contain the aromatic stack, depending on the site of cleavage. Furthermore, a form of factor VII lacking both the Gla domain and the first EGF domain has also been produced recombinantly,[98–100] and it fails to bind to TF.[98] Factor VII mutants have been produced, in which the activation cleavage site has been mutated so that it cannot be activated by the usual activators,[101] which demonstrated that zymogen factor VII had no detectable enzymatic activity. In addition, the active site serine (Ser344) has been mutated to alanine, generating a form of factor VIIa without enzyme activity which has been useful in investigating the conversion of factor VII to VIIa.[12]

STRUCTURE OF THE TISSUE FACTOR AND FACTOR VIIA COMPLEX

The x-ray crystal structure of the complex of soluble TF and active site–inhibited factor VIIa has been solved to a resolution of 2.0 Å,[86] and the structure of a similar complex inhibited with a modified version of bovine pancreatic trypsin inhibitor has been solved to a resolution of 2.1 Å.[102] The structure of TF in these complexes is very similar to that of soluble TF alone, confirming the relative rigidity of this molecule. Within the complex, factor VIIa is a highly elongated protein that forms extended contacts with TF (see FIGURE 11.4). The main sites of contact on factor VIIa are located in the first EGF domain and the protease domain, with additional points of contact within the second EGF domain and the aromatic stack region of the Gla domain. The sites of contact on TF are located on both fibronectin type III domains and the interfacial region between them.

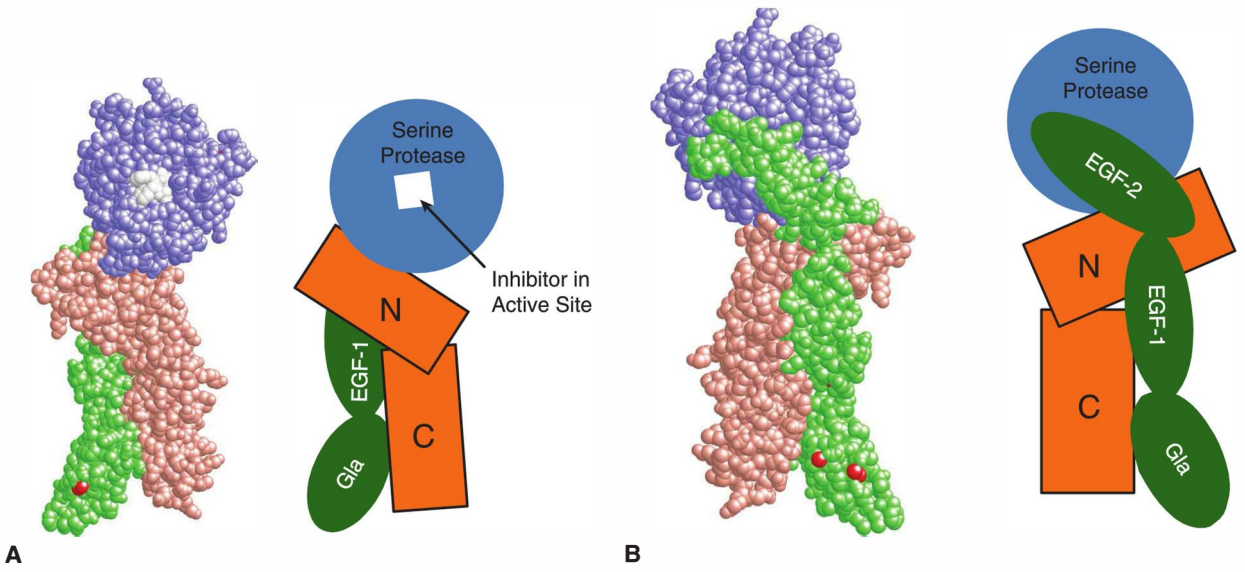

FIGURE 11.4 Two views of the x-ray crystal structure of the complex of soluble TF and active site–inhibited factor VIIa **(left)** and diagrammatic representations of the domain organizations of TF and factor VIIa **(right)**. **A:** "Front" view of the TF:VIIa complex, with the active site facing the viewer. **B:** "Back" view of the complex, with the active site facing away from the viewer. The membrane surface would be located at the bottom of each complex. TF is shaded *brown*, whereas factor VIIa is shown in *green* (light chain) and *blue* (heavy chain). In addition, the tripeptidylchlormethylketone inhibitor in the active site of factor VIIa is colored *white* and calcium ions are *red*. For the TF representation, *N* and *C* refer to the *N*-terminal and *C*-terminal fibronectin type III domains, respectively. For the factor VIIa representation, the Gla domain is labeled *Gla* and the EGF-like domains are labeled *EGF-1* or *EGF-2*. The serine protease domain and the inhibitor are also labeled. The two views of the TF:VIIa complex were generated with the program RASMOL,[424] using the atomic coordinates file 1DAN[86] from the Protein Data Bank.

Several studies have used domain swapping and site-directed mutagenesis to identify the binding sites on TF and factor VIIa that permit them to interact with each other,[103–106] revealing that some contacts are important for stabilizing the binding of TF to factor VIIa (chiefly with the first EGF domain of factor VIIa), whereas others appear to be important in enhancing the enzymatic activity of factor VIIa (chiefly with the protease domain of factor VIIa). These findings are reviewed in detail elsewhere.[105,106]

The portion of the Gla domain located at the bottom of the factor VIIa molecule (as shown in FIGURE 11.4) contains three solvent-exposed hydrophobic residues that are proposed to be inserted into the hydrophobic core of the PL bilayer.[107] The carboxy-terminus of soluble TF is also located near the bottom of the structure, as presented in FIGURE 11.4; in intact TF, this part of the molecule is linked to the membrane anchor by a short peptide sequence. Therefore, in the views given in FIGURE 11.4, the membrane surface should be located at the bottom of the complex, with TF linked to the membrane by its membrane-anchoring domain and with VIIa interacting with PL head groups through the Gla domain. Fluorescence resonance energy-transfer experiments indicate that the TF:VIIa complex is oriented almost perpendicular to the membrane, with the active site of factor VIIa located approximately 78 Å above the membrane surface.[108,109] This same orientation was observed in detailed molecular dynamics simulations of the TF:VIIa complex on membranes.[110]

MODULATION OF FACTOR VIIA ACTIVITY BY TISSUE FACTOR

When factor VIIa binds to TF, its ability to catalyze the hydrolysis of small peptidyl-amide or peptidyl-ester substrates is enhanced 20-fold to 100-fold, depending upon the substrate.[42,111–113] This is usually accomplished by an increase in the catalytic rate constant (k_{cat}) and, in some cases, by a decrease in the Michaelis-Menten constant (K_m), indicating that TF is an allosteric activator of factor VIIa.

Substrate hydrolysis by serine proteases is a multistep process,[114] and the mechanism by which TF enhances the enzymatic activity of factor VIIa is not completely understood. Studies with a slowly hydrolyzed model substrate have shown that TF enhances the rate of substrate hydrolysis by factor VIIa chiefly by enhancing substrate affinity of factor VIIa rather than by altering the rates of any of the subsequent catalytic steps.[115]

A wealth of structure–function studies combining site-directed mutagenesis and x-ray crystallography have enhanced the understanding of the allosteric activation of factor VIIa by TF.[116,117] Insights into the mechanism of allosteric activation of factor VIIa have also been obtained by designing mutants of factor VIIa that have enhanced enzymatic activity in the absence of TF.[118,119] One of the most surprising results comes from the crystal structure of zymogen factor VII, which exhibits extensive β-strand re-registration relative to the crystal structures of factor VIIa.[100]

Serine protease zymogens are converted to active enzymes by limited proteolysis, whereupon the newly generated amino-terminus folds back into the protein to stabilize the oxyanion pocket.[120] It has been proposed that free factor VIIa exists in two states in equilibrium with each other: one in which the protein is catalytically active and a second, zymogen-like state, in which the amino-terminus of the heavy chain is not properly folded back into the catalytic domain.[121,122] Surprisingly, in the crystal structure of factor VIIa obtained in the absence of TF or any inhibitor in the active site, the amino-terminus of the factor VIIa heavy chain was still folded back into the catalytic domain and the oxyanion pocket was intact.[99]

Free factor VIIa proteolytically activates factors IX and X extremely slowly, but when it binds to TF that has been incorporated into suitable PL vesicles, its activity is enhanced many 1,000-fold.[123–125] (Factors IX and X will bind to PL vesicles only if they contain negatively charged PLs, most particularly phosphatidylserine; therefore, TF is most often reconstituted into vesicles containing a mixture of phosphatidylserine and the neutral PL, phosphatidylcholine.) There are three main reasons for this rate enhancement. First, the reversible binding of factors IX and X to PL surfaces raises the concentration of these substrates in the vicinity of the enzyme TF:VIIa, which reduces the apparent K_m. Second, TF is an allosteric activator of factor VIIa enzymatic activity. Third, TF appears to provide an extended binding site (exosite) for macromolecular substrates, as identified by site-directed mutagenesis.[97,126,127]

The fact that most coagulation reactions are restricted to two-dimensional surfaces substantially complicates the interpretation of their enzyme kinetics.[128–130] In addition, coagulation reactions can occur *in vivo* under conditions of stasis or under conditions of considerable blood flow. Andree and Nemerson[131] have published extensively on the effects of flow on the kinetics of factor X activation by the TF:VIIa complex.

It has long been known that resting, intact cells have much lower procoagulant activity than do cells that have been damaged, lysed, or treated with calcium ionophore.[132] Although present on the surface of intact cells, TF becomes fully active only when the membrane properties of the cell are altered,[133,134] a process that is sometimes referred to as decryption of "encrypted" cell-surface TF. There are several potential explanations for TF encryption/decryption. The first is that most cells restrict the distribution of aminophospholipids (such as phosphatidylserine) to the inner leaflet of the plasma membrane.[135] Exposure of negatively charged PLs on the outer leaflet is necessary for efficient binding of substrate (i.e., factor IX or X), so the membrane asymmetry of intact cells will tend to limit the activity of TF. When cells are lysed, damaged, or treated with calcium ionophore, PL asymmetry is lost, resulting in the exposure of phosphatidylserine and phosphatidylethanolamine on the outer leaflet of the plasma membrane, thereby enhancing the activity of TF:VIIa. Another potential explanation in some cell types is that TF may associate with caveolae, areas of the cell surface with altered lipid composition.[136,137] It has been also proposed that dimerization or oligomerization of TF in the membrane may reduce its activity and that damage or lysis of cells may foster the formation of TF monomers with enhanced activity.[138]

Finally, it has been proposed that Cys186 and Cys209 of TF participate in an unusual "allosteric" disulfide bond that can readily be broken or re-formed (i.e., reduced or reoxidized) to switch TF from a predominantly signaling form to a highly procoagulant form on cell surfaces.[139] It has further been proposed that extracellular protein disulfide isomerase (PDI) may regulate TF encryption/decryption via this allosteric Cys186–Cys209 bond, and also derivatization of these Cys residues via S-nitrosylation or glutathionation.[32] These proposals have been disputed by others, in reports that the Cys186–Cys209 bond of

TF is not required for coagulant activity and that PDI appears not to play a direct role in TF encryption/decryption.[31,33,140]

TF:VIIa has an extremely restricted substrate specificity; its main natural substrates appear to be factors VII, IX, and X. Factor X is the favored substrate under most *in vitro* conditions, although activation reactions taking place on intact cells can show a preference for either factor IX or factor X[125,141–143] for reasons that are not clear. When factor IX is activated, two peptide bonds must be cleaved. TF:VIIa complex catalyzes the cleavage of one of these bonds (i.e., at Arg180) more efficiently than the other (i.e., at Arg145), and, furthermore, factor Xa and TF:VIIa complex can synergize to activate factor IX to IXa more efficiently.[144]

Factor VII is a substrate for factor VIIa[145] in an autoactivation reaction enhanced by TF.[12] This reaction requires that the TF:VIIa and TF:VII complexes encounter each other by lateral diffusion in the plane of the membrane.[13] Autoactivation of factor VII might be important in initiating the clotting cascade, but its precise role is yet to be elucidated.

The TF:VIIa complex can cleave multiple peptide bonds in factors V and VIII, including both activation and inactivation cleavage sites,[146,147] although the physiologic significance of this is uncertain.

TISSUE FACTOR PATHWAY INHIBITOR

In 1947, Thomas[148] and Schneider[149] showed that blood contained an inhibitor of TF-initiated coagulation. Ten years later, Hjort[150] described many of the *in vitro* properties of the inhibitor, but it took an additional 30 years before the inhibitor was isolated.[151] Previously called antithromboplastin, anticonvertin, TF inhibitor, extrinsic pathway inhibitor, or lipoprotein-associated coagulation inhibitor, in 1991 the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis settled on the currently used name, tissue factor pathway inhibitor (TFPI).

STRUCTURE OF TFPI ISOFORMS

The TFPI gene spans about 90 kb on the long arm of chromosome 2 (q32) and contains 10 exons (GenBank: NC_000002.11).[152,153] All the splice junctions between exons are of the same type (type I), suggesting that the TFPI gene was assembled during evolution through a process of gene duplication and exon shuffling. Two alternatively transcribed isoforms of TFPI have been identified. TFPIα (GenBank NM_006287.4) is the originally isolated form of TFPI. The mature TFPIα protein contains an acidic amino-terminus, followed by three tandem Kunitz-type protease inhibitory domains, and a basic carboxyterminus (FIGURE 11.5). With posttranslational modifications, including *N*- and *O*-linked glycosylation and partial phosphorylation of Ser2, the molecular mass of TFPIα is approximately 43 kDa.[154,155] In TFPIβ (GenBank NM_001032281.2), an alternative carboxyterminus is inserted at residue 182, which directs the attachment of a glycosyl phosphatidylinositol (GPI) anchor (FIGURE 11.6). The protein mass of TFPIβ is less than that of TFPIα (22 vs. 32 kDa), but it migrates on SDS-PAGE at the same apparent molecular weight as TFPIα due to additional *O*-linked carbohydrate.[156] In human umbilical vein endothelial cells and endothelial-like cell lines (EAhy926, ECV304), the ratio of TFPIα/TFPIβ mRNA varies between 5 and 10.[157]

Kunitz-type inhibitors act by a standard mechanism in which the inhibitor feigns to be a good substrate, but, after the enzyme binds, subsequent cleavage between the P_1 and P_1' amino acid residues of the inhibitor occurs slowly or not at all. The P_1 residue is an important determinant of the specificity and activity of these inhibitors. In kinetic terms, Kunitz-type inhibitors produce slow, tight-binding, competitive, and reversible enzyme inhibition. In the reaction, the Kunitz inhibitor forms an initial "encounter" complex (EI) with the enzyme, and this complex then "slowly" isomerizes to a much tighter form (EI*). "Slow" implies that the final degree of inhibition does not occur immediately and "tight-binding" means that the inhibition occurs at a concentration of the inhibitor that is near to that of the enzyme being inhibited. The Kunitz-2 domain of TFPI mediates factor Xa binding and inhibition, whereas the Kunitz-1 domain is necessary for the inhibition of factor VIIa in the TF:VIIa complex.[158] The Kunitz-3 domain of TFPIα lacks protease inhibitory activity.[159] TFPI also inhibits trypsin and chymotrypsin reasonably well and inhibits cathepsin G, plasmin, and activated protein C (APC) poorly; the physiologic significance of these other inhibitory reactions, however, is obscure.

DISTRIBUTION OF TFPI

The microvascular endothelium is felt to be the major source of TFPI *in vivo*. Northern blot analysis shows the highest TFPI mRNA levels in the placenta and lung and the lowest in the brain.[160] Studies of normal tissues have detected TFPI protein in the endothelium of the microvasculature, smooth muscle cells, monocytes/macrophages, megakaryocytes/platelets, mesangial cells, fibroblasts, microglia, cardiomyocytes, and mesothelial cells.

Plasma TFPI

The plasma concentration of TFPI is approximately 1.6 nM (~70 ng/mL). Most of the circulating TFPI is bound to lipoproteins (~80%), and manipulations that alter the levels of low-density lipoprotein (LDL), a major carrier of TFPI, also affect TFPI levels.[161–163] TFPI circulating with LDL is 34 kDa and appears to represent a substantially truncated form, which lacks a large portion of Kunitz-3 domain and the *C*-terminal domain of TFPIα.[161,164] The 41 kDa form of TFPI that is bound to high-density lipoprotein seems to represent a similar carboxy-truncated form that is linked in a mixed disulfide bond with monomeric apolipoprotein-AII. Whether the lipoprotein-associated TFPIs are extensively truncated forms of TFPIα or modified forms of TFPIβ has not been established, and the mechanism underlying the association of 34 kDa with LDL has not been determined. Nonlipoprotein-bound or "free" TFPI is 20% of total plasma TFPI and is a combination of forms of TFPIα with limited carboxyterminal truncation produced by an as yet to be unidentified protease(s) (10%) and full-length TFPIα (10%). The concentration of full-length TFPIα is reduced in the plasmas of patients with factor V or protein S deficiency, and binding interactions between full-length TFPIα and these proteins have been demonstrated.[165,166] Whether these interactions affect the expression, proteolytic degradation, or clearance of TFPIα remains to be determined. Oral contraceptives and hormone replacement therapy reduce plasma levels of TFPI.[167–170]

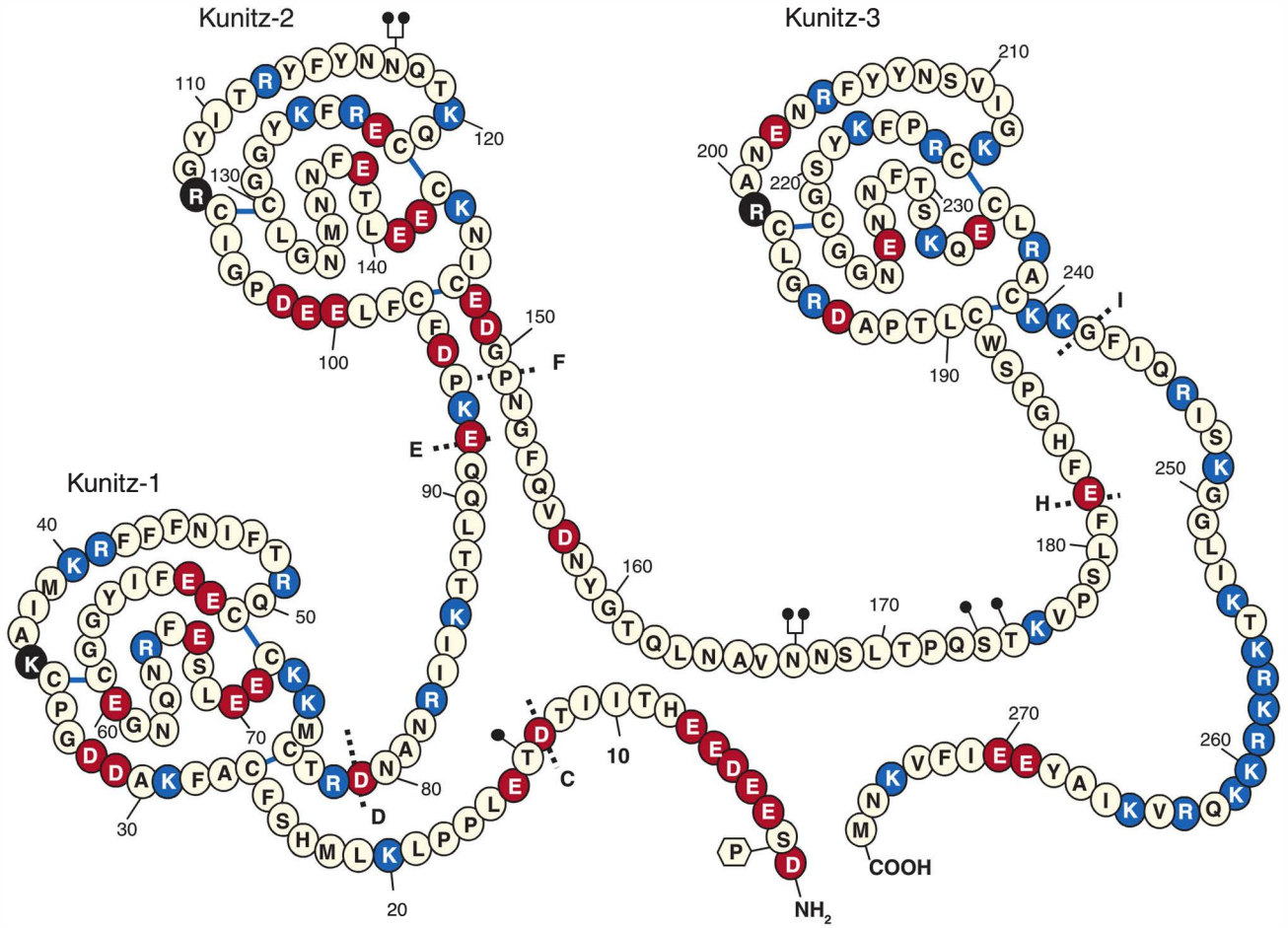

FIGURE 11.5 Structure of TFPIα. Amino acids are identified by the single-letter code (*encircled*). Positively charged amino acids are shown in *red*, negatively charge amino acids in *blue*, and neutral amino acids in *white* (histidine residues are considered uncharged). The Kunitz domains are labeled and the basic P_1 residues at the active site cleft for each domain is shown in *black*. *N*-linked glycosylation sites are denoted at N117 and N167 by and *O*-linked glycosylation sites are denoted at T14, S174, and T175 by . Partial phosphorylation at Ser2 is also shown . The sites of introns in the TFPI gene are labeled with *dotted lines* and *capital letters*. (Figure created by Thomas Girard.)

Platelet TFPI

TFPIα is expressed by megakaryocytes, stored in platelets at a site separate from α granules, and released in response to thrombin and other agonists.[171,172] In "coated" platelets produced by dual agonist stimulation (e.g., thrombin/collagen), a proportion of the released TFPIα remains bound and functional at the platelet surface. Similar to other ligands detected on the surface of coated platelets, transglutaminase inhibitors prevent this retention of surface TFPIα.[172] The full-length TFPIα carried by platelets is 8% to 10% of the total TFPI in blood, which is comparable to the quantity of soluble full-length TFPIα in plasma. Therefore, it is likely that platelets are a major source of full-length TFPIα, the most anticoagulantly active form of TFPI (see below), at local sites of coagulation where platelets aggregate. On the other hand, polyphosphates released from the dense granules of platelets reduce the anticoagulant effect of TFPIα in plasma coagulation assays, apparently in part through their enhancement of factor V activation.[173,174]

Cell-Associated TFPI

Several reports have documented the low-affinity, heparin-inhibitable binding of TFPIα to cells, the internalization and degradation of TFPIα through the action of LDL-receptor–related protein (LRP), and the rapid clearance of TFPIα in animals.[98,175–183] These studies used recombinant TFPIα (rTFPIα) that lacked the post-translational modifications found in mammalian TFPIα. In contrast, the rTFPIα produced by a mammalian cell line (mouse C127) does not bind to cells or interact with LRP in the same fashion as *Escherichia coli* rTFPIα and is cleared from plasma at a 10-fold slower rate.[184] Therefore, the studies that used nonmammalian-derived TFPI should probably be viewed with considerable caution.

A substantial fraction of the TFPI produced by endothelial cells remains at the cell surface, associates with caveolae, and is released by phosphatidylinositol-specific phospholipase C (PIPLC). These are properties of GPI-anchored proteins. The PIPLC-induced release of TFPIβ is consistent with its possession of an intrinsic GPI anchor.[157] It has been reported that

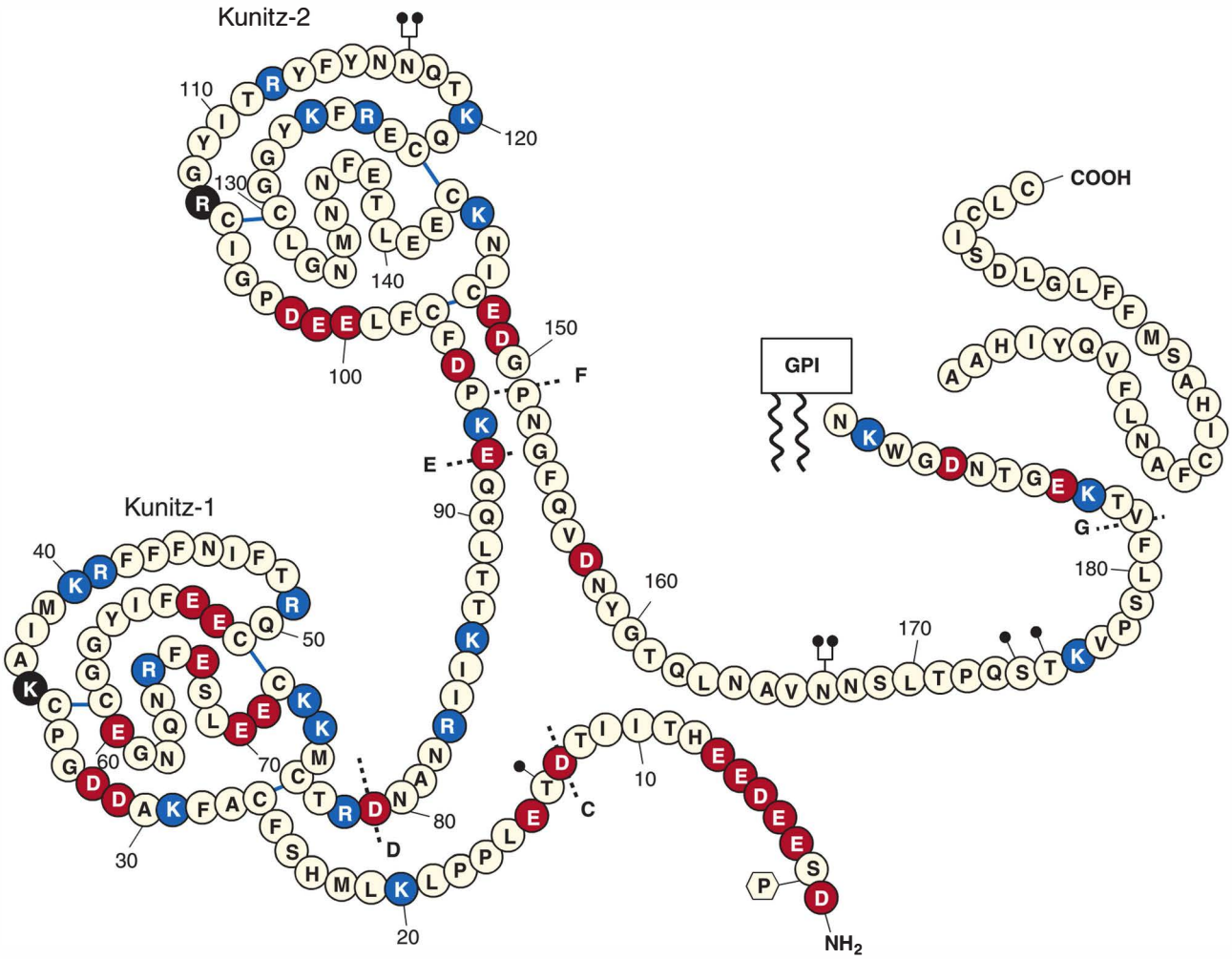

FIGURE 11.6 Structure of TFPIβ. Symbols as denoted in the legend of FIGURE 11.5. The GPI anchor is shown attached to mature TFPIβ along with the released carboxyterminal peptide. (Figure created by Thomas Girard.)

cell-surface TFPIα is also released by PIPLC treatment.[185,186] Since the amino acid sequence of TFPIα does not contain the canonical motifs to direct the attachment of a GPI anchor, this implied that the cell-surface binding of TFPIα may involve its interaction with a separate GPI-anchored coreceptor(s), which controlled its cellular trafficking and surface expression.[187] Subsequent work, however, has shown that it is TFPIβ that is released by PIPLC from endothelial cells and placental microsomes.[188] In endothelial cells induced to produce TF, GPI anchorage of TFPI serves to redistribute TF:VIIa to caveolae in a factor Xa–dependent manner.[136,185,189] GPI anchorage, however, may not be a requirement for TF:VIIa inhibition by cell-surface TFPI as chimeric forms of rTFPI anchored by GPI or transmembrane domains produce similar levels of TF:VIIa inhibition.[190] Endothelial cell–associated TFPI represents a substantial reservoir of TFPI *in vivo*, suggesting that the regulation of TF-mediated procoagulant and cell-signaling activities in the endothelium is a critical physiologic role of TFPI. Whether TFPI is attached to the surface of other TFPI-expressing cells (e.g., vascular smooth muscle cells, monocytes/macrophages) in the same manner as it is in endothelial cells remains to be determined.

Releasable TFPI

Heparin, thrombin, and shear increase the expression and release of TFPI from cells in culture.[157,187,191–196] The release of TFPI induced acutely by these agonists appears to involve the redistribution of TFPI from stores located near the plasma membrane (perhaps caveolae) to the cell surface, with the subsequent release into the media of a portion of the total cellular TFPI. During this process, the TFPI available at the surface of the cells remains unchanged or increases. The parenteral administration of heparin or low molecular weight heparin, but reportedly not the heparin pentasaccharide (fondaparinux), rapidly increases the circulating levels of total TFPI in plasma 1.5- to 3.0-fold.[197–200] It is full-length TFPIα that is released in this process, raising the plasma concentration of full-length TFPIα 10- to 20-fold.

Degradation of TFPI

In vitro experiments have shown that TFPIα can be proteolytically degraded by a variety of proteases, including thrombin, plasmin, neutrophil elastase, cathepsin G, factor Xa (when at molar excess over TFPIα), cell-derived matrix metalloproteinases, and

bacterial omptins.[201–207] Plasmin-mediated degradation of TFPI in plasma and on the monocyte surface has been demonstrated in patients following thrombolytic therapy and indirectly on the cells of the lung in septic baboons.[208,209] The degradation of TFPI by neutrophil proteases, especially elastase, has been shown to enhance microvascular coagulation, thereby limiting the tissue dissemination of bacterial pathogens, but also increasing vascular thrombosis.[210] This cleavage of TFPI by neutrophil elastase is facilitated by neutrophil-derived externalized nucleosomes, which form neutrophil extracellular traps that serve to colocalize elastase and TFPI. In tissue culture cells, APC cleaves TFPI between its Kunitz-1 and Kunitz-2 domains in an endothelial protein C receptor–dependent process and thereby reduces cell-surface TFPI activity.[211]

FUNCTIONS OF TFPI

TFPI limits coagulation both through the inhibition of factor Xa, a process enhanced by protein S, and through factor Xa–dependent feedback inhibition of TF:VIIa (FIGURE 11.7). TFPI-mediated inhibition of factor Xa generation by TF:VIIa and the inability to rapidly produce additional factor Xa through the action of factor IXa:factor VIIIa helps explain the bleeding seen in hemophilia.[212–214] Thus, neutralization of TFPI

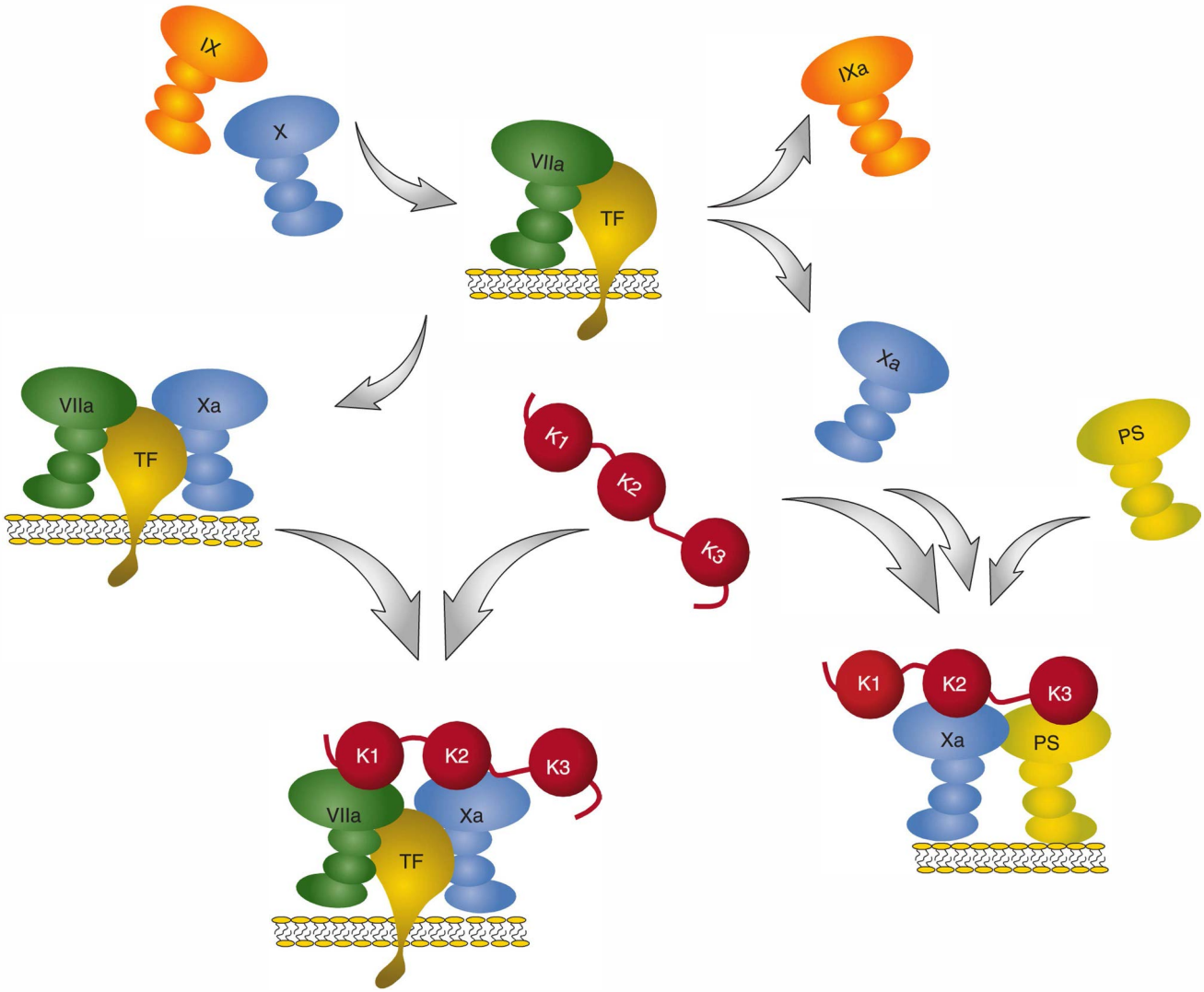

FIGURE 11.7 Functions of TFPIα. The TF:VIIa catalytic complex on a PL surface activates factors X and IX **(top)**. TFPI **(center)** binds to the transient ternary TF:VIIa:Xa complex **(left)** produced during the activation of factor X, forming a quaternary TF:VIIa:Xa:TFPI inhibitory complex **(bottom)** in which the Kunitz-1 and Kunitz-2 domains of TFPI bind and inhibit factor VIIa and factor Xa, respectively. On the right, factor Xa, protein S, and TFPI are shown forming an inhibitory complex at a PL surface in which the Kunitz-2 domain binds and inhibits factor Xa and the Kunitz-3 and carboxyterminal domains of TFPIα are required for interactions with protein S and PLs. Direct inhibition of factor Xa by TFPI and an alternative two-step pathway for TF:VIIa inhibition in which a factor Xa/TFPI complex binds to TF:VIIa producing the final quaternary inhibitory complex are not shown. Also not depicted are the factor VIIa/TF and factor Xa inhibition produced by cell membrane-bound TFPIα and TFPIβ. The Kunitz-1, Kunitz-2, and Kunitz-3 domains of TFPI are labeled K1, K2, and K3, respectively. (Figure created by Thomas Girard.)

action has been explored as a potential therapeutic modality for hemophilia.[215–217]

Factor Xa Inhibition

The inhibition of factor Xa by TFPI is through its Kunitz-2 domain,[158,159] but other parts of the TFPI molecule are important for this inhibitory effect. The carboxyterminal domain of TFPIα is required for rapid and efficient factor Xa inhibition, and cleavage of TFPI between Kunitz domains 1 and 2 (e.g., by neutrophil elastase) dramatically reduces the ability of TFPI to inhibit factor Xa.[159,204,218,219] The inhibition of factor Xa by TFPI in the presence of physiologic calcium ion concentrations is enhanced by procoagulant PLs.[218] The basic carboxyterminal region of the TFPIα molecule is important for this effect and presumably permits the simultaneous interaction of factor Xa and TFPIα with the PL surface. Under these conditions, the kinetic inhibitory constants (Kis) for the EI and EI* complexes of TFPI with factor Xa are 2 to 4 nM and 50 pM, respectively.[218,220] The TFPIβ isoform lacks the carboxyterminus of TFPIα and demonstrates relatively weak inhibition of factor Xa when tested in soluble phase.[221] The inhibition of factor Xa by TFPIβ, however, is likely to be significantly enhanced when it is bound to a PL surface via its GPI anchor.

Protein S substantially enhances the inhibition of factor Xa by TFPIα in the presence of PLs and calcium ions by increasing the affinity of the initial EI between factor Xa and TFPIα 10-fold, to near the concentration of full-length TFPIα in plasma (FIGURE 11.7).[220] An optimal effect requires the carboxyterminal domain of TFPIα and appears to involve at least in part a direct interaction of protein S with the Kunitz-3 domain of TFPIα.[222] As protein S binds factor Xa in a calcium ion- and PL-dependent fashion,[223] the most straightforward explanation for the potentiating effect of protein S on factor Xa inhibition by TFPIα is that an association between protein S and TFPIα serves to increase TFPIα's interactions with PLs and with factor Xa at PL surfaces.

Heparin and other polyanions accelerate factor Xa inhibition by TFPIα. The heparin dose response exhibits an optimum, suggesting that the polyanion forms a template upon which factor Xa and TFPI simultaneously bind.[218,224] Basic residues within the carboxyterminus and Kunitz-3 domains of TFPIα are required for optimal heparin binding, and progressive carboxyterminal truncation of the TFPI molecule produces forms with decreasing affinity for heparin.[224,225] Rather than a specific binding epitope, charge density on the glycosaminoglycan appears to be most important for TFPI binding.[226]

Tissue Factor:Factor VIIa Inhibition

Factor Xa–dependent inhibition of TF:VIIa by TFPI requires the Kunitz-1 domain and involves the formation of a quaternary TFPI-factor VIIa–TF-factor Xa (TFPI:VIIa:TF:Xa) complex (FIGURE 11.7).[212,227] The final quaternary complex could be produced in a two-step process in which TFPI first binds factor Xa and then the factor Xa–TFPI complex binds and inhibits TF:VIIa. That this two-step pathway can occur has been documented *in vitro*. Kinetic studies, however, strongly suggest an alternative pathway in which TFPI interacts with the ternary complex of TF:VIIa:Xa, which forms transiently during the activation of factor X and from which the product factor Xa dissociates relatively slowly.[228] As a result, TF:VIIa inhibition is extremely rapid, and the concentration of active factor Xa that escapes regulation depends linearly on the quantity of available TF. That protein S significantly enhances the formation of the factor Xa–TFPI complex, but does not increase the inhibition of TF:VIIa by TFPI, is consistent with this alternative pathway.[229] The Gla domain of factor Xa and Lys165–Lys166 of TF, which are structures required for the optimal recognition of factor X by TF:VIIa, are also important for the inhibition of TF:VIIa by factor Xa–TFPI.[227,230,231]

Full-length TFPIα inhibits factor Xa much faster than carboxyterminal truncated forms of TFPI.[159,219,224] In contrast, full-length and carboxyterminal truncated forms of TFPIα appear to inhibit factor X activation by TF:VIIa at comparable rates.[232–234] GPI-anchored TFPIβ is the preeminent regulator of TF/VIIa on cells expressing both TFPI and TF.[156] The requirement of factor Xa for the inhibition of TF:VIIa by TFPI is not absolute and high concentrations of TFPI will inhibit TF:VIIa in the absence of factor Xa.[159,235] This factor Xa-independent inhibition of TF:VIIa by TFPI, however, is of uncertain physiologic relevance.

Anticoagulant Activity

The anticoagulant activity of TFPI in plasma assays is due to its inhibition of factor Xa and not its inhibition of TF:VIIa. The exogenous addition of TFPIα to plasma inhibits the coagulation induced by factor Xa, the X-coagulant protein of Russell viper venom, and TF to a similar extent.[219] An optimal effect requires the presence of protein S and is dependent on the Kunitz-3 and carboxyterminal domains of TFPIα.[222] In plasma containing physiologic concentrations of full-length TFPIα (~0.16 nM), a similar effect of protein S and TFPI on thrombin generation can be demonstrated, but only at low concentrations of TF (<14 pM) or factor Xa (250 pM).[236] At higher concentrations of TF, the rate and extent of factor Xa generation produced by factor IXa:factor VIIIa as well as TF:VIIa is presumably sufficient to overwhelm the factor Xa inhibition mediated through TFPIα and protein S. When the coagulation response to higher concentrations of TF is limited by activation of the protein C pathway (e.g. addition of APC or thrombomodulin to plasma), however, the effect of protein S–dependent TFPI inhibition of factor Xa is again apparent.[236,237,238] Indeed, the combination of the actions of APC and TFPIα, enhanced by their mutual cofactor protein S, serves to synergistically limit coagulation.

While the full-length form of TFPIα is required for optimal factor Xa inhibition and the anticoagulant effect of TFPI in one-stage coagulation assays,[219,222,239] the TF:VIIa inhibition produced by the carboxyterminal truncated forms of TFPI, present at 10-fold higher concentrations than full-length TFPIα, in plasma is demonstrable in two-stage assay systems.[239] It seems likely that the attachment of the carboxyterminal truncated forms of TFPI to large lipoprotein particles in plasma would hinder the recognition of cellular TF:VIIa, but this has not been directly tested.

The critical importance of the regulation of TF-induced coagulation by TFPI has been demonstrated in gene-disrupted mice that express isoforms of TFPI lacking the Kunitz-1 domain (TFPIΔK1). These isoforms cannot inhibit TF:VIIa and presumably possess dramatically reduced anti–factor Xa activity.[204] The homozygous TFPIΔK1 mice die *in utero* of disseminated intravascular coagulation and a consumptive coagulopathy,[240] but can be rescued by concomitant factor VII or TF

deficiency.[241,242] The anticoagulant properties of TFPIα have been shown to limit pathologic processes that are dependent on TF's procoagulant activity, such as thrombosis and intimal hyperplasia following vascular injury,[243,244] and cancer metastasis[245] in animal models.

Inhibition of TF Cell Signaling

The cell-signaling effects of TF are produced not only indirectly through the actions of thrombin (and perhaps factor Xa) generated downstream in the coagulation cascade but also directly through TF-dependent cell-surface complexes (see section, *Tissue Factor as a Signal Transducer*). Factor VIIa bound to TF, including cryptic TF and factor Xa in the transient TF:VIIa:Xa complex, initiates cell signaling by cleaving and activating protease-activated receptor (PAR)-2.[246,247] TF-dependent signaling through PAR-2 enhances the inflammatory response, angiogenesis, and cellular migration and appears to play an important role in tumor growth and development.[57,248–259] The target of TFPI action is the TF:VIIa:Xa complex, and PAR-2 activation produced by this complex is suppressed by TFPI. The PAR-2 signaling induced by factor VIIa bound to cryptic TF is presumably not affected by TFPI as this complex does not engage factor X(a).[247]

Anti-TF antibodies, active site blocked factor VIIa (VIIai), and TFPIα were shown to reduce the inflammatory response, coagulation changes, and mortality in a baboon model of *E. coli* sepsis.[58,260,261] Treatment with active site blocked factor Xa (Xai) prevented thrombin generation and the resultant coagulopathy, but did not affect mortality in the same baboon model.[262] These results are consistent with the notion that upstream, TF-dependent signals play an important role in inflammation in general and in the pathophysiology of sepsis in particular. A subsequent large phase III clinical trial in sepsis, however, failed to demonstrate an effect of rTFPIα on mortality.[263] The dose of rTFPIα administered in the trial was limited due to hemorrhagic complications noted at higher doses in a prior phase II clinical trial[264] and produced plasma levels (~4 nM) that were >10-fold lower than those that had been shown to be efficacious in the baboon model of *E. coli* sepsis.[261] In the phase III clinical trial, the lower dose of rTFPIα minimally reduced thrombin generation and, in contrast to the baboon studies, did not affect the inflammatory response assessed by interleukin-6 (IL-6) levels. Similarly, preclinical studies had shown that rTFPIα limited inflammation and mortality in animal models of acute lung injury, but a subsequent large clinical trial in severe community-acquired pneumonia, which also used reduced doses of rTFPIα, did not detect an effect on mortality.[265]

TF Independent

Some studies have suggested roles for TFPIα in biologic processes unrelated to its inhibition of the actions of TF, for example bacterial growth,[266–268] angiogenesis,[269–271] and lipoprotein metabolism.[272] In most cases, the observed effect of TFPIα appears to be mediated through its basic carboxyterminal peptide.[266,267,270,272] The levels of full-length TFPIα or carboxyterminal peptide required to elicit these responses, however, are considerably higher than those likely to be encountered *in vivo*. Therefore, additional investigation will be required to determine the physiologic relevance of these observations.

DEFICIENCY OF TFPI

Severe deficiency of TFPI has not been described in humans and may be incompatible with survival. Homozygotic TFPIΔK1 gene disruption in mice produces embryonic lethality[240] and heterozygotic disruption increases thrombosis and intimal hyperplasia following vascular injury,[273] atherosclerosis in the apoE-null mouse model,[274] and the thrombotic phenotype of mice with factor V_{Leiden} or thrombomodulin deficiency.[275,276] Although TFPI certainly plays a key role in the regulation of TF-initiated coagulation, a clear relationship between plasma levels of TFPI and thrombosis has been difficult to demonstrate in humans. In this regard, it must be noted that circulating TFPI represents only a fraction of the total intravascular TFPI that is readily available (see sections, *Cell-Associated TFPI* and *Releasable TFPI*). Two studies, however, have reported a weak association of low levels of plasma TFPI (≤10th percentile) with venous thrombosis[170] and arterial vascular disease.[277] Several polymorphisms within the TFPI gene have been described, but convincing evidence for a relationship between TFPI genotype and thrombotic risk is lacking.

Other Inhibitors of TF:VIIa

In the presence of heparin and under conditions of saturating TF, antithrombin also inhibits the TF:VIIa complex with a second-order rate constant of 5.6×10^3/M/s.[278,279] The heparin enhancement of antithrombin inhibition appears to involve the allosteric mechanism, since the heparin pentasaccharide (fondaparinux) produces a similar effect.[280] Antithrombin and heparin, however, only modestly affect factor X activation by TF:VIIa under the more physiologic conditions of limiting TF.[281,282] This is due to the competition of the substrate, factor X, for TF:VIIa and the exchange of uninhibited factor VIIa for the inhibited factor VIIa–antithrombin complex at TF-binding sites.[283,284] Antithrombin with heparin likely plays a more important role in regulating TF-induced coagulation by inhibiting the activation of factor VII by factor Xa, thrombin, factor IXa, and other proteases. Thus, in reactions containing zymogen factor VII, rather than factor VIIa, the activation of factor X is dramatically delayed in the presence of antithrombin and heparin.[281,282] Plasminogen activator inhibitor-1 and protein C inhibitor have also been shown to inhibit VIIa in the TF:VIIa complex, but the physiologic role of these reactions is not clear.[285,286]

Several artificial inhibitors of TF:VIIa complex have been developed. These include a potent inhibitor consisting of the light chain of factor Xa linked to the first Kunitz domain of TFPI[287] and other Kunitz-type inhibitors that have been engineered for increased reactivity with TF:VIIa.[288,289] An inhibitor with enhanced potency for factor VIIa was created by linking a modified Kunitz-type inhibitor with a mutated form of soluble TF.[290] In addition, an inhibitor that targets TF:VIIa complex, termed *NAPc2*, has been cloned from hookworms[291] and has been evaluated in clinical studies as a novel antithrombotic agent.[292] Blocking antibodies to TF or factor VIIa have been utilized in animal models of sepsis and thrombosis, in which they reduce the severity of coagulopathy or thrombosis.[58,293–295] Active site–blocked factor VIIa (factor VIIai) has also been successfully used as a selective inhibitor of TF function in animal models of thrombotic disease.[59,260,296–298] Novel peptide inhibitors that bind to exosites on factor VII/VIIa and allosterically inhibit factor

VIIa have been reported.[299] Several studies have reported that blocking TF:VIIa complex is effective in decreasing thrombosis without causing bleeding side effects associated with other antithrombotic agents.[298,300–302] This suggests that inhibitors directed at TF or factor VII may have advantages over conventional antithrombotic drugs, an area that will doubtless continue to be the subject of considerable research interest in the future. Indeed, several lead compounds for the development of small molecule inhibitors of TF:VIIa have been reported.[302–304]

DIAGNOSTIC AND THERAPEUTIC USES OF TISSUE FACTOR AND FACTOR VIIA

The commonly employed prothrombin time (PT) clotting assay uses a source of TF as the procoagulant agent. Typically, PT reagents are made from homogenates of brain, lung, or placenta (of human, rabbit, or bovine origin) and are often referred to as *thromboplastins*. Because these reagents have varying amounts of TF, PLs, and contaminants, they have varying potencies. A major application of the PT assay is to monitor dosage for oral anticoagulant therapy, but the results obtained with this assay may vary according to the sensitivity of the thromboplastin reagents. To control for differing thromboplastin sensitivities, a standardization system for these reagents has been developed—the international sensitivity index (ISI)—which is used in conjunction with a normalization method, termed the international normalized ratio, for reporting prolongations of the PT in patients receiving oral anticoagulant therapy.[305] Recombinant thromboplastin reagents are now commercially available that are made from purified, recombinant TF that has been reconstituted into PL vesicles. Because the recombinant reagents have a defined composition, their ISI values can be adjusted readily by the manufacturer to more closely resemble international reference standards for thromboplastins[306] (see Chapter 50).

A clotting test has been developed that uses soluble TF in place of full-length TF[4] to measure trace levels of circulating factor VIIa. This assay takes advantage of the fact that soluble TF does not promote the conversion of factor VII to VIIa,[307] so clotting times are dependent upon the preexisting levels of plasma factor VIIa.

Soluble TF has also been used as the toxic payload in experimental antineoplastic agents. In this approach, soluble TF is linked to targeting molecules in order to deliver it to tumor vasculature, where it can kill tumors by infarction.[308]

Recombinant human factor VIIa has been developed for use as a pharmaceutical agent, termed *eptacog alfa*, and has been marketed under the brand names NovoSeven or NiaStase. It is approved for use in treating bleeding episodes in patients with hemophilia who have intractable inhibitors (blocking antibodies) to factor VIII and who therefore fail to respond to factor VIII replacement therapy.[309] It is also being investigated as a more general hemostatic agent[310] and has seen increasing "off-label" use to control bleeding in nonhemophilic patients.[311] High levels of plasma factor VIIa permit the bypass of the factor VIIIa:factor IXa part of the clotting cascade in a manner that is not fully understood. High levels of factor VIIa may do this by enhancing the activity of TF that is exposed at sites of vascular injury or by directly activating factor X (especially in the presence of negatively charged PLs at the site of injury) in a TF-independent manner.[312] Newer-generation variants of recombinant factor VIIa with increased intrinsic enzymatic activity[313] or longer circulating half-lives[314] are under development and testing, which may have advantages for treating bleeding.

ROLES OF FACTOR VII/VIIA AND TISSUE FACTOR IN PROTHROMBOTIC STATES

The blood clotting system is composed of multiple hemostatic factors that can be subdivided into procoagulant, anticoagulant, and fibrinolytic arms, which must act in a balanced fashion so that blood clots are formed only for the purpose of stopping blood loss following injury. Certain hereditary conditions and disease states can predispose individuals to episodes of thrombosis and are often called *prothrombotic* or *hypercoagulable* states. Alterations in the quantity or activity of a number of hemostatic factors—including factor VII and TF—have been reported to be associated with altered risk of thrombotic disease.

Several methods exist for quantifying plasma factor VII levels, including measurements of total factor VII mass (factor VII + VIIa), factor VII coagulant activity (FVII:C), or factor VIIa. Total factor VII mass is typically quantified by measuring factor VII antigen levels (FVII:Ag)[315] or by the coupled amidolytic assay (FVII:Am), an activity assay in which factor VII is quantitatively converted to factor VIIa.[316] FVII:C assays measure the procoagulant activity of factor VII in a plasma sample in a clot-based assay. Because some of the factor VII is converted to VIIa during the test, FVII:C assays measure an aggregate of both factor VII and factor VIIa.[317,318] Factor VIIa levels can be measured using a soluble TF-based clotting assay[4] or a commercial ELISA in which active factor VIIa reacts with a biotinylated enzyme inhibitor.

Elevated plasma factor VII was initially identified as a risk factor for thrombotic disease in several epidemiologic studies,[319,320] particularly when fatal events were analyzed in long-term follow-up.[321] Other epidemiologic studies have reported correlations between elevated plasma factor VII:C or VIIa levels and angina pectoris, transient ischemic attacks, diabetes, uremia, and peripheral vascular disease.[322–331] These studies are complicated by the fact that the activation state of plasma factor VII is influenced by plasma lipid levels.[15,16,332,333] On the other hand, other studies have failed to find a relation between factor VII levels and thrombotic disease.[334,335] Population studies have reported that factor VII levels are unrelated to the degree of carotid artery thickness or other manifestations of vascular disease.[336–340]

Several studies have directly investigated the relation between plasma factor VIIa levels and risk of thrombotic disease. Some studies have found significant positive correlations,[341] whereas a few have found significant negative correlations.[342,343] In some studies, factor VIIa levels were found to be significantly related to the risk of thrombotic disease only in women.[341,342] The relation between variation in plasma factor VIIa levels and risk of thrombotic disease is therefore currently unclear.

Naturally occurring polymorphisms have been identified in the factor VII gene. This includes a substitution of Gln for Arg353 in the protease domain of factor VII[344] as well as mutations in the promoter region of the gene.[345–348] These polymorphisms are associated with variation in factor VII levels, and, furthermore, some of these markers are in strong linkage disequilibrium with each other.[335,347,349] Some studies[350] have found factor VII genotype to be associated with thrombotic risk, but

others[351,352] have not obtained similar results, and this area remains controversial.[335,349]

BLOOD-BORNE TISSUE FACTOR

TF antigen has been detected at low levels in plasma and urine, and these levels increase significantly in certain pathologic states, such as disseminated intravascular coagulation, thrombotic thrombocytopenic purpura, diabetic microangiopathy, glomerulonephritis, and certain leukemias.[353–356] Whole blood TF activity levels increase following endotoxin challenge and in other settings in which intravascular activation of the blood clotting system can occur, such as sickle cell disease.[357–359]

Giesen et al.[360] reported that TF-containing microparticles, along with monocytes and granulocytes that were positive for TF antigen, accumulated in thrombi formed *ex vivo* by flowing blood over collagen-coated surfaces. Because the perfusions lasted only 5 minutes, there was clearly insufficient time for *de novo* synthesis of TF by leukocytes, and therefore this protein must have come from a preexisting source. Furthermore, these studies suggested that blood-borne TF may play an important role in thrombus formation. Subsequent studies from the same laboratory[361] and other studies have continued to demonstrate that blood-borne TF can be detected at low levels in blood and that it appears to accumulate in thrombi, where it can play an active role in thrombus propagation (reviewed by Morrissey,[362] Morel et al.,[363] and Giesen and Nemerson[364]).

The source of blood-borne TF is currently a matter of intense research. The TF-bearing microparticles shed from leukocytes seem to be a likely source. Monocytes are capable of synthesizing TF following exposure to inflammatory mediators, but low levels of leukocyte TF expression in nonpathologic settings could contribute to blood-borne TF microparticles. TF has been reported to exist in multiple forms intravascularly—as a soluble protein; as a membrane protein in microparticles; and on the surface of, or within, monocytes, granulocytes, and platelets.[360,365–370] Not all of these reports agree with each other, and, in particular, the results concerning the presence of TF in platelets and granulocytes are controversial.[371–373]

HEREDITARY DEFICIENCIES IN TISSUE FACTOR

Human deficiencies in TF have not been reported, leading to the presumption that TF is essential for life. When the TF gene was inactivated in mice, heterozygotes were normal and healthy, but the homozygous TF-null mice died between days E8.5 and E11.5 of embryonic development.[374,375,376] The TF-null embryos exhibited abnormalities in the yolk sac vasculature by approximately day E9.5, and their death was attributed to bleeding from both embryonic and extraembryonic vessels[374,375] and subsequent wasting owing to insufficiency in vitelloembryonic circulation.[376] Some investigators have suggested that these developmental abnormalities may be caused by nonhemostatic roles of TF that are essential for normal blood vessel development[377] (see section, *Nonhemostatic Functions for Tissue Factor and Factor VII*). Interestingly, genetic background influences the morbidity of TF-null mice: In a 129/Sv background, all TF-null embryos died by midgestation, whereas in a C57BL/6 background, two populations of null embryos were observed, one that died at midgestation and another that died at birth.[378]

Although TF-null mice die during embryogenesis, mice in which only the cytoplasmic domain of TF has been deleted develop normally and show no bleeding tendency.[379,380] In addition, fibroblasts from these mice show normal signal transduction when factor VIIa binds to the mutant TF. On the other hand, these mice have reduced immunoinflammatory responses, macrophage function, and susceptibility to arthritis[381]; and they have blunted leukocyte responses to bacterial endotoxin challenge and decreased susceptibility to death from endotoxemia.[382]

TF-null mice can be rescued by using a human TF "minigene" that is expressed at only 1% the level of the endogenous murine TF gene.[383] These low-TF mice develop normally but have shorter life spans than do wildtype mice. Female low-TF mice may have fatal bleeding episodes during pregnancy, and low-TF mice exhibit frequent spontaneous hemorrhage, deposition of hemosiderin, and fibrosis in cardiac tissue.[384] Interestingly, mice engineered to express extremely low levels of factor VII also show significant hemosiderin and fibrosis in their hearts.[384] Mice lacking the cytoplasmic tail of TF did not exhibit the same phenotype as low-TF mice, again excluding a role for the cytoplasmic tail in the fibrosis and early death associated with low-TF mice. TF levels are much higher in cardiac muscle than in skeletal muscle,[48] suggesting that TF plays a role in protecting the myocardium from ongoing vascular damage and subsequent hemorrhage. Interestingly, TF has been implicated in cardiomyopathy as a result of increased expression of mRNA and antigen in cardiomyocytes during myocardial ischemia.[385]

HEREDITARY DEFICIENCIES IN FACTOR VII

Hereditary deficiency in factor VII is a rare, recessive autosomal disorder in humans.[386] Individuals with severe factor VII deficiency may exhibit life-threatening bleeding episodes, although the correlation between factor VII:C levels and bleeding tendency is not particularly strong. The molecular basis for inherited factor VII deficiency has been determined for a number of individuals, resulting in the identification of a variety of nonsense, missense, and splice-site mutations throughout the factor VII coding region, as well as a few small deletions and at least one mutation in the promoter region that causes reduced factor VII levels[387] (see Chapter 53).

Factor VII knockout mice develop normally *in utero* but die neonatally from massive hemorrhage.[388] This finding parallels that of a splice-site mutation in the human factor VII gene, which results in an in-frame deletion of the region encoding the first EGF domain of factor VII in the mature mRNA.[389] A human infant who is homozygous for this mutation had no detectable plasma factor VII activity and no developmental abnormalities, but died from severe postnatal bleeding. Taken together, these results indicate that a total lack of factor VII is not compatible with life.

NONHEMOSTATIC FUNCTIONS FOR TISSUE FACTOR AND FACTOR VII

Tissue Factor as a Signal Transducer

A role for TF as a signaling molecule is now well documented and appears unconnected to its role in coagulation. Initial reports established that binding of factor VIIa to TF evoked intracellular calcium responses in a variety of cell types.[390,391]

These observations were followed by reports of phosphorylation of several mitogen-activating protein (MAP) kinases, such as p38 and JNK, in response to binding of factor VIIa to TF.[392,393] Activation of the Src family members, c-Src, Lyn, and Yes, by TF:VIIa complex in fibroblasts triggers activation of the downstream GTPases Rac and Cdc42 by phosphatidylinositol 3-kinase,[394] ultimately resulting in p38 MAP kinase activation and cytoskeletal reorganization. Interestingly, these responses depend on the catalytic activity of factor VII because factor VIIai (which binds tightly to TF but which has no enzymatic activity) or blocking anti-TF antibodies abolish these effects.[391–393,395]

A body of evidence shows that TF:VIIa can transduce signals by activation of PAR-1 and PAR-2.[396,397] However, TF:VIIa readily activates downstream coagulation proteases, such as factors IXa and Xa, and, indirectly, thrombin. Because some of these proteases are potent PAR activators, it has been a matter of some speculation whether TF:VIIa directly or indirectly activates PARs. Some groups have reported that factor X is required to generate an intracellular response through TF:VIIa.[246,247] Riewald and Ruf[247] have proposed that newly activated factor X remains bound to TF:VIIa long enough for it to be presented within a ternary TF:VIIa:Xa complex to PARs. Factor Xa in this transient ternary complex is therefore proposed to activate PAR-1 and PAR-2 more efficiently than either TF:VIIa or factor Xa alone. One report has shown that the combination of factor VIIa and factor Xa, but not factor VIIa alone, strongly induced migration of breast cancer cells by MAP kinase phosphorylation.[398] On the other hand, certain studies have employed specific factor Xa inhibitors, such as tick anticoagulant protein, or thrombin inhibitors, such as hirudin, in their experiments to show that neither factor Xa nor thrombin is necessary for TF signaling.[392,399,400] These differences in apparent mechanism could potentially arise from the use of different cell types or different concentrations of factor VIIa.

Both TF and PAR-2 are specifically upregulated in response to inflammatory stimuli.[401,402] Ahamed and Ruf[403] showed that phosphorylation of the TF cytoplasmic domain was induced by PAR-2 but not PAR-1 upon ternary TF:VIIa:Xa complex formation. Other coagulation proteases failed to induce phosphorylation of the TF cytoplasmic domain in endothelial cells, suggesting that PAR-2 activation distinguishes between upstream and downstream coagulation protease signaling. The reported effects of PAR-2 and TF suggest that these systems could act in concert to control cell migration. Indeed, it was recently demonstrated that binding of TF to factor VIIa resulted in increased cell migration in a breast cancer cell line because of specific upregulation of interleukin-8 through PAR-2.[255]

The function of the cytoplasmic domain of TF in signaling is not completely defined. It is not essential for induction of gene expression by TF:VIIa,[404] but both intra- and extracellular domains of TF are required for the prometastatic function of TF, at least in some model systems.[405,406] Serine residues in the cytoplasmic tail of TF are phosphorylated by protein kinase C–mediated events.[35] Chimeric molecules, generated from extracellular and transmembrane parts of the interleukin-2 receptor and the cytoplasmic domain of TF, interacted with actin-binding protein-280 (ABP-280) by the cytoplasmic domain of TF.[407] Ser253 and Ser258 in the cytoplasmic tail of TF were crucial for this interaction. ABP-280 is fundamental for normal cell migration and implicates TF in cytoskeletal organization.

Binding of factor VIIa to TF on human fibroblasts can enhance chemotaxis by 100-fold.[400] This stimulation takes place via platelet-derived growth factor-B isoform through activation of phospholipase C. Adhesion and migration of mononuclear phagocytes on endothelial cells have been shown to be TF dependent.[408] TF-based peptides and antibodies to TF inhibited this migration. This evidence, along with the role of the TF cytoplasmic tail in binding of ABP-280 and the association of TF with PAR-2, demonstrates a possible function for TF in adhesion and migration. These findings may provide an explanation for the value of inhibiting TF:VIIa activity in reducing neointima formation in animal models with restenosis.[297]

Tissue Factor in Angiogenesis and Tumor Development

A role for TF in both embryonic and tumor angiogenesis has been described extensively. It is likely that TF contributes to angiogenesis by both clotting-dependent and clotting-independent pathways.[409] Clotting-dependent pathways are likely to involve the production of the downstream protease thrombin, activation of PAR-1 (and possibly other PARs), and subsequent signal transduction. Clotting-independent pathways may involve phosphorylation of the cytoplasmic tail of TF.[410]

A significant relation exists between TF expression and tumor vascularization.[411,412] In a mouse xenograft model, transfection of tumor cells with antisense TF or injecting specific antibodies to TF reduced tumor growth and vascularization.[413,414] Factor VIIai has been shown to have antimetastatic properties, demonstrating the importance of factor VIIa proteolytic activity in the metastatic process.[406] Overexpression of TF in tumor cells contributes to an angiogenic phenotype by both upregulating the expression of the proangiogenic molecule vascular endothelial growth factor (VEGF) and downregulating the antiangiogenic protein thrombospondin.[410,413] Significant correlations exist between expression of TF and VEGF in human tumors.[411,415,416] Embryos lacking VEGF or TF have similar histopathologies.[417,418] Together, these findings suggest that VEGF and TF might regulate similar functions *in vivo*. One study, using TF mutants lacking the serine residues in the cytoplasmic domain, showed that this domain of TF was essential in VEGF regulation.[419] This report was not substantiated by others,[420] and some investigators have suggested that upregulation of VEGF requires generation of factor Xa.[421,422] Despite conflicting reports on the mechanism involved, it is clear that TF can indeed upregulate VEGF. The converse is also true, that is, VEGF upregulates TF expression by Egr-1 on endothelial cells.[423] The wealth of information derived from mouse genetics and clinical studies has led to the idea that TF plays direct and indirect roles in tumor development and neovascularization.

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CHAPTER

12 Factor V and Factor VIII

RANDAL J. KAUFMAN, PHILIP J. FAY, LAURA POPOLO, AND THOMAS L. ORTEL

Factor V and factor VIII are two homologous nonenzymatic proteins that play a critical role in blood coagulation, an essential process that involves the sequential activation of plasma proteases in response to blood vessel injury. Factor V and factor VIII circulate in plasma in inactive forms that are devoid of coagulant activity. In their active state, factors Va and VIIIa serve as cofactors for their respective proteases (factor Xa and factor IXa), resulting in a striking enhancement of their catalytic activity.

To maximally express their function, factor VIIIa and factor Va need to assemble into enzyme complexes. Factor VIIIa together with factor IXa, calcium ions, and phospholipid membranes forms the *intrinsic Xase complex*. This complex catalyzes the proteolytic conversion of the serine protease factor X to factor Xa. Subsequently, factor Xa, factor Va, Ca^{2+}, and phospholipid membranes form the *prothrombinase complex* that converts prothrombin to thrombin. The thrombin-mediated conversion of soluble fibrinogen to insoluble fibrin generates a stable platelet–fibrin clot at the site of vascular injury. Thus, factor V and factor VIII catalyze pivotal steps in the coagulation cascade. This is also underscored by the clinical findings, which indicate that conditions of factor V or factor VIII deficiency lead to parahemophilia and hemophilia A, respectively.

The activation of factor V and factor VIII consists of proteolytic cleavage catalyzed by thrombin or factor Xa. The activation process has been the subject of intense investigation during the last two decades. Since proteolysis is an irreversible protein modification, it needs to be spatially and temporally controlled. Efficient inactivation mechanisms targeting the cofactors exist and are essential for normal hemostasis. One key reaction in down-regulating coagulation is the inactivation of factor Va by the anticoagulant-activated protein C (APC). Cleavage of factor Va at specific sites converts it into factor Vai with a complete loss of cofactor activity. Factor VIIIa is also a substrate of APC, but the intrinsic instability of factor VIIIa may make this reaction irrelevant from a biologic perspective.

Although the knowledge of factor V and factor VIII and their regulation has progressed significantly over the last two decades, the molecular mechanisms by which protein cofactors factor V and factor VIII accelerate substrate cleavage remains to be fully defined. The difficulties in having homogeneous preparations of these factors were partially overcome by the advent of recombinant DNA technologies. However, the dynamics and interactions of these factors with partners (ions, membranes, activators and inhibitors, plasma proteins) are many, and their analyses require a reductionist approach and a rather complex process of critical integration and interpretation of the results obtained. The molecular models that were recently built on the basis of the crystal structures of factor VIII, factor Vai, and their interacting proteins (proteases and substrate-proteases) provide a background to gain insight into the mechanistic details of how the structure of these factors changes to fulfill their function.

FROM GENE TO PROTEIN

Factor V and Factor VIII: Historical Background

Coagulation factor V and factor VIII were discovered in the 1930s and 1940s. Quick[1] first reported the identification of a labile factor in plasma that was necessary for the rapid conversion of prothrombin to thrombin. At approximately the same time, Owren[2] identified a patient with a severe bleeding disorder (parahemophilia), who lacked this factor. This protein, which was the fifth coagulation factor to be discovered, was designated factor V. Subsequent work established that activated factor V functions as a nonenzymatic cofactor in the *prothrombinase* complex.[3] The observation that a factor V mutation is the most common risk factor for venous thrombosis[4] has drawn attention to this protein, leading to many advances in the field.

Factor VIII identification was tightly linked to characterization of hemophilia A, a severe bleeding disorder caused by deficiency of factor VIII. Transfusion of whole blood was shown to successfully treat a hemophilia-associated bleeding episode by 1840.[5] In 1911, Addis[6] demonstrated that normal plasma can shorten the clotting time of hemophilic blood. It was 25 years later when Patek and Taylor described the role of factor VIII in hemostasis and designated the factor as "antihemophilic globulin."[7] In the 1950s, plasma concentrates and then in 1965, cryoprecipitates were developed for treatment of bleeding episodes associated with hemophilia A. In the early 1980s, factor VIII was purified from human plasma[8] and subsequently the gene was cloned. At approximately the same time, the human factor V gene was isolated. Over the past three decades, considerable efforts have been made defining the cofactor activity and its regulation.

Organization of Factor V and Factor VIII Genes

The genes encoding human factor V and factor VIII,[9,10] named *F5* and *F8*, respectively, have an almost identical exon–intron organization.[11,12] *F5* is localized to human chromosome 1q21–25.[13] The gene spans approximately 80 kilobase (kb) pairs of DNA and consists of 25 exons and 24 introns (FIGURE 12.1). The exons range in size from 72 to 286 base pairs (bp) with the exception of exon 13, which spans 2,820 bp. The introns range in size from 448 bp to 11.4 kb. The length of *F5* mRNA is approximately 7 kb.[14–16] At the present time, there is no evidence for alternative splicing of *F5* transcripts, and the mechanisms

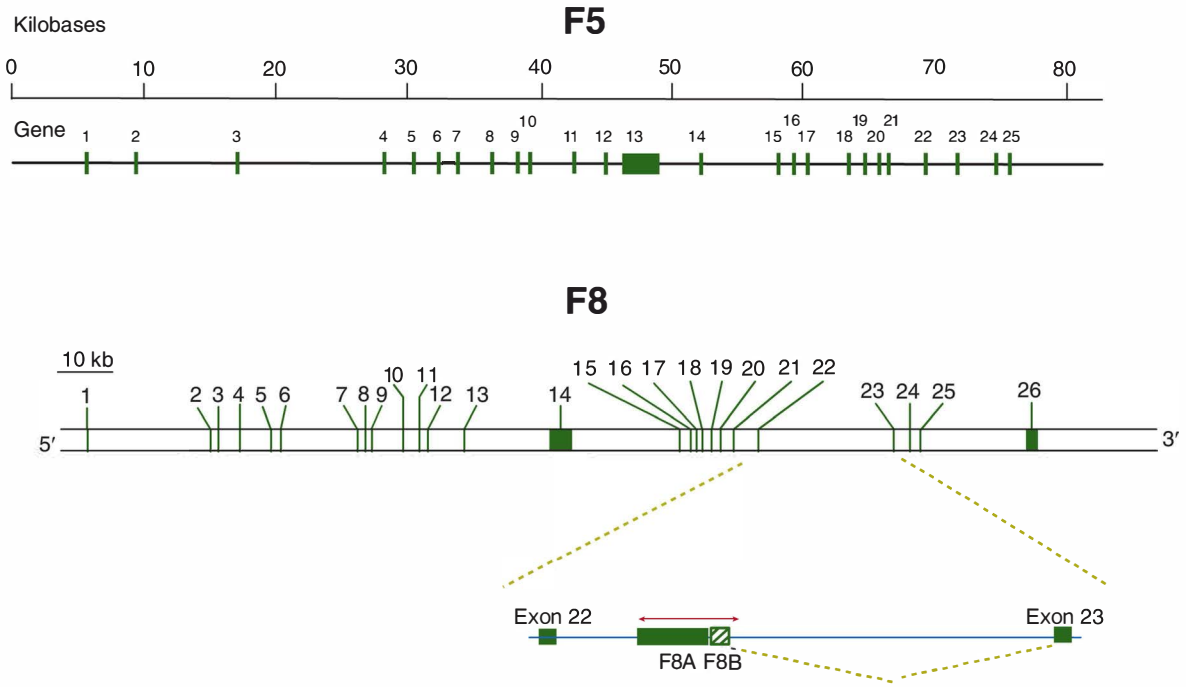

FIGURE 12.1 Features of the factor V and factor VIII encoding genes. The top line shows the scale of *F5* genomic sequence. The second line shows the intron–exon organization of *F5* with the exons being represented by *green boxes*. For *F8*, the bar represents the scale, and the bottom of the figure shows the intron–exon organization with exons represented by *green boxes*. The genomic region between exons 22 and 23 is enlarged to show the direction of transcription of *F8A* and *F8B* (see text).

responsible for the control of transcription and translation remain unknown. A rich report of this gene and the surrounding genomic region is available at NCBI (http://www.ncbi.nlm.nih.gov/gene/2153).

F8 is localized on human X chromosome (Xq28). The gene is 186 kb long (~0.1% of the DNA of the X chromosome) and contains 26 exons and 25 introns (FIGURE 12.1). The exon length varies from 69 to 262 bp except for exon 14 that is 3,106 bp and the last exon 26 that has 1,958 bp. There are some large intervening sequences including IVS22 that is 32 kb long. *F8* mRNA is approximately 9 kb, of which the coding sequence covers 7,053 nucleotides. In the gene, a CpG island within IVS22 is associated with two additional transcripts. One transcript of 1.8 kb is produced abundantly in a wide variety of cells. The orientation of this transcript is opposite to that of factor VIII and contains no intervening sequence.[17] This cDNA of 1.7 kb, termed factor VIII–associated gene A (*F8A*), is conserved in the mouse.[18] The second transcript of 2.5 kb, named factor VIII-associated gene B (*F8B*), is transcribed in the same direction as factor VIII, and after a short exon that may encode for eight amino acids, it utilizes exons 23 to 26 of the *F8* gene[19] (FIGURE 12.1). The two transcripts *F8A* and *F8B* originate within 122 bases from each other. The sequences of *F8A* and *F8B* along with a few kilobases of surrounding DNA are also present in two other areas of the X chromosome approximately 400 kb telomeric to *F8*.[17,20] The function of these transcripts and their potential protein products is unknown. Transgenic mice with a deletion of the *F8B* gene showed particular eye abnormalities, suggesting that migration of neural crest cells might have been perturbed during eye development.[21] For a detailed report and graphical view of *F8* and its genomic context, see http://www.ncbi.nlm.nih.gov/gene/2157.

Features of Factor V and Factor VIII Proteins

F5 and *F8* encode large precursor proteins of 2,224 and 2,351 amino acids, respectively. At the *N*-terminal, both precursors have a signal peptide, of 19 and 28 amino acids, respectively, that is cleaved upon translocation in the lumen of the endoplasmic reticulum (ER). Factors V and VIII share a common organization of three structural domains that occur in the same order A1-A2-B-A3-C1-C2 (FIGURES 12.2 and 12.3) and a high degree of amino acid identity (about 40%) over the entire length except for the B domains where there is no similarity. The B domain is encoded by an unusually large single exon spanning 3.1 and 2.8 kb in FVIII and FV, respectively. In general, the B-domain sequence is about 900 amino acids in length and rich in flexible amino acids (e.g., serine, threonine, glycine, and proline) and *N*- and *O*-glycosylation sites. A unique feature of both B domains is their unusual number of *N*-linked oligosaccharides. The factor VIII and factor V B-domains have 18 and 25 *N*-linked glycosylation sites, respectively. In the human factor V B-domain, two tandem repeats of a 17-amino acid sequence and 31 tandem repeats of a nine–amino acid sequence are present (FIGURE 12.2). The consensus sequences for these two types of repeats are SQDTGSPSXMPRPWEDXP and [T,N,P]LSPDLSQT. The significance of the tandem repeats structures that are present in the B domain of factor V, but not factor VIII, remains to be determined.

The three A-type domains present in factor V and factor VIII are approximately 330 amino acids in length, and are

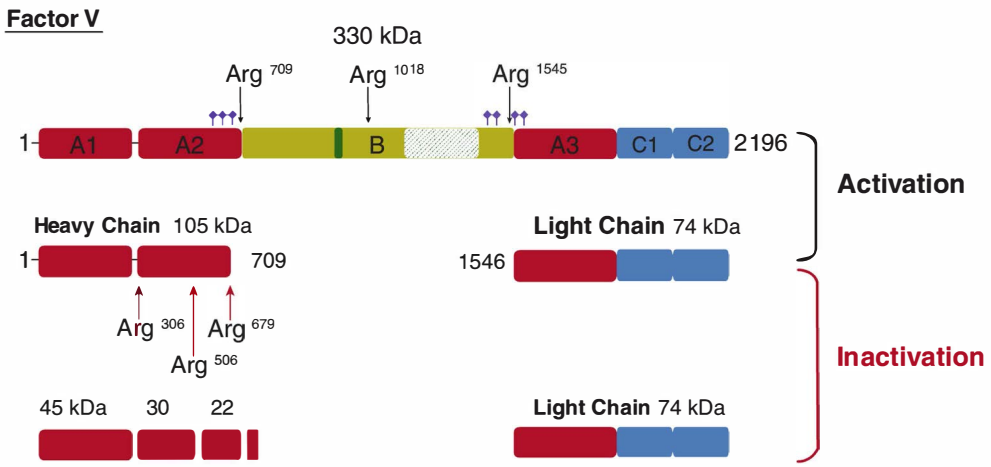

FIGURE 12.2 Domain structure and proteolytic activation/inactivation of human factor V. The structural domains of the single-chain factor V are depicted in the top line by the labeled *boxes*: (i) a triplicated A domain of approximately 330 amino acids; (ii) a unique B domain of 890 amino acids; and (iii) a duplicated C domain of approximately 150 amino acids. The tandem repeats of 2 × 17 amino acids are represented by the *green box* and the 9 × 31 amino acids by the *dashed green box*. The location of potential disulfide bonds and asparagine-linked glycosylation sites is not shown for simplicity (see Accession number P12259 at UniprotKB: http://www.uniprot.org/uniprot/). The *violet diamonds* indicate the location of putative sulfotyrosines. The thrombin cleavage sites are indicated by the *black arrows* at the top of the figure. The location of the APC cleavage sites are indicated by *red arrows* at the bottom of the scheme. The apparent molecular mass of the intermediates and final products following activation by thrombin and inactivation by APC are indicated. The heavy chain and light chain of thrombin-activated factor V (Va) form a calcium-dependent heterodimer. The numbering refers to the amino acid sequence devoid of the signal sequence.

about 30% identical to the triplicated A-type domains in the copper-binding proteins ceruloplasmin and hephaestin and to the single A domain of ferroxidase.[9,10,16,22] The C-type domains in factor V and factor VIII are approximately 150 amino acids in length and are homologous with discoidin 1, a lectin that mediates cell adhesion and migration in the single-cell organism *Dictyostelium discoideum*. Discoidin 1 domains are found in a growing family of proteins implicated in cell adhesion and/or developmental processes.[23,24]

In addition to these domains, in factor VIII there are three acidic amino acid–rich regions (a1, a2, a3) that reside at the junction of the A1/A2 (a1: residues 336 to 372), A2/B (a2: residues 711 to 740), and B/A3 (a3: residues 1649 to 1689) domains (FIGURE 12.3). Moreover, both factor V and factor VIII have been shown to bind copper and calcium ions.[25,26]

Within the secretory pathway, factor VIII is processed to a heterodimer of the heavy-chain fragment of variable size (apparent molecular weight ~90 to 200 kDa)[7,8] in a metal-dependent association with the light chain of 80 kDa (FIGURE 12.3). The A1, A2, and B domains occur in the heavy chain whereas the A3, C1, and C2 domains occur in the light chain. In plasma, human factor VIII is present at a concentration of approximately 1 nM (150 ng/mL) and is stabilized by association with a 50-fold molar subunit excess of von Willebrand factor (vWF). Human factor V circulates in the plasma as single-chain glycoprotein of high apparent molecular weight (~330 kDa) (FIGURE 12.2). Its concentration is approximately 30 nM (10 μg/mL).

The analysis of homologies of the primary sequences of factors V and factors VIII from different species suggests that these genes evolved from an ancestral ferroxidase gene by triplication of the A domain, insertion of the B domain, and addition of the C domains to the primordial cofactor gene. Subsequently, the factor V and factor VIII genes possibly evolved by duplication and divergence of amino acid residues within the B domain, although the high number of *N*-glycosylation sites were preserved, while amino acid sequences within the A and C domains were conserved.

Biosynthesis of Factor V and Factor VIII

Factor V Expression

The main site of factor V biosynthesis is the liver. Human megakaryocytes also have been shown to contain factor V mRNA and to express factor V[27] but it is believed that most or all of platelet factor V originates from the plasma pool by endocytosis.[28,29] As uptake of factor V by megakaryocytes has not been demonstrated, the origin of platelet factor V in humans remains controversial.[30] However, total factor V is distributed into two pools: 20% is contained in platelet granules whereas the remaining 80% is in the plasma compartment. The storage of factor V within platelets appears to be important because, when platelets aggregate to form the primary hemostatic plug, they also provide both a surface for assembly of the *prothrombinase* complex as well as high local concentrations of released platelet factor V. In addition, residual platelet factor V supports thrombin generation in patients with severe factor V deficiency and mild bleeding symptoms.[31] The synthesis of factor V by several other cell types, including bovine aortic endothelial cells, bovine vascular smooth muscle cells, and human mesangial cells, has also been reported, although the physiologic importance of synthesis at these sites remains unknown.

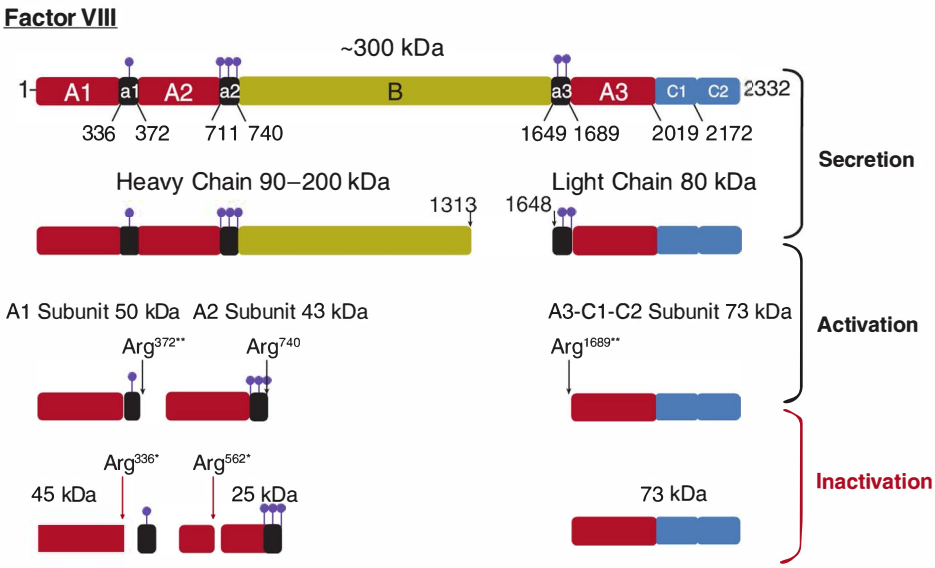

FIGURE 12.3 Domain organization and proteolytic activation/inactivation of human factor VIII. The structural domains of the full-length factor VIII are depicted in the *top line* by the labeled *boxes*: (i) a triplicated A domain of about 330 amino acids, (ii) a unique B domain of 980 amino acids, and (iii) a duplicated C domain of 150 amino acids. The *black boxes* represent regions enriched in acidic amino acids. The ball-stick symbol (*violet*) represents the positions of sulfotyrosine residues that were experimentally determined (residues 346, 718, 719, 723, 1664, 1680). The location of free cysteine residues, disulfide bonds, and asparagine-linked glycosylation sites is not shown (see Accession number P00451 at UniprotKB Database: http://www.uniprot.org/uniprot/). Within the Golgi compartment in the cell, factor VIII is cleaved at two sites within the B domain to generate heavy-chain polypeptides of variable size (90 to 200 kDa) and the 80-kDa light chain. The cleavage sites for activation by thrombin (*black arrows*) and inactivation by APC (*red arrows*) are shown at the point of the processed products. The apparent molecular mass (kDa) of the intermediates and final products are indicated. The two cleavages required for thrombin activation (**) and the sites of proteolytic inactivation by APC (*) are indicated. The numbering refers to the amino acid sequence devoid of the signal sequence.

Factor VIII Expression

Although the natural cell type that produces factor VIII has not been definitively identified, liver transplantation studies in factor VIII-deficient dogs[32] and several hemophiliac patients[33] strongly implicate the liver and/or the reticuloendothelial system as primary sites of factor VIII synthesis. Initial immunochemical localization by light microscopic[34] or electron microscopic[35] examination detected the factor VIII antigen in hepatocytes. However, RNA hybridization analysis detected factor VIII mRNA in hepatocytes as well as in many other cells and tissues.[36] More recent analysis of factor VIII mRNA by quantitative reverse transcription polymerase chain reaction identified highest levels in liver, followed by kidney.[37] Factor VIII mRNA and protein were concentrated in hepatic sinusoidal endothelial or Kupffer cells, with significantly less in hepatocytes.[37] In addition, isolated sinusoidal endothelial cells were shown to produce factor VIII.[38] Also a part of factor VIII is contained in α granules of platelets together with factor V.[39] Since there are no known natural established cell lines that express factor VIII, it has not been possible to study the biosynthesis of factor VIII in its native host cell.

Trafficking and Modification within the Cell

Factor V and factor VIII are abundantly modified glycoproteins secreted into the blood. The factor VIII biosynthetic pathway was proposed based on analysis of factor VIII expression in transfected cell lines, such as Chinese hamster ovary cells (FIGURE 12.4). On synthesis, factor VIII is translocated into the lumen of the ER where the signal peptide is cleaved and the protein is subjected to *N*-linked core glycosylation (FIGURE 12.4 steps 1 to 3, see also Chapter 5 "Cell Biology, Protein Processing, and Cell Signaling"). A significant portion of newly synthesized factor VIII is retained in the ER through interactions with two protein chaperone systems designed to prevent the exit of unfolded proteins from the ER. First, factor VIII is bound to BiP, the most abundant ER protein[40] (FIGURE 12.4, step 4). BiP expression is induced by glucose deprivation, inhibition of *N*-linked glycosylation, or the presence of malfolded protein within the ER. In addition, high-level factor VIII expression can also induce transcription of the BiP gene indicating the induction of the unfolded protein response (for details, see Chapter 5).[41] The level of BiP in the cell inversely correlates with the efficiency of factor VIII secretion.[42,43] BiP exhibits a peptide-dependent adenosine triphosphatase activity. Factor VIII dissociation from BiP and secretion requires an unusually high level of intracellular adenosine triphosphate (ATP).[44] Because factor VIII interacts with BiP, and factor V does not, it was possible to identify a primary BiP-binding site by analysis of the secretion of factor VIII and factor V chimeric proteins. This study identified a hydrophobic beta-sheet within the factor VIII A1 domain that resides adjacent to a proposed ligand, Cys310, for binding copper ion that was also recently confirmed by the crystal structure of FVIII (see further FIGURE 12.6).[26,45,46] Exposed hydrophobic residues are

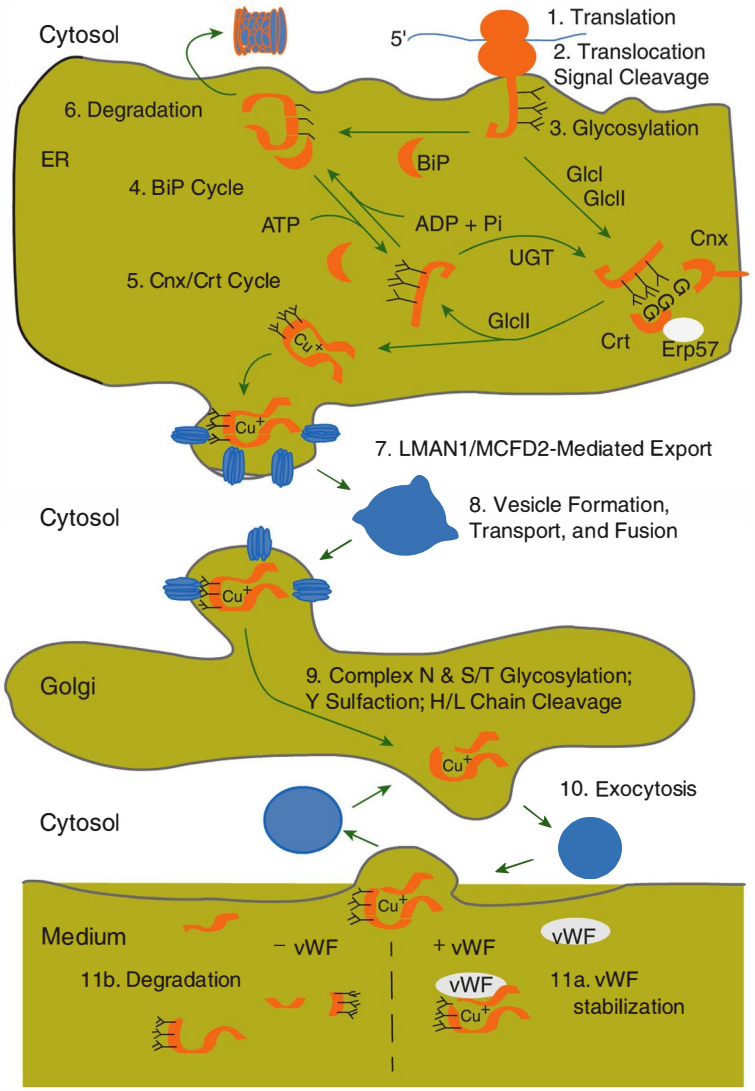

FIGURE 12.4 Synthesis, processing, and secretion of factor VIII in mammalian cells. The figure depicts the pathway of biosynthesis of factor VIII from the primary translation product that is translocated into the lumen of the ER to the medium as determined by expression in culture cells. For detail in the description see text under "Trafficking and modification within the cell."

preferred sites of BiP binding. Substitution of phenylalanine at residue 309 with serine within the hydrophobic sheet increased the secretion efficiency of factor VIII several-fold and this correlated with a reduced requirement for ATP for secretion.[45] These studies suggest that BiP may have a role in copper ion binding to factor VIII. Finally, a portion of factor VIII forms high molecular weight aggregates immediately after synthesis.[47] These aggregates require ATP for disassembly and secretion.[47]

The second chaperone system that retains factor VIII in the ER recognizes the structure on *N*-linked oligosaccharides on factor VIII. Rapid trimming of the two outermost glucose residues of the *N*-linked glycans (*N*-acetylglucosamine$_2$-mannose$_9$-glucose$_3$) prepares protein-bound *N*-glycans for association with the two homologous ER lectins calnexin (CNX) and calreticulin (CRT) and exposure to the glycoprotein-specific oxidoreductase ERp57 to catalyze proper disulfide bond rearrangement (see Chapter 5).[48,49] Release from CNX/CRT depends on glucosidase II cleavage of the innermost glucose residue. Although the modalities of misfolded protein domain recognition and ER retention are not known,[50,51] the enzyme UDP-glucose:glycoprotein glucosyltransferase (UGT) and the CNX/CRT chaperone system are thought to play central roles.[52,53] Current models[52–56,57] propose that UGT reglucosylates non-native polypeptides thereby preventing their release from the CNX/CRT chaperone system and their exit from the ER. Factor VIII interacts with both CNX and CRT, although their roles in factor trafficking within the ER are not known.[58]

Formation of disulfide bonds by oxidation of a pair of cysteine residues is another crucial modification for proteins destined to be secreted and contributes to the stabilization of the structure. Factor VIII contains eight disulfide bonds and factor V has seven putative disulfide bonds deduced by similarity. The enzyme PDI1 plays the predominant function as catalyst of oxidative folding in the ER. Interestingly, it has been recently reported that treatment with antioxidants improves secretion of factor VIII from cultured cells suggesting that folding of complex multidomain proteins can generate oxidative stress.[59]

Improperly folded proteins are selectively extracted from the ER and directed for degradation by the cytosolic proteasome in a process called ER-associated degradation (*ERAD*, step 6 in FIGURE 12.4). A significant portion of factor VIII within the ER never transits to the Golgi compartment, but rather is

degraded through ERAD (see Chapter 5). Factor VIII within the ER is retrotranslocated and degraded by the cytosolic 26S proteasomal machinery. Recently, *E*R *d*egradation *e*nhancing *m*annosidase-like (EDEMs) proteins were shown to recognize oligosaccharide structures trimmed by ER mannosidase I and mediate their transfer to the cytosol for ERAD.[60]

Finally, studies on combined deficiency of factors V and VIII indicate that oligosaccharides are also important for trafficking of factor VIII and V from the ER to the Golgi compartment through interaction with the cargo receptor complex LMAN1/MCFD2 (FIGURE 12.4, step 7). The sum of these findings supports the notion that oligosaccharide processing plays a central role in directing factor VIII trafficking within the secretory pathway.

The portion of factor VIII that is secretion competent transits to the Golgi apparatus, where the majority of factor VIII is processed at two sites within the B domain after residues 1313 and 1648 to generate the heavy chains (90 to 200 kDa) and the light chain (80 kDa) (FIGURES 12.3 and 12.4). Also within the Golgi apparatus, factor VIII is further processed by (a) modification of the asparagine-linked high mannose-containing oligosaccharides to complex types, (b) addition of carbohydrate to multiple serine and threonine residues within the B domain (*O*-glycosylation), and (c) addition of sulfate to six tyrosine residues within the heavy and the light chains[61] (FIGURE 12.4, step 9).

Factor VIII is finally secreted by exocytosis (FIGURE 12.4, step 10). In the plasma, vWF stabilizes factor VIII through noncovalent association. *In vitro* studies demonstrate that vWF can alter intracellular transport and secretion of factor VIII from the cell. vWF promotes the association of the light and heavy chains of factor VIII upon secretion from the cell and results in stable accumulation of factor VIII activity in the conditioned medium of factor VIII-producing cultured mammalian cells[62,63] (FIGURE 12.4, step 11a). In the absence of vWF in the conditioned medium, the factor VIII heavy and light chains are secreted into the medium as dissociated chains that are subsequently degraded (FIGURE 12.4, step 11b).

In vitro reconstitution experiments demonstrated that vWF can directly promote reassembly of isolated heavy and light chains of factor VIII,[62,64] suggesting a possible role of vWF in facilitating factor VIII assembly. The effect of vWF in promoting factor VIII heavy and light chain assembly and stable secretion in cell culture systems may reflect the role of vWF in regulating levels of factor VIII activity *in vivo*.[65–68] However, it is not known whether vWF also regulates factor VIII protein synthesis and/or secretion *in vivo*.[69] At present, there are no natural cell types that are known to express both factor VIII and vWF.[70] It is proposed that a portion of factor VIII synthesized is coexpressed with vWF from an endogenous endothelial bed and secreted as a complex upon stimulation,[71] although this has not been directly demonstrated *in vivo*.

Metal Ions in Factor V and Factor VIII

Both factors V and VIII bind calcium and copper ions and ion-dependent interactions appear vital for both their structure and function. The recently reported three-dimensional (3-D) structures of these factors identified the position of the high-affinity ion-binding sites and provided new interpretations on the role of ions in factor V and VIII structure and function. Adams et al.[72] solved the structure of bovine factor Vai (A1/A3-C1-C2) (FIGURE 12.5). The two A domains sit on a platform formed by the two C domains, which are aligned in an edge-to-edge fashion, a feature that was found to be present also in the crystal structure of a recombinant single-chain B-domain–deleted human factor VIII (FIGURE 12.6B) and in an intermediate-resolution crystallographic structure of a recombinant form of factor VIII consisting of a heterodimer of the A1-A2 and A3-C1-C2 subunits.[73]

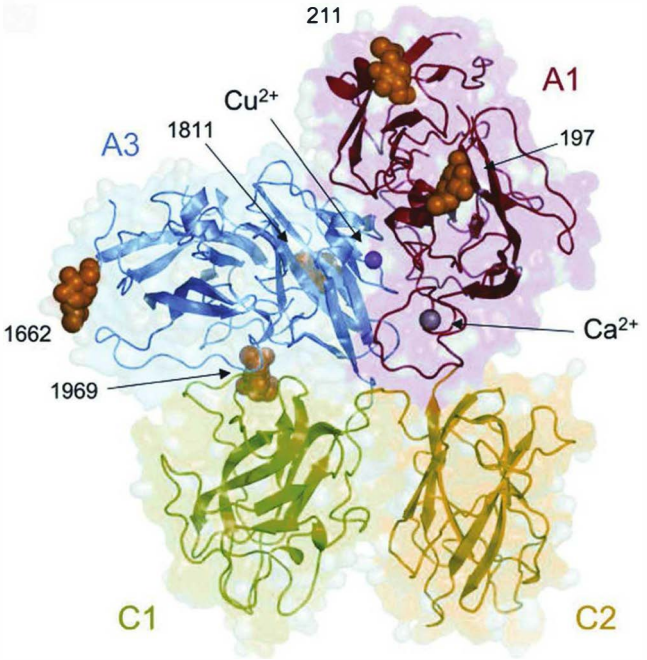

FIGURE 12.5 The 3-D structure of bovine factor Va_i. Domain arrangement in a ribbon representation of bovine factor Va_i indicating the positions of the metal ions within the A1 and A3 domains (Ca^{2+}: *gray* and Cu^{2+}: *pink*) and of five *N*-linked chains (*orange*) at residues N197, N211, N1811, N1662, and N1969. The van der Waals representation in the background shows the surface contour of the protein. The C domains are side-by-side and predict a role in membrane binding (see text). (Adapted from Adams TE, et al. The crystal structure of activated protein C-inactivated bovine factor Va: implications for cofactor function. *PNAS*. 2004;101:8918–1923. Use with permission © 2004 by PNAS).

A high-affinity calcium-binding site and a copper-binding site were identified in factor Vai. Chain association is required for factor V function and is dependent on a divalent cation. Although calcium ion was believed to bridge the heavy and light chains,[74] the structure revealed that the high-affinity calcium-binding site is located entirely in the A1 domain[74] (FIGURE 12.5). The side chains of both Asp111 and Asp112, along with the main-chain carboxyl oxygens of Lys93 and Glu108, coordinate the calcium ion. The structural studies clearly indicate that chain association cannot directly be attributed to the coordination of calcium but rather calcium may play a role in the maintenance of the A1-C3 domain interface.

The role of copper ion in factor V has always been uncertain. A single copper ion-binding site is located in the A3 domain of bovine factor Va_i with ligands His1802, His1804, and Asp1844

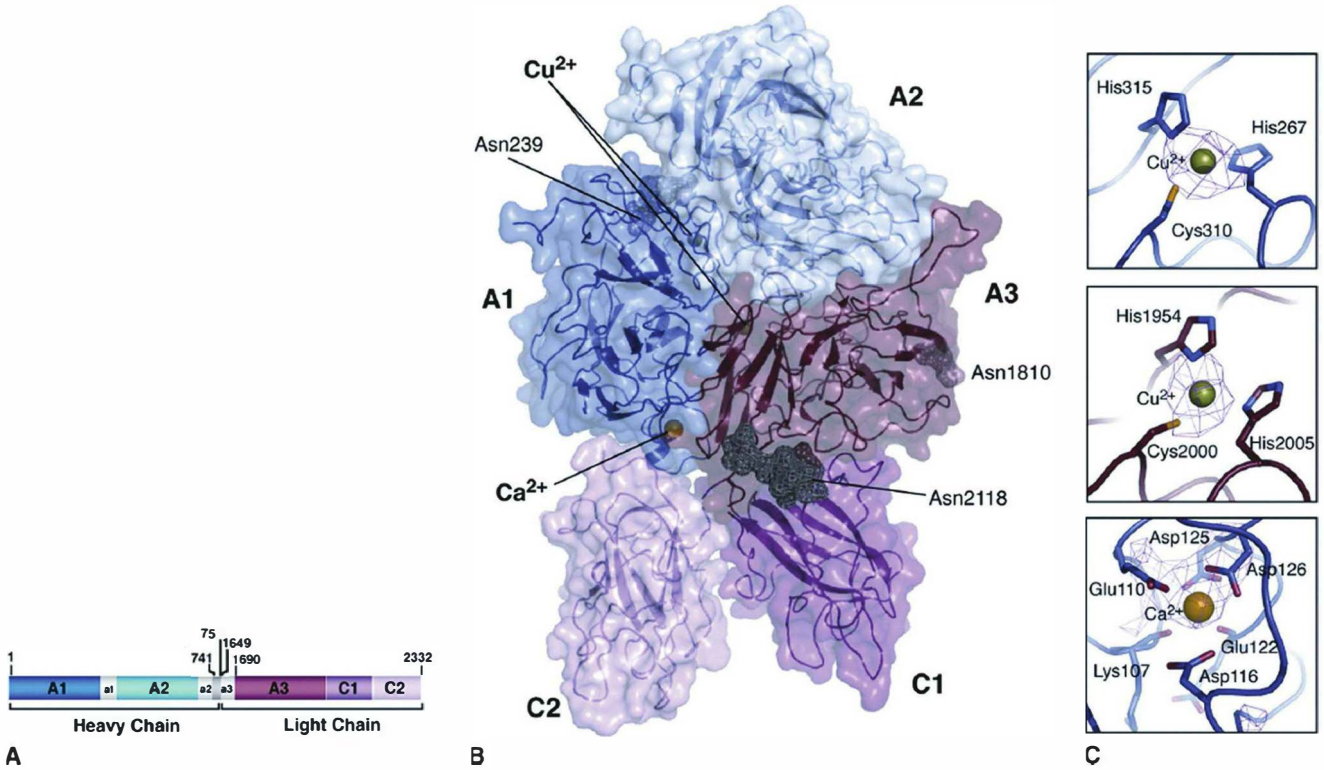

FIGURE 12.6 Three-dimensional structure of B-deleted domain factor VIII. **(A)** Domain organization of the B domain-deleted human factor VIII used for x-ray crystallography. **(B)** Structure of B-domain-deleted factor VIII. The A1 domain and a2 acidic regions are colored in *dark blue*. The A2 domain and the a2 acidic region are in *light blue*. The A3 domain, the C1 domain, and the C2 domain are colored *dark red, dark pink*, and *light pink*, respectively. The a3 acidic region is disordered and not included in the structure. Two Cu^{2+} ions are located internally within the A1 and A3 domains (*green*), one Ca^{2+} (*yellow*) is bound within the A1 domain and three *N*-linked oligosaccharide chains (*gray*) are indicated by *arrows*. **(C)** The amino acids that define the ion-binding sites are shown in detail (for a description, see text). (Adapted from Ngo JC, et al. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure* 2008;16:597–606. Use with permission © 2008 by Elsevier Ltd).

arranged in a trigonal planar coordination geometry.[72] Recently, Song et al.[74] used recombinant factor V mutants to demonstrate that Asp111 in the heavy chain was important for the metal ion binding and for interactions between the heavy and light chains of the active cofactor. However, the structure of factor Vai suggested that copper ion provides additional stabilization of the A1-A3 interface rather than directly linking the two domains.

The crystal structures of factor VIII revealed one calcium-binding site and two copper-binding sites. The position of the calcium-binding site in the A1 domain confirms the region in the A1 domain identified in factor V as a calcium-binding site. In the structure of factor VIII, the calcium is liganded by the carboxyl groups of Glu110, Asp116, Asp125, and Asp126 and the backbone carbonyl of Glu122 and Lys107 as observed for factor V[74] (FIGURE 12.6C). It is noteworthy that mutations of these residues (Asp125Ala and Asp126His) were associated with mild/moderate hemophilia A, whereas mutations at the remaining residues (Glu110Val, Asp116Tyr, Glu110Val, Lys107Thr, and Glu122Lys) were related with severe hemophilia A (http://hgdb.org.uk/). Recent mutational data further support the existence of this calcium-binding site in both factors Va and VIIIa.[74] The calcium ion is unlikely to play a role in directly bridging the light and heavy chains but could be involved in the maintenance of the interface between C2 and A1 domains in a given conformation. In addition, the intermediate-resolution x-ray crystal structure predicts a second calcium-binding site within the A2 domain utilizing residues Asp538 and Asp542.[73]

While previous results indicated a role of calcium in promoting the active conformation of factor VIII, copper ion was primarily involved in enhancing intersubunit affinity.[75] The two copper-binding sites are within the A1 and A3 domains (FIGURE 12.6B). In the A3 domain, the site is composed of His1954, Cys2000, and His2005 and is dispensable for cofactor function.[76] However, a His1954Leu mutation associated with mild hemophilia A has been reported (http://hgdb.org.uk/). The second site in the A1 domain (composed of ligands His267-Cys310-His315) is essential for cofactor function (FIGURE 12.6C). Electron paramagnetic spectroscopy suggests that cupric ion (Cu^{+}) occupies this site.[26] The mutation of Cys310Ser destroyed factor VIII activity and yielded secreted heavy and light chains that were not associated, supporting a requirement for this copper ion-binding site in heavy and light chain association. Interestingly, mutations of Cys310Phe and His267Pro have been reported in patients with severe hemophilia A, suggesting the

importance of this second copper-binding site (http://hgdb.org.uk/). However, this second copper site in the A1 domain localizes near the interface of the A1 and A3 domains rather than at the interface suggesting that a role for copper in enhancing interchain affinity is indirect.

Copper alone is insufficient to generate cofactor activity but in the presence of calcium does marginally increase the specific activity of factor VIII.[64,77] Recombining purified factor VIII chains in buffers containing Ca^{2+} or Mn^{2+} yields active factor VIII.[62,64] These ions do not contribute to the interchain affinity but rather promote cofactor activity by modulating the conformation of the heterodimer.[75]

FUNCTION AND REGULATION OF FACTOR V AND FACTOR VIII

Cofactor Activities of Factor VIII and Factor V

Factor VIII acts to increase the rate of conversion of factor X to factor Xa by the protease factor IXa. The K_m for factor X activation in the presence of factor IXa and calcium is decreased several orders of magnitude by phospholipid due to concentration of enzyme and substrate on the plane of the phospholipid surface (Table 12.1). Factor VIIIa dramatically increases the V_{max} of factor X activation by factor IXa by 100,000-fold[78] and consequently the catalytic efficiency (Table 12.1). Factor VIIIa accelerates the proteolysis of factor X by interaction with both factor X and factor IXa on a phospholipid surface to facilitate a conformational change in the active site of factor IXa that favors catalysis.[79]

In a similar manner, factor Va enhances the activation of prothrombin to thrombin by the serine proteinase factor Xa in the presence of calcium ions and a procoagulant phospholipid surface.[3] As shown in Table 12.1, the acceleration of prothrombin activation by phospholipids results from a 1000-fold decrease in the K_m for prothrombin.[80,81] The major contribution of factor Va is a 3,000-fold increase in the V_{max} for prothrombin activation[80–82] (Table 12.1). The observed increase in V_{max} is due, in part, to the fact that factor Va acts as a receptor for factor Xa resulting in an increased concentration of factor Xa at the membrane surface. Overall, the main effect of factor Va is to enhance the catalytic efficiency of prothrombin activation probably by properly orienting factor Xa and prothrombin.

Table 12.1

The effect of cofactors on the rate of activation of factor X and prothrombin

Components	K_m	V_{max}	Catalytic Efficiency[a]
Factor X Activation			
IXa	300	0.002[b]	1
IXa, Ca^{2+}	181	0.011	8
IXa, Ca^{2+}, PL	0.06	0.002	5.8×10^3
IXa, Ca^{2+}, PL, VIIIa	0.06	500	1×10^9
Prothrombin Activation			
Xa	130	0.061[c]	1
Xa, Ca^{2+}	84	0.68	1.7
Xa, Ca^{2+}, PL	0.06	2.30	8.3×10^3
Xa, Ca^{2+}, PL, Va	0.21	1900	2×10^6

[a]The catalytic efficiency (V_{max}/K_m) for a mixture is compared to that of the enzyme alone.
[b]V_{max}: mol factor Xa min^{-1} mol factor IXa^{-1}.
[c]V_{max}: mol thrombin min^{-1} mol factor Xa^{-1}.
PL, phospholipids.
Data from bovine proteins and adapted from Gilles JG, Jacquemin MG, Saint-Remy JM. Factor VIII inhibitors. *Thromb Haemost* 1997;78:641–646.

Regulation of Factor VIII Cofactor Activity

Activation

The secreted heterodimer factor VIII is activated by limited proteolysis (for review, see Ref.[83]). Upon treatment of intact factor VIII with thrombin, there is a rapid >30-fold increase and subsequent first-order decay of procoagulant activity. Significant data has also characterized the activation of factor VIII by factor Xa, and other observations suggest that Factor XIa[84] and VIIa[85] can also catalyze this reaction. The physiologic activator(s) of factor VIII has (have) not been rigorously determined. Thrombin is the best characterized activator of factor VIII and maximal activation by thrombin is greater than that observed with factor Xa[86].

Thrombin cleaves peptide bonds after Arg372, Arg740, and Arg1689. Procofactor activation coincides with proteolysis of both the heavy and light chains of factor VIII as depicted in FIGURE 12.3. Cleavage within the heavy chain at Arg740 generates a 90-kDa polypeptide that is subsequently cleaved at Arg372 to generate polypeptides of approximately 50 and 43 kDa.[87] These polypeptides are designated as the A1 (residues 1 to 372) and A2 (residues 373 to 740) subunits, respectively, owing to their domainal derivations (FIGURE 12.3). Concomitantly, the 80-kDa light chain is cleaved at Arg1689 to generate a 73-kDa polypeptide[88] designated as the A3-C1-C2 subunit (residues 1690 to 2332). The cleavage of selected bonds is likely to lead to the appropriate structural changes that impact cofactor function. Significant data support that thrombin anion-binding exosites I and II are involved in recognition of factor VIII.[89,90,176,177] The corresponding binding sites on FVIII are thought to be the regions rich in acidic amino acids that contain the posttranslational modified amino acid tyrosine sulfate and border each of the cleavage sites[91] (FIGURE 12.3). The tyrosine-sulfate residues appear to enhance thrombin cleavage at adjacent sites, but do not affect factor Xa cleavage.[92] These observations suggest that thrombin may utilize tyrosine-sulfate residues to facilitate interaction and/or cleavage. In support of this contention are data showing that a mutation at a tyrosine sulfation site (Tyr346 to Cys) yielded a factor VIII that was defective in activation by thrombin[93] and is associated to mild hemophilia A. However, conflicting data show that mutations of individual Tyr residues to Ala in the acidic region designated as a2 resulted in little, if any, effect on rates of thrombin-catalyzed cleavage or activation of factor VIII.[94] Thus the contribution of sulfated Tyr residues to the activation mechanism may be restricted to selected regions in the procofactor.

Numerous studies have correlated the appearance of the 50-, 43-, and 73-kDa polypeptides with peak factor VIII activity.[87,95,96] Other studies examined the role of the individual cleavage sites in the expression of FVIIa cofactor activity. Mutagenesis studies showed that cleavage after residue 740 was not required for cofactor activity.[97,98] In contrast, mutation at either Arg372 or Arg1689

yielded molecules that were not cleaved by thrombin at the mutated site and were not susceptible to thrombin activation.[97] The importance of cleavage at residues 372 and 1689 for factor VIII activation was demonstrated *in vivo* by the identification of hemophilia A patients having missense mutations that prevent cleavage at either Arg372 or Arg1689.[99–102] These findings indicate that activation requires cleavage at both residues 372 and 1689, but does not appear to require a specific sequential order for cleavage at these sites. Cleavage at Arg1689 releases factor VIII from the inhibitory influence of vWF[103] and appears to additionally increase the activity of factor VIIIa.[104,105] Cleavage at Arg372 is essential to expose a functional factor IXa-interactive site(s) in the A2 domain that is cryptic in the unactivated molecule.[106]

Examination of apparent rates of thrombin cleavage at the three sites in factor VIII shows that Arg740 > Arg1689 > Arg372. Thus, the step essential to forming cofactor activity, cleavage at Arg372 to liberate the A1 and A2 subunits, is rate limiting. Cleavage at Arg^{740} and Arg^{1689} appears to facilitate cleavage within the heavy chain as mutations at these sites slows subsequent cleavage at Arg^{372}. However, the different magnitude of the effects produced by Arg1689Gln or Arg740Gln mutations on the reduction of the rate of cleavage of the light chain indicates that the docking of Arg740 represents the preferred pathway.[107,108]

Factor VIIIa is a heterotrimer of A1, A2, and A3-C1-C2 subunits[105,109,110] (FIGURE 12.3). The A1 and A3-C1-C2 subunits retain the metal ion linkage and can be isolated as a stable A1/A3-C1-C2 dimer. Conversely, the A2 subunit is associated with the A1/A3-C1-C2 dimer in a primarily electrostatic interaction and readily dissociates from the dimer at physiologic pH and ionic strength (see below).

In an attempt to probe individual residues that contribute side chain hydrogen-bonding interactions to the association of A2 subunit in the cofactor, Wakabayashi et al. mutated 30 polar or charged residues to Ala at the borders of the A2 and A1, and A2 and A3 domains that were spatially separated by <2.8 Å.[111,112] Results from that study showed that a number of the point mutations exhibited more rapid decay rates (5- to 40-fold) in factor VIIIa activity, hence showed reduced affinity for A2 subunit, suggesting a role for these residues in A2 subunit retention in the heterotrimer. Recent results have shown that replacing acidic residues such as E272, D519, E665, and E1984 with Ala or Val to yield hydrophobic interactions at the interfaces yielded factor VIII variants with as much as eightfold increases in the stability of factor VIIIa, as judged by reduced rates of activity decay.[113] Furthermore, these mutations appeared to have an additive effect in that variants where more than one acidic residue was replaced with Ala or Val showed further increases in cofactor stability.[112]

Aside from identification of cleavage sites, the structural alterations that transform "procofactor" factor VIII into active cofactor, factor VIIIa, are not well characterized. Circular dichroism spectral analysis indicates that purified factor VIIIa shows significant reduction in random coil and increase in beta-sheet structures compared with factor VIII;[114] however, it is unclear what contribution the B domain makes to these gross conformational differences. Zero-length cross-linking studies suggest formation of a new salt bridge between the *N*-terminal half of A2 subunit and the *C*-terminal region of A1 subunit following thrombin cleavage.[115] Furthermore, new hydrogen-bonding interactions involving A2 domain appear to form following conversion of the procofactor to the active cofactor.[111] Taken together, these results suggest that activation of factor VIII resulting in limited proteolytic cleavage is accompanied by changes in the protein conformation.

Finally, the cellular surface profoundly affects the procoagulant activity of factor VIII. Negatively charged phospholipids are usually confined to the inner leaflet of cellular membranes and are exposed upon cellular lysis at sites of injury.

Formation of the Xase Complex

Interaction with Factor IXa

The proteolytically activated form of factor VIII, factor VIIIa, serves as a cofactor for the serine protease factor IXa in the conversion of factor X to factor Xa. This complex of enzyme and cofactor, assembled on an anionic phospholipid surface, is referred to as the intrinsic factor Xase. The phospholipid surface is primarily involved in reducing molecular interactions to a 2-D space, thereby markedly decreasing the K_m for factor X. The role of surface also appears to contribute to the K_{cat} effect.[116] The association of factor VIIIa and factor IXa is complex and not fully understood. This interaction has been studied by homology modeling, analysis of naturally occurring hemophilia A and B mutations, mutagenesis, cross-linking, and inhibition with synthetic peptides. All these studies came to the conclusion that the interaction sites are localized in the A2 domain (residues 558 to 569, residues 484 to 509, residue Asp527, and residues 708 to 715) and the A3 domain (residues 1811 to 1818).[114,117,118,119,120,121–124] The molecular model of factor VIII[46], although not the activated form, mapped the potential factor IXa binding sites on the A2 domain at residues 558 to 665 and the region around Asp712 and in the A3 domain in the region of residues 1811 to 1818. All these regions appear on the model to be solvent exposed and thus likely accessible to binding factor IXa (see further FIGURE 12.7).

Interaction with Factor X

Little information is available on the interaction of factor VIIIa with factor X, the substrate for factor Xase complex. Analyses employing solid-phase binding assays[125] and fluorescence anisotropy[126] localized a factor X interactive site to the acidic *C*-terminal region (residues 337 to 372) of the A1 subunit. The affinity of this association was determined to be 1 to 3 μM; however, these experiments were performed in the absence of phospholipid vesicles, which may contribute to the affinity of the interaction. Zero-length cross-linking studies revealed that the site of interaction on factor X was in the serine protease-forming domain of the heavy chain but was not in that portion of the sequence representing the activation peptide.[127] Factor VIIIa reduces the K_m of factor Xase complex for factor X by several-fold,[128,129] suggesting a direct or indirect contribution to substrate affinity. Interestingly, deletion of the *C*-terminal region of A1 following proteolysis at Arg336 by factor Xa or APC (see below) resulted in an approximately fivefold increase in the K_m for factor X.[130] This increase in K_m from approximately 40 to 200 nM could potentially depress factor Xase complex activity in plasma since the latter K_m value is approximately twice the plasma concentration of factor X. Furthermore, evidence indicates that factor X increases the affinity of factor IXa for factor VIIIa by approximately 10-fold.[131] Evaluation of a functional K_d for the factor VIIIa–factor IXa interaction as measured by rates of factor Xa generation yields a value of approximately 70 pM. One interpretation of these data is that the enhanced affinity for the binary interactions reflects interdependent associations involving the three proteins: cofactor, enzyme, and substrate.

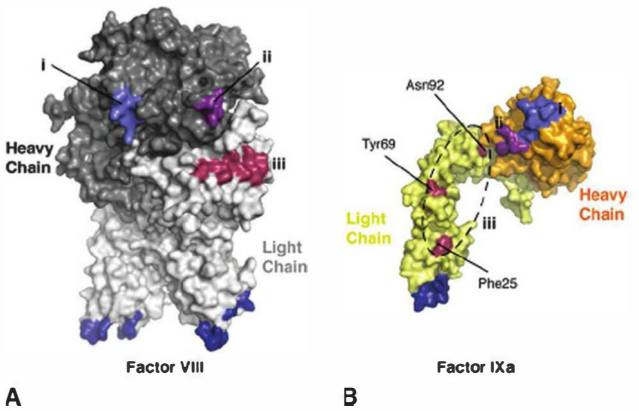

FIGURE 12.7 Binding interface between factor VIII and factor IXa. Models of the binding interface of factor VIII **(A)** and factor IXa **(B)**. The crystal structure of factor VIII shown in Fig. 12.6 and the structure of the porcine factor IXa backbone were used to construct a model of the factor IXa-factor VIIIa complex, the following constraints derived from previous studies: (i) residues 558 to 565 of factor VIII interact with the 330–339 helix of factor IXa (both in *light blue*), (ii) residues 707 to 712 of factor VIIIa bind to factor IXa residues 301 to 303 (*purple*), and (iii) residues 1811 to 1819 of factor VIII interact with the light chain of factor IXa (*red*). Phe25 in the Gla domain of factor IXa is juxtaposed with the light chain, and the area that interacts with residues 1811 to 1819 is highlighted with a *dashed line*. The putative phospholipids–binding sites in the C1 and C2 domains of factor VIII and the Gla domain of factor IXa that forms noncovalent interactions with the phosphoserine of the phospholipid bilayer are indicated (*dark blue*). [Adapted from Ngo JC, et al. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure* 2008;16:597–606. Use with permission © 2008 by Elsevier Ltd].

From the above studies, it appears that all three A domains of factor VIII participate in interprotein interactions essential for the efficient conversion of substrate to product. Although progress has been made in the identification and initial characterization of intermolecular binding sites, the mechanism by which factor VIIIa modulates the active site of factor IXa to greatly stimulate factor Xa generation is not known. Fluorescence studies using active site-modified factor IXa have yielded insights into the factor VIIIa–factor IXa interaction. Fluorescence energy transfer studies show that binding of factor VIIIa does not markedly alter the distance separating the active site of factor IXa and the phospholipid surface.[79] However, binding factor VIIIa did alter the emission intensity and anisotropy of the fluorescent probes used to modify the factor IXa, indicating a change in probe environment. Duffy et al.[117] showed that the fluorescence anisotropy of active site-labeled factor IXa was increased in the presence of factor VIII and that a further incremental increase was observed upon thrombin activation of factor VIII. While both the A1/A3-C1-C2 dimer and the isolated A2 subunit somewhat increased anisotropy of a similarly labeled factor IXa molecule, factor VIIIa-like increases in anisotropy values required reconstitution of the cofactor from the isolated subunits.[132] Interestingly, in the absence of factor VIIIa, substrate factor X shows little effect on the anisotropy of the labeled factor IXa.[133] However, inclusion of factor X produced a maximal increase in the factor VIIIa-dependent anisotropy. These results indicate that a primary role of factor VIIIa is to alter the conformation around the active site of factor IXa and its orientation relative to substrate factor X.

Interaction with Phospholipids

Phospholipids interact with substrates, enzymes, and cofactors to play a critical role in the assembly and functional activity of the coagulation protease complexes. Negatively charged phospholipids are required for factor VIIIa-mediated enhancement of the activation of factor X.[78,134,135] *In vivo*, negatively charged phospholipids are likely provided by activated platelets and damaged endothelial cells. Factor VIII and factor V bind phosphatidylserine (PS) by both hydrophobic and electrostatic interactions.[136–141] However, factor V does not efficiently compete with binding of factor VIII to phospholipid vesicles composed of 15% phosphatidyl-L-serine.[134] Under equilibrium conditions, factor VIII can bind phospholipid vesicles containing 15% to 25% PS with an apparent K_d of 2 to 4 nM.[117,134,142] Saturation occurs between 170 and 385 moles of phospholipid per mole of factor VIII,[134] although the process involves both rapid and slow interactions.[143] Factor VIII binding to phospholipid involves stereoselective recognition of the O-phospho-L-serine moiety of PS.[142] Since the composition of PS exposed on the platelet membrane surface can increase from 2% to 13% after stimulation,[144] the increase in PS content could account for the ability for factor VIII to specifically bind the surface of thrombin-activated platelets.

Addition of negatively charged phospholipids to the factor VIII–vWF complex dissociates factor VIII from vWF.[145,146] Interestingly, thrombin-treated factor VIII does not bind vWF with high affinity[147–149] but binds phospholipid with a 10-fold increased affinity compared with that of the procofactor, factor VIII.[150] This effect is thought to result from a conformational change within the C2 domain following activation, based upon the capacity of the anti-C2 monoclonal antibody, ESH8, to lock the conformation of C2 into the low-affinity form. The phospholipid-binding site(s) within factor VIII appears primarily localized to the light chain,[151,152] and antibody inhibition studies suggest that binding is likely modulated by the C2 domain.[153]

A high-resolution x-ray structure (1.5 Å) of the factor VIII C2 domain was determined.[154] It predicts a β-sandwich core (40 × 30 Å) with two pairs of hydrophobic residues extending from adjacent loops that contribute to phospholipid binding. The two hydrophobic spikes comprising Met2199/Phe2200 and Leu2251/Leu2252 are proposed to penetrate the lipid bilayer and associate with the aliphatic hydrocarbon chains. On the other hand, a ring of basic residues (Arg2215, Arg2220, Lys2227, and Lys2249) localized above the hydrophobic residues contributes electrostatic interactions. A number of point mutations resulting in hemophilia A that map to the C2 domain tend to cluster at the protein core region rather than at putative membrane–binding sites. One explanation proposed for this observation[155] is that the binding energy reflects the additive nature of several side chains at the membrane interface. For this reason, single-point mutations within this flexible region may be tolerated. Conversely, mutations within the protein core of the C2 domain

could disrupt folding and interfere with secretion, thereby producing loss of function.

The intermediate-resolution x-ray crystal structures of factor VIII indicate that the C1 and C2 domains are aligned such that both domains may interact with the phospholipid surface.[46,73] A similar alignment was noted for factor Vai.[72] Recent results comparing isolated factor VIII C2 domain with a C1 C2 protein showed enhanced affinity of the latter in platelet binding suggesting direct and/or indirect roles for C1 in this interaction.[156] In a recent report, Wakabayashi et al.[157] demonstrated that a factor VIII variant lacking the entire C2 domain possessed a marked (~15-fold) reduction in affinity for phospholipid membranes, supporting a direct role for the C1 domain in this interaction. Furthermore, deletion of C2 did not appear to grossly alter the rate of procofactor activation by thrombin, the affinity of factor VIIIa for factor IXa, the K_m of Xase for substrate factor X, or k_{cat} for factor Xa generation. However, this deletion did significantly destabilize the cofactor, such that when combined with several modest defects in various intermolecular interactions, it resulted in a significant reduction in the thrombin-generating capacity. Overall, these observations show pronounced membrane-dependent cofactor activity in the absence of C2 domain, yet indicate this domain was essential in contributing to maximal cofactor function.

Modeling of the Factor Xase Complex

The crystal structure of factor IXa shows it to be an elongated molecule analogous to a tulip with the γ-carboxyglutamic acid (Gla)-rich, epidermal growth factor-like (EGF), and serine protease domains representing the bulb, stem, and flower, respectively[158] (FIGURE 12.7B). The Gla domain binds the protease to the phospholipid surface, whereas the active site is located high above the membrane surface (>70 Å).[79,125] The A3-C1-C2 subunit of factor VIIIa associates with the phospholipid surface via residues in the C1 and C2 domains. Thus, it is reasonable to speculate the high-affinity binding sites in factors VIIIa and IXa involve a surface proximal interaction between the A3 domain of factor VIIIa and the Gla–EGF domains of factor IXa. Furthermore, the A2 subunit directly modulates the active site of factor IXa.[159] Therefore, a surface distal, weaker-affinity interaction likely occurs between the A2 subunit and the serine protease domain. Based upon biochemical and mutational results described above for interactive regions in factors VIII and IXa, Ngo et al.[46] proposed a molecular model of the factor Xase complex using the crystal structure of IXa and VIII. FIGURE 12.7 shows the structures of factor VIII and IXa with interprotein interactive sites highlighted. A membrane-bound model of factor Xase is illustrated in FIGURE 12.8 and visualizes a putative complex of the two proteins. Unfortunately, this model does not consider what changes occur upon activation of factor VIII to factor VIIIa. Furthermore, interactions of specific residues within these structures are not identified. This latter information will require results obtained following cocrystallization of the cofactor and proteinase. However, the 3-D model does provide a basis for predictions of intra- and intermolecular interactions.

Inactivation

Factor Xase has been regarded as "self-damping,"[160] and the labile nature of this complex reflects the decay of factor VIIIa. The affinity of A2 subunit for the A1/A3-C1-C2 dimer (K_d ~ 260 nM)[161,162] greatly exceeds the concentration of factor VIII in plasma (~1 nM). Thus, one would predict that activation of factor VIII would be followed by immediate and near complete dissociation of A2 subunit. The dissociation rate

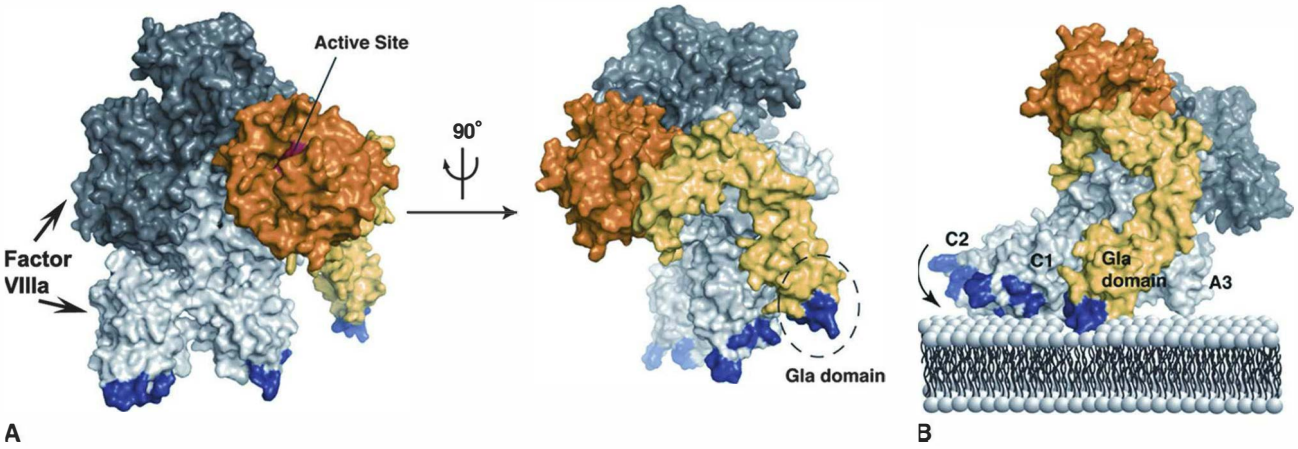

FIGURE 12.8 Model of the factor VIIIa–factor IXa complex. **(A)** Front and scale views of the complex of factor VIIIa (*dark gray*: heavy chain; *light gray*: light chain) and factor IXa (*orange*: heavy chain; *light green*: light chain). Four putative membrane–binding sites, including the bases of C1 and C2 domains, the A3 domain loop of factor VIIIa, and the Gla domain of factor IXa are shown in *blue*. The active site of factor IXa is indicated (*red*). **(B)** View of the possible interaction of the factor VIIIa–factor IXa complex with phospholipids membrane surfaces. The model suggests that upon binding with factor IXa, factor VIII orients in a bent position and C2 domain undergoes a conformation change to align its membrane-binding site with the Gla domain to interact with the membrane simultaneously. The active site of factor IXa faces the solution. (Adapted from Ngo JC, et al. Crystal structure of human factor VIII: implications for the formation of the factor IXa-factor VIIIa complex. *Structure* 2008;16:597–606. Use with permission © 2008 by Elsevier Ltd).

constant for loss of A2 subunit in human factor VIIIa (~0.35/minutes) is markedly faster than that observed with porcine factor VIIIa (~0.12/minutes), indicative of a higher affinity interaction in the latter.[162] Indeed, reconstitution of factor VIIIa using a porcine A2 subunit plus human A1/A3-C1-C2 yielded a hybrid factor VIIIa molecule possessing threefold higher specific activity than factor VIIIa reconstituted from the human subunits.

However, an effect opposing dissociation of subunits is the capacity for factor IXa to stabilize the labile factor VIIIa heterotrimer. Lollar et al.[163] determined that at low factor VIIIa (<1 unit/mL) and factor IXa (5 nM) and in the presence of phospholipid, porcine factor VIIIa was stabilized from spontaneous decay. It was subsequently determined that factor IXa transiently enhances the reconstitution of factor VIIIa from isolated subunits by a mechanism consistent with reducing the intersubunit dissociation rate constant.[164] One mechanism by which factor IXa stabilizes factor VIIIa may involve interaction of the enzyme with sites on the A2 subunit and A3 domain to in effect tether the labile complex. However, prolonged reaction of factor VIIIa with factor IXa results in proteolysis in the A1 subunit and subsequent inactivation of the cofactor. Sequence analysis revealed that factor IXa cleaves the A1 subunit at Arg336,[165,166] thereby altering interaction of A1 with the A2 subunit to yield a low-activity conformation. A secondary, slow reacting site at Arg1719 was also identified; however, cleavage at this site is not inactivating.[167] This factor VIII–directed proteolytic activity coupled with the interfactor VIIIa subunit stabilizing activity provides factor IXa mechanisms for regulating its own activity by modulating the activity of its cofactor. Thus a situation of "reciprocal regulation" appears to exist and may influence the activity of intrinsic factor Xase.

APC is a potent anticoagulant and its importance as a regulator of blood coagulation is apparent from predisposition to thrombosis in individuals with protein C deficiency (see Ref. [168] for review). The anticoagulant effect is phospholipid and Ca^{2+} dependent and results from the selective inactivation of factor Va and factor VIIIa. Cleavage of factor VIIIa occurs at Arg562,[121] bisecting the A2 subunit and Arg336[87,121] preceding the *C*-terminal acidic region of the A1 subunit (FIGURE 12.3). The former cleavage occurs within a factor IXa interactive site and effects the factor VIIIa-dependent modulation of the factor IXa active site, as judged by fluorescence anisotropy.[126] The latter cleavage likely influences the affinity of the A1/A3-C1-C2 dimer for A2 subunit as well as affects the interaction of factor VIIIa with substrate factor X. APC-catalyzed cleavage appears to show a preferred pathway. The human enzyme cleaves the A1 subunit initially.[169] The rate of cleavage at Arg336 is approximately 25-fold faster than at Arg562, and cleavage at either site is independent of the other.[170] Factor IXa selectively protects cleavage of factor VIIIa at Arg562; however, this effect is abrogated by inclusion of protein S,[171] a cofactor for APC. APC-catalyzed inactivation of factor VIIIa may be secondary to its inactivation by subunit dissociation. Comparison of rates of cofactor inactivation attributable to APC action suggests factor Va is the primary substrate, since the majority of factor VIIIa activity lost was not the result of proteolysis.[169] Furthermore, studies using a reconstituted coagulation model show greater degradation of factor Va compared with factor VIIIa by the APC pathway.[172]

APC resistance is one of the major causes of hereditary thrombophilia that results from an Arg to Gln mutation in the APC cleavage site in factor V at residue 506.[4,173,174] The APC-resistant phenotype was first reported as a poor anticoagulant response to APC in an activated partial thromboplastin time assay (called the APC resistance ratio).[173] It is possible that APC resistant mutations in factor VIII may also lead to hereditary thrombophilia. Analysis of mutant factor VIII protein demonstrated that cleavage at either Arg336 or Arg562 only partially inactivated factor VIII and that resistance to cleavage at both Arg336 and Arg562 was necessary to detect a poor anticoagulant response in an activated partial thromboplastin time assay.[175] These results show that a single mutation in either factor APC cleavage site to yield cleavage resistance would not be detected as a poor anticoagulant response *in vitro*. In addition, a search did not identify any mutations at Arg336 or Arg562 in factor VIII in 125 patients with venous thrombosis.[176] The sum of these studies indicates that it is unlikely mutations in factor VIII APC cleavage sites will result in thrombophilia.

Factor Xa also catalyzes inactivating cleavages in factor VIIIa, and since this protease is a product of factor Xase, it suggests a potential to participate in its down-regulation. Factor Xa cleaves factor VIIIa at Arg336[87] and Lys36[177] in the A1 subunit. Loss of activity due to cleavage at the latter site is less well understood than cleavage at Arg336, but appears to result in an altered conformation of this subunit affecting interaction with A2 subunit.[177] The interaction of protease with the factor VIII substrate appears to require residues in the C2 domain[178] and the *C*-terminal region of A1 subunit.[177] Binding to the latter site may make use of a heparin-binding exosite[177] recently identified in factor Xa.[179] Overall, the relative contributions of spontaneous A2 subunit dissociation and proteolysis mediated by APC, factor Xa, or factor IXa, in limiting factor VIIIa *in vivo* remain unclear.

Regulatory Role of vWF

The plasma levels of factor VIII and vWF are maintained at a fairly constant ratio of 1 molecule of factor VIII to 50–100 vWF subunits. For example, patients with type 1 von Willebrand disease who have a 50% reduction in vWF have a corresponding 50% reduction in factor VIII. Infusion of vWF into vWF-deficient patients elicits an immediate rise in circulating levels of factor VIII. Studies of clearance kinetics upon infusion of factor VIII/vWF complex or pure factor VIII clearly established the stabilizing influence of vWF on factor VIII in the circulation.

In addition to increasing factor VIII survival, vWF plays a critical role in directly regulating factor VIII activity.[180] vWF prevents factor VIII binding to phospholipids[145] and activated platelets,[181] and inhibits factor VIII from activation by factor Xa[182] and inactivation by APC.[121,183,184] Furthermore, vWF appears to protect factor VIII from imidoacetate-induced degradation by blocking loss of bound calcium in the procofactor.[185] On the other hand, vWF does not interfere with activation by thrombin,[147,186] and actually serves as a cofactor in facilitating the thrombin-catalyzed cleavage of factor VIII light chain, thereby enhancing rates of factor VIIIa dissociation from vWF.[187] It is likely that vWF mediates its inhibitory effects on factor VIII activity by preventing factor VIII interaction with phospholipids, a reaction required for both factor Xa and APC-mediated cleavage of factor VIII. However, there is evidence that binding sites for vWF and APC in the factor VIII C2 domain may overlap,[184] supporting a mechanism of inhibition by direct competition. It is proposed that vWF binding to platelet receptor glycoprotein Ib (GPIb) brings factor VIII to the vicinity of

platelets adhering to damaged endothelium.[181] After proteolytic activation of factor VIII, factor VIIIa is released from vWF permitting its interaction with the platelet surface.

Recently, a reciprocal effect, that of factor VIII regulating vWF, has been observed. Proteolytic cleavage of large vWF multimers by the metalloproteinase, ADAMTS13 is important for normal hemostasis. Failure to process these multimers due to deficiency or defect in the proteinase results in the generation of microthrombi, a characteristic feature of thrombotic thrombocytopenic purpura. Factor VIII serves as a cofactor in the proteolytic cleavage of vWF, increasing the rate of ADAMTS13-catalyzed cleavage at the Tyr1605-Met1606 bond in vWF by approximately 10-fold.[188] The a3 segment of factor VIII that forms a primary binding site of vWF appears critical for this interaction.

The interaction of factor VIII with vWF is mediated by a major factor VIII–binding site within the first 272 amino acids of the mature vWF molecule[189–191] with residues 78 to 96, residues around 53, and the amino-terminus providing crucial roles.[189,192–195] Moreover, processing of vWF propeptide in the Golgi, essential for multimerization, is required for generation of high-affinity factor VIII–binding sites.

High-affinity binding to vWF requires both the amino-terminal and carboxy-terminal ends of the factor VIII light chain.[124,196,197] A vWF-binding site on factor VIII was localized to residues 1673 to 1684 (a3), a region removed by thrombin cleavage at residues 1689 (see FIGURE 12.3). Phospholipid and vWF compete for binding to factor VIII.[142,145,146] The two hydrophobic spikes (Met2199/Phe2200 and Leu2251/Leu2252) participate in binding to phospholipid membranes and also contribute to the binding of vWF. Furthermore, an isolated C1-C2 fragment demonstrated higher affinity for vWF than isolated C2.[198] Taken together, these data suggest a complex intermolecular interaction that occurs over an extended interface involving the amino-terminal region of vWF and both ends of the factor VIII light chain.

In the circulation, factor VIII(a) catabolism is mediated by low-density lipoprotein receptor–related protein (LRP), a hepatic clearance receptor. The association of factor VIII with vWF reduces the binding of factor VIII to LRP by >90% contributing to reduce its clearance. Both factor VIII and vWF bind to macrophages with subsequent rapid uptake and degradation of the internalized protein.[199] Chemical inactivation of macrophages resulted in a doubling of factor VIII levels in vWF-null mice. These results suggest a prominent role for macrophages in the clearance of the factor VIII/vWF complex.

Regulation of Factor V Cofactor Activity

Activation

As a single-chain protein, factor V expresses very little, if any, procoagulant activity.[82] Procoagulant activity is expressed following limited proteolysis at discrete sites in the molecule. Thrombin is considered the physiologic activator of factor V. Thrombin cleaves three peptide bonds at Arg709, Arg1018, and Arg1545 within the B domain of factor V (FIGURE 12.2). Thrombin-activated factor V is a calcium-dependent heterodimer consisting of an *N*-terminal heavy chain (A1-A2) of 105 kDa and a light chain (A3-C1-C2) of 74 kDa.[200–202] The heavily glycosylated B domain is not necessary for procoagulant activity and is released during activation by thrombin.[201–203] The use of snake venom proteases and factor V mutants has provided important insights into the functions of the individual thrombin cleavage sites.[204–207] Thrombin cleavage at Arg709 is kinetically favored over cleavage at Arg1018 and Arg1545. Thrombin cleavage at Arg709 results in little or no increase in cofactor activity.[183,204–206] Cleavage at both Arg709 and Arg1018 releases the amino-terminal portion of the B domain resulting in further partial activation of the cofactor.[204,206] Cleavage at Arg1545 and release of the carboxyl-terminal fragment of the B domain from the light-chain fragment is required for maximal activation.[204–207] Cleavage at Arg709 and Arg1018 is necessary for maximal rates of factor V activation by thrombin.[204,205] Factor V mutants, in which cleavage at Arg709 and/or Arg1018 are blocked, are resistant to thrombin cleavage at Arg1545. In contrast, these mutants can be fully activated by the snake venom protease RVV-V, which cleaves factor V at Arg1018 and Arg1545.[204,205,207] These observations indicate that release of the B domain from the heavy chain is not absolutely required for maximal expression of procoagulant activity.

Thrombin cleavage of factor V at Arg709 and Arg1545 requires interactions with anion-binding exosite I on thrombin.[90] Factor V residues Glu666-Glu672 have been shown by crystal structure to bind to exosite 1 on thrombin, with the aromatic side chain of Phe668 inserting into a hydrophobic pocket.[208] Modeling experiments indicate that the sequence between this binding site and Arg709 of factor V wraps around thrombin in productive thrombin-factor V complexes that cover a large surface area to engage the active site. An acidic region in factor V, Asp695-Tyr698, is also required for efficient thrombin cleavage at Arg709.[209] The sequence of this peptide (DYDYQ) contains the consensus sequence for tyrosine sulfation and is the likely site of sulfation in the factor Va heavy chain.[210] Tyrosine sulfation of factor V is required for efficient thrombin cleavage at both Arg709 and Arg1545.[88] The factor V B domain contains a similar acidic region Glu1508-Tyr1515, which is the likely site of sulfation in the factor V B domain.[88,210] This peptide (EDDYAEIDY) may also play a role in binding to exosite 1 and is required for efficient cleavage by α-thrombin and formation of the factor Va light chain.[211] Consistent with this hypothesis, deletion of the entire factor V B-domain (amino acids 710 to 1545) results in a molecule that is resistant to thrombin cleavage and activation.[212] In contrast, factor V molecules with deletions of amino acid residues 811 to 1491 or 709 to 1476 were efficiently cleaved by thrombin and fully activated.[204,213–215]

Characterization of the B-domain deletion mutant lacking amino acid residues 811 to 1492 and mutated in thrombin cleavage sites Arg709 and Arg1545 indicated that removal of this portion of the B domain leads to constitutive but partially active single-chain cofactor; however, cleavage at Arg1545 is essential for complete activation of factor V.[204] Consistent with this observation, thrombin cleavage at Arg1545 appears to be required for optimal factor Xa–binding affinity.[206,207] These results suggest the presence of the B domain in factor V is necessary to inhibit procoagulant activity prior to proteolytic processing. Recent truncation analyses identified a discrete region of the B domain that stabilizes the profactor state.[216] The interaction between factor Va and prothrombin is mediated by the heavy chain of the cofactor, and this interaction is independent of calcium ions.[217] Factor V can also be activated by factor Xa.[205,218] After several studies by different groups, data of Orfeo et al.[219] concluded that direct activation of factor V by factor Xa at physiologically

relevant concentrations does not appear to be a significant contributor to factor Va formation.[219]

Several other enzymes (factor XIa, calpain, monocyte-derived cathepsin G, plasmin, neutrophil elastase snake venom protease NN) also can activate factor V. In contrast to the activators discussed above, these enzymes produce complex proteolytic cleavage pattern and/or can only partially activate factor V. Recent studies indicate that factor XIa is capable of activating factor V at plasma concentrations during the preincubation phase of a PTT assay.[84]

Formation of the Prothrombinase Complex

Formation of the prothrombinase complex requires the presence of a membrane surface to which the individual components bind and subsequently assemble into a functional complex. Activated platelets, platelet microparticles, and damaged vascular cells provide this surface *in vivo* (for a review, see Ref. [220]). The interaction of factor V with cellular surfaces appears to be mediated, at least in part, by binding to acidic phospholipids (e.g., PS). These phospholipids are normally sequestered in the inner leaflet of the cellular membrane but become exposed following platelet activation and microparticle formation.

The primary membrane-binding sites for factor V are contained within the light-chain fragment (A3-C1-C2). Structural data indicate that membrane-binding sites for PS membranes in the C1 and C2 are each composed of a common distorted jelly-roll β-barrel structure with three variable loops, or "spikes," located at the membrane-binding surface.[72,221] The C1 and C2 domains in factor Va are aligned in an edge-to-edge orientation, making it possible for the variable loops in both domains to interact with a membrane surface.[72] In contrast, the factor V A3 domain, which was also proposed to interact with phospholipids membranes, rests on top of the platform formed by the C1 and C2 domains.[72]

Factor Va interacts with factor Xa in the presence of calcium ions on a phospholipid membrane surface to accelerate the conversion of prothrombin to thrombin. The affinity of factor Xa for phospholipids increases when factor Va is bound to a membrane surface containing acidic phospholipids. Binding of factor Xa to factor Va allosterically alters the active site of factor Xa and, in addition, repositions the active site of the enzyme to the proper distance above the membrane for prothrombin activation.[222] The binding of factor Xa to factor Va also results in modulation of the binding of prothrombin to substrate-binding exosites distinct from the active site of factor Xa. Further studies also suggested that PS may allosterically regulate interactions between factor Va and factor Xa.[223]

More recently, mutagenesis studies localized a key factor Xa-binding surface to residues Arg501, Arg 510, Arg 511, Asp513, Asp577, and Asp578 in the factor Va A2 domain.[224] Discrete regions within the factor Va heavy chain also regulate the activation of prothrombin by the prothrombinase complex, including residues 334 to 335,[225,226] residues 659 to 663,[227] and residues 695 to 698.[226–228]

Prothrombin does not bind to factor V, indicating that the cofactor must be activated before it binds prothrombin.[229] Sedimentation equilibrium analysis revealed that the factor Va heavy chain and prothrombin form a 1:1 complex with a dissociation constant of 10 μM.[217] Proteolysis of the factor Va heavy chain by APC results in the loss of its ability to bind prothrombin.[229] A prothrombin-binding site was localized to an acid peptide containing residues 697 to 709 at the carboxyl terminus of the factor Va heavy chain.[207,209] A pentapeptide from this region, spanning Asp695 to Gl699, preferentially interferes with the initial cleavage of prothrombin at Arg320 by prothrombinase, shifting activation of prothrombin through prethrombin 2 rather than meizothrombin.[228] The binding of prothrombin to factor Va appears to involve proexosite 1 in the catalytic domain of prothrombin[230,231] as well as the kringle 1 and kringle 2 domains.[232]

The ability of factor Va to bind to factor Xa is lost when factor Va is proteolytically cleaved by APC. Conversely, APC cleavage of factor Va at Arg506 decreases the affinity of the cofactor for factor Xa,[233] whereas APC cleavage at Arg306 leads to a total loss of factor Xa binding.[234]

Inactivation

Activation of the protein C pathway leads to inactivation of factor Va and down-regulation of the *prothrombinase* complex. APC is a vitamin K–dependent serine protease that inactivates factor Va by limited proteolysis of the factor Va heavy chain. Efficient inactivation of factor Va requires a protein cofactor, protein S, and a phospholipid or platelet membrane surface. An APC-binding site on the factor Va heavy chain includes residues 311 to 325, which overlap with a binding site for factor Xa.[235] APC cleaves human factor Va heavy chain at Arg306, Arg506, and Arg679 (FIGURE 12.2). There is an additional APC cleavage site present at Lys994 in the B domain of human factor V (not shown in FIGURE 12.2).[236] Binding sites for APC have been localized to the factor Va light chain.

Characterization of factor V_{Leiden} has helped to define the roles of the APC cleavage sites at Arg306 and Arg506. Factor V_{Leiden} lacks the APC cleavage site at Arg506 due to substitution of glutamine at this position (R506Q). An initial rapid phase of factor Va inactivation correlates with APC cleavage at Arg506 and is followed by a slower phase that results in complete inactivation of the cofactor.[233] APC cleavage at Arg506 results in a partial loss of cofactor activity because of a 50-fold decrease in the affinity of the cofactor for factor Xa.[233] The second, slow phase of inactivation seen during inactivation of factor Va and FVa_{Leiden} correlates with APC cleavage at Arg306. APC cleavage at Arg306 releases the heavy-chain fragment corresponding to the A2 domain (amino acids 307 to 709), generating an A1/A3C1C2 heterodimer that expresses no cofactor activity.[237] The role of APC cleavage at Arg679 has not been completely defined. APC cleavage at Arg679 occurs more slowly than APC cleavage at Arg506 or Arg306 and appears to play a minor role in the inactivation of normal factor Va.[233,238]

Rapid cleavage of factor Va by APC at Arg306 is completely membrane dependent.[234] APC cleavage of factor Va at Arg506 can occur in the absence of phospholipid membranes;[236] however, the membranes do enhance the rate of this cleavage. In contrast, the slow cleavage of factor Va at Arg679 does not appear to be enhanced on the membrane surface.[233,236]

Protein S is a vitamin K–dependent plasma protein that acts as a cofactor promoting inactivation of factor Va by APC. Protein S selectively promotes APC cleavage of factor Va at Arg306 while having no effect on the rate of APC cleavage at Arg506.[239]

Formation of the *prothrombinase* complex by addition of factor Xa protects factor Va from inactivation by APC. Rosing et al.[239] demonstrated that factor Xa selectively inhibits APC cleavage at Arg506 while having no effect on the rate of APC cleavage at Arg306. These findings suggest that the binding sites

for factor Xa and APC overlap. The inhibitory effects of factor Xa can be partially overcome in the presence of protein S because of the enhanced rate of APC cleavage at Arg306.[239] More recently, Norstrøm et al.[240] confirmed the protective effect of factor Xa at Arg506, but found that APC cleavage at Arg306 was actually stimulated in the presence of factor Xa. In addition, protein S can counteract the protective effect of factor Xa against cleavage at Arg506.[240] In the presence of both factor Xa and protein S, inactivation of factor Va and FVa_{Leiden} can proceed at similar rates because the contribution of APC cleavage at Arg506 to inactivation of the cofactor is minimal under these conditions.[239] These observations may explain the relatively mild prothrombotic risk associated with the FV_{Leiden} mutation.

Prothrombin also protects factor Va from inactivation by APC.[241,242] Prothrombin impairs cleavages at both Arg306 and Arg506, and this inhibition was seen in the presence and absence of protein S.[241] In particular, cleavage at Arg306 was more pronounced in the presence of protein S, indicating that prothrombin impaired APC function as well as the cofactor function of protein S.[241] The gla domain of prothrombin linked to kringle domains 1 and 2 is necessary for the ability of prothrombin to protect factor Va from APC cleavage; once activated, thrombin is not protective.[242]

GENETICS AND PATHOLOGY

Factor V–Associated Diseases

Some mutations in factor V have been reported to lead to a bleeding disorder (parahemophilia) whereas others increase the risk of developing thrombosis (APC-resistance) with no mutations or polymorphisms at the factor V thrombin cleavage sites.

Deficiency of Factor V: Parahemophilia

Congenital factor V deficiency, also called parahemophilia, is a rare disorder that has an estimated incidence of 1 in 10^6. More than 200 cases have been reported in the literature.[243] Parahemophilia is characterized by low levels of functional factor V activity in plasma and platelets and is inherited in an autosomal recessive manner. Clinical manifestations include ecchymoses, epistaxis, oral bleeding, menorrhagia, and bleeding following surgery or trauma. Rare patients also have had thromboembolic events, including deep and superficial venous thromboses, pulmonary embolism, stroke, and myocardial infarction.[244] Most patients with congenital factor V deficiency have low antigenic levels of factor V; however, approximately 17% of patients were found to have a discrepancy between their functional and antigenic levels.[245] Plasma factor V levels have not correlated well with the hemorrhagic tendency, and several authors have suggested that the severity of bleeding symptoms may be more closely correlated with platelet than with plasma factor V levels.[246,247] The molecular basis for factor V deficiency has been established in 120 patients (see Table 12.2). Factor $V_{New\ Brunswick}$ (A221V) is a missense mutation in the factor V heavy chain that results in the expression of a factor V molecule with reduced specific activity caused by an increased rate of dissociation of the heavy and light chains of factor Va.[248,249] Several missense or splicing mutations impair secretion of factor V.[250–252] Five patients had severe factor V deficiency caused by homozygous nonsense or frameshift mutations in the B domain.[253–255] These mutations predict the synthesis of truncated factor V molecules lacking a portion of the B domain and all of the light chain. In contrast, factor V–deficient mice generated by homologous recombination have a phenotype characterized by fatal hemorrhage and an incomplete block in embryogenesis.[256] Transgenic rescue of factor V–deficient mice indicates that factor V levels of <0.1% are sufficient for survival.[257] Survival of patients with homozygous nonsense mutations may be explained by ribosomal slippage and trace expression of factor V.[257,258]

Table 12.2

Pathogenic *F5* mutations causing factor V deficiency[a]

Nucleotide substitution: missense codon	52
Nucleotide substitution: nonsense codon	19
Nucleotide substitution: splicing error	10
Small deletions (up to 20 bp)	28
Small insertions (up to 20 bp)	7
Gross deletions	2
Other rearrangements	2

[a]Compiled from http://www.isth.org/default/index.cfm/publications/registries-databases/mutations-rare-bleeding-disorders/and references.[288,289]

APC-Resistant Factor V and Risk of Thrombosis

As mentioned above, one of the genetic risk factors for thrombosis is factor V_{Leiden} that is partially resistant to inactivation by APC due to a Gln substitution at the APC cleavage site Arg^{506}. Patients who are heterozygous for this mutation have a three- to eightfold increased risk of developing thrombosis whereas the risk for homozygous individuals is 50- to 80-fold higher compared to normal individuals.

Factor VIII-Associated Disease: Hemophilia A

Unlike factor V, no factor VIII mutation has been described so far that is linked to a prothrombotic state. Although APC resistance is strongly correlated with enhanced risk to develop thrombosis, no mutations at the corresponding APC inactivation sites have been observed in factor VIII in patients affected by venous thrombosis. However, high levels of factor VIII are associated with an increased risk of venous thrombosis. To date, no genetic variations in the *F8* gene have been identified that could account for this phenotype.

The *F8* gene is located on the human X chromosome and therefore hemophilia A is a classic example of X-linked recessive inheritance. It occurs almost exclusively in males for they only have one X chromosome; females with one abnormal copy of the *F8* gene are carriers because the other X chromosome contains a normal copy of the gene. The frequency of the disorder is 1 in 5,000 to 10,000 male births and there is no particular ethnic group that has unusually lower or higher incidence of the disease. Severe hemophilia A was in the past a genetically lethal disease in which affected males produced few offspring. Therefore, nearly one-third of the mutant alleles would be lost in each generation. Haldane in 1935 predicted that in order to maintain a constant frequency in the population about one-third of cases would be the result of novel mutations. The prediction was proven to be correct since a large number of different mutations

exist in the *F8* gene and many patients carry a *de novo* mutation not present in the X chromosome of their mothers. One notable exception to the Haldane prediction is the case of the common inversion within the *F8* gene (see below).

The severity and frequency of bleeding in the patients correlate with the level of factor VIII activity in plasma.[259] About 50% to 60% of patients have severe hemophilia A with factor VIII activity <2% of normal; these patients have frequent spontaneous bleeding into joints, muscles, and internal organs. Moderately severe hemophilia A occurs in about 25% to 30% of patients; the factor VIII activity is 2% to 10% of normal and there is bleeding after minor trauma. Mild hemophilia occurs in 15% to 20% of patients and is associated with factor VIII activity of 20% to 30% where bleeding occurs only after significant trauma or surgery. Individuals with factor VIII activity >30% do not have symptoms of hemophilia A. Of particular interest for understanding the mechanism of factor VIII action is a category of patients with considerable amount of factor VIII protein in their plasma (at least 30% of normal) but the protein is nonfunctional. Approximately, 5% of patients belong to this category termed CRM (cross-reacting material) positive.[260] CRM reduced is another category in which the factor VIII antigen and activity are equally reduced.

F8 Gene Defects

The *F8* gene from more than 4,500 hemophilia A patients has been examined for molecular defects using a variety of methods. The human genome database (HGMD: www.hgmd.org) provides a comprehensive list of the *F8* mutations. Another database was created and is updated to 2007 (HAMSTeRS http://hadb.org.uk/ and [261]). In the HAMSTeRS database more than a thousand mutations have been classified. For each mutation, the degree of severity of the disease and for some also the levels of factorVIII antigen and/or activity present in the plasma of the corresponding patient are reported. In Table 12.3 the gene defects are classified according to the type of mutation. The point mutations are further divided into missense, nonsense, and nucleotide substitutions that cause splicing errors. Missense mutations are the most represented both in the point mutation category and in the overall mutations reported in Table 12.3.

Table 12.3

Pathogenic *F8* mutations causing hemophilia A summary of unique mutations (regardless of recurrence)

Nucleotide substitution: missense codon	583
Nucleotide substitution: nonsense codon (stop)	131
Nucleotide substitution: splicing error	95
Small deletions (up to 50 bp)	197
Small insertions (up to 50 bp)	80
Gross deletions	135

Source: The Haemophilia A Mutation, Structure, Test and Resource (HAMSTeRS) http://hadb.org.uk/. Latest available summary January 1, 2007.
Data refer only to exons 1–26 of *F8* gene.
Permission to use obtained by Geoffrey Kemball-Cook.

The missense mutations are spread throughout the gene with only four mutations in the B domain.

Hemophilia A also originates from gross DNA rearrangements, known as common partial inversions of *F8* gene. The most common cause of severe hemophilia A is a partial inversion up to and including exon 22 of the *F8* gene. In 1993, two studies found that approximately 40% of patients with severe hemophilia A had a partial inversion of *F8* gene.[262,263] A second inversion was identified in intron 1 of *F8*.[264] The mechanism of inversion is intrachromosomal or intrachromatid recombination between homologous regions. For the first inversion, the involved regions include the *F8A* gene in intron 22 and one or two other homologous regions located more than 400 kb 5' (telomeric) to *F8*. For intron 1, the inversion is mediated by a 1041 bp duplication that maps within intron 1 and approximately 140 kb telomerically near the VBP1 gene. Other rearrangements such as duplications, chromosomal translocation, and insertion of retrotransposons (LINE elements) or other mobile elements (*Alu* sequences) were reported in a few cases of hemophilia A.

Aside from the common intron 22 inversion, the frequencies of the different types of genetic defects in severe and mild to moderate hemophilia are shown in FIGURE 12.9 which refer to tables and references updated to August 2007 [http://hadb.org.uk/WebPages/Database/Download]. The number of genetic defects that are taken into consideration is rather large being 984 and 417 for severe and for mild to moderate hemophilia A, respectively. For severe hemophilia A, other than the intron 22 inversion, the largest class of genetic defects is represented by small deletions (<50 bp) that amount to 29% of all the classified mutations. Missense mutations are the second largest class (25%), whereas large deletions (>50 bp) and nonsense (stop) have a similar frequency (14% to 13%), and splice mutations are the least abundant class (6%). Interestingly, in mild to moderate hemophilia A, the missense mutations constitute the predominant class (80%).

The splice site mutations in FIGURE 12.9 include mostly nucleotide substitutions. Donor or acceptor splicing sites are affected, new sites are created, or cryptic splicing sites are activated. These mutations are distributed in all the introns with only one reported mutation in introns 8, 21, and 25 out of 101 total mutations. A few rare mutations (5 out of 101) are within exons or at the intron/exon boundaries. These mutations may affect enhancers or silencers of splicing sites and may cause abnormal pre-mRNA splicing.[265] Nonsense mutations comprise 13% of the mutations associated with severe hemophilia A. These mutations cause premature translation termination of the polypeptide chain and may also reduce the *F8* mRNA level through nonsense-mediated mRNA decay (NMD). Small deletions or insertions predominantly result in frameshift. Together, they constitute 409 unrelated mutations out of 984 severe hemophilia A mutations and represent the largest class of mutations (42%) (FIGURE 12.9A). Moreover, 150 out of 304 small deletions and 98 out of 140 insertions were found as expected in the large exon 14.

Missense Mutations and Factor VIII Protein

Missense mutations reported in Table 12.3 result in amino acid substitutions in the mature protein and are important for understanding the function of the protein and the pathophysiology of the disease. Amino acid substitutions can affect the local protein structure and have only mild effects on the folding/stability/function of the molecule. Alternatively, some amino acid replacements can have both local effects and

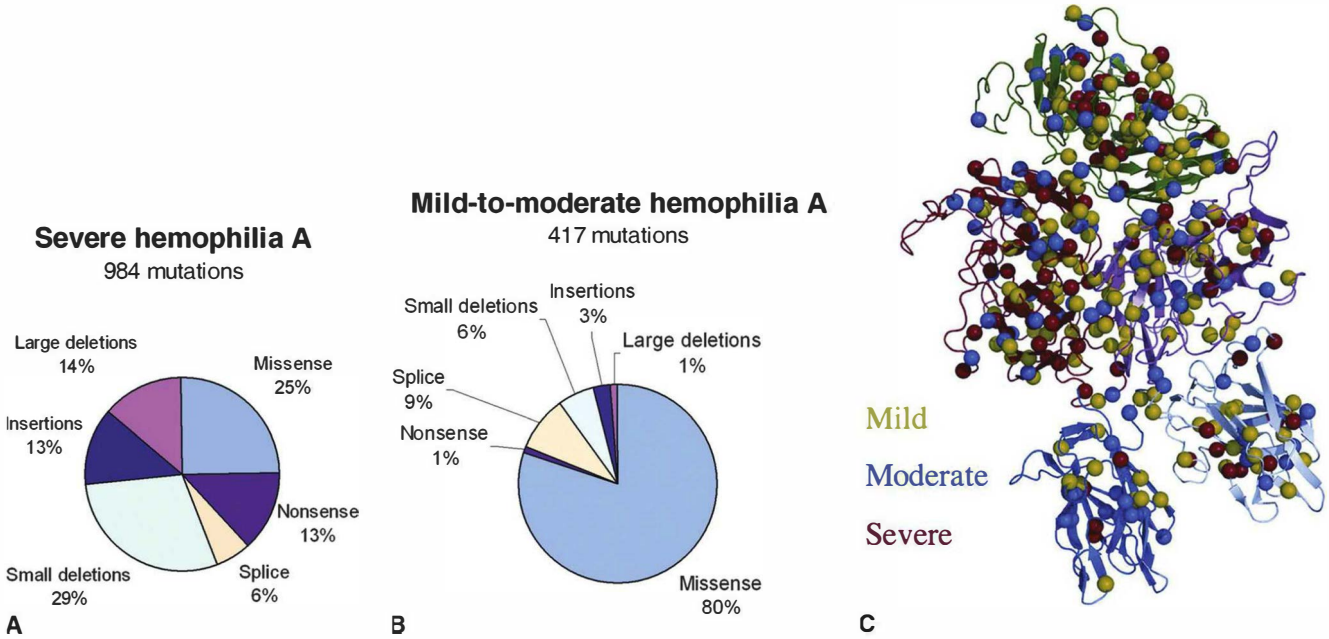

FIGURE 12.9 Factor VIII gene defects in severe and mild to moderate hemophilia A. The frequency of gene defects in severe **(A)** and mild to moderate hemophilia A **(B)**, excluding the intron 22 inversion, was elaborated from data deposited in the HAMSTeRS database (updated August 2007). Only data for which a classification of the degree of severity was reported were taken into consideration. Missense mutations refer to the amino acid number of protein devoid of the signal peptide. Where a range from severe to mild of symptoms was reported, the missense mutation was assigned to severe. Small deletions (50 bp or less), large deletion (>50 bp), insertions (all reported insertions), and splice mutations (all reported splice mutations). **(C)** Missense mutations in the factor VIII structure related to hemophilia A. The structural domains of the protein are shown and the amino acid substitutions are depicted with different colors depending on the severity of the disease/symptoms as defined by criteria of the HAMSTeRS Database (http://hadb.org.uk/). (Use with permission from *Blood*, Shen BW, Spiegel PC, Chaing C, et al. The tertiary structure and domain organization of coagulation factor VIII. *Blood* 2008;111:1240–1247 © 2008 by The American Society of Hematology)

long-range effects that may affect folding and cause instability/dysfunction of the entire molecule. Expression level, secretion efficiency, and/or half-life in the plasma can also be affected. Moreover, amino acid substitutions can occur at critical sites, for example, thrombin cleavage sites or sites of interaction with the substrate or other proteins. For example: (a) mutations in patients with CRM-positive hemophilia A that affect thrombin cleavage (mutations R372H, R372C, and S373L, abolish cleavage in the heavy chain[99,102,266] and mutations R1689C and R1689H abolish thrombin cleavage in the light chain[101,267]); (b) Y1680F substitution at the site of tyrosine sulfation that is required for high-affinity vWF interaction, observed in patients with moderate CRM-reduced hemophilia A,[268] decreases binding affinity to vWF presumably because the Phe residue cannot be sulfated;[92,269] (c) two other CRM-positive mutations, I566T and M1722T, produce severe hemophilia A by creating new *N*-glycosylation sites N564 and N1770, respectively, in the A2 and A3 domains[270] where factor VIII is present at normal levels in plasma, but is inactive. When the plasma from either patient is deglycosylated, factor VIII activity is restored to a significant degree. The glycosylation in the I566T mutant factor VIII causes steric hindrance for interaction with factor IXa.[271]

In a recent study, the missense mutations associated to hemophilia A were mapped onto the 3-D structure of the B domain–deleted factor VIII heterodimer[73] (FIGURE 12.9C). Hemophilia A-associated missense mutations appear to be distributed uniformly along the entire polypeptide chain, regardless of either severity of the symptoms or the structural domain of the protein.

Hypotheses regarding how the amino acid changes affect the structure, function, and secretion of the protein can be tested by expression of the mutant factor VIII in cultured cells. Not only do these studies provide insight into the amino acids and contacts essential for cofactor function[271–273] but this knowledge can also be used to engineer FVIII variants that have improved clinical utility for therapy of hemophilia A[274] (for a review, see Ref. [275]). An elegant example of this type of approach is a recent demonstration that altering contacts at the A1–C2 interface by introducing a disulfide bond or by filling a cavity with a bulky hydrophobic group increases interchain affinity and functionally enhances factor VIII stability.[276]

Combined Factor V and VIII Deficiency (F5F8D)

Combined factors V and VIII deficiency (F5F8D) is an autosomal recessive disease that was described in 1954 by Oeri et al.[277] Patients with this disorder exhibit factor V and factor VIII antigen levels in the range of 5% to 30% that correlates with the

activity measurements. Bleeding is similar to that observed in other coagulation factor deficiencies. The levels of all other plasma proteins that have been measured appear normal in these patients, including those of the homologous copper ion–binding protein ceruloplasmin. Although there have been several reports of individuals who simultaneously inherited mutations in both the factor VIII and factor V gene,[278] this is rare (~$1/10^{10}$).

Homozygosity mapping and positional cloning approaches were used to identify the defective genes in F5F8D. Essentially, all individuals with F5F8D have mutations in *LMAN1* (lectin, mannose-specific, also known as *ERGIC-53*) (~70% of the families)[279] or *MCFD2* (multiple coagulation factor deficiency gene 2)(~30% of the families).[280] At least 32 mutations in *LMAN1* have been reported throughout all 13 exons of the gene.[281,282] The mutations would be expected to result in a complete loss of function of the protein. In contrast, 16 mutations have been reported in *MCFD2*, the majority being splice site or missense mutations and would be expected to produce protein products. The diversity of the mutations suggests that F5F8D evolved through multiple independent genetic events. Interestingly, some mutations are uniquely associated with selective populations, such as two mutations in *LMAN1* account for all Jewish F5F8D patients. One mutation is most common in Sephardic Jews while the other mutation is unique to Middle Eastern Jews,[279] suggesting that in some populations, founder mutations may account for all or the majority of individuals with F5F8D.

LMAN1 encodes a 53-kDa type-1 transmembrane protein of the ER–Golgi intermediate compartment (hence, ERGIC-53) that has a luminal carbohydrate recognition domain that selectively binds mannose in a Ca^{2+}-dependent manner. LMAN1 forms homohexamers in the cell that cycle between the ER and the Golgi compartment.[283,284] *MCFD2* encodes a 16-kDa monomeric lumenal protein that has two EF-hand domains that bind Ca^{2+}. MCFD2 interacts with LMAN1 in a Ca^{2+}-dependent manner, and missense mutations that disrupt Ca^{2+} binding in MCFD2 disrupt LMAN1 interaction and cause F5F8D.[285] Analysis of factor VIII functional and physical interaction with LMAN1/MCFD2 suggests that the oligosaccharides within the B domain are the most important feature for LMAN1/MCFD2 interaction.[286,287] Most data support the hypothesis that factor V and factor VIII have evolved to utilize LMAN1/MCFD2 to facilitate their trafficking from the ER to the Golgi complex. Although there are several reports that describe alternative cargo for the LMAN1/MCFD2 trafficking complex, more definitive studies are required to substantiate these findings.[285]

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Full Reference Lists can be found online

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CHAPTER

13 Structure and Function of von Willebrand Factor

SANDRA L. HABERICHTER AND ROBERT R. MONTGOMERY

von Willebrand disease (vWD) is the most common hereditary bleeding disorder with a prevalence estimated by some to be 1% or greater.[1,2] Numerous studies have demonstrated the autosomal inheritance pattern for vWD (FIGURE 13.1). vWD is caused by decreased levels or defective function of von Willebrand factor (vWF) protein. vWF is a large, multimeric glycoprotein that functions to bind platelets at the site of vascular injury, to promote platelet–platelet interaction, and to serve as the carrier protein for coagulation factor VIII (FVIII). vWF is synthesized as a precursor protein comprised of a large propeptide (vWFpp) and even larger mature vWF protein (refer to Table 13.1 for terminology).

MOLECULAR AND STRUCTURAL BIOLOGY

Over the past 20 years, investigators have gained much insight into the structure and function of vWF through the delineation of its molecular and cellular biology. Such studies have helped us develop assays for laboratory diagnosis and understand the clinical manifestations, , the classification of vWD and its variants, and the rationale for therapy.

Molecular Biology

The coding sequence for human vWF was identified in a cDNA library derived from endothelial mRNA by several independent groups in 1985.[3,4–6] This cDNA sequencing demonstrated an 8.7-kb mRNA with an open reading frame that encodes a 2,813–amino-acid protein, including a signal peptide of 22 amino acids, a large propolypeptide of 741 amino acids, and a mature vWF molecule containing 2,050 amino acids. FIGURE 13.2 demonstrates the relationship of this cDNA to the vWF protein. Using the current numbering system, the initiating methionine codon is defined at nucleotide number one, and this methionine is also identified as amino acid number one. Analysis of the protein structure suggested areas of internal homology that have been termed A, B, C, and D domains.[7,8] The A repeats have sequence similarity to complement Factor B, type VI collagen, chicken cartilage matrix protein, and the "I-domain"–containing integrin α-subunit (Mac-1, VLA2, LFA, p 150, 95).[9,10] Areas of the C repeat are similar to segments of procollagen and thrombospondin.[11] Several functions of vWF have been mapped to specific domains. The D′ and D3 domains are important in FVIII binding, and the D3 domain is also essential for multimerization. The A1 domain contains the site for glycoprotein GPIbα binding. vWF binding to collagen occurs through interaction with the A1 and A3 domains. The C1 domain contains an RGDS sequence that allows vWF to bind glycoprotein GPIIb/IIIa. The site for proteolysis by the vWF-cleaving protease, ADAMTS13, is located within the A2 domain.[12]

After the coding sequence for vWF was identified, the entire human vWF gene has been cloned and much of its structure defined. The gene is about 178 kb long and contains 51 introns.[13] The localization and sequence of the intron–exon boundaries of these 51 introns have been determined. The gene for vWF has been localized to the short arm of chromosome 12, although a pseudogene, representing approximately the middle third of the vWF gene, has been identified and mapped to chromosome 22.[14,15] The presence of this pseudogene can cause problems in determining the genetic structure of some of the vWD variants, but various strategies have been developed to avoid this problem.[16] This pseudogene has about 98% homology to its corresponding portion of the authentic vWF gene on chromosome 12.[14]

Various genetic alterations of the vWF gene have been reported and include deletions, insertions, point mutations, alternative splice junctions, and premature transcription termination sites. Many of the molecular mutations reported are included in exon 28.[17] This exon encodes the region of vWF that interacts with platelet glycoprotein Ib (GPIb) and encodes the portion of the vWF protein containing the important *N*-terminal multimerization site(s)[7] and an important site where a metalloprotease cleaves vWF between Y1605 and M1606.[18] Most of the type 2A and 2B mutations have been localized within exon 28.[17] Type 2N mutations are clustered within the D′ and D3 domains of vWF.[19–23] Although a number of patients with type 1 vWD do not appear to have identifiable mutations in the vWF coding region, mutations that have been identified in type 1 vWD do not appear to cluster in any one domain, but are located throughout the vWF protein.[24–26]

Within intron 40, there are repetitive elements of variable length that have been termed VNTR (variable number of tandem repeats) or STR (short tandem repeats).[27,28] These repetitive elements can be amplified to define the allelic inheritance for unusual genetic phenotypes. These STRs may also be helpful for intrauterine diagnosis using amniocytes or chorionic villi cells with subsequent gene amplification of segments of the vWF gene using the polymerase chain reaction. Caution in amplifying this region must be exercised because of instability of this locus.[29]

von Willebrand Factor Promoter

Significant progress has been made in defining the upstream regulatory elements that control vWF expression. Several consensus sequences for *cis*-acting elements have been identified in the immediate upstream promoter region and first exon including

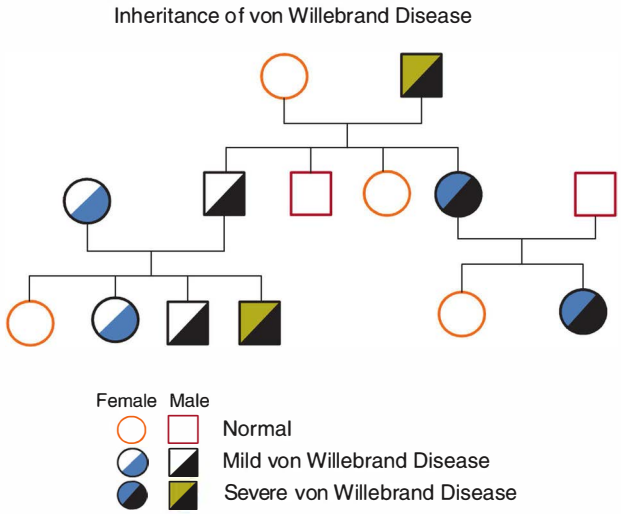

FIGURE 13.1 The autosomal inheritance of von Willebrand disease (vWD) is illustrated in this figure. Because low von Willebrand factor (vWF) has a frequency of 1% to 2%, some of these individuals are asymptomatic. Those with symptoms are said to have mild type 1 vWD. Patients with type 3 vWD are homozygous and inherit null alleles from each parent. (Used by permission, Montgomery RR.)

two GATA-binding consensus sequences.[30] The endothelial cell–specific expression appears to be regulated by a repressor–derepressor mechanism and includes both an NF1-binding site and an Oct-1-binding site.[30–34] A more complex mode of transcriptional regulation through vascular bed–specific signaling pathways has been recently defined. A 2,200 base pair 5′ flanking sequence together with the first intron and exon direct expression to microvascular endothelial cells of skeletal muscle and heart.[35] Recently, cell type–specific regulation of vWF expression by the E4BP4 transcriptional repressor has been identified.[36] Within the heart, a cardiomyocyte-dependent signaling pathway through platelet-derived growth factor (PDGF) has been defined.[37] Several single-nucleotide polymorphisms at nucleotides -1793, -1234, -1185, and -1051 have been associated with plasma vWF:Ag levels.[38,39] Thus, vWF gene is regulated by both cell-specific elements as well as the environment in which these cells are growing.[31,40]

Biochemistry

Numerous laboratories have purified normal human vWF, and there is general agreement concerning its structure and function relationships.[41–47] It is composed of a series of high molecular weight multimers that range between approximately 600,000 (vWF dimer) and 20 million Da. Using low-concentration agarose gels (0.65% to 1%), these multimers can be resolved, with the lowest band representing the dimer. Using high-concentration agarose gels (2% to 3%), each of these individual multimers can be resolved into several additional bands that are thought to result from subunits that have undergone partial proteolytic cleavage or contain variable amounts of modified carbohydrate.

When the disulfide bonds are reduced, a single predominant band is seen on polyacrylamide gel electrophoresis with an apparent molecular weight estimated to be approximately 220,000, plus some smaller bands caused by proteolytic degradation.[48] The complete protein sequence has been determined

Table 13.1

Terminology

vWD	The autosomally inherited bleeding disorder with a reduced amount or function of vWF
vWF	The glycoprotein that is abnormal or present in reduced amounts in patients with vWD
vWF:Ag	vWF antigen, the detection and quantitation of vWF by immunoassay
vWF:Rco	Ristocetin cofactor, the detection or quantitation of vWF using the antibiotic ristocetin, which induces vWF binding to platelets
vWF multimers	The multiple molecular forms of vWF comprising *C*-terminal–linked dimers assembled through subsequent *N*-terminal multimerization to produce high molecular weight vWF multimers
vWFpp	The large propeptide of vWF normally cleaved from pro-vWF during intracellular processing to produce a free vWFpp molecule (also previously called VW AgII) and mature vWF
FVIII	Factor VIII, the protein that is abnormal or reduced in patients with hemophilia A; vWF serves as an FVIII carrier protein in plasma, and, therefore, if vWF is reduced or its binding to FVII is abnormal, patients with vWD have reduced FVIII levels
Ag	Factor VIII coagulant antigen, factor VIII antigen is measured by immunoassay

vWD, von Willebrand disease; vWF, von Willebrand factor.
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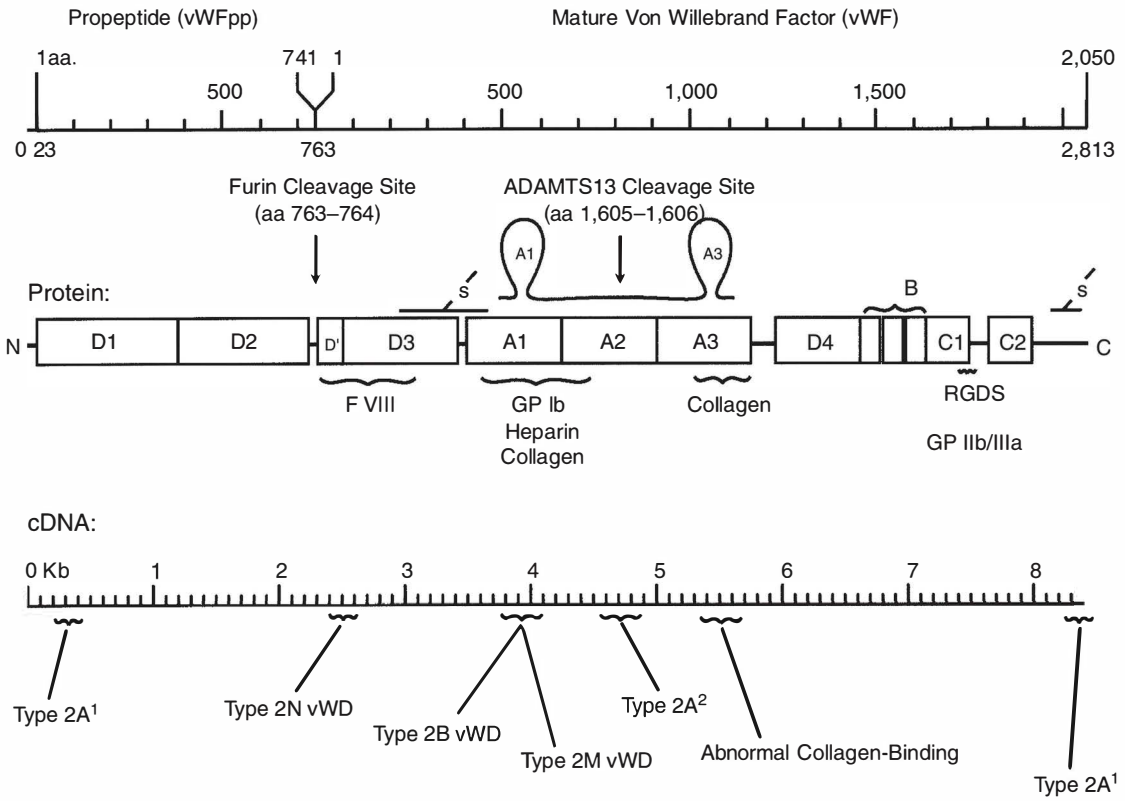

FIGURE 13.2 The von Willebrand factor (vWF) protein is initially synthesized as a 2,813–amino acid pro-vWF molecule whose synthesis is directed by an 8.5-kb RNA. The pre-pro-vWF is composed of a 22–amino acid signal peptide, a 741–amino acid propeptide (vWFpp), and a 2,050–amino acid mature vWF monomer. The pro-vWF is composed of A, B, C, and D repeats. Various functional domains have been identified and contain the sites of interaction with factor VIII, platelet glycoprotein GPIb, GPIIb/IIIa, collagen, and heparin. On the basis of these functional domains, various genetic mutations have been mapped to discrete regions of the cDNA as illustrated. Two protease domains are illustrated: furin cleavage site (cleaves the vWFpp from vWF) and ADAMTS13 cleavage site that cleaves the A2 domain. This ADAMTS13 cleavage is increased by some vWF mutations that cause type 2A vWD caused by increased vWF proteolysis. Cleavage by ADAMTS13 is absent in patients with thrombotic thrombocytopenic purpura (TTP) either because of a deficiency of the enzyme (hereditary TTP) or an autoantibody that blocks its cleavage of vWF (acquired or sporadic TTP). Type 2 mutations are generally located in specific regions along the vWF protein. Types 2A, 2B, and 2M are primarily located within exon 28 that encodes for the A1 and A2 regions of vWF. The two different types of 2A are those that have increased proteolysis 2A[2] and those with abnormal multimer synthesis 2A[1]. Mutations with abnormal collagen binding are clustered in the region encoding the A3 domain. (Used by permission, Montgomery RR.)

and is identical to the protein sequence derived from the cDNA sequence with a molecular weight of 225,663 for just the amino acid sequence of the mature vWF.[3–5,49] The carbohydrate component is estimated to add approximately 10% to 15% to the molecular mass; thus, the true molecular weight of the monomer is approximately 255,000, and its migration in polyacrylamide gel underestimates its true molecular weight.

The vWF multimers are extraordinarily large; the biggest are considerably larger than some virus particles. Electron microscopy of the vWF molecule after rotary shadowing reveals a flexible filamentous structure with irregularly spaced nodules that have been reported from several groups.[50,51,52] These filaments are approximately 20 to 30 Å wide and as long as 11,500 Å, with an average length of 4,780 Å; for comparison, fibrinogen is 475 Å long. Under native conditions, these long filamentous strands are presumed to be wound together in a compact molecule. During activation, shear stress, or other *in vivo* modifications, portions of the vWF may be unwound, allowing exposure of multiple interactive sites that may participate in platelet adhesion. Decreasing the size of the multimers by partial reduction of disulfide bonds results in a dramatic decrease in the platelet-agglutinating activity.[50,53,54]

This molecular weight-dependent heterogeneity of vWF is important when one compares the results of assays based on immunologic and platelet-agglutinating activities. Although immunoassays recognize large and small multimers, estimates of vWF activity using the ristocetin cofactor (vWF R:Co) assay predominantly measure the function of high molecular weight multimers.[54,55,56,57] In contrast, estimates of vWF activity using botrocetin-induced platelet binding correlate better with the immunoassays but may not represent functional activity *in vivo*.[58] Individuals with hereditary conditions such as type 2A

vWD, in which there is a reduction in multimeric size, often have more severe bleeding symptoms than individuals with a similar reduction in protein concentration but normal multimeric size (type 1 vWD); thus, *in vivo*, multimeric size may correlate with function.[55]

CELL BIOLOGY

Although vWF is synthesized both in the megakaryocyte and the endothelial cell, most of our understanding of cellular biosynthesis is derived from the endothelial cell because of the ease of *in vitro* endothelial cell culture.[59,60] Studies on megakaryocytes are limited.[61] Studies using transformed cell lines, and inference from the study of platelets, suggest that platelet vWF production is similar to that in endothelial cells. One difference has been seen in dogs whose platelets have been shown by several laboratories to lack vWF,[62] although one report identified vWF in canine platelets.[63]

Cellular Processing

Endothelial cells and platelets store vWF in secretory granules that are termed Weibel-Palade bodies (endothelial cells) and α-granules (megakaryocytes and platelets).[60,64,65] These secretory granules can be induced to release their contents by a variety of agonists such as calcium ionophore A23187, thrombin, adenosine diphosphate (ADP)/epinephrine (platelets), and histamine (endothelial cells).[66–68] *In vivo*, the vasopressin derivative desmopressin acetate (DDAVP) can induce the release of vWF into plasma.[69] The cell from which it is released is presumably the endothelial cell, as evidenced by a lack of reduction in platelet vWF after DDAVP therapy and a loss of Weibel-Palade body vWF on immunostained tissue sections obtained after DDAVP administration.[70] Much of our understanding of these endothelial cell events comes from expression studies in model cell lines and is summarized in the following paragraphs.[45,46,71,72]

vWF is synthesized as a 2,813–amino acid pre-pro-vWF molecule and contains a 22–amino acid signal peptide, 741–amino acid propeptide, and 2,050–amino acid mature vWF protein (FIGURE 13.3). vWF is subjected to extensive intracellular modifications. In the endoplasmic reticulum (ER), the signal peptide is removed, disulfide bonds are formed, and protein folding occurs. vWF is a cysteine-rich protein, containing 234 cysteine residues. While early studies indicated these cysteines were all involved in disulfide bonds, more recent studies using more sensitive techniques suggest that there may be a number of reactive free sulfhydryls in plasma vWF.[73,74] Although a number of disulfide linkages have been mapped, the majority of disulfide mapping in vWF has not been accomplished.[75–77] The process of protein folding and disulfide bonding has to be remarkably complicated given the large number of cysteines in vWF. The pro-vWF forms *C*-terminal dimers in the ER. This results in a pro-vWF *C*-terminal–linked dimer, the protomeric species that later forms larger vWF multimers. This dimerization is dependent upon the last 151 amino acids of the mature vWF protein.[78,79] These *C*-terminal sequences may serve a role in retaining monomers in the ER until they are either dimerized or degraded. The *N*-terminal portion of vWF is less important for dimerization and the large vWF propeptide is not required for dimerization to occur.[80–82]

Upon transport through the Golgi compartments, vWF undergoes carbohydrate modification. The mature vWF subunit is heavily glycosylated with 10 *O*-linked and 12 *N*-linked oligosaccharides.[49,83,84] This glycosylation accounts for approximately 18% to 19% of the total protein mass. Sulfation of the vWF molecule has also been demonstrated.[85] In the Golgi, the *C*-terminal–linked pro-vWF dimers form *N*-terminal–linked multimers that may exceed 20 million Da in size. The vWF propeptide (vWFpp), a protein initially described in plasma as VW AgII, is also proteolytically cleaved in the *trans*-Golgi. Processing of pro-vWF into vWFpp and the mature vWF is carried out by a paired amino acid–cleaving enzyme (PACE), or furin, that cleaves the propeptide from the mature vWF.[86,87] The site of propeptide cleavage is targeted by the sequence motif Arg-Xxx-Arg/Lys-Arg at the *C*-terminal end of the propeptide.[87] Because patients with hereditary persistence of pro-vWF have normal multimers, cleavage of the propeptide does not appear to be a prerequisite for multimerization or secretion.[88] The cleavage of propeptide appears to be essentially completed in the Golgi, prior to regulated storage.[80,89,90] Both portions of the molecule, the high molecular weight multimerized vWF and the cleaved propeptide, are stored in the Weibel-Palade body secretory granule.

vWF Multimerization and Regulated Storage

The assembly of vWF into *N*-linked multimers is a complex process involving interchain disulfide bonds. The first interchain disulfide bonds are formed during dimerization and involve cysteine residues located in the *C*-terminus region. Multimerization of *C*-terminal dimers involves cysteine residues located in the *N*-terminus region (D′ and D3 domains).[45,91] *C*-terminal dimerization is independent of multimerization. Although both processes involve the formation of disulfide bonds, *C*-terminal dimerization of vWF is completed in the ER, while *N*-terminal multimerization is carried out in the Golgi. The two processes are compartmentalized implying different enzymes or mechanisms regulate these steps. In the ER, neutral pH and the presence of necessary oxidoreductase enzymes such as protein disulfide isomerase promote disulfide bonding.[92,93] The acidic pH and lack of chaperones in the Golgi provide a desolate environment for disulfide bonding. However, ER-to-Golgi transport vesicles are unlikely to be able to accommodate the very large vWF multimers; thus, the formation of the large multimers is delayed until transport to the Golgi is complete. Initial glycosylation of the vWF protein and a low pH in the *trans*-Golgi network have been shown to be important for normal multimerization of vWF.[46] The acidic pH in the *trans*-Golgi network appears to be of particular importance for multimer formation. The assembly of functional vWF multimers from purified vWF subunits can be triggered *in vitro* under low pH conditions, similar to the pH in the Golgi.[73] The large propeptide plays an essential role in the vWF multimerization process. Deletion of either of the D1 or D2 propeptide domains, or the entire propeptide, results in a complete loss of normal vWF multimerization.[82,94] When the propeptide and the "mature" vWF molecule (propeptide-deleted) were expressed as two separate gene constructs, normal multimerization was observed indicating the propeptide can function *in trans* to facilitate multimerization.[82] The propeptide may facilitate vWF multimerization through areas of the propeptide that contain vicinal

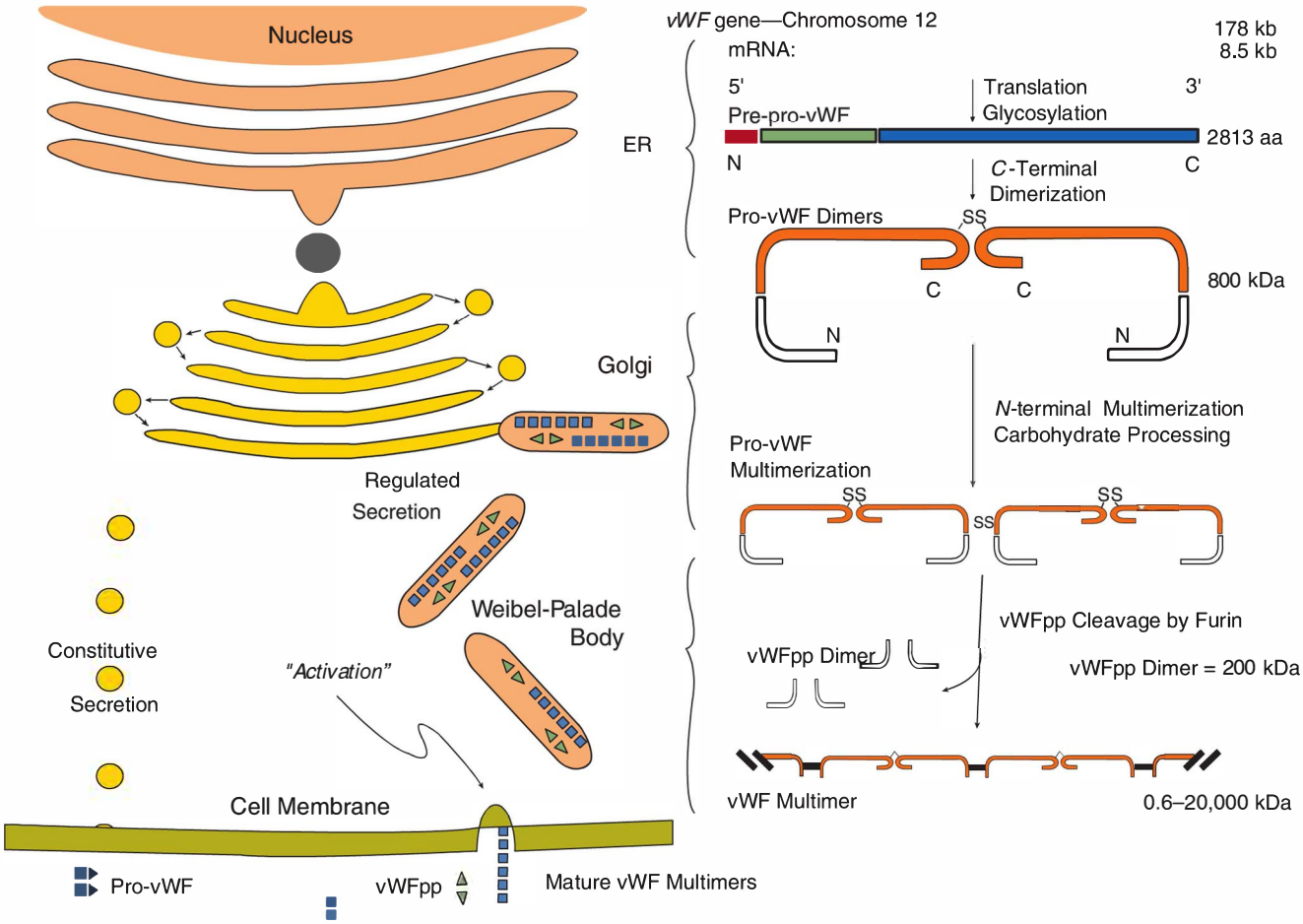

FIGURE 13.3 The sites of cellular biosynthesis are represented schematically in this illustration of endothelial cell biosynthesis. The von Willebrand factor (vWF) protomer is synthesized as a pre-pro-vWF dimer that is a *C*-terminal disulfide-bonded dimer within the endoplasmic reticulum (ER). The 22–amino acid signal peptide is then cleaved. The carbohydrate is then processed into complex sugars within the Golgi compartment. *N*-Linked multimerization by virtue of the self-association of the vWF propeptide (vWFpp) occurs in the acidic compartment of the late-Golgi, and the vWFpp is cleaved from the mature vWF by furin. A pH-dependent association between vWFpp and vWF enables the packaging of both proteins into storage granules termed Weibel-Palade bodies. After activation of the endothelial cell, the regulated secretion of these proteins occurs, but at the neutral pH in plasma, there is no longer an association of vWFpp with vWF. A similar pathway is assumed to occur in megakaryocytes, but the α-granule contains more different types of proteins. In the absence of vWF, the Weibel-Palade body is not formed, but the α-granule is still present. (Used by permission, Montgomery RR.)

cysteine motifs similar to disulfide isomerase.[95] The propeptide was shown to act as an oxidoreductase, thus promoting the multimerization of vWF in the Golgi apparatus.[96] Additional studies identified two amino acids in the D3 domain, Cys-1099 and Cys-1142, that participate in this process.[76]

Both vWF and vWFpp are stored for regulated secretion in Weibel-Palade bodies (endothelial cells) and alpha-granules (megakaryocytes/platelets). The Weibel-Palade body was first described nearly 50 years ago by Weibel and Palade.[97] Weibel-Palade bodies are large cigar-shaped granules that are the hallmark of endothelial cells. This secretory organelle is used for the storage of vWF (amongst other proteins) in endothelial cells and can be very rapidly triggered to release its contents.[98,99] Weibel-Palade bodies store components that are important to hemostasis, inflammation, and angiogenesis. vWF is the main component protein within Weibel-Palade bodies and plays an important role in the biogenesis of this organelle as discussed below.

The large vWF propeptide also plays a critical role in the regulated storage of vWF. vWF expression studies utilizing the AtT-20 and HEK293 cell lines have examined the regulated storage of vWF.[80,94,100,101,102] After the propeptide is furin cleaved in the Golgi, the propeptide and vWF remain noncovalently associated and are trafficked to regulated storage in a propeptide-dependent process. Expression studies have shown that deletion of either of the D domains abolishes the sorting of vWF to storage granules.[94] When the entire propeptide is deleted (Δpro), regulated storage of the "mature" vWF protein is also abolished. In contrast, the propeptide sorts to endogenous storage granules when it is expressed alone in AtT-20 cells. When the vWF propeptide and mature vWF (propeptide-deleted vWF or Δpro) are expressed together as two separate gene constructs, normal

granular storage of vWF is reestablished.[80,103] The propeptide contains the necessary sequence or conformation for sorting to storage granules and secondarily cotraffics mature vWF multimers through noncovalent association.[80] The nature of the noncovalent association between propeptide and mature vWF has been further defined. Two amino acids, arginine 416 within the propeptide and threonine 869 in the mature vWF protein are essential for noncovalent association and regulated storage of vWF.[104] The putative sorting signal for the propeptide may been located in the D2 domain. By using a series of propeptide truncation cDNA constructs (FIGURE 13.4), the sorting signal has be localized within amino acids 387 to 545 of the propeptide. The sorting signal within the vWF propeptide has been used to traffick an unrelated, nonsecretory granule protein (C3α) to the regulated storage pathway in AtT-20 cells and endothelial cells.[90]

Although the propeptide plays a critical role in both the multimerization and storage of vWF, the two events are clearly independent. Expression of vWF with disrupted vicinal cysteine motifs demonstrated a lack of vWF multimerization but maintenance of vWF regulated storage.[95] This was further demonstrated by expression studies utilizing human and canine vWF cDNA constructs. The canine propeptide was able to facilitate multimerization of human propeptide-deleted vWF (Δpro) but could not traffic the human vWF to regulated storage.[80] In a study utilizing the cross-species storage difference, a large number of human/canine chimeric constructs were expressed and examined for multimerization and regulated storage. No correlation was found between vWF multimerization and granular trafficking.[104] In many cases, normally multimerized vWF was not stored in granules and conversely, several vWF species that did not form multimers were trafficked to storage granules. A number of vWF mutations have been identified in vWD patients that disrupt vWF multimerization but did not affect the regulated storage of vWF.[101,105] There have been few patient mutations reported that maintain normal vWF multimerization but eliminate regulated storage and secretion.[102,106,107]

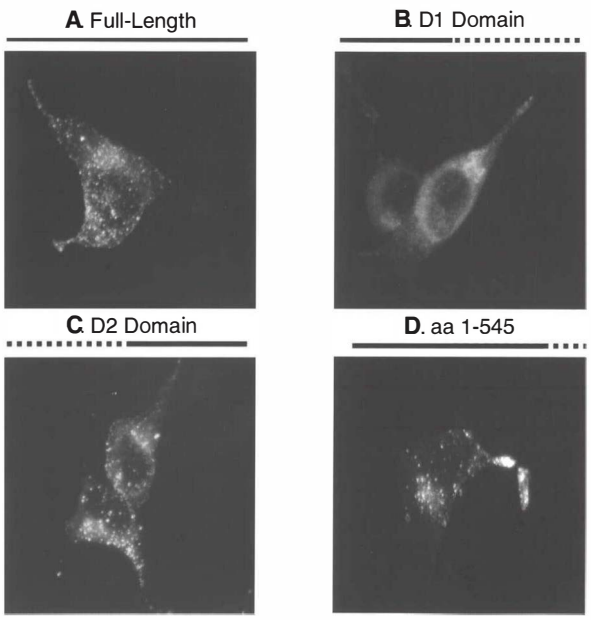

FIGURE 13.4 At T-20 cells were transiently transfected with von Willebrand factor (vWF) propeptide truncation expression plasmids, immunofluorescently stained, and examined by confocal microscopy. **A:** Cells expressing full-length propeptide demonstrate a granular staining pattern. **B:** Cells expressing only the D1 domain (amino acids 1 to 386) of the propeptide demonstrate a diffuse staining pattern with no granules detected. **C:** Cells expressing only the D2 domain (amino acids 386 to 763) of the propeptide demonstrate a granular staining pattern similar to full-length propeptide. **D:** Granular staining of a propeptide consisting of amino acids 1 to 545. The putative vWF propeptide sorting signal lies in the D2 domain within the region of amino acids 387 to 545. (Used by permission, Montgomery RR and Haberichter SL.)

The regulated storage of vWF in endothelial cell Weibel-Palade bodies and platelet alpha-granules is important physiologically. Patients with type 1 vWD are often treated with the vasopressin analog DDAVP (1-desamino-8-D-arginine-vasopressin). Administration of DDAVP results in a rapid increase in plasma vWF levels resulting from release of vWF from Weibel-Palade bodies.[108,109] vWF plays an essential role in the formation of Weibel-Palade bodies in endothelial cells. In the vWF-deficient mouse (e.g., type 3 vWD), no Weibel-Palade bodies can be detected in endothelial cells and the membrane protein P-selectin is routed to lysosomes.[110] Similarly, cultured endothelial cells harvested from type 3 vWD dogs demonstrated a lack of Weibel-Palade bodies.[111] Expression studies using the endothelium from the type 3 vWD dogs have demonstrated that expression of vWF protein is necessary for the formation of Weibel-Palade bodies that contain the membrane protein, P-selectin (FIGURE 13.5).[111] Both propeptide and mature vWF are necessary for formation of Weibel-Palade bodies: neither the propeptide nor mature vWF (Δpro) were sufficient for granule formation. Expression studies using *C*-terminal truncations of vWF showed that a vWF construct consisting of vWFpp through the vWF A1 domain stored vWF as tubules that correlated with the elongation of Weibel-Palade bodies.[112] More recently, vWF tubule assembly has been demonstrated *in vitro* using only purified vWFpp and D–D3 domains of vWF, narrowing the required domains for Weibel-Palade body formation to the vWF D1–D3 domains.[113] These studies also indicated that vWF may serve an active role in the recruitment of the leukocyte receptor, P-selectin to the Weibel-Palade body membrane.[114] While vWF is believed to be the most abundant protein in the Weibel-Palade body, additional components include P-selectin, the tetraspanin CD63, IL-8, osteoprotegerin, α1,3-fucosyltransferase VI, endothelin-1, eotaxin, and t-PA.[115–120,121,122,123] Although the site of synthesis of FVIII is unclear, FVIII can be stored together in a Weibel-Palade body when expressed with vWF.[124] Thus, vWF-dependent Weibel-Palade body biogenesis has significant biologic implications.

Although there are many similarities between endothelial cell Weibel-Palade bodies and platelet alpha-granules, there also several differences. While the hallmark characteristic of the endothelial cell Weibel-Palade body is its elongated, cigar shape, alpha-granules are not elongated. While both granules contain vWF and P-selectin, the alpha-granule contains several proteins not found in Weibel-Palade bodies such as fibrinogen, coagulation factor V (FV), platelet factor 4, thrombospondin-1, and fibronectin.[125] Unlike endothelial cell Weibel-Palade bodies, platelets do not require the synthesis of vWF to form the alpha-granule. In vWF-deficient mice[110] or dogs (Haberichter,

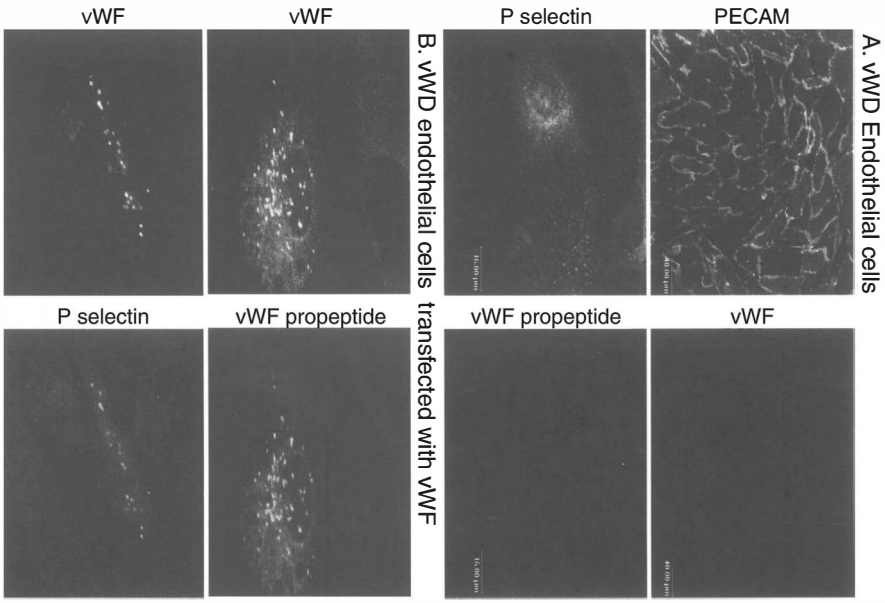

FIGURE 13.5 **A:** Endothelial cells were harvested from the aortas of dogs with type 3 von Willebrand disease (vWD), cultured, immunofluorescently stained, and examined by confocal microscopy. The cells were platelet/endothelial cell adhesion molecule (PECAM) (CD31)-positive, indicating that a homogenous population of endothelial cells had been cultured as shown. The cells were also found to be vWF-negative as shown. Staining for P-selectin was faint and diffuse with many small granules, most likely lysosomes. The vWF propeptide could not be detected in these cells. Therefore, no apparent Weibel-Palade bodies were detected in the canine vWD aortic endothelial cells. **B:** The canine vWD aortic endothelial cells were transiently transfected with a vWF expression plasmid. Immunostaining of cells expressing vWF demonstrated a granular distribution of vWF and vWF propeptide as shown. Dual staining for vWF and P-selectin revealed that the vWF was colocalized with P-selectin in granules. Therefore, in the absence of vWF, no Weibel-Palade bodies are formed in endothelial cells. However, vWF expression induces the formation of Weibel-Palade bodies, and the Weibel-Palade body distribution of endogenous P-selectin is reestablished. (Used by permission, Montgomery RR and Haberichter SL.)

unpublished data), P-selectin-containing alpha-granules are still present in platelets and megakaryocytes. Therefore, vWF is required for the biogenesis of the Weibel-Palade body but not the alpha-granule.

On appropriate agonist stimulation, endothelial cells and platelets release both vWF and the propeptide from Weibel-Palade bodies and α-granules, respectively. The cleaved propeptide remains associated with vWF under acidic conditions similar to those observed in the late *trans*-Golgi network and Weibel-Palade bodies, but dissociates from vWF when exposed to the higher pH found in plasma. The propeptide self-associates as a noncovalent dimer in plasma.[126] vWF can be secreted both luminally into plasma and abluminally into the subendothelial cell matrix.[71] Although the propeptide is also secreted, its extracellular function has not been delineated.

Proteolysis and Clearance of vWF

Once secreted, vWF multimers circulate in plasma at a concentration of approximately 10 μg/mL with a half-life of about 12 hours. The free propeptide circulates in normal human plasma at a concentration of about 1 μg/mL with a half-life of 2 to 3 hours. Little is known regarding the mechanisms underlying the normal clearance of vWF from circulation.[127] Increased clearance from the circulation has been identified as one mechanism causing type 1 vWD (type 1C).[128,129,130] In these patients, vWF has a significantly decreased half-life. Mutations in vWF have been associated with this phenotype including R1205H, C1130G/F/R, C1149R, W1144G, S2179F, and C2671Y.[128,129,131,132] In these patients, clearance was not dependent on multimer size as all multimers, small and large, disappeared quickly. Patients with this phenotype can be identified by an increased ratio of steady-state plasma vWFpp to vWF:Ag (FIGURE 13.6).[129] The characteristic elements of the type 1C phenotype include a substantially reduced vWF:Ag, reduced vWF half-life, diminished vWF multimer satellite bands, an apparently enhanced DDAVP response, *vWF* gene mutation, and increased vWFpp/vWF:Ag ratio.[131] An experimental model has been developed to examine clearance of human vWF from mouse circulation.[133] These studies supported the role of the D′-D3 domains in regulation of clearance. Carbohydrate modification and ABO blood group may also be important in vWF clearance.[134–137] The relevance of this mechanism to the normal clearance of vWF is not known.

There is a marked effect of ABO blood type on the vWF antigen (vWF:Ag) levels seen in normal individuals.[138,139] A study of more than 1,300 normal blood donors revealed that patients with group A, B, and AB have a much greater mean vWF:Ag plasma concentration than blood group O individuals. The A, B, and H antigen (the unmodified H antigen is present in persons with blood group O) has also been identified in vWF. The primary effect of this posttranslational carbohydrate modification appears to result in altered vWF

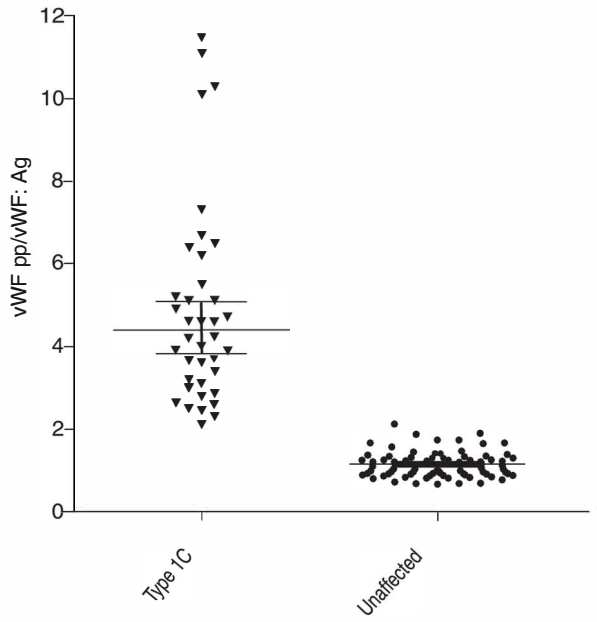

FIGURE 13.6 Steady-state plasma vWFpp/vWF:Ag in type 1C vWD. In patients with increased vWF clearance, plasma levels of vWF:Ag are markedly reduced while steady-state plasma levels of vWFpp are not reduced. Shown here are vWFpp/vWF:Ag ratios for a number of type 1C vWD patients with vWF mutations including R1205H, C1130F/G/R/Y, W1144G, and S2179F. The mean vWFpp/vWF:Ag in these patients was 4.9, in contrast to unaffected family members and normal controls with a vWFpp/vWF:Ag ratio of 1.2. The steady-state vWFpp/vWF:Ag ratio is useful in identifying vWD patients with reduced vWF survival. (Used by permission, Haberichter SL.)

survival *in vivo*. A study by Gallinaro et al. demonstrated a moderately reduced half-life of vWF in type O individuals.[135] Type O individuals have an increased ratio of vWFpp/vWF:Ag (1.33 ± 0.28 for type O and 1.03 ± 0.21 for non-O) supporting a reduced vWF survival in these individuals (Habericher, *unpublished data*). Table 13.2 lists the normal ranges for the different blood types reported in one study.[138] The effect of ABO blood type on vWF is now recognized as a disease-modifying gene for vWD.

Degradation of vWF is accomplished by the vWF-cleaving protease, ADAMTS13, a member of the "*a d*isintigrin-like *a*nd *m*etalloprotease with *t*hrombo*s*pondin repeats."[140,141–143] vWF is proteolytically processed by ADAMTS13 by cleavage of the Tyr[1605]–Met[1606] bond located within the A2 domain.[144,145] vWF is resistant to ADAMTS13 cleavage under static conditions but is rapidly cleaved when subjected to high shear stress. The high shear conditions most likely expose the cleavage site. vWF released from endothelial cell Weibel-Palade bodies consists of ultralarge vWF multimers. If the ultralarge vWF multimers are released directly into plasma, platelets can spontaneously aggregate resulting in thrombosis.[146,147] The ultralarge multimers form long string-like structures that remain tethered to the endothelial cell surface by interaction of the vWF with the membrane protein, P-selectin.[148] ADAMTS13 cleaves the tethered ultralarge vWF multimers rapidly under flow conditions.[149,150] Recently, the binding of platelet GPIbα to the vWF A1 domain was shown to stimulate the cleavage of the adjacent A2 domain by ADAMTS13.[151] A number of mutations in ADAMTS13 have been identified that affect its synthesis or function and cause a congenital form of thrombotic thrombocytopenic purpura (TTP).[143,152] In contrast, a number of mutations have been identified in vWF that increase the susceptibility of vWF to proteolysis by ADAMTS13 resulting in a loss of high molecular weight multimers in patient plasma and defective platelet binding (type 2A vWD).[18,153] The role of ADAMTS13 in normal degradation of vWF multimers is not well defined.

vWF is subjected to extensive posttranslational modifications, and mutations in the vWF gene can affect its intracellular processing. In the ER, protein misfolding (perhaps a result of aberrant disulfide bonding) can lead to intracellular retention and degradation resulting in reduced vWF secretion and low plasma vWF levels. Defective vWF multimerization results in reduced platelet binding as seen in type 2A vWD. Aberrant processing of carbohydrates may affect vWF function or its clearance from plasma. Defects in any of the many vWF intracellular processing steps may result in vWF intracellular retention, lack of secretion, defective regulated storage and secretion, increased clearance or degradation, or a functionally defective protein.

vWF FUNCTION

vWF serves several functional roles in hemostasis related to the initiation of hemostasis. During vascular injury, vWF first adheres to exposed subendothelial matrix proteins, including various types of collagens.[66,54] This tethered vWF then recruits circulating platelets in order to initiate platelet adhesion by

Table 13.2

Influence of ABO blood group on von Willebrand factor: values in volunteer blood donors

ABO Type	*N*	Geometric Values	Geometric Mean ± 2 SD
O	456	74.8	(35.61 ± 57.0)
A	340	105.9	(48.02 ± 33.9)
B	196	116.9	(56.82 ± 41.0)
AB	109	123.3	(63.82 ± 38.2)

The groups were statistically significantly different from each other as follows: O vs. A, B, AB, $P < 0.01$; A vs. AB, $P < 0.01$; B vs. A, $P < 0.05$.
Used by permission, Montgomery RR.

binding to the GPIb receptor found on platelets.[155,156] After the adhesion event, platelets become activated and platelet aggregation ensues, primarily by use of fibrinogen binding. vWF also supports platelet aggregation through binding to glycoprotein IIb/IIIa, but usually when fibrinogen levels are low, or the local concentration of vWF is high.[157,158] vWF also serves as the carrier protein for coagulation factor VIII (FVIII), protecting it from proteolytic degradation in plasma.[159] It has been suggested that since FVIII will ultimately participate in clot formation by binding to the activated platelet's exposed phosphatidylserine, vWF may play a role in the delivery of the necessary FVIII to the site of clot formation.

Interaction with Platelets

vWF is known to bind to subendothelium, perhaps in part due to its interaction with collagen.[160–162] Thus, in the normal hemostatic system, an intact endothelium precludes the interaction between matrix-bound vWF and platelets. After vessel injury, however, this bound vWF can be induced to interact with the GPIb receptor on platelets.[163] *In vitro*, this interaction with platelets can be mimicked in platelet-rich plasma by the addition of the antibiotic ristocetin, by the venom protein botrocetin, or by subjecting the platelets to high shear stress, all of which promote the binding of vWF to GPIb platelet.[158,164,165] vWF can also bind to a second receptor on platelets, the GPIIb/IIIa complex.[157,166,167] The GPIIb/IIIa receptor normally is the physiologic binding site for fibrinogen during *in vitro* aggregation, but in the absence of fibrinogen or after the local concentration of vWF is increased (platelet or endothelial cell secretion), vWF also may promote this platelet aggregation process.[164] Although the physiologic stimuli for the interaction of vWF with platelets are not fully defined, vWF may undergo a conformational change caused either by interaction with subendothelial matrix or by shear stress.[163,168,169] As described in more detail below, shear-induced platelet aggregation or adhesion can be inhibited by monoclonal antibodies directed against both GPIb and GPIIb/IIIa, suggesting that both receptors are physiologically relevant.[168,170–177]

Effects of Ristocetin on Binding of vWF to Platelets

The interaction of vWF with platelets is the biologic event that reflects a major interaction of vWF with the hemostatic system. Much of our knowledge about the normal interaction of vWF with platelets is derived from several assays that identify a functional abnormality in blood from patients with vWD: ristocetin-induced (or botrocetin-induced) platelet aggregation and agglutination, *ex vivo* interaction of platelets with blood vessel segments from which the endothelium has been denuded, and shear-induced platelet adhesion or aggregation.

Ristocetin, an antibiotic introduced into clinical practice in the late 1950s was observed to produce thrombocytopenia in recipients and was therefore withdrawn from clinical use. Approximately 10 years later, ristocetin was shown to aggregate normal platelets *in vitro* but to cause little or no aggregation of platelets from patients with vWD.[178,179] This observation permitted the development of a quantitative assay, the ristocetin cofactor assay.[180] If fixed platelets, which lack metabolic activity, are used, the response to ristocetin is termed *ristocetin-induced platelet agglutination*.[181] When ristocetin is used with metabolically active platelets in platelet-rich plasma, however, the overall response is termed *ristocetin-induced platelet aggregation* (*RIPA*). vWF binding to platelets induced either by ristocetin (GPIb) or thrombin (GPIIb/IIIa) is agonist dependent. Modified vWF molecules such as type 2B vWF, or normal vWF in which the negatively charged sialic acid residues are removed (asialo vWF), can bind directly to platelets even without any agonist activation.[182–184] In contrast, vWF protein harboring the D1472H polymorphism commonly found in African Americans has reduced ristocetin-induced platelet binding thought to be a result of decreased ristocetin binding to vWF, rather than a true type 2M defect.[185]

vWF Interactions Under High Shear Stress

To simulate more closely the conditions *in vivo* and to permit the differentiation between initial platelet adhesion to a surface in the subsequent platelet–platelet aggregation, a number of research models have been developed to study the effects of shear stress. Early studies on shear stress used the wall of an inverted rabbit aorta that demonstrated the adherence of isolated platelets and subsequent platelet aggregate formation under the control of vWF and fibrinogen.[186,187] The participation of vWF in this process was highly dependent on high shear forces (1,300 to 5,200/second).[188,189] A number of laboratories developed techniques to define the interaction between vWF and GPIbα, the necessary initial step that tethers platelets to damaged vascular surfaces.[190,191] The immobilization of plasma vWF onto exposed subendothelial surfaces is thought to be the predominant mechanism that initiates platelet response in areas of vascular injury exposed to elevated shear forces. Shear forces in the circulation uncoil globular vWF proteins while they are bound transiently to vascular surfaces.[192] The binding of GPIbα to the A1 domain of vWF is the main interaction that can tether platelets to a surface, even under conditions of high flow velocity.[193–195] Furthermore, the stable attachment of platelets requires the synergistic binding of integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ to their respective substrates. The initial binding of platelets to vWF tethered to exposed subendothelial matrix proteins slows the rolling of platelets so that other platelet receptors such as $\alpha_2\beta_1$ can further immobilize the platelet. Under abnormally elevated shear conditions (>5,000/second), vWF is thought to participate in the binding to $\alpha_{IIb}\beta_3$ in addition to GPIbα.[165,196,197] Subsequent studies have demonstrated a role for vWF, fibrinogen and possibly fibronectin, and CD40 ligand in the support of thrombus development.[110,190,191,198–200] Under physiologic arterial shear rates, vWF is responsible for the immobilization of platelets on the surface. Irreversible attachment of these platelets to the surface requires both vWF and fibrinogen and involves both receptors GPIbα and $\alpha_{IIb}\beta_3$.

Interaction with Platelet Glycoprotein Ib

The primary platelet receptor for vWF is GPIb. The GPIb complex on the platelet surface is comprised of at least four proteins: GPIbα, GPIbβ, GPIX, and GPV.[201–205] The site of interaction on the GPIb complex is GPIbα, but GPIbα is not trafficked to the platelet surface unless the other components of the complex are present. Within vWF, the site for

interaction with GPIb is the A1 domain. Although the sites for interaction with GPIb are within the A1 loop, the sites for ristocetin interaction are just outside the A1 loop. The interaction of platelet GPIb and the vWF A1 domain is particularly important as this interaction supports activation-independent platelet tethering even under conditions of elevated flow velocities. This bond has a fast dissociation rate and is considered "selectin-like."[206] *In vivo*, the stimulus for platelet adhesion to vWF is recognized to be shear. Under shear, vWF is unraveled and the binding sites for platelet GPIb are exposed, initiating the immobilization of circulating platelets.[207,208] Platelets tethered to the vessel wall through vWF–GPIb interaction move slowly in the direction of flow. This slow motion allows the interaction of other platelet receptors to the vessel wall, such as the interaction of the activated integrin GPIIb/IIa with collagen receptors.

There are three types of mutations in vWF that impact the binding of vWF to GPIb including "gain-of-function" mutations that cause type 2B vWD, "loss-of-function" mutations that cause type 2M vWD, and gain of function mutations within GPIbα that cause platelet-type of pseudo-vWD.[58,209–213] The crystal structure for the vWF A1 domain has been solved and both type 2B and 2M mutations are found to cluster together.[214,215] More recently, it has been reported that the A1 loop of vWF has an active conformational state that allows it to easily bind to GPIb.[216–219] This "activated" vWF can be identified using a llama-derived antibody fragment that only binds to the A1 loop when vWF is in an active conformation. This "activated" vWF conformation is identified in TTP, type 2B vWD, and antiphospholipid syndrome.[216,217,219]

Interaction with Platelet Glycoprotein IIb/IIIa (Integrin $\alpha_{IIb}\beta_3$)

In vitro, if one uses washed platelets and purified vWF, platelet agonists such as ADP and thrombin can induce the binding of vWF to GPIIb/IIIa.[166,167] This binding is blocked by monoclonal antibodies to GPIIb/IIIa but not by antibodies to GPIb.[126,164] In the presence of normal concentrations of fibrinogen, however, fibrinogen, not vWF, binds to this receptor.[164] This may be in part a result of the fibrinogen concentration being much greater than the concentration of vWF (3 mg/mL vs. 10 μg/mL). In clinical conditions in which fibrinogen is reduced in concentration, vWF can be demonstrated to bind to this receptor and may partially support platelet aggregation in patients congenitally deficient in fibrinogen (afibrinogenemia).[220] *In vivo*, at local sites at which platelets and endothelial cells may participate in hemostasis, secretion of vWF from α-granules and Weibel-Palade bodies may increase dramatically the local concentration of vWF and facilitate vWF interaction with GPIIb/IIIa. In studies on shear-induced platelet aggregation both in a cone viscometer as well as in flow chambers, vWF appears to participate with both the GPIb receptor and the GPIIb/IIIa receptor.[221,222]

Identification of the specific sites of interaction between vWF and platelets has been greatly facilitated by both the delineation of vWF genetic structure and by using specific vWF fragments, synthetic peptides, and recombinant proteins. A crucial interaction site for GPIIb/IIIa is the Arg-Gly-Asp (RGD) sequence at amino acid positions 1744 to 1746 on the *C*-terminal end of the vWF subunit.[223,224] Initial studies indicated that the GPIb binding site for vWF was localized to a disulfide-bonded loop at amino acids 463 to 921 of vWF, which is encoded by exon 28 of the vWF gene.[225,226]

Subendothelial Binding

Types I, III, and VI collagen are known to support platelet adhesion and aggregation.[227] Several putative collagen-binding proteins have been identified on platelets including integrin $\alpha 2\beta 1$, GPVI, and GPIV.[228–232] However, only $\alpha 2\beta 1$ and GPVI have a clear role in collagen-mediated platelet binding. Platelets can also bind to fibrinogen/fibrin, fibronectin, thrombospondin, and laminin.[207] The relative importance of each remains unclear. vWF plays a unique role in platelet adhesion under flow conditions. The mechanism of vWF interaction with components of the blood vessel wall has been extensively studied. Endothelial cells not only secrete vWF but also deposit vWF on the abluminal surface in close association with the internal elastic lamina.[233,234] vWF binds to type VI collagen via the A1 domain, while the A3 domain does not appear to play any role in this interaction.[235] vWF also binds to types I and III collagen and these interactions have been localized to the A1 and A3 domains of vWF.[236] Several mutations have been identified in the A3 domain that result in defective collagen binding to types I and III collagen including S1731T, W1745C, S1783A, and H1786D.[237–240] While vWF binding to type VI collagen appears to occur at low shear rates, the relative contributions of vWF binding to types I, III, and VI collagen *in vivo* have yet to be determined.[240,241] vWF binding to collagen is dependent upon multimer size—only the largest molecular weight multimers bind efficiently. Therefore, the vWF–collagen binding assay may be used to identify lack of high molecular weight multimers as found in types 2A and 2B vWD. Another subendothelial component that may function as a binding site for vWF is the elastin-associated microfibril; extracts of this material cause platelets to aggregate in the presence of vWF, which requires the presence of platelet GPIb receptor.[242,243] Further studies are necessary to fully delineate the various interactions of vWF required for normal hemostatic function.

Interaction with Factor VIII

Antihemophilic factor, Factor VIII, circulates in plasma associated with vWF; this led investigators to conclude that vWF and Factor VIII were the same molecules.[244–249] Other investigators established that these were two independent molecules that circulated together in plasma as an associated complex.[245–249] While the initial name for vWF was factor VIII–related antigen, this is now an outdated and incorrect term (see Table 13.1).

Early studies on patients with vWD demonstrated a reduced level of FVIII.[250–252] When cryoprecipitate was infused into patients with vWD, there was a modest initial increase in FVIII that increased to even higher levels over several hours and then slowly declined. This contrasts sharply with the pattern seen in patients with hemophilia infused with the same product, who have a rapid increase in FVIII with a normal 12-hour half-life.[159,252] Furthermore, early studies demonstrated that after cryoprecipitate or plasma prepared from patients with hemophilia containing no FVIII was infused into patients with vWD, it resulted in a paradoxic increase in plasma FVIII in the patient's plasma.[251–253] Our current understanding is that these two proteins associate

through a noncovalent interaction.[254,255] The infused vWF associates with the endogenously produced FVIII to protect this FVIII from rapid degradation and removal, normalizing the short half-life of the endogenously produced FVIII.[256,257]

Factor VIII is synthesized as a large precursor protein containing repeated domains A1-A2-B-A3-C1-C2. Factor VIII circulates in plasma as a heterodimer of heavy chain A1-A2-B, and light chain A3-C1-C2, in complex with vWF.[258] The binding site on FVIII for vWF was initially localized to the carboxyl terminus of the light chain. Together, the acidic region of the FVIII light chain and the C2 domain form a high-affinity binding site for vWF.[259] Mutations have been identified in hemophilia A patients that affect FVIII binding to vWF including Y1680F, S2119Y, R2150H, and P2300S.[260–262] Within vWF, the binding site for FVIII has been localized to the *N*-terminus region of vWF within the D′-D3 domains.[263] A number of mutations have been identified in type 2N vWD patients with reduced FVIII binding including R760C, R763G, Y795C, Q1053H, C788R/Y, C804F, D879N, C1060R, and C1225G.[264]

It is generally assumed that vWF is synthesized in endothelial cells and megakaryocytes[7,41,43,265] and that FVIII is synthesized in hepatocytes, although expression of FVIII in murine sinusoidal and human microvascular lung endothelial cells has been reported.[266–269] When the drug DDAVP is administered, vWF is released from endothelial cells, and FVIII is found to increase in parallel to the vWF, although the source of this released FVIII remains controversial. It has been demonstrated that if FVIII is produced in a cell that also synthesizes and stores vWF (AtT-20 cells or endothelial cells), FVIII follows vWF into storage granules.[270] In addition, patients with severe hemophilia A who are administered recombinant FVIII for prolonged periods have no increase in their FVIII after administration of DDAVP.[271] Furthermore, the patient with severe vWD who is given only vWF, which results in normalization of FVIII, has been found to have no change in plasma FVIII after the administration of DDAVP. Thus, FVIII and vWF must both be endogenously synthesized for FVIII to be released from storage sites. A study by Lollar and coworkers demonstrates that sinusoidal endothelial cells synthesize and store FVIII together with vWF in *ex vivo* cultured cells.[269] Understanding the mechanism of vWF and FVIII association may be important for the most appropriate transfection strategy in the gene therapy of hemophilia A. The functional reason for the association of FVIII with vWF is not entirely clear, but one could theorize that vWF binding and delivery of FVIII to the phospholipid surface of platelets might be coordinated *in vivo*. Recent studies have demonstrated the potential for targeting FVIII expression to megakaryocytes and platelets for efficient delivery of FVIII to the site of platelet plug formation.[272,273]

Animal Models of vWD

Several animal species have been found to have vWD similar to the human disorder (canine and porcine). A "knockout" mouse model of vWD has also been developed.[110] Porcine vWD has been identified for more than 20 years.[274] This model was used to study both liver and bone marrow transplantation as a means of correcting endothelial (liver) or platelet vWF (bone marrow). Bone marrow corrected the platelet vWF but resulted in only a minimal increase in plasma vWF. Bleeding symptoms decreased and there was partial correction of the bleeding time. Liver transplantation caused a near normalization of plasma vWF levels and a normalization of the bleeding time. Such studies suggest that plasma vWF is primarily derived from endothelial cell vWF synthesis, but that platelet vWF also provides some benefit even if it is not accompanied by an increase in plasma vWF.[275] Canine models of vWD are also available as either type 2 or type 1 vWD.[63,276,277] Such models will be useful in studying gene therapy of vWD. The canine type 2 models are important for studying the variant forms, which are not present in the porcine model. Studies on the porcine and murine model of vWD suggested a potential role for vWF in the development of atherosclerosis and endocarditis.[278] Studies on human vWD have not yet identified a similar protection from atherosclerosis.[279]

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CHAPTER

14

Structure and Function of Vitamin K–Dependent Coagulant and Anticoagulant Proteins

KANAGASABAI VADIVEL, AMY E. SCHMIDT, VICTOR J. MARDER, SRIRAM KRISHNASWAMY, AND S. PAUL BAJAJ

Physiologic coagulation is initiated when, upon vascular damage, blood comes into contact with tissue factor (TF) expressed by cells in the extravascular compartment.[1,2,3–7] TF exposed at the injury site complexes with factor VIIa (FVIIa, ~100 pM) as well as with factor VII (FVII, ~10 nM).[8,9] The FVIIa/TF complex activates both factors X (FX) and IX (FIX), leading to thrombin (IIa) generation and fibrin formation (FIGURE 14.1). In positive feedback loops, factor Xa (FXa), factor IXa (FIXa), and FVIIa/TF generate additional FVIIa/TF from FVII/TF.[10–15] Similarly, factor XIa (FXIa), formed by the activation of FXI by thrombin, produces additional FIXa for sustained coagulation.[16,17,18,19,20] FXa initially formed by FVIIa/TF generates small amounts of thrombin, which activates platelets as well as factor V (FV) and factor VIII (FVIII).[21] FIXa and FVIIIa assembled on the surface of the activated platelets convert additional FX to FXa, which complexes with FVa to further augment thrombin generation.[21] In addition to the four procoagulant proteins (FVII, FIX, FX, and FII), three other vitamin K–dependent (VKD) proteins, protein C (PC), protein Z (PZ), and protein S (PS), control the process by acting as anticoagulants. Activated PC (APC) generated by the action of thrombin/thrombomodulin complex inactivates FVa and FVIIIa,[22,23] PZ serves as a cofactor for inhibition of FXa by the protein Z–dependent inhibitor (PZI),[24] and PS serves as a cofactor for inactivation of FVa and FVIIIa by APC.[25,26,27] Other VKD proteins have been described, including osteocalcin (bone metabolism),[28] periostin (matrix homeostasis), and Gas6 (endothelial homeostasis),[29,30] plus others of unknown function.[31] This chapter focuses on the structural and functional aspects of the VKD proteins involved in hemostasis and thrombosis.

MOLECULAR ORGANIZATION OF THE VKD PROTEINS

Domain Organization

Zymogen FVII, FIX, FII, PZ, and PS circulate as single-chain molecules, whereas FX and PC circulate as two-chain molecules.[32,33–39] FVII, FIX, FX, and PC have similar structural architecture, in which an *N*-terminal γ-carboxyglutamic acid (Gla)-rich domain is followed by two epidermal growth factor (EGF)-like domains and a *C*-terminal serine protease domain (FIGURE 14.2).[40–45] PZ has similar domain organization, but it contains a pseudoprotease domain without catalytic activity at the *C*-terminus.[37,38] PS consists of six domains, an *N*-terminal Gla domain, a thrombin-sensitive region (TSR), four EGF-like domains, and a sex hormone globulin binding domain.[46–48] Prothrombin (FII) has a distinct domain architecture, in which the *N*-terminal Gla domain and the *C*-terminal protease domain are linked by the two kringle domains.[49]

Zymogen Activation

The VKD zymogens are synthesized in the liver and secreted into the blood; the molecular mass and plasma concentrations are shown in Table 14.1 (see also Chapter 9).

FVII consists of an *N*-terminus Gla domain (residues 1 to 38), a short hydrophobic segment (residues 39 to 45) followed by two EGF-like domains (residues 47 to 84 and 31 to 85), and a *C*-terminus latent protease domain (residues 153 to 406)[12,34,50] (FIGURE 14.2A). FXa, FIXa, and the FVIIa/TF complex activate FVII by cleaving a single peptide bond between Arg152 and Ile153 located in the connecting region between the EGF2 and the protease domain,[15,51–53] leading to a two-chain molecule (FIGURE 14.3A), a light chain of 152 residues, and a heavy chain of 254 residues linked by a single disulfide bond between Cys-135 and Cys-262.

FIX consists of *N*-terminus Gla domain (residues 1 to 40), a short hydrophobic segment (residues 41 to 46), two EGF-like domains (residues 47 to 84 [EGF1] and 85 to 127 [EGF2]), an activation peptide region (residues 146 to 180), and a latent serine protease module (residues 181 to 415) (FIGURE 14.2A).[33,54,55] Activation of FIX requires two peptide bond cleavages, one at Arg145–Ala146 and Arg180 and one at Val-181, releasing a 35-residue activation peptide (FIGURE 14.3A).[32,56] The resulting FIXa consists of a light chain containing Gla, EGF1, and EGF2 domains (residues 1 to 145) and a heavy chain containing the protease domain (181 to 415) held together by a single disulphide bond.

FX consists of a light chain (amino acids 1 to 139) and a heavy chain (amino acids 143 to 448) held together by a single disulfide bond between Cys-132 and Cys-c302 (FIGURE 14.2A).[35] The release of a 52-residue activation peptide after cleavage at Arg194–Ile195 (by FVIIa/TF, FIXa/FVIIIa, or Russell's viper venom protease) in the heavy chain leads to the generation of FXa (FIGURE 14.3A).[57] The light chain of FXa contains *N*-terminus Gla domain (residues 1 to 39), a hydrophobic stack of residues (residues 40 to 45), EGF1 domain (residues 46 to 84), and EGF2 domain (residues 85 to 128), and the heavy chain contains the protease domain (residues 195 to 448).

Prothrombin (FII) is the most abundant VKD protein in plasma and circulates as a single-chain molecule consisting of an

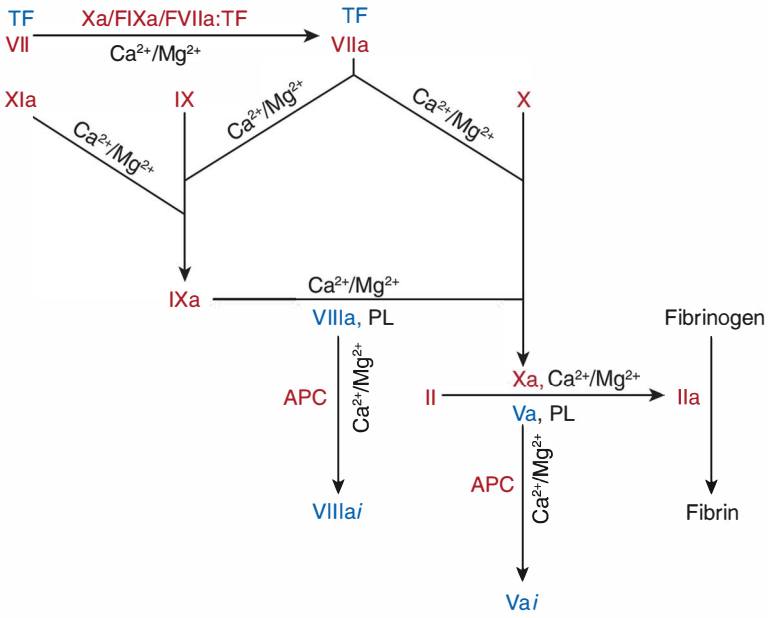


FIGURE 14.1 The role of VKD coagulant and anticoagulant proteins in hemostasis. The FVIIa/TF (extrinsic tenase complex) converts zymogens FX and FIX into their respective active FXa and FIXa forms. In positive feedback loops **(top left)**, FXa, FIXa, and FVIIa/TF activate FVII/TF. FIXa bound to FVIIIa (intrinsic tenase complex) activates FX to FXa on the phospholipid (PL) surfaces, FXa then binding to FVa (prothrombinase complex) to convert prothrombin (II) to thrombin (IIa). APC inactivates FVa (to FV*ai*) and FVIIIa (to FVIII*ai*). Activation of PC to APC by the IIa/-thrombomodulin complex and that of FXI to FXIa by IIa are not shown. Under physiologic conditions, all activation and inactivation processes involving VKD enzymes are Ca^{2+}/Mg^{2+} dependent. Although Ca^{2+} alone can function in these reactions, free Mg^{2+} can substitute for Ca^{2+}. Enzymes and inactive precursors are shown in *red*, cofactors in *blue*.

N-terminal Gla domain, two kringle domains, and a latent serine protease domain (FIGURE 14.2A).[49,58,59] *In vitro*, thrombin cleavage of prothrombin at Arg155–Ser156 generates prothrombin fragment-1 (PF1) and prethrombin-1 (PT1) (FIGURE 14.3B).[60,61] PF1 consists of Gla domain (residues 1 to 40) and kringle-1 domain (residues 65 to 155), whereas PT1 consists of prothrombin fragment-2 (PF2, residues 156 to 271), and prethrombin-2 (PT2), which contains the A chain (residues 272 to 320) and B chain (residues 321 to 579) of the *C*-terminus latent protease domain (FIGURE 14.3C). Cleavage of PT1 by FXa at Arg271–Thr272 releases PF2 from PT2. Proteolysis at Arg320–Ile321 by FXa then results in the two-chain active thrombin, which cleaves itself at R284-T285 to generate stable α-thrombin.[32,62,63] Physiologic activation of prothrombin by the prothrombinase complex (FXa, Ca^{2+}/Mg^{2+}, PL, FVa) occurs by sequential proteolysis at Arg271–Thr272 and Arg320–Ile321.[32,63,64] Initial cleavage at R320-I321 between the A and B chains is the preferred pathway under physiologic conditions and generates the active intermediate meizothrombin that retains all four domains of prothrombin. The alternative initial cleavage at Arg271–Thr272 generates inactive precursor PT2 (residues 272 to 579) and a combined PF1 and PF2 (residues 1 to 271). Cleavage of PT2 by FXa at Arg320–Ile321 generates a two-chain active thrombin composed of a 49 residue A chain and 259 residue B chain. This two-chain molecule, further processed by thrombin which cleaves chain A at Arg284, generates a stable α–thrombin with 36 residues A chain (FIGURE 14.3B).

APC circulates as a disulfide-linked two-chain zymogen PC.[65,66,67] The *N*-terminal light chain contains the Gla domain and two EGF-like domains (EGF1 and EGF2), and the heavy chain contains the latent serine protease domain (FIGURE 14.2B).[22,68,69] Although α–thrombin can activate PC to APC, the rate is extremely slow for a physiologically significant reaction. The physiologic activator of PC is the thrombin/thrombomodulin complex at the endothelial surface.[23,70,71] During conversion of PC to APC, a short 12-peptide fragment is removed from the heavy chain *N*-terminus to generate the active protease (FIGURE 14.3A).[60]

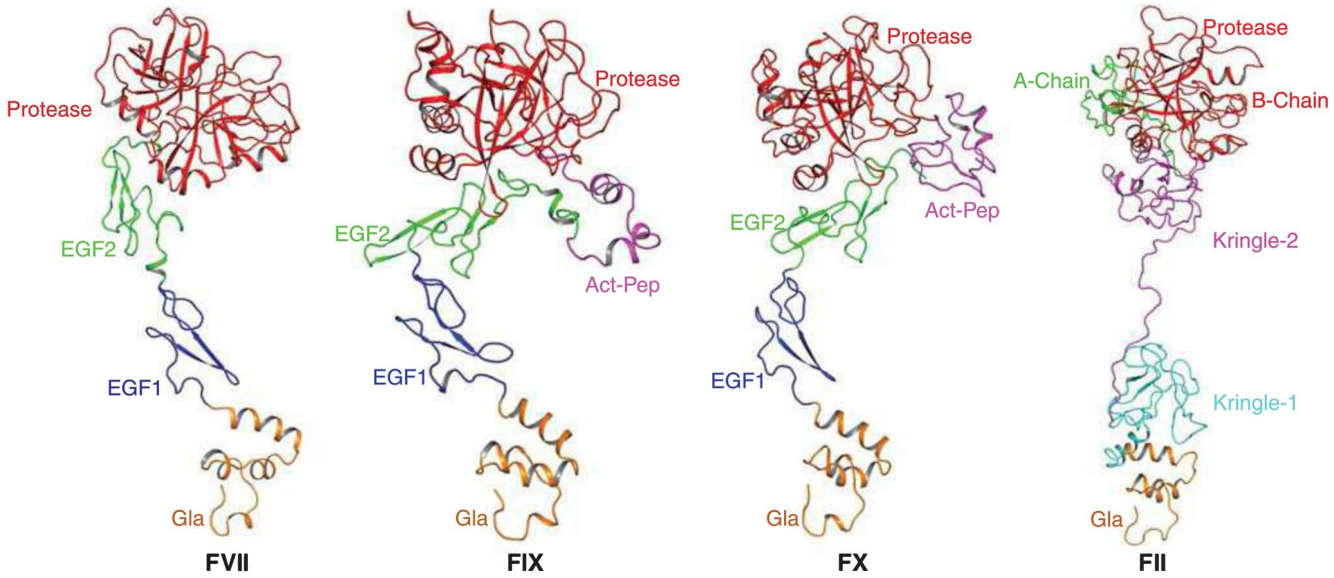
Cofactor Participation

The catalytic activity of each VKD proteolytic protein is augmented by binding to their respective cofactors (Table 14.2). TF functions as a cofactor for FVIIa in the activation of FX and FIX. TF is an integral membrane protein and consists of an extracellular region, a transmembrane region, and a cytoplasmic region, which is structurally homologous to the class 2 cytokine receptor family.[72] The extracellular region is made up of two fibronectin type-III domains that interact with all FVIIa domains.

FIXa requires FVIIIa as a cofactor for effective activation of FX. FVIII circulates in plasma in an inactive form consisting of a heavy chain (Mr varies between 90,000 and 200,000) that contains A1, A2, and B domains and a light chain (Mr ~ 80,000) that contains A3, C1, and C2 domains.[73,74] The activated form of FVIII is generated by the removal of B domain, either by FXa or by thrombin.[75] Similarly, FVa acts as a cofactor for FXa to augment prothrombin activation. FV (Mr ~ 330,000) circulates in plasma as a single-chain molecule having a similar domain organization as FVIII.[76,77] The release of domain B by the action of thrombin generates FVa,[78] a two-chain molecule consisting of a heavy chain (A1-A2 domains) and a light chain (A3-C1-C2 domains) (see Chapter 12).

PZ circulates in complex with PZI, as a single-chain molecule,[79] consisting of an *N*-terminus Gla domain (residues 1 to 41), two EGF-like domains (EGF1, residues 42 to 82; EGF2, residues 83 to 139), and a pseudocatalytic domain (residues 149 to 360) (FIGURE 14.2B). The typical active site residues of the serine proteases, His57 and Ser195 (chymotrypsin numbering), are replaced with Lys and Asp, respectively, in the *C*-terminal domain.[37]

PS circulates as a single-chain molecule[80] consisting of an *N*-terminus Gla domain (residues 1 to 41), a unique TSR (residues 42 to 75), four EGF-like domains (EGF1, residues 76 to 114; EGF2, residues 115 to 159; EGF3, residues 160 to 201; EGF4, residues 202 to 242), and a *C*-terminus sex hormone binding globulin (SHBG) domain (residues 243 to 635), which is completely different from other VKD proteins.[80] The SHBG



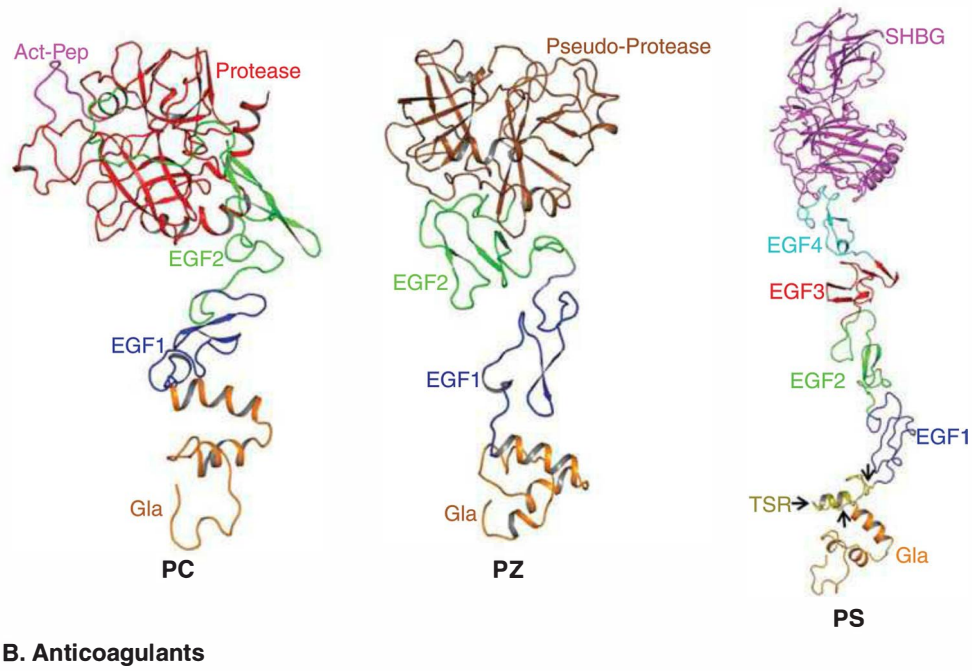


FIGURE 14.2 Modeled structures of the zymogen forms of coagulant **(A)** and anticoagulant **(B)** VKD proteins. The Gla domains are shown in *orange*, EGF1 domains in *blue*, EGF2 domains in *green*, and protease domains in FVII, FIX, FX, II, and PC in *red*. Kringle domains 1 and 2 of FII are colored *deep teal* and *magenta*, respectively, and the segments that become A and B chains in α-IIa are colored *green* and *red*, respectively. Activation peptide regions in FIX, FX, and PC are colored *magenta*. The pseudoprotease domain of PZ is colored *brown*. The thrombin-sensitive region (TSR), the EGF3, EGF4, and SHBG domains in PS are colored *yellow*, *red*, *deep teal*, and *magenta*, respectively; the SHBG domain in PS contains two laminin G-type binding subdomains.

region consists of two laminin G–like domains. PS functions as a cofactor for the anticoagulant APC and enhances the inactivation FVa and FVIIIa.[27,81,82]

POSTTRANSLATIONAL PROTEIN PROCESSING

γ-Carboxylation and Propeptide Cleavage

The propeptide region contains the carboxylase recognition site. In FIX, site-specific mutagenesis of Phe(−16) to Ala and Ala(−10) to Glu inhibits γ-carboxylation.[83,84] Naturally occurring Ala(−10) to Thr or Val and Asn(−9) to Lys mutations lead to warfarin hypersensitivity where severe depression of FIX occurs despite mild reductions in other VKD proteins.[85–87] Synthetic FIX propeptides with Thr(−10) or Gly(−10) have reduced carboxylase affinity compared to Ala(−10),[87] and mutation of Leu(−6) to Asp in FIX leads to impaired γ-carboxylation in tissue culture.[88] Similar studies on FII show that the propeptide residues at −18, −17, −15, and −10 are important for γ-carboxylation.[89] A DNA construct in which the propeptide region was placed adjacent to a region of thrombin rich in Glu residues resulted in γ-carboxylation of the Glu residues to Gla.[90] Moreover, deletion mutants of proprotein C lacking residues −17 to −12 were also defective in γ-carboxylation.[91] The NMR structure of synthetic FIX propeptide shows that the γ-carboxylase recognition site is likely within

Table 14.1
Properties of VKD zymogens

Zymogen	Number of Amino Acids	Molecular Mass (kDa)	Plasma Concentration (μg/mL)	Plasma Concentration nM
FVII	406	50,000	0.5	10
FIX	415	57,000	5	90
FX	448	59,000	10	180
FII	579	72,000	100	1390
PC	419	62,000	4	65
PZ	360	62,000	2–3	60
PS	635	69,000	25	360

an α–helical region that includes a hydrophobic surface similar to prothrombin.[92,93] These data support a concept that the propeptide region itself is sufficient to support γ-carboxylation.

Synthetic peptides with a complete propeptide sequence of FII were able to have their respective Glu residues γ-carboxylated efficiently with a K_m of approximately 3 μM,[94,95] whereas peptides with a shortened propeptide region or without the γ-carboxylase recognition site were poorly γ-carboxylated.[94,95] Moreover, a synthetic peptide based upon the propeptide sequence of FX stimulates γ-carboxylation of a pentapeptide Phe–Leu–Glu–Glu–Leu approximately eightfold.[96] Thus, it is feasible that the catalytic activity of carboxylase is regulated by binding to its normal substrate.

Importantly, the Gla domains also play a role in γ-carboxylase binding. In FIX, linkage of the propeptide to the Gla domain increased its affinity approximately 20-fold for γ-carboxylase.[97] Moreover, the Gla domain was found to play an important role in the rate of release of FIX from γ-carboxylase as the k_{off} of fully

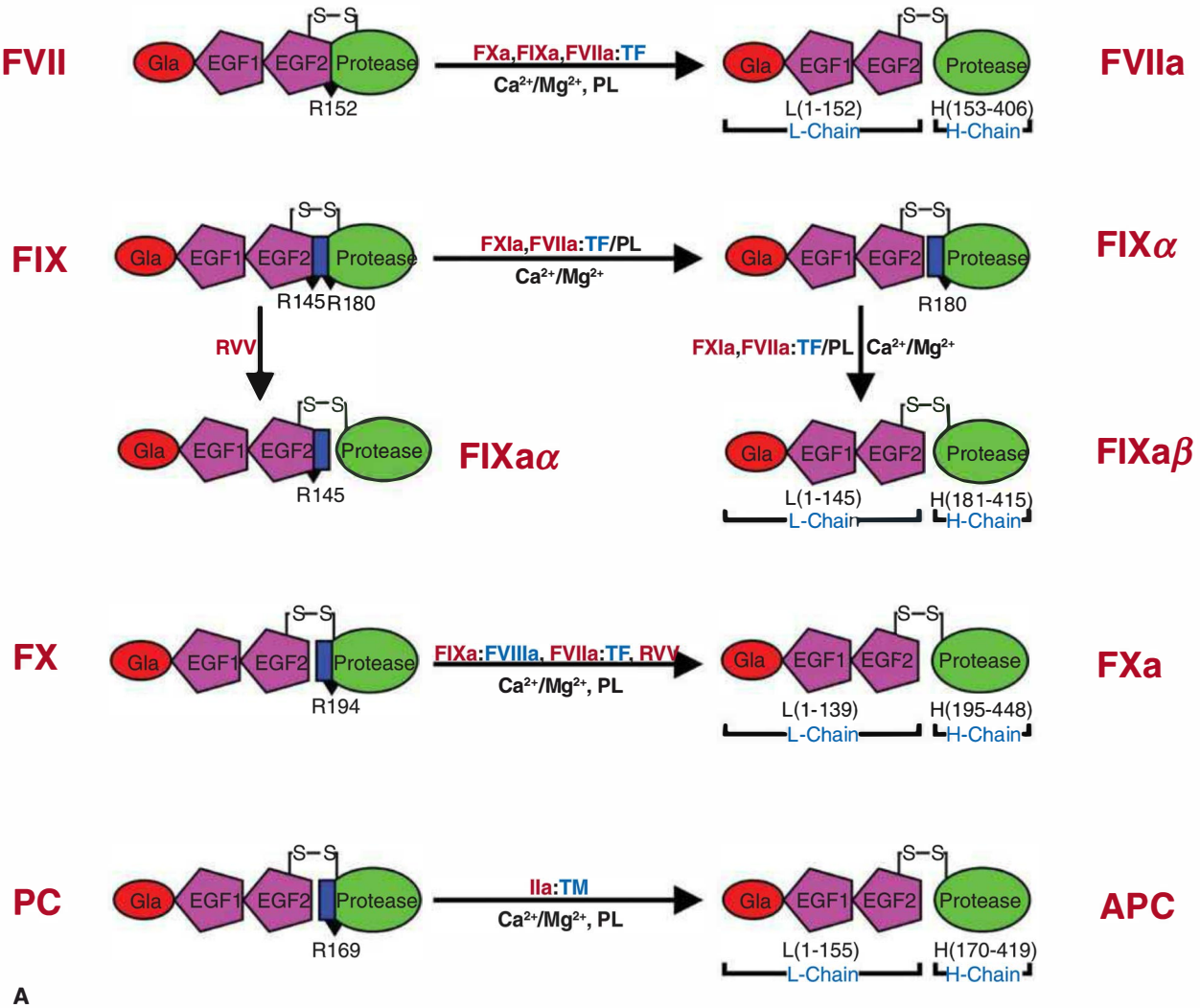


FIGURE 14.3 Schematic representation of zymogen activation. **A**: FVII, FX, and PC are converted to active forms by cleavage at a single residue at R152, R194, and R169, respectively. FIX is activated by FXIa and FVIIa/TF by sequential cleavages at R145 and R180 and by RVV by cleavage at R180. The activation peptide regions in FIX, FX, and PC are shown as *blue rectangles*, and the cleavage sites are marked with *black triangles*.

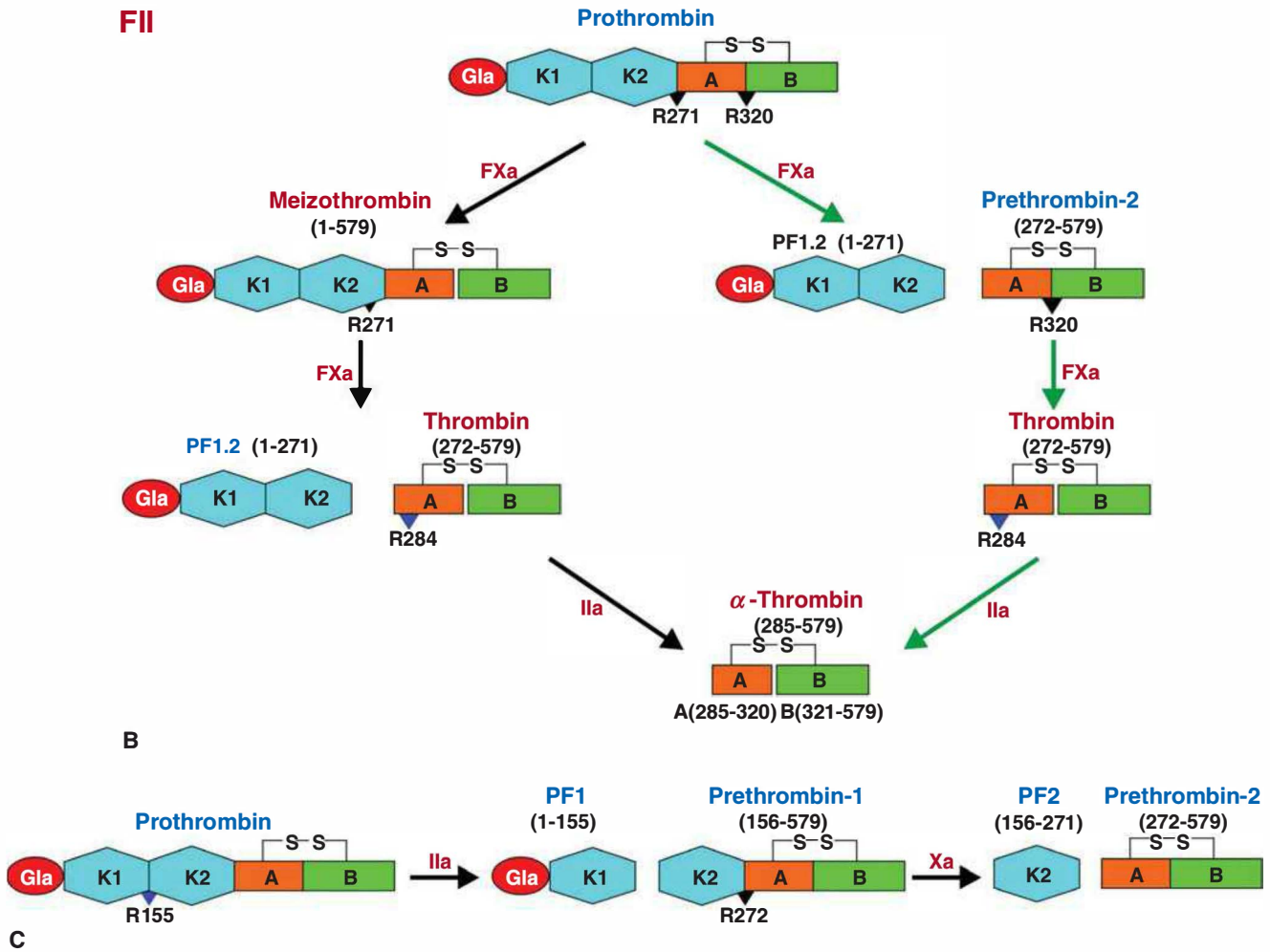


FIGURE 14.3 *(Continued)* **B**: Physiologic generation of thrombin from prothrombin. FXa converts prothrombin to thrombin, by proteolytic cleavages at R272 and R320, which occurs by two pathways. In one pathway (*black arrows*), cleavage at R320 yields meizothrombin, which is then cleaved at R271 to yield thrombin and PF1.2. In the second pathway (*green arrows*), cleavage at R271 yields PT2 and PF1.2. The generated PT2 is cleaved at R320 by FXa to yield thrombin. Thrombin generated by either pathway is further cleaved at R284 by thrombin itself to yield stable α-thrombin. FXa cleavage sites are indicated by *black triangles* and the thrombin cleavage site by the *blue triangle*. **C**: Thrombin-initiated conversion of prothrombin to thrombin starts with cleavage at R155, yielding PF1 and PT1. FXa cleavage at R272 in PT1 yields PF2 and PT2, which is converted to thrombin. Gla, γ-carboxyglutamic acid–rich domain; EGF1, epidermal growth factor–like domain 1; EGF2, epidermal growth factor–like domain 2; K, kringle domain; PF1, prothrombin fragment-1; PF2, prothrombin fragment-2; PF1.2, prothrombin fragment-1 and fragment-2.

Table 14.2
Cofactors for VKD enzymes

Cofactor	Enzyme	Substrate
TF	FVIIa	FX, FIX
FVIIIa	FIXa	FX
FVa	FXa	FII
PS	PC	FVa, FVIIIa
PZI	PZ	FXa

γ-carboxylated FIX Gla domain was approximately threefold faster than the k_{off} of noncarboxylated Gla domain.[97] Absence of γ-carboxylation at two or more sites results in a prothrombin molecule that is an ineffective substrate for the prothrombinase complex.[98] Thus, the prothrombin deficient in γ-carboxylation cannot bind Ca^{2+} and fails to undergo conformational changes that occur with Ca^{2+} binding. Moreover, the molecules are defective in binding to the PL surface on which the prothrombinase complex assembles.

The propeptide is cleaved from each VKD protein by PACE/furin,[99] which recognizes a consensus sequence Arg–X–Arg/Lys–Arg at positions −4 to −1 and cleaves after the −1 position Arg residue. In FIX, this sequence includes three basic residues,

Arg(−4)–Pro–Lys–Arg(−1)–Tyr(+1). Hemophilic mutations at the −4, −2, and −1 positions (P4, P2, P1) cause a severe bleeding tendency due to dysfunctional FIX.[100–102] FIX of hemophiliacs with Arg(−4) to Trp, Gln, or Leu react normally to antibodies that bind in the presence of Mg^{2+}, indicating that γ-carboxylation has occurred, but the FIX mutant proteins require more than 5 mM Ca^{2+} for binding to the PL surfaces.[103] Similarly, site-specific mutagenesis of Arg(−1) to Thr reduced propeptide cleavage by more than 99%, and nonconservative mutations at Pro(−3) and Leu(−6) also reduced propeptide cleavage in FIX.[104] Recombinant profactor IX with[105] full γ-carboxylation does not bind to PL membrane and is not activated by FXIa unless the propeptide is cleaved by PACE/furin. Thus, a free *N*-terminus is essential for proper conformational folding of the Gla domain, and acylation of Tyr(+1) is sufficient to prevent FIX binding to PL.[103]

β-Hydroxylation

The VKD proteins undergo posttranslational modification *via* hydroxylation of Asp and Asn residues by aspartyl β-hydroxylase.[106,107] FIX has β-hydroxylation at Asp64 in the EGF1 domain.[108] However, only one-third of the Asp64 residues are hydroxylated,[109] inhibition of hydroxylation in recombinant FIX does not decrease activity,[110] and the hydroxyl group is not necessary for Ca^{2+} binding.[111,112] Bovine FVII contains a β-hydroxyaspartic acid in the EGF1 domain at Asp63; however, Asp63 in human FVII is not β-hydroxylated.[113] PS contains a β-hydroxyaspartic acid at residue 95 in the first EGF domain, and the other three EGF domains contain β-hydroxylated Asn residues, which are incompletely hydroxylated.[114,115] Asp63 is hydroxylated in FX,[116] and Asp71 in PC,[117] whereas Asp64 is β-hydroxylated in PZ.[118]

Signal Peptide and Its Cleavage

In FIX, the hydrophobic signal peptide residue Ile30 is important as the hemophilic mutation to Asn results in no detectable FIX antigen.[100] FIX signal peptide cleavage occurs between Thr18 and Cys19 and mutation of Cys19 to Gly, Arg, Trp, or Tyr is associated with hemophilia B.[100] When the signal and propeptide sequences of FIX are substituted for those of PC, full γ-carboxylation occurs,[119] indicating that the signal and propeptide sequences are not specific among homologous VKD proteins (see Chapter 9).

Glycosylation, Sulfation, and Phosphorylation

Glycosylation occurs either *via* Asn or Ser/Thr residues, forming amide linked (*N*-linked) or *O*-glycosidically linked (*O*-linked) carbohydrate chains. Human FVII contains two *N*-linked carbohydrate chains attached to the residues Asn145 and Asn322[113] and two *O*-linked carbohydrate chains attached to the residues Ser52 and Ser60 in the EGF1 domain. Ser52 is linked to disaccharide (Xyl–Glc) or trisaccharide (Xyl_2–Glc),[120] whereas Ser60 is linked to a fucose.[121] Alanine mutations at Ser52/Ser60 revealed that these *O*-glycosylation sites do not affect TF binding or the proteolytic activity of FVIIa/TF complex toward FX or FIX, even though the mutant FVIIa had reduced activity.[122]

Bovine FIX contains 26% carbohydrate[123] and human FIX contains 17%,[124] oligosaccharides primarily *N*-linked to consensus Asn–Xxx–Thr/Ser sequences (four in bovine and two in human). The two sites of *N*-linked glycosylation in FIX are Asn157 and Asn167, both within the activation peptide region.[33] FIX from all species, all besides humans have an *N*-linked glycosylation at Asn260, all have Asn157, all but porcine have Asn167, and the sequences of ovine, bovine, and porcine predict an Asn172 site.[125] Notably, FIX activity is preserved following sialic acid removal with neuraminidase.[126] In the first EGF domain of FIX, Ser53 has *O*-linked di- and trisaccharides [Xyl_1- or Xyl_2-Glc-*O*-Ser][120] and Ser61 contains a tetrasaccharide [NeuAc-Gal-GlcNAc-Fuc-*O*-Ser].[127] Of note is the fact that FIX proteins from other species such as porcine do not have homologous Ser residues, so *O*-linked carbohydrate moieties in the EGF1 domain are unlikely to have functional importance. In the FIX activation peptide, the hydroxyl groups of Thr159 and Thr169 are glycosylated.[128] Plasma-derived FIX has an *O*-linked oligosaccharide at Thr172, sulfation of Tyr155, and phosphorylation of Ser158.[129] Importantly, of all the activation peptide modifications in FIX, only the equivalent of Ser158 phosphorylation and *N*-linked glycosylation at Asn157 are conserved in other species. Lastly, the catalytic serine protease domain has no posttranslational modifications.[129,130]

Human FX contains two *N*-linked carbohydrate chains attached to residues Asn181 and Asn191[35] and two *O*-linked carbohydrate chains attached to Thr159 and Thr171.[131] The *O*-glycosylation sites are found in the activation peptide region, which is removed during activation to FXa. Human FX contains neutral bi-, tri-, and tetra-antennary complex type oligosaccharides without a fucose residue at the *N*-glycosylation sites and disialylated Galβ1-3GlaNAc sequences at the *O*-linked sites.[132] The removal of the activation peptide or the Asn191 to Ala mutation affects the activation of FX by FVIIa/TF[133,134] and FIXa/FVIIIa[133] marginally. However, in another study, the carbohydrates contribute to the cofactor-dependent recognition of the zymogen by both the extrinsic and intrinsic Xase complexes.[135] Thus, the physiologic role of carbohydrate chains in FX remains unclear.[133–135] Human FII contains approximately 10% carbohydrates attached through the three *N*-linked glycosylation sites at Asn78 and Asn100 in the kringle-1 domain of PF1 and Asn102 of the thrombin B chain.[136] Partial deglycosylation at Asn102 using neuraminidase, β-galactosidase, and β-*N*-acetylglucosaminidase suggests that the carbohydrates at this site had no effect on thrombin functions.[137]

Similar to prothrombin, human PC contains only *N*-linked glycosylation sites. The *N*-linked glycosylation in PC is highly variable with several glycosylation variants present in human plasma that contain bi-, tri-, or tetra-*N*-linked carbohydrates attached to Asn97, Asn248, Asn313, and Asn329.[68,138] Mutational studies carried out at each glycosylation site of PC indicate that Asn97 glycosylation is essential for the efficient secretion of PC and affects the degree of core glycosylation at Asn329.[139] All glycosylation variants of PC can be activated; however, they have different rates of activation as well as different anticoagulant activities. Elimination of all the glycosylation sites in PC increases the anticoagulant activity two- to threefold.[140] Elimination of only the *N*-linked site at Asn313 yields a 2.5-fold increase in the rate of PC activation due to a decrease in K_m.[139] A polylactosamine on PC has been implicated in inhibiting cell adhesion through E selectin and may represent a potential anti-inflammatory function.[140]

Human PZ contains nine potential carbohydrate sites, of which five are *N*-linked sites and the other four are *O*-linked sites. The potential *N*-linked oligosaccharide are Asn59, Asn185, Asn193, Asn266, and Asn292, and the potential *O*-linked oligosaccharide sites are Ser53, Ser72, Ser196, and Ser275.[38] Ser53 is linked to either Xyl–Glc or the Xyl_2–Glc.[120] The functional role of

carbohydrate chains in PZ is unknown. PS contains three potential *N*-linked glycosylation sites at residues Asn458, Asn468, and Asn489 in the SHBG domain.[27,141] Asn to Gln mutagenesis revealed that Asn458 mutant affects cofactor activity while the Asn468 mutant affects γ-carboxylation.[142,143]

VKD CRYSTAL STRUCTURES

The coagulation process is regulated by VKD proteins that appeared to be evolved from the common ancestor. These proteins share high degree of sequence and structural homology; however, each VKD protein performs its function in a precise, controlled manner. Among the VKD proteins, FVIIa is the only one whose complete structure has been determined.[144,145] FVIIa adopts an extended conformation, where the Gla domain is on one end of the molecule and the catalytic domain is on the other. The binding of FVII/FVIIa to TF makes FVII/FVIIa somewhat more rigid and less flexible.[146] The sequence similarity and domain organization of FIXa, FXa, and APC indicate that these proteins may assume similar conformations.[147,148,149,150–154,155]

Crystal structures of IIa and its different forms as well as of FII fragments have been determined; these include PF1,[156,157] PT1,[158] meizothrombin des-F1,[159,160] PF2,[161] PT2,[162–164] and native and mutant thrombins.[165,166–175] The EGF2-protease segment of PZ in complex with ZPI has been reported,[176,177] but for PS, only the NMR structures of EGF3–EGF4 domains have been reported.[178]

Gla Domains

The common structural element among all VKD proteins is their *N*-terminal Gla domain, the glutamic acid residues within the first 40 residues being converted to Gla residues by carboxylase. The binding of Ca^{2+} and Mg^{2+} divalent metal ions to the Gla domain induces a structural transition from an unfolded, nonfunctional form to a folded functional form. In its functional form, the Gla domain binds to phosphatidylserine-exposed PL membranes with the aid of solvent-exposed hydrophobic residues in the ω-loop. Prothrombin, FVII, APC, and PS each contain seven divalent metal binding sites in their Gla domains, whereas FIX, FX, and PZ contain more than seven sites. In addition to membrane binding, Gla domains serve as cofactor and/or substrate binding recognition sites.

FVII/VIIa. Seven divalent metal binding sites[144] have been identified in the Gla domain of FVII/FVIIa and are necessary for stabilizing its functional fold. Among the 10 Gla residues present in FVII/FVIIa, 9 are associated with the metal ions. In the crystal structure of FVIIa/TF solved in the presence of Ca^{2+} only (PDB id 1DAN), all seven sites in the Gla domain are occupied by Ca^{2+} ions,[144] whereas in the presence of Ca^{2+}/Mg^{2+} (PDB id 2A2Q,[145]), three of the seven Ca^{2+} ions at positions 1, 4, and 7 are replaced by Mg^{2+} (compare FIGURE 14.4A,B). The fold of the Gla domain ω-loop in FVIIa is different between these two structures (FIGURE 14.4C). Folding of the ω-loop under near physiologic concentrations of Ca^{2+}/Mg^{2+} is similar to 2A2Q

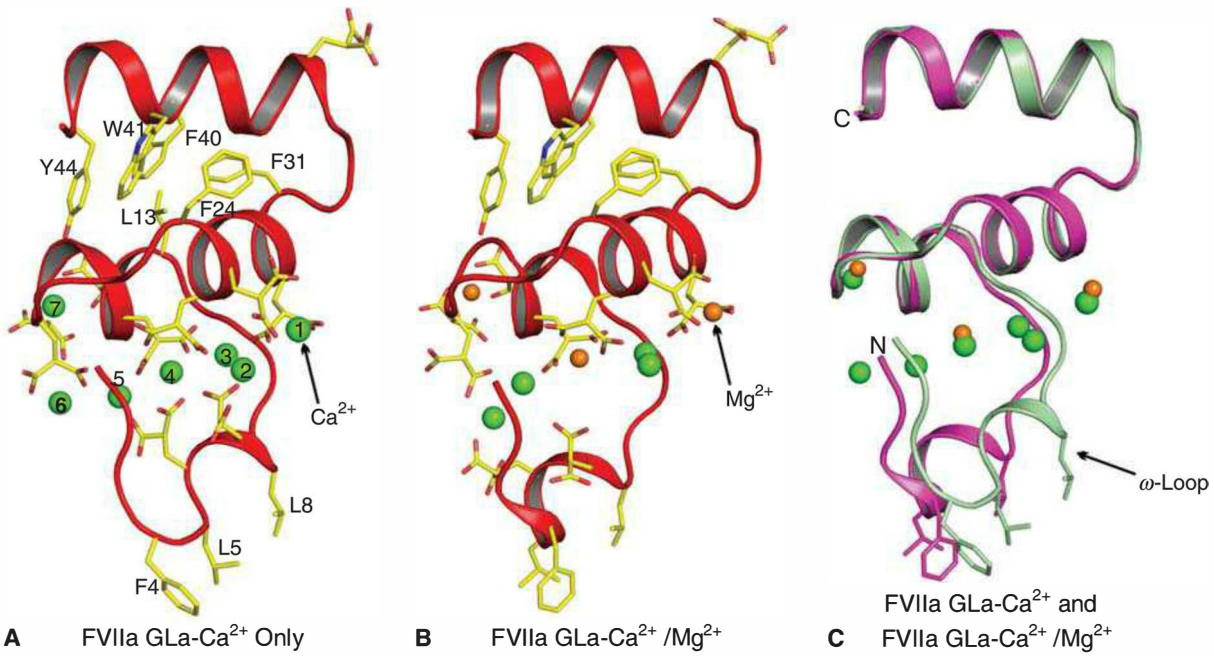


FIGURE 14.4 Structure of the FVIIa Gla domain in the presence of Ca^{2+} only, and with Ca^{2+} and Mg^{2+}. **Left**: Structure of the Gla domain in the presence of Ca^{2+} only.[144] The Gla domain of FVIIa contains seven metal binding sites, and all are occupied by Ca^{2+} in this structure. **Center**: Folding of the Gla domain in the presence of Ca^{2+}/Mg^{2+}.[145] In this structure, the external (numbers 1 and 7) and the central (number 4) metal sites are occupied by Mg^{2+} while the other sites are occupied by Ca^{2+}. **Right**: The superimposed structures shown in "A" and "B." Although the divalent metal ions occupy similar positions in the two structures, the fold of the ω-loop is different between the two structures. The membrane binding residues Phe4, Leu5, and Leu8 in the ω-loop as well as the Gla residues and the hydrophobic residues in the *C*-terminal helix of the Gla domain are shown in stick representation. The Ca^{2+} (*green*) and the Mg^{2+} (*orange*) are shown as *spheres*. The numbering system used is that of Tulinsky et al.[156]

structure, with identical positions for four Ca^{2+} and three Mg^{2+} (PDB id 3TH2,[179]). Further, the folding of the ω-loop under 45 mM Ca^{2+}/5 mM Mg^{2+} is similar to that reported in the presence of Ca^{2+} only (PDB id 3TH4,[179]); however, positions 1 and 7 are occupied by Mg^{2+} (PDB id 3TH4). Thus, it would appear that four Ca^{2+} and three Mg^{2+} ions are bound to the Gla domain in circulating FVII/FVIIa.

The portion of the Gla domain located at the bottom of FVIIa (FIGURE 14.5A) contains three solvent-exposed hydrophobic residues (Phe4, Leu5, and Leu8) present in the ω-loop that are hypothesized to be inserted into the PL membrane.[180,181] The carboxy terminus of sTF is also located near the bottom of the structure; in intact TF, this part of the molecule is linked to the membrane anchor by a short peptide sequence. Therefore, the membrane surface should be located at the bottom of the Gla domain, with TF linked to the membrane by its membrane-anchoring domain and the FVIIa interacting with PL head groups through the Gla domain. Fluorescence resonance energy transfer experiments indicate that the FVIIa/TF complex is oriented almost perpendicular to the membrane, with the active site of FVIIa located approximately 78 Å above the membrane surface.[182]

In addition to membrane binding, FVIIa Gla domain is involved in binding to the *C*-terminal region of TF, a mainly hydrophobic interaction involving the hydrophobic residues present in the Gla *C*-terminal helix. The FVIIa Gla domain also plays a role in FIX and FX substrate interaction where it serves the exosite recognition sites for macromolecular substrates. Biochemical and modeling data suggest that residues Asp33, Ala34, Gla35, Arg36, Leu39, Trp41, Ile42, and Lys48 in the FVIIa–Gla domain are involved in substrate interaction.[183–187]

FIX/IXa. The Gla domain of human FIX contains 12 Gla residues,[188] the first 10 of which are at homologous positions of other VKD proteins. The FIXa Gla domain undergoes a transition to an ordered structure on addition of Ca^{2+} or Ca^{2+}/Mg^{2}, as seen in x-ray diffracted crystals[189,190]; NMR studies indicate a very similar structure for FIX Gla domain in solution.[191,192] The coordination of the Ca^{2+} within the FIX Gla domain differs slightly from those in FVII, FX, and FII (FIGURE 14.5A).[193,194] The FIX Gla domain binds to eight Ca^{2+}, five of which are internal and three are on the surface and contribute to surface charge.[189,190] The three surface sites bind to Mg^{2+} as well.[189] Of the Gla residues, 8 of the first 10 that are homologous to the other VKD proteins are essential for activity. Of the 10, hemophilia B is associated with mutations in all but Gla15,[100] which coordinates to Ca^{2+}-7.[189,190] Gla21 mutations result in an inactive protein.[193] This is interesting because Gla21 coordinates to Ca^{2+}-6[189] and Ca^{2+}-6 coordinates to lysophosphatidylserine, displacing a water molecule from the coordination sphere in PF1.[190] In contrast, the nonhomologous Gla36 and Gla40 do not contribute to activity[194]; however, they provide all four ligands in coordinating to Ca^{2+}-8 or Ca^{2+}-9[193], indicating that Ca^{2+}-8 or Mg^{2+}-8 (FIGURE 14.5A) is not necessary for FIXa activity.

Antibodies that bind to the FIX Gla domain in the presence of divalent metal ions indicate the presence of both Ca^{2+} and Ca^{2+}/Mg^{2+}-dependent epitopes, and the latter are localized to the carboxy terminus of the domain.[195] Mg^{2+} binding to the Gla domain are important for FIX activity.[196,197] Comparison of immunoreactivity of FIX molecules substituted with portions of the FVII Gla domain indicates that Gla33, Thr39, and Gla40 are involved in a high affinity where Mg^{2+} can bind.[198] The ω-loop in FIXa is important in binding to negatively charged PL surface, involving Lys5 (FIGURE 14.5A) binding to the glycerol phosphate backbone and the carboxyl group of phosphatidylserine coordinating to Ca^{2+}-5 and Ca^{2+}-6 as has been shown for PF1.[157,199] Further, Mg^{2+} binding to the Gla domain enhances binding of FIX to PL at physiologic Ca^{2+}.[200]

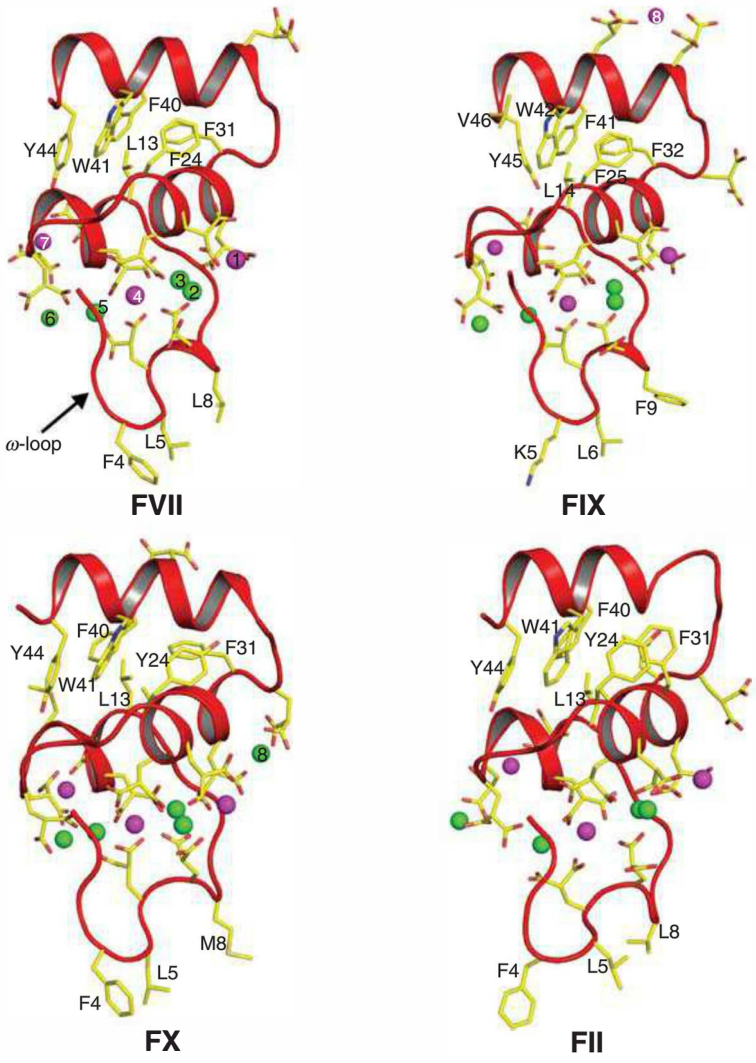
Gla20 and Gla21 are in a conserved 4–amino acid disulfide loop that is buried with hydrophobic interactions to an aromatic stack composed of Phe41, Trp42, and Tyr45 near the carboxy terminus of the domain. Hemophilic or recombinant mutations of the Cys residues inactivate the protein.[201] Recombinant FIX without the aromatic stack can be activated by FXIa but has low specific activity; mutagenesis of Phe41 to Val or Asp similarly affects activity whereas Tyr41 retains 64% of FIX activity.[202] The Gla domain of FIX is important for activation by both FVIIa/TF[203] as well as by FXIa.[204,205] The Gla domain of FIXa has been implicated in binding to TF in the FVIIa/TF:FIXa ternary complex[187,206] and binds directly to FXIa.[205] The Gla domain of FIXa also binds to the FVIIIa C2 domain (A3-C1-C2),[207,208] most likely involving Phe25 and Val46. Mutation of Gly12 to Arg disrupts FVIIIa binding[209]; however, this is most likely due to a structural impairment rather than from a direct binding effect. More studies are needed to elucidate the exosites on FIXa important in binding to FVIIIa.

Cultured endothelial cells have specific receptors that bind FIX and FIXa,[210,211] allowing intrinsic FX activation without the need for platelets; this binding may also occur *in vivo*.[212] As is the case with the PL, binding of the Gla domain of FIXa involves residues 3 through 11[213] although collagen may account for some of the binding.[214] Using protein fragments to inhibit ^{125}I-labeled FIX binding to cultured endothelial cells, determinants in the first EGF-like domain of FIX were demonstrated, whereas binding to PL was only competed with the Gla domain peptides.[215] From domain exchange experiments with FIX and PC, optimal endothelial cell binding appears to involve the entire FIX Gla domain including its aromatic stack.[216] In kinetic studies, FVIIa activated FIX, but not FX, directly on the endothelial surface, and FIX activation was about one-sixth as rapid as compared to observed with stimulated platelets.[217]

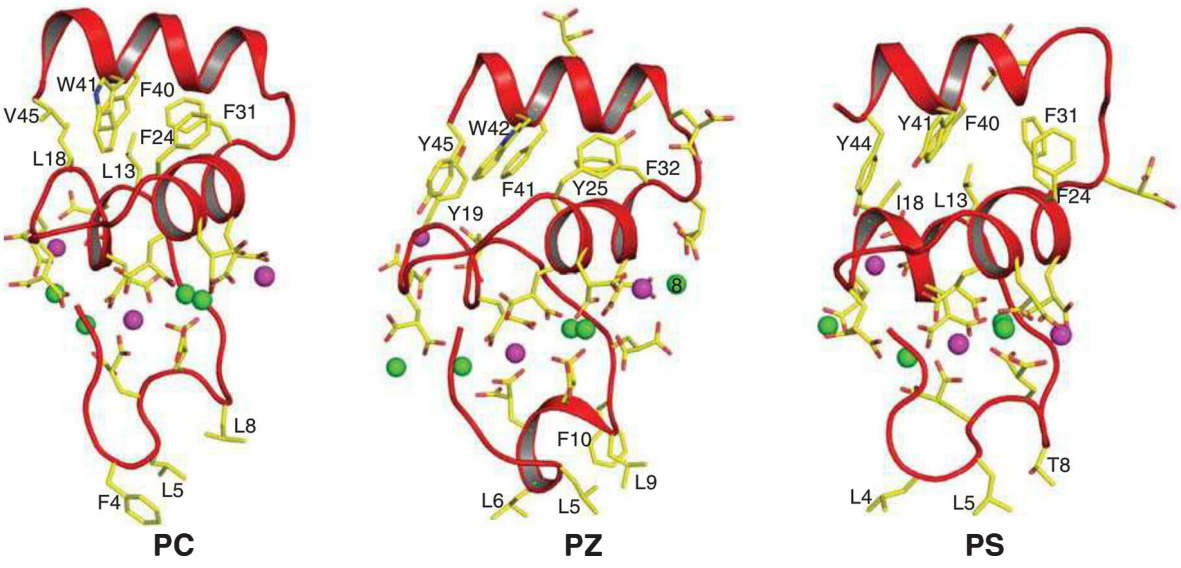
In vivo, there is a basal low level of FIX activation by FVIIa,[218] presumably at the endothelial cell surface. Mutation of Lys5 to Ala or Val10 to Lys considerably reduces the affinity of FIX for endothelial cells, whereas Lys5 to Ala increases FIX affinity for bovine vascular endothelial cells threefold.[213] The endothelial cell–binding site for FIX involves two sites on collagen IV that are 50 and 98 nm from the *C*-terminus of collagen IV.[214,219] Further, FIX binds *in vivo* to the endothelial cell collagen IV surface,[220] and this binding may play an important role in regulating the concentration of FIX in the blood.

The distance from a rhodamine-labeled lipid surface to a fluorescein-labeled FIXa active site is at least 70 Å,[221] a value consistent with the molecular dimensions of the crystallographic structure if lipid binding involves the Gla domain and the balance of the protein extends vertically above the surface.[147] Electron crystallographic studies of two-dimensional (2-D) crystals of human FIX zymogen bound to a lipid layer support this orientation.[222]

FX/Xa. The human FX Gla domain contains 11 Gla residues and binds 8 Ca^{2+} ions (FIGURE 14.5A),[223] 7 of which have been observed in the crystal structure of the Gla domain of bovine FX. The Ca^{2+}-8 was not seen in the crystal structure and is



A. Coagulants Gla Domains with Ca^{2+}



B. Anticoagulants Gla Domains with Ca^{2+}

FIGURE 14.5 Structures of the Gla domains of coagulant **(A)** and anticoagulant **(B)** VKD proteins in the presence of Ca^{2+}. FVII, FII, PC, and PS contain seven metal binding sites, while FIX, FX, and PZ contain more than seven sites in their respective Gla domains. The Gla residues and the membrane binding hydrophobic residues at the *C*-terminal helix of the domain are shown in stick representation. The Ca^{2+} metal sites, which cannot be replaced by Mg^{2+} are shown as *green spheres* and those which could be replaced by Mg^{2+} are shown in *magenta*. In circulation, the depicted Mg^{2+} sites are most likely not occupied by Ca^{2+} and are likely specific for Mg^{2+}. The metal binding sites in the Gla domain of PZ and of PS are modeled based upon other VKD proteins.

modeled based upon the structure of the Sr^{2+} bound Gla domain of bovine PF1.[224] An additional Ca^{2+} site is in the Gla domain of bovine FX involving Gla residues 35 and 40; residue 35 is Asp in human FX Gla domain, which precludes binding of Ca^{2+} at this site. Ca^{2+} sites at positions 1, 4, and 7 were occupied by Mg^{2+} in the crystal structure of Gla domain of human FX in the presence of Mg^{2+} only.[152] Mutation of Gly11 to Val observed in FX deficient patients disrupts the activation of FX by either FVIIa/TF or FIXa/FVIIIa[225]; this mutation could partially impair folding of the ω-loop. Another mutation (Gla7 to Gly) may impair binding of Ca^{2+} at sites 2 and 3 and proper folding of the Gla domain.[226] The Gla domain of FXa is also important in the activation of FVII/TF to FVIIa/TF.[227]

Prothrombin (II). The first x-ray structure of the Gla domain was obtained for bovine PF1.[156] The Ca^{2+} ions are necessary to stabilize the Gla domain fold of FII.[156,228] Human FII contains 10 Gla residues and binds 7 Ca^{2+} in the absence of Mg^{2+} and 4 in the presence of Mg^{2+}.[145,229] Based upon the crystal structures of the FVIIa and FIXa Gla domains,[145,189] positions 1, 4, and 7 can be occupied by Mg^{2+} (FIGURE 14.5A). All Gla residues except Gla6 are important for proper function in FII.[230] Ca^{2+}/Mg^{2+} binding to the Gla domain are required for insertion of designated hydrophobic amino acids from the ω-loop into the PL surface.[112,156]

PC/APC. The Gla domain of PC/APC contains nine Gla residues and binds seven Ca^{2+} ions (FIGURE 14.5B)[68,187] in the absence of Mg^{2+}, and four in the presence of Mg^{2+}. Mutations of Gla residues 7, 20, 26, and 29 results in nearly complete loss of APC anticoagulant activity. Mutation of Gla25 decreases activity by 75%, but Gla6, 14, and 19 are not critical for APC functions.[231–234,235,236] Mutations at the hydrophobic residues near the *N*-terminus, particularly Leu5,[233] disrupt PL binding, and Arg15 is important in Ca^{2+} binding, as is the disulfide bond for function.[236] His10 is largely responsible for the 10-fold higher membrane binding affinity of human PC over bovine PC, which has a Pro at this position.[237] Endothelial protein C receptor (EPCR) can substitute for PL in promoting PC activation by binding to the Gla domain, but such binding requires near complete γ-carboxylation.[238–240] Binding of PC to EPCR increases PC activation *via* decreasing the K_m.[241,242] Soluble EPCR inhibits PC activation by blocking PC binding to cellular EPCR.[242] Human, but not bovine[243] FVa can potentiate PC activation on endothelium,[244,245] and human FVa can accelerate PC activation in solution.[245]

PZ. The human PZ Gla domain contains 13 Gla residues, which is the highest for any VKD protein.[37,38] Similar to FIX Gla domain, PZ has an additional residue at position 4 (FIGURE 14.5B).[37] The ω-loop of PZ contains four hydrophobic residues, unusual for the VKD proteins, while the others contain only three hydrophobic residues. Ca^{2+} binding studies performed with PZ and Des-Gla PZ revealed that only the Gla domain has Ca^{2+} binding sites, whereas the EGF-like domains and the pseudoprotease domain do not.[246] Direct Ca^{2+} binding studies performed from 0 to 0.8 mM Ca^{2+} indicate that the Gla domain of PZ contains only four sites[247]; however, from the positions of the Gla residues and the similarity with FIX and PF1, PZ might contain more than seven metal binding sites (FIGURE 14.5B). Two point mutations (Gln or Lys) have been reported in patients at residue Glu30, affecting PZ secretion from transfected cells.[247–249] PZ functions as a cofactor for PZI inhibition of FXa by binding to the PL through its Gla domain.[24] PZ has nearly 100-fold slower membrane binding and dissociation kinetics as compared to PS and PC because of the unique Gla residue at position 11.[250]

PS. Human PS contains 11 Gla residues and may contain seven metal binding sites (FIGURE 14.5B) and binds to PL membrane with higher affinity than PZ or PC.[251] Chemical modifications in the Gla residues of PS affect PL binding and also self association.[252] The Gla domain of PS binds to PL membrane in a Ca^{2+} dependent manner, but high Ca^{2+} (10 mM) disassociates the Gla domain from PL.[253] The TSR region in PS may affect Ca^{2+} binding and disrupt the structure of the Gla domain.[254] The Gly11Asp and Thr37Met missense mutations are reported in patients with PS deficiency, and mutagenesis studies reveal that Gly11 to Asp mutation affects its cofactor activity, while the Thr37 to Met mutation affects PS secretion.[255] Mutation at Gla36 affects its cofactor activity by 89% for FVa inactivation and completely for FVIIIa.[256] PS enhances the binding of APC to PL membrane,[257] and the Gla domain of PS is implicated in plasma clotting assays.[257]

EGF-Like Domains

EGF domains are mainly involved in protein–protein interactions and include human EGF receptor family ligands such as EGF and Transforming Growth Factor-α (TGF-α), low-density lipoprotein receptor, matrix proteins, the transmembrane receptor Notch, and proteins involved in blood coagulation and fibrinolysis.[107] The EGF domain contains approximately 45 amino acids and the fold is primarily stabilized by the three signature intradisulfide bond patterns as 1–3, 2–4, and 5–6. The EGF domain has two antiparallel β-sheets, which are referred to as major (*N*-terminal) and minor sheets (*C*-terminal). FVII, FIX, FX, PC, and PZ each contain two EGF-like domains, whereas PS contains four EGF-like domains in tandem. The first EGF domain in FVII, FIX, FX, and PC is a Ca^{2+} binding type domain, with functional significance, restricting Gla domain movement, which is necessary for the biologic activity. The first EGF domain in PZ or PS lacks the Ca^{2+} binding site and may be more flexible.

FVII/VIIa. The EGF domains present in FVIIa make strong interactions with TF. The main sites of contact on FVIIa are located in the first EGF domain and the protease domain, with additional points of contact within the second EGF domain and the aromatic stack region of the Gla domain. The sites of contact on TF are located on both fibronectin type III domains and the interfacial region between them,[144,145,258,259] Binding studies with proteolytic fragments and domain swapping experiments indicate that FVII EGF domains are important in binding to TF.[259,260] The *N*-terminus of the EGF1 domain contains a strong Ca^{2+} binding site, which is important for cofactor interaction and proteolytic function.[261,262] The Ca^{2+}-bound structure of EGF1 (FIGURE 14.6) shows Ca^{2+} coordinated to the main chain carbonyl oxygen of Gly47 and Gln64 as well as to the side chain carboxyl group oxygen of Asp46 and Asp63. Site-directed fluorescence experiments reveal that complex formation between sTF and FVIIa is initiated upon Ca^{2+} binding to EGF1.[263] The structural similarity between the Ca^{2+}-bound and Ca^{2+}-free structures suggest that Ca^{2+} binding is responsible for the increased affinity of FVII/FVIIa for TF.[264] Site-directed mutagenesis experiments in which Ca^{2+} binding residues Asp46 and Asp63 are replaced with Asn abolished Ca^{2+} binding and reduced FVIIa proteolytic activity.[265] The Asn57 to Asp

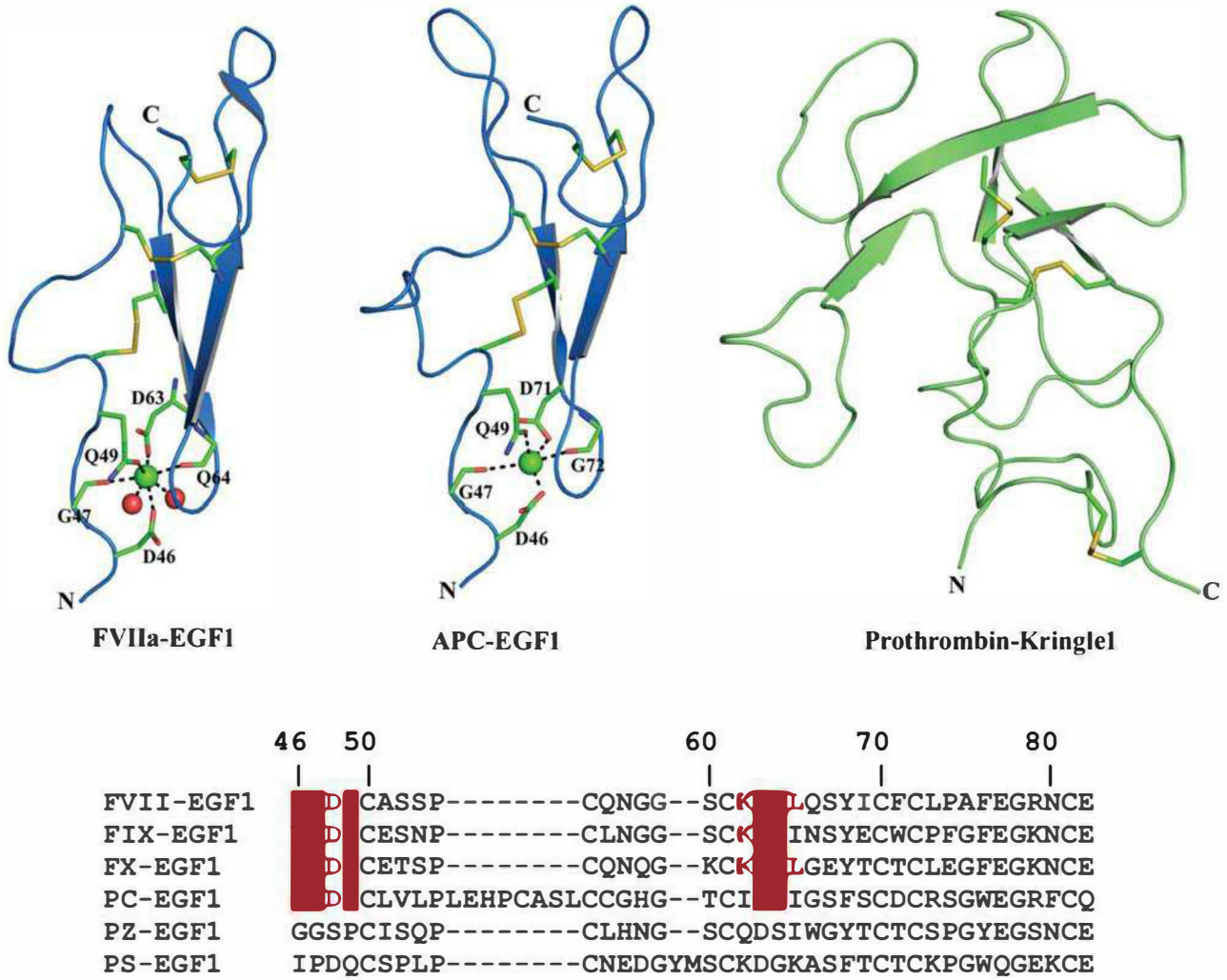


FIGURE 14.6 Cartoon representation of the EGF1 domains and the prothrombin kringle domain-1 (K1). The FVIIa EGF1 domain is on the **left**, APC EGF1 domain at the **center**, and K1 domain of prothrombin fragment-1 (PF1) is on the **right**. The Cys residues which form disulfide bridges are shown in stick representation. Ca^{2+} ions are shown as *green spheres*. The Ca^{2+} binding loops are similar in FVII, FIX, and FX, whereas APC has an eight-residue insertion in its Ca^{2+} binding loop. The multiple sequence alignment of EGF domains of FVII, FIX, FX, PC, PZ, and PS are shown at the **bottom**. The residues that serve as ligands for Ca^{2+} binding are highlighted in *red*; Mg^{2+} cannot replace Ca^{2+} in these domains. PZ or PS does not contain a Ca^{2+} binding site in the EGF1 domain.

mutation in EGF1 affects the FVIIa secretion as well as its activity.[265] Two other EGF2 mutations (Arg110 to Cys and Asp123 to Tyr) reduce FVII secretion.[266] FVII EGF domains also play a role in FX activation.[267] A peptide sequence from the EGF2 domain of FVII inhibits TF-dependent FX activation, most pronounced for a constrained sequence corresponding to residues 91 to 102.[267] These results suggest a direct role for the EGF2 domain of FVII, and in particular its loop I, in the formation and function of the FVII/TF/FX complex.[267]

FIX/IXa. The FIX EGF1 domain extends from the aromatic stack to EGF2, which lies under the catalytic domain.[147,148] The two FIX EGF domains interact through a salt bridge between Glu78 and Arg94 and by a hydrophobic patch.[268,269] There is more restriction of rotation between the two hydrophobic patches in FIXa than in FXa.[270] The interface between the EGF2 domain and the serine protease domain has extensive contacts involving aromatic residues.[270] The structure of the EGF fragment by 2-D–NMR[271] indicates that antiparallel β-pleated sheets observed in crystals also exist in solution. The first EGF domain of FIX was crystallized with Ca^{2+} bound to the Ca^{2+} site.[272] Similar to FVII, the first EGF domain in FIX binds a single Ca^{2+} with high affinity,[111,273–276] whereas the second EGF domain does not bind Ca^{2+}. Ca^{2+} in the EGF1 domain of FIX coordinates to the side chains of residues Asp47, Gln50, and Asp64 and to the backbone carbonyls of residues Gly48 and Asp65[273]; Val46Glu or Gln50Glu mutations do not affect binding of Ca^{2+} to the EGF1 domain.[276] Tyr69 to Cys produces severe hemophilia B[100] and site-specific mutagenesis indicates that this residue is important for activity but not for Ca^{2+} binding or β-hydroxylation.[277] Gly60 to Ser is a mild hemophilic mutation,[100] and modeling predicts misfolding

due to steric clash with the EGF1 Cys73–Cys82 disulfide bond.[278] Pro55 to Ser or Leu also causes mild hemophilia, caused by impaired FX activation.[279]

Domain exchange among homologous VKD factors has provided further evidence for key structural elements of the EGF-like domains. Lin et al.[280] found that replacing the first EGF-like domain of FX into FIX had little effect on activity and that substitution of both EGF-like domains with those of FX retained only 4% FIX activity. Conversely, replacing both the Gla and first EGF-like domain of FIX into FX led to a requirement of higher concentrations of FVa for FX activity[281]; therefore, cofactor binding was suboptimal but did not require specific FX sequences within the replaced segment. When the EGF-like domains of FIX were substituted with those of PC, the resulting APC had only 10% activity.[282] The first EGF-like domain of FVII has also been substituted for that of FIX in a recombinant protein[283]; this species is of particular interest because FVIIa/TF activates FIX, and this portion of FVII participates in TF binding. Curiously, the chimeric protein had twice the specific activity of wild-type FIX, enhanced binding to FVIIIa, and even enhanced activity in canine hemophilia B. Monoclonal antibody that recognizes the *C*-terminal part of the EGF1 domain, particularly Trp72 and Lys80, blocks FIX activation by FXIa, marginally decreases activation by the FVIIa/tTF complex, and slightly decreases the k_{cat} for FX activation.[284] Further, the Fab fragment of this EGF1 monoclonal antibody has 10-fold higher affinity for FIXa as compared to FIX,[285] indicating that the conformational changes that occur in the protease domain during activation and removal of the activation peptide are linked to the EGF1 domain and that the FIXa EGF1 domain is not involved in binding to FVIIIa.

The FIX EGF1 domain is required for activation by the FVIIa/TF complex but not by FXIa.[286,287] Further studies by Zhong et al.[288] revealed that the EGF1 domain of FIX interacts with TF in the FVIIa/TF. Recently, Ndonwi et al.[227] reported that EGF1 domain of FIXa is important for the feedback activation of FVII/TF to FVIIa/TF. Several studies have emphasized the importance of Ca^{2+} binding to the FIXa EGF1 domain for FVIIIa binding on PL.[111,268] However, Gln50 to Pro mutation only reduced affinity for FVIIIa in the presence of PL,[289] and replacement of the FIX EGF1 domain with that of PC resulted in approximately 80-fold decreased affinity for FVIIIa in the presence of PL and a minor difference without PL.[289] Monoclonal antibody to the EGF1 domain showed that the antibody does not affect FVIIIa binding to FIXa.[285] This study and others question whether the hydrogen bond between Glu78 in the EGF1 domain and Arg94 in the EGF2 domain is crucial for the interaction of FIXa with the light chain of FVIIIa, as hypothesized by Christophe et al.[268] Therefore, it appears that the EGF1 domain does not directly bind to FVIIIa but rather correctly positions the EGF2 and protease domains for proper interactions.

Within the second EGF-like domain of FIX, dysfunctional hemophilic mutations[100] cluster in the loop before the second disulfide bond and the main β-pleated sheet; mutations in the third disulfide loop are predominantly associated with near-normal specific activities. The EGF2 domain has been implicated in binding to FVIIIa, as well as in binding to platelets. Individual and complete replacement of the FIXa EGF2 loops with comparable FVIIa loops did not alter FVIIIa binding, but the K_d for assembly of the FX activating complex on platelets was increased for the replacement mutants.[288] Therefore, residues 88 to 109 in the FIXa EGF2 domain are important in assembly of the FX activating complex on platelets.[290] In studies of FIXa EGF2 mutants, the Leu84–Thr87 linker region had a substantial effect on FX activation in the presence of FVIIIa, but binding of the A2 subunit was marginally affected.[291] A decrease in FX activation was found with the purified protein from a patient with hemophilia with an Asn92His mutation.[292] Chang et al.[293] used scanning Ala mutagenesis of the EGF2 domain and proposed that residues 89 to 93 are involved in binding to FVIIIa and residues 102 to 108 are involved in binding to FX. Therefore, the EGF2 domain most likely plays a role in FVIIIa binding, tenase complex assembly, and mediating binding to the platelet surface. Replacement of the Asn89–Lys91 loop region decreased the activation of FX in the presence of FVIIIa by only a small amount. Other studies suggest that the EGF2 domain may bind to either the A2 subunit, the A3 subunit, or to both the A2 and A3 subunits of FVIIIa. Earlier studies have shown that the 1811–1818 region of the A3 subunit of factor VIIIa may bind to FIXa[294]; however, it is unclear as to which region of FIXa it binds to. For FIXa, cleavage of the Arg145–Ala146 bond appears to enhance binding of the FVIIIa light chain.[295]

FX/FXa. The two FX EGF-like domains keep the protease domain about 61 Å away from PL surface in the absence of cofactor FVa and 69 Å away in the presence of cofactor.[296] The first EGF domain binds a single Ca^{2+} ion, and residues Asp46, Gly47, Gln49, Hya63 (β-hydroxy aspartate), and Gly64 serve as ligands.[297] Ca^{2+} affinity is approximately 10- to 20-fold higher for the Gla–EGF1 pair than for the isolated EGF1 domain.[298] The *N*-terminal FX EGF1 domain is required for activation of both prothrombin and FVIIa/TF.[299] The EGF2 domain protects the surface-exposed hydrophobic residues in the FX protease domain and thereby stabilizes its structure.

PC/APC. Similar to FVII, FIX, and FX, APC contains two EGF-like domains, the first of which (EGF1) binds a single Ca^{2+} ion[300] (FIGURE 14.6). Residues Asp46, Gly47, Gln49, Asp71, and Gly72 serve as ligands for Ca^{2+}. PC EGF1 is different from the other VKD proteins in that there is an eight-residue insertion at the *N*-terminal end that contains extra cysteine residues (FIGURE 14.6), the functional significance of which is unknown. Mutation of the β-hydroxylated Asp71 to Glu in EGF1 resulted in loss of Ca^{2+} binding and a 90% reduction in anticoagulant activity.[300,301] Mutation of E149 to Ala in the linker peptide region after EGF2 increased anticoagulant activity and enhanced binding to PS.[302]

PZ. Multiple sequence alignment of EGF1 domains of VKD proteins indicates that neither PZ nor PS has the Ca^{2+}-site (FIGURE 14.6).[303] Similar to other VKD proteins, PZ EGF2 domain strongly interacts with the pseudoprotease domain, stabilizes its fold,[176] and keeps it at an appropriate height from PL surface to allow the ZPI associated with PZ to effectively inhibit FXa. In the modeled complexes of PZ/ZPI:FXa, the EGF2 domain is in close proximity to the FXa protease domain.[176]

PS. PS contains four EGF domains in tandem, the last three of which bind Ca^{2+} (FIGURE 14.6). The *C*-terminus EGF domain has 5,000-fold higher affinity for Ca^{2+} than the second EGF domain.[304] In the NMR structure of the EGF3–EGF4 fragment of PS, the EGF3 domain adopts a bent conformation, in contrast to the extended conformation of the other EGF domains in the presence of Ca^{2+}. Conformational switching could have functional importance and involvement in protein–protein interactions of PS.[178] Mutations in the PS EGF domain confirm that this section of the molecule has multiple functions.[305] EGF1 is involved in APC interaction.[306,307] The naturally occurring

mutation at residue Thr103 to Asn in EGF1 causes type II deficiency.[308] Deletion of PS EGF2 results in a comparable loss of its cofactor activity, indicating that EGF2 has a functional role rather than serving as just a spacer.[309] The Lys155 to Glu mutation in the PS EGF2 domain has low APC cofactor activity and also lacks the PS-mediated inhibitory effect on the prothrombinase complex and is associated with thrombotic disease.[310,311] The PS EGF domains also mediate interaction with FXa.[304]

Kringle Domains

Prothrombin contains two adjacent kringle domains between the Gla and the protease domains. PF1 contains the K1 domain, which was the first blood coagulation protein fragment structure determined by x-ray crystallography (FIGURE 14.6).[228] The Gla and the kringle-1 domains are linked by a 34 residue α–helical segment. The folding of the kringle is largely determined by close van der Waals contacts between the interloop cysteine disulfide bonds, which are buried in the interior and are inaccessible to solvent. K1 has potent antiangiogenic activity, which inhibits Lewis lung carcinoma tumor growth and metastasis[312] and is involved in FVa binding, during the assembly of the prothrombinase complex.[313] PF2 contains the K2 domain and has several interesting functional assignments.[312,314,315] K2 binds Ca^{2+} and is the primary region that mediates the interaction with FVa.[316–318] The interaction between K2 domain and FVa may be responsible for altering the conformation of FII, making it more accessible to FXa.[319]

Catalytic Domains

FVII, FIX, FX, FII, and PC contain a serine protease domain (S1 family of the SA clan)[320] at the *C*-terminus. These proteases mainly contain β-strands with the catalytic triad residues His, Asp, and Ser (FIGURE 14.7). The catalytic domain is folded into two six-stranded antiparallel β-barrel structure subdomains. The active and substrate recognition sites are formed at the interface between the two β-barrel domains. The inactive zymogen is converted to the active enzyme by the proteolytic cleavage of a single peptide bond at the *N*-terminus, releasing an activation peptide, as inferred from studies with trypsin.[321] The newly formed *N*-terminus residue is then inserted near the active site

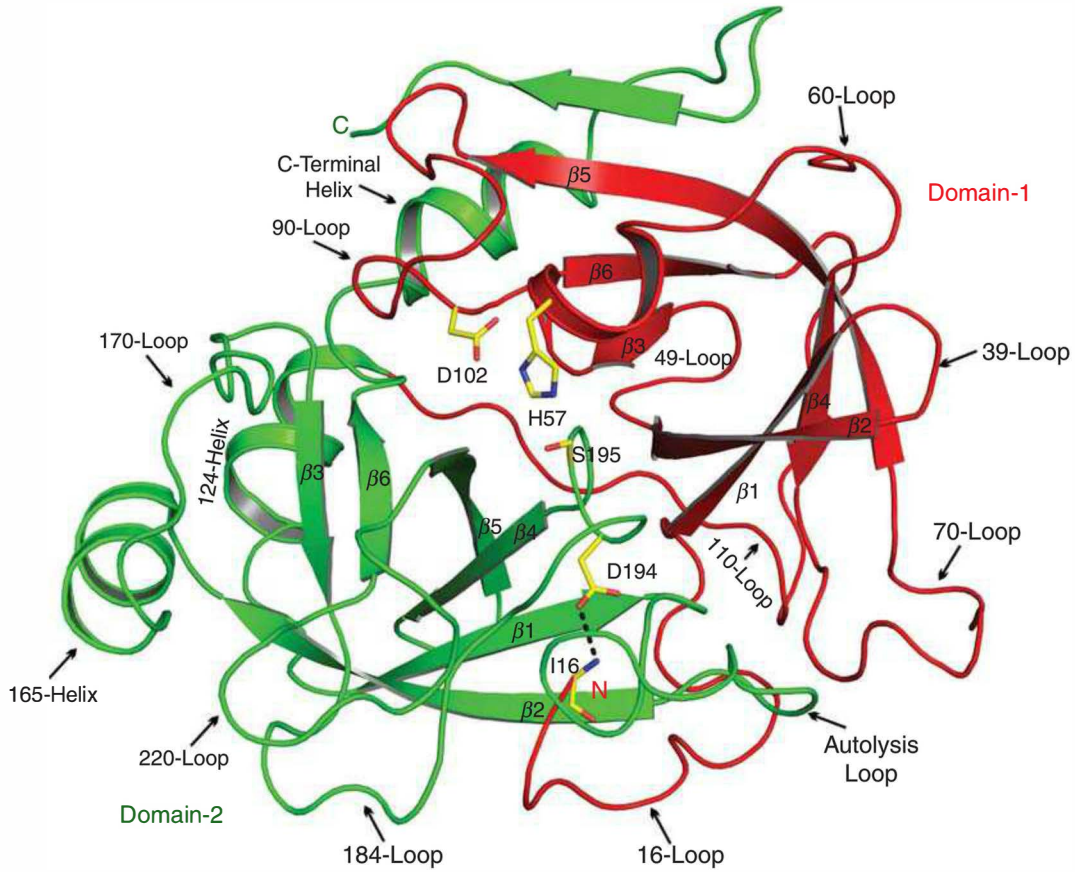


FIGURE 14.7 Cartoon representation of the FVIIa serine protease domain. The protease domain is primarily made up of β-strands with a few α-helical segments, folded into two subdomains, each of which consists of a six-stranded antiparallel β-barrel. Domain-1 is shown in *red* and domain-2 in *green*. The catalytic triad and the substrate binding cleft are formed between the two β-barrel domains. The active site residues H57, D102, and S195 (chymotrypsin numbering) as well as D194 and I16 are shown in stick representation. Upon conversion from the inactive zymogen form to the active form, the 16-loop, autolysis loop, 184-loop, and the 220-loop undergo a large conformational change. Upon activation, a salt bridge is formed between residue 16 and residue 194, shown by a *black dashed line*.

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pocket and makes a salt bridge with the side chain of Asp [c194], where [c] indicates chymotrypsin numbering. During the activation process, the residues in the c16-loop (residues 16 to 19, activation loop), c142-loop (residues 142 to 152, autolysis loop), c184-loop (residues 184 to 193), and the c220-loop (residues 216 to 223) undergo large conformational changes and restructure the protease domain to its functional form. In the functional form, the nucleophilic attack on the substrate peptide carbonyl moiety by Ser [c195], utilizing the charge relay system between the triad residues His [c57], Asp [c102], and Ser [c195], leads to the cleavage of the peptide bond.[321] The VKD proteins differ from trypsin in that activation alone is not sufficient enough to convert into physiologic active forms, requiring additional cofactors to perform biologic functions. The structure-based multiple sequence alignment of the VKD proteins is shown in FIGURE 14.8,[322] with differences primarily due to variations in loop regions, the greatest dissimilarity occurring in the autolysis loop. The secondary structural regions and few loop regions share similarity among the VKD proteins. The 70-loop, 90-loop, the autolysis loop, and their nearby regions provide the substrate recognition site. The different substitutions of amino acids together with the structural variations generate different substrate specificity and selectivity among the VKD proteins.

FVIIa. FVII is converted to FVIIa *via* proteolysis of a peptide bond between Arg^{152} [c15] and Ile^{153} [c16] without release of an activation peptide. The catalytic domain of FVIIa adopts a typical conformation of other serine proteases in which the active site catalytic triad residues His^{193} [c57], Asp^{242} [c102], and Ser^{344} [c195] are located at the interface between the two β-barrels. The FVIIa protease domain contains several hydrophobic residues on the surface that make contact with EGF2. FVIIa contains one Ca^{2+}, one Na^{+}, and two Zn^{2+} binding sites in the protease domain that play functional roles[145,323–325] (FIGURE 14.9A). The Ca^{2+} is coordinated to the carbonyl groups of residues Glu^{212} [c72] and Glu^{215} [c75], and side chains of Glu residues 210 [c70] and 220 [c80] present in the c70-loop (residues 210 to 220).[144,145] The Ca^{2+} binding to the c70-loop induces the conformational change and increases catalytic activity.[324,326] The Na^{+} binding site is located between the c184-loop and c220-loop. The back bone carbonyl oxygens of Tyr^{332} [c184], Ser^{333} [c185], His^{373} [c224], and Thr^{370} [c221] serve as ligands for Na^{+}.[145] The binding of Zn^{2+} to FVIIa has a negative effect on proteolytic activity, and affinity for sTF.[327] Residues His^{216} [c76], Glu^{220} [c80], and Ser^{222} [c82] provide ligands for the first Zn^{2+} site, and residues His^{257} [c117], Asp^{219} [c79], Gly^{209} [c69], and Lys^{161} [c24] provide ligands for the second Zn^{2+} site.[145] Overlapping Ca^{2+} and Zn^{2+} sites could explain Ca^{2+} reversal of the inhibitory effect of Zn^{2+}.[325]

The main natural substrates of FVIIa/TF are FIX and FX.[327–330] When FIX is activated, two peptide bonds must be cleaved; FVIIa/TF complex catalyzes the cleavage of Arg145 more efficiently than Arg180.[205,331] Residue Lys^{341}[c192] also plays an important role in substrate recognition.[332] The primary physiologic inhibitor of FVIIa is tissue factor pathway inhibitor (TFPI) complexed to FXa; however, FVIIa can also be inhibited by AT in the presence of heparin.[333,334] Truncated forms of FVII have been produced in which the Gla domain has been removed by limited proteolysis[335,336] or by expression of the truncated recombinant protein.[337] A form of FVII lacking both the Gla domain and the first EGF domain fails to bind to TF effectively.[338–340] A FVII mutant has been produced in which the activation cleavage site has been mutated so that it cannot be activated by its usual activators.[341] This mutant had no detectable enzymatic activity indicating that zymogen FVII is inactive.

The FVIIa residues at the *C*-terminus of the c126 helix (Arg^{266}–Val^{277} [c126–c129F]) and the following loop as well as the residues at the *N*-terminus of c165 (Thr^{307}–Gln^{312} [c165–c170]) helix and those of the preceding loop interact with TF[144,145] (FIGURE 14.10). The side chain of residue Met^{306} [c164] is implicated in allosteric activation of FVIIa. Substitution of Met^{306} [c164] by Asp essentially abolishes the TF-induced allosteric activation of FVIIa.[342] The FVIIa regions that play an important role in preventing the transition from the zymogen-like to the active enzyme-like molecule and how TF binding promotes this transition is under intense investigation.[343–348] The binding of TF induces an allosteric effect on FVIIa, which is then lead to a fully functional form of FVIIa. According to one proposed pathway, the binding of TF to FVIIa stabilizes the intramolecular interactions of TF binding helix (residues 307 to 312 [c165–c170]),[344] triggering the initiation of the allosteric effect at *C*-terminal of the helix, which is then transferred to the activation loop 3, leading to proper formation of the S1–S3 substrate sites.[349–351] In the second pathway, TF binding induces allosteric changes through the *N*-terminal side of the TF binding helix, where it stabilizes the hydrophobic interactions between Leu^{305} [c163] and Phe^{374} [c225], inducing conformational change in the activation loop 3, and the oxyanion hole containing loop and activation pocket at the S1 site. These transitions lead to the insertion of *N*-terminus Ile^{153} [c16] into the activation pocket.[351] These conformational changes in the protease domain result in the proper formation of the substrate recognition subsites.

When FVIIa binds to TF, its ability to catalyze the hydrolysis of small peptidyl-amide or peptidyl-ester substrates is enhanced approximately 20- to 100-fold,[324,352–355] primarily by enhancing substrate affinity of FVIIa rather than by altering the rates of any of the subsequent catalytic steps.[356] Higashi et al.[357] have proposed that free FVIIa is structurally more zymogen-like, and when bound to TF, it is more active enzyme-like. Alanine scanning mutational studies of surface residues in the protease domain of FVIIa support this concept.[358]

A defining feature of each serine protease is the presence of an oxyanion-binding site, generally known as the oxyanion hole[359]; however, it is absent in *p*AB-FVIIa/sTF (PDB id 2A2Q) or the benzamidine-FVIIa/sTF (PDB id 2AER) structures.[145] Earlier observations of an absent oxyanion hole were attributed to the unusual nature of the amidinophenylurea-based inhibitor, in which the amidino NH groups of the inhibitor induced approximately 180 degrees rotation of the Lys^{341}–Gly^{342} [c192–c193] peptide bond.[360] A similar peptide bond flip at c192–c193 has also been observed with sulfonamide[361] and phenylglycine amide inhibitors.[362] Thus, the abnormal conformation of the c192–c193 peptide bond in FVIIa/sTF is not related to the presence of the unusual inhibitors in the active site, but rather, it is likely an inherent property of the FVIIa active site.[145]

Binding of the substrate/inhibitor at the active site of FVIIa/TF provides energy for the flipping of the Lys^{341}–Gly^{342} [c192–c193] peptide bond and formation of the competent oxyanion hole.[145] The substrate/inhibitor induced formation of the oxyanion hole is also supported by the structures of the FVIIa EGF2/protease domain (PDB id 1KLI and 1KLJ) obtained in the absence of TF.[363] In this structure, the bottom of the S1 region of active site is occupied by benzamidine and top of the S1 site is occupied by a sulfate ion.[363] Here, occupancy of the sulfate oxyanion in the S1 site most likely induced 180 degrees flip of the c192–c193 peptide bond during crystallization. These

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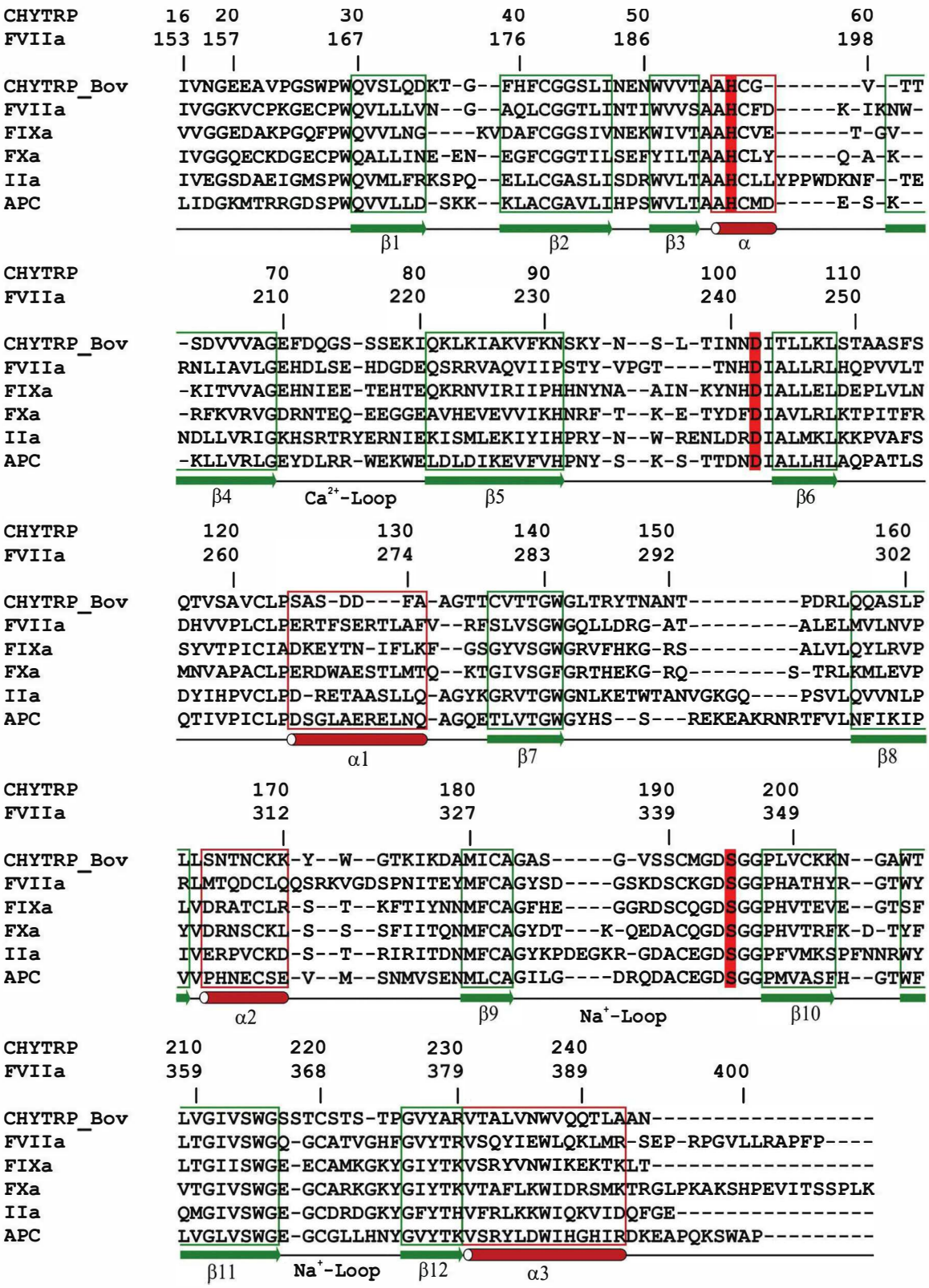

FIGURE 14.8 Structure-based multiple sequence alignment of the protease domains of coagulant and anticoagulant VKD proteins. The multiple sequence alignment of the protease domain of bovine α-chymotrypsin, FVIIa, FIXa, FXa, IIa, and APC was performed by structure-based alignment using the program Chimera. The secondary structural regions, strands, and helices are indicated below the sequences with *arrows* and *cylinders*, respectively. The chymotrypsin numbering and the FVII numbering are given. Ca^{2+} and Na^{+} binding loops are indicated, and the active site residues are highlighted in *red*; IIa does not have a Ca^{2+} binding site in its protease domain.

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observations lend support to a concept that the oxyanion hole in FVIIa is induced by the substrate/inhibitor and not by TF.

FIXa. The heavy chain of FIXa is comprised of the serine protease domain, which contains the catalytic triad, His^{221} [c57], Asp^{269} [c102], and Ser^{365} [c195]. Following activation peptide cleavage, a new amino-terminus Val^{181} [c16] is generated. As in other serine proteases, the amino group of Val^{181} [c16] must insert near the active site to form a salt bridge with the side chain carboxylate of Asp^{364} [c194] for conversion of zymogen FIX to enzyme FIXa. This converts the hydroxyl side chain of Ser^{365} to a reactive species, capable of hydrolyzing the activation cleavage site of FX. Lack of appropriate FIX cleavage as in Arg^{180} or Val^{181} mutation leads to severe hemophilia B.

As with other serine proteases, catalysis of FX proceeds by an acyl intermediate from the peptide bond being cleaved to the active hydroxyl group of Ser^{365} [c195] in FIXa with charge contributions from the other catalytic triad residues, His^{221} [c57] and Asp^{269} [c102] (FIGURE 14.9B). Substrate specificity is greatly restricted in FIXa,[364] perhaps related to variances in the c99-loop.[365,366] This loop restricts catalytic activity toward FX in the absence but not in the presence of FVIIIa.[366] In FIXa crystal structures 1PFX and 1RFN, Tyr^{266} [c99] has two different conformations.[147,148] In the 1RFN structure with *p*AB in the S1 site, access to the S2 site is hindered by Tyr^{266} [c99], whereas in the 1PFX structure Tyr^{266} side chain is rotated such that it is positioned to interact with Phe of PPAck inhibitor in the S4 site. Sichler et al.[367] have shown that mutation of Tyr^{259} [c94], Lys^{265} [c98], and Tyr^{345} [c177] produced a FIXa molecule with approximately 7,000-fold increased activity and altered specificity. It has been proposed that surface loop 199-204 [c30-c40] of FIXa is involved in macromolecular substrate interaction in the absence but not in the presence of FVIIIa.[368] The c99-loop, c60-loop, and

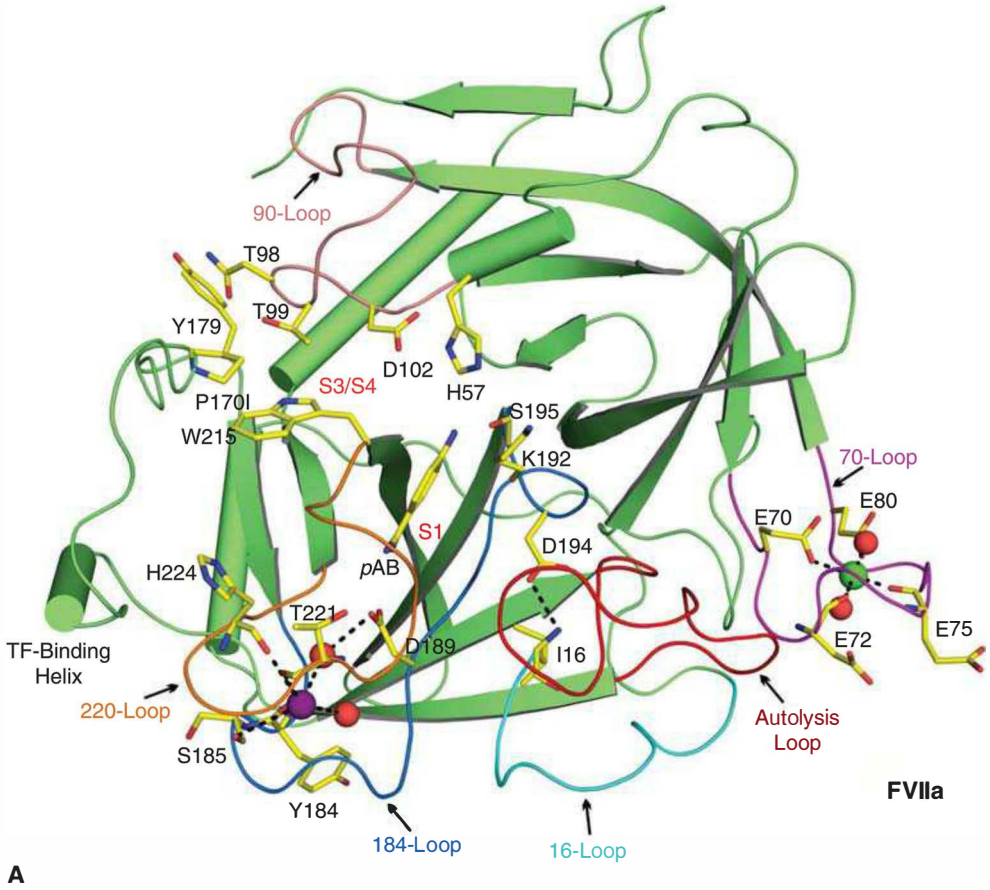

FIGURE 14.9 Ca^{2+} and Na^{+} binding sites in the protease domains of VKD coagulant and anticoagulant proteins. In each protease, the residues coordinated to Ca^{2+} and Na^{+} are shown in stick representation. Loops that are affected by Na^{+} and Ca^{2+} binding are marked; secondary structural elements, β-strands and helices are depicted as *arrows* and *cylinders*, respectively; Ca^{2+}, Na^{+}, and the water molecules are shown as *green*, *purple*, and *red spheres*, respectively; coordination bonds and the hydrogen bonds are represented by *black dashed lines*. The chymotrypsin numbering scheme is used to label the residues. The PDB ids used to draw the figures are: 2A2Q for FVIIa, 2D1J for FXa, 1SG8 for thrombin and 3F6U for APC. ***A*, FVIIa**: The side chains of E70 and E80, and main chain carbonyl oxygens of E72 and E75 serve as ligands for Ca^{2+}. Ca^{2+} binding stabilizes the 70-loop, 16-loop, and the autolysis loop. The main chain carbonyl oxygens of Y184 and S185 from the 184-loop, and T221 and H224 from the 220-loop serve as Na^{+} ligands. The Na^{+} binding stabilizes the 184-loop and 220-loop and the substrate S1 binding pocket. In the structure shown, the S1 site is occupied by *p*AB and its amino groups are hydrogen bonded to D189 and S195. The locations of the TF binding helix and of the substrate sites S3/S4 are marked.

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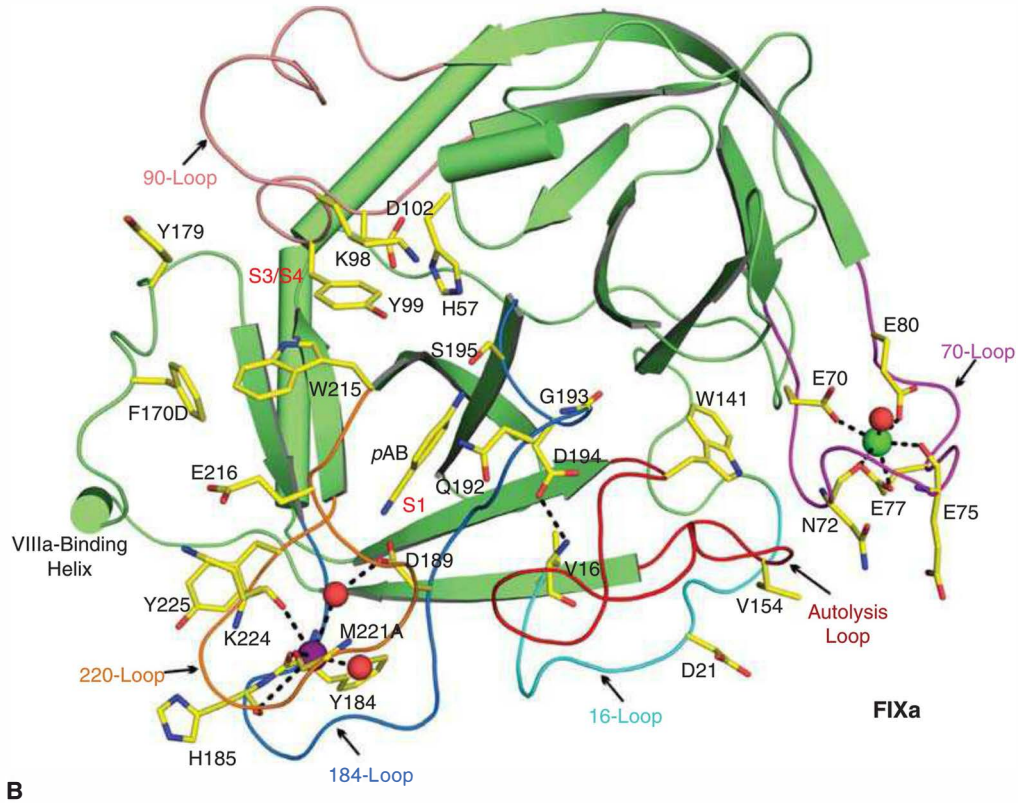
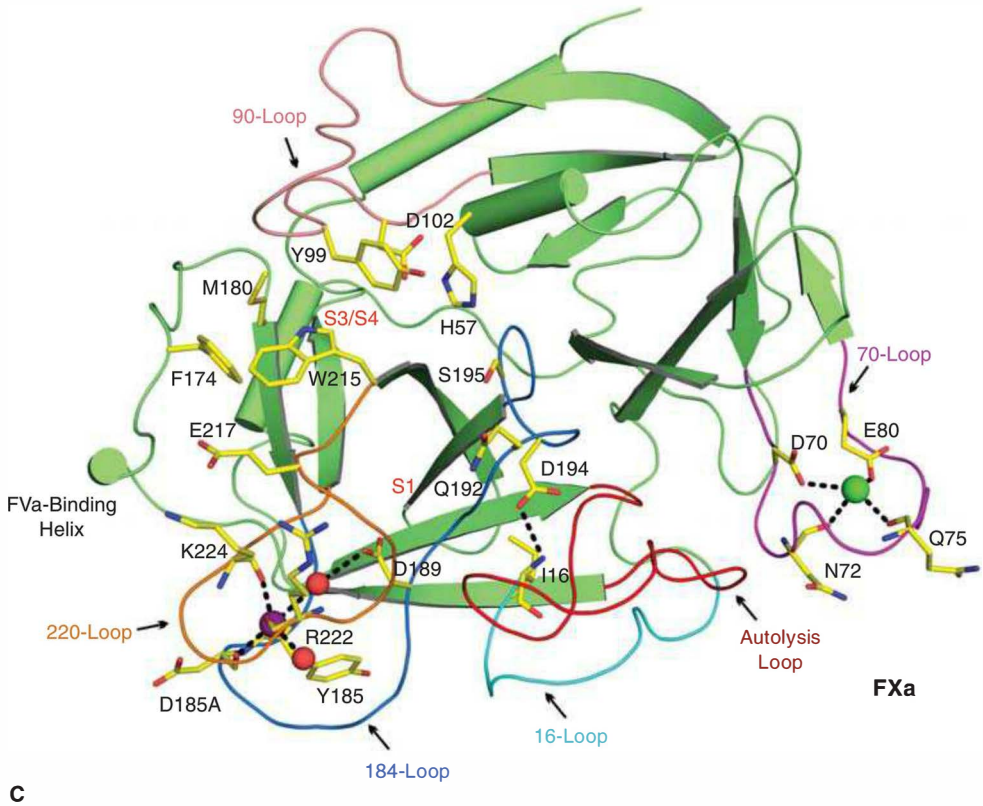


B



C

FIGURE 14.9 *(Continued)* ***B*, FIXa**: The side chains of E70, E77, and E80 and the main chain carbonyl oxygen of N72 and E75 serve as ligands for Ca^{2+}. The main chain carbonyl oxygens of Y184 and H185 from the 184-loop, and M221A and K224 from the 220-loop serve as Na^{+} ligands. In the structure shown, the S1 site is occupied by *p*AB and its amino groups are hydrogen bonded to D189 and S195. ***C*, FXa**: The side chain of D70 and E80 and the main chain carbonyl oxygen of N72 and Q75 serve as ligands for Ca^{2+}. The main chain carbonyl oxygens from residues Y185 and D185A from the 184-loop, and R222 and K224 from the 220-loop served as a Na^{+} ligands.

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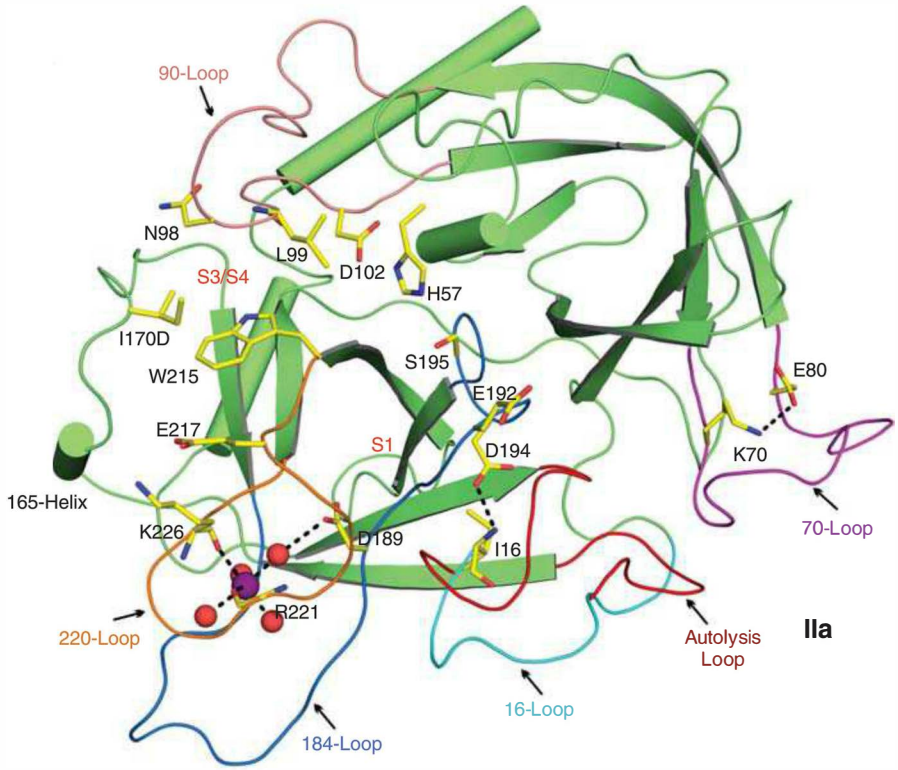
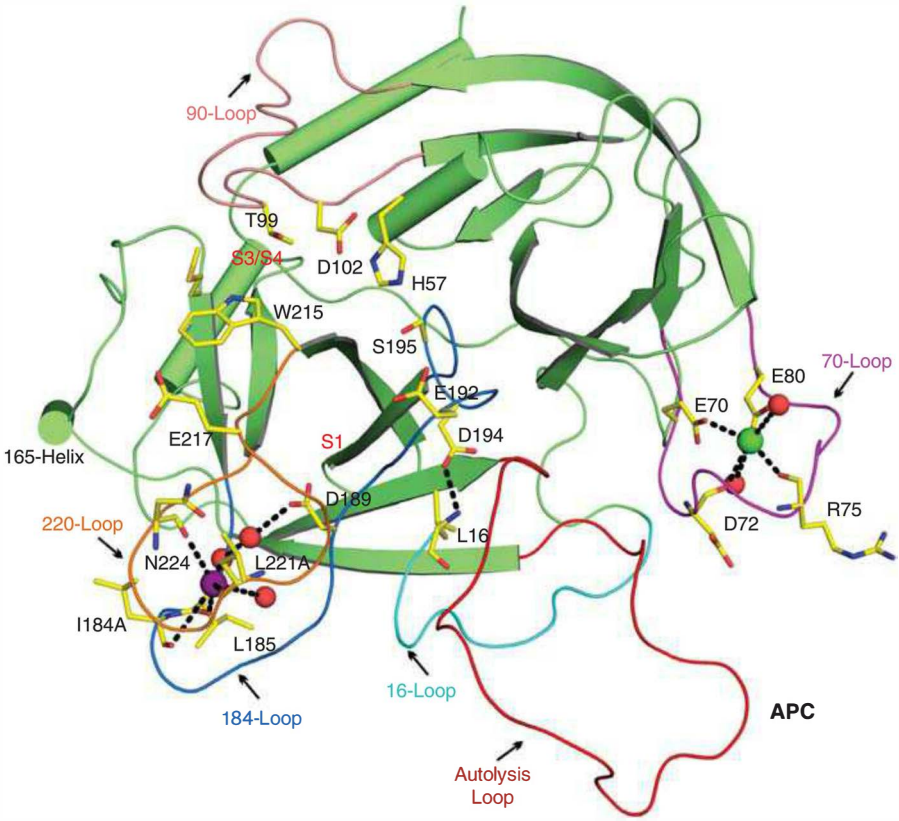


D



E

FIGURE 14.9 *(Continued)* ***D***, **Thrombin**: Thrombin does not have a Ca^{2+} site in its protease domain; instead, the 70-loop is stabilized by forming a hydrogen bond between the side chains of K70 and E80. The main chain carbonyl oxygens from residues R221 and K226 from the 220-loop and four water molecules serve as Na^{+} ligands. ***E***, **APC**: The side chains of E70 and E80, and the main chain carbonyl oxygens of D72 and R75 serve as ligands for Ca^{2+}. The main chain carbonyl oxygens from residues I184A and L185 from the 184-loop, and L221A and N224 from the 220-loop served as Na^{+} ligands.

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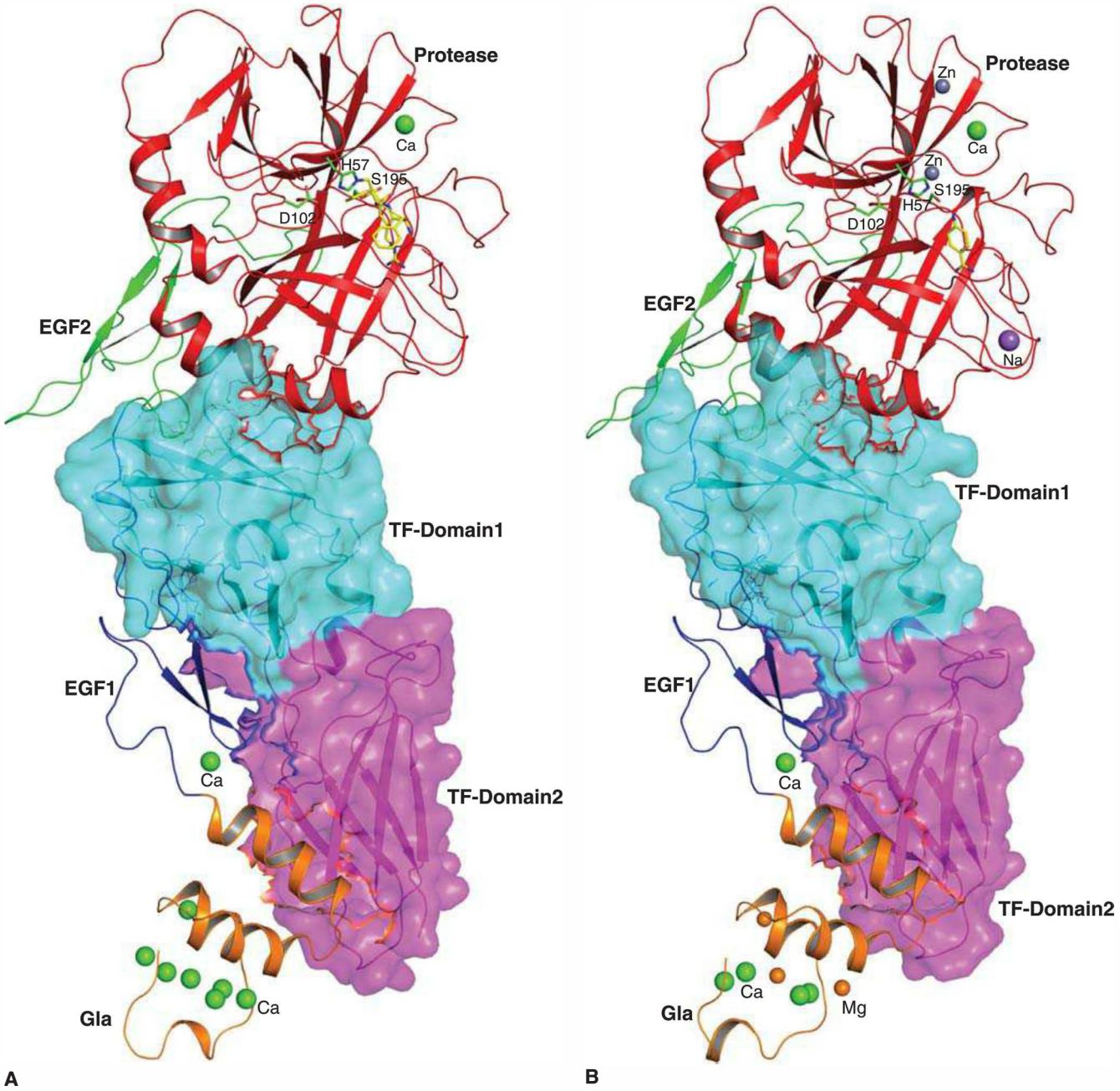

FIGURE 14.10 Interactive regions between FVIIa and the extracellular domain of TF (sTF). The FVIIa/sTF complex in the presence of Ca^{2+} only (pdb id 1DAN) is shown in (**A**) and in the presence Ca^{2+},Mg^{2+}, Na^{+}, and Zn^{2+} (pdb id 2A2Q) is shown in (**B**). All domains in FVIIa are involved in sTF interaction. The Gla domain of FVIIa binding sites are localized in *C*-terminal domain of TF. The EGF1 domain of FVIIa binding sites are localized at the interface between TF domain-1 and domain-2. The EGF2 and the protease domain of FVIIa binding sites are localized in TF domain-2. The active site triad residues H57, D102, and S195 as well as the bound inhibitors are shown in stick representation. FVIIa is shown in cartoon representation and the sTF is shown in surface representation. FVIIa Gla, EGF1, EGF2, and the protease domain are colored *orange*, *blue*, *green*, and *red*, respectively. The sTF domain-1 is colored *cyan* and the domain-2 is colored *magenta*. The Ca^{2+}, Mg^{2+}, Zn^{2+}, and Na^{+} are shown as *green*, *orange*, *black*, and *purple spheres*, respectively.

the residue Tyr [c177] of FIXa is implicated in modulating the catalytic activity.[367–369] A sequential activation mechanism has been proposed for FIXa that depends on its cofactor FVIIIa and its substrate FX. Binding of FVIIIa to the c162-helix of FIXa[289] might release the steric hindrance caused by Tyr [c177] but not by the Lys [c98].[149,369] Further, apart from the cleavage site, an unidentified site on FX binds to the Lys [c98] and completes the opening of the entrance to the S1 site in FIXa.[369,370] Thus,

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cofactor and macromolecular physiologic substrate act in concert to potentiate the catalytic activity of FIXa. Mutations of the catalytic triad residues produce hemophilia with normal circulating levels of dysfunctional protein.[100–102] Gly^{207} to Glu mutation within the His^{221} disulfide loop eliminates a hydrogen bond that stabilizes the active site.[370]

Trypsin-like serine proteases bind their substrates' Arg side chain to the S1 binding pocket with Asp^{359} [c189] at the bottom. Mutation of Asp^{359} to Gly in factor IXa causes hemophilia B^{100} most likely due to defective activation of FX. Mutations of Val^{307} are predicted to disrupt this binding pocket as well.[371] Ser^{360} [c190] is important in substrate binding to the S1 site as it allows an additional H-bond to be formed with the P1 Arg or Lys residue, thus stabilizing the substrate residues.[339] Mutation of Gly^{363} [c193] also causes hemophilia B.[372] Gly^{363} [c193] is involved in formation of the oxyanion hole in FIXa and other serine proteases. Mutation to any other residue most likely repositions the backbone amide of residue 363 [c193] such that it is pointing away from the oxyanion hole and unable to stabilize the developing oxyanion of the tetrahedral intermediate.[372] FX binding includes secondary binding sites such as one that is partially disrupted with an $Ile^{397}Thr$.[373,374] This is a common recurrent mutation and represents a founder effect.[103] A recombinant $Arg^{338}Ala^{375}$ was created to examine a potential thrombin inactivation cleavage site.[376] The recombinant Ala^{338} protein had threefold enhanced activity, but this was due to enhanced k_{cat} for FX cleavage, indicating involvement of this mutation in substrate interaction as opposed to stabilization from cleavage. Arg^{338} is in a Cys^{336}–Cys^{350} disulfide loop, and these data suggest this region can affect catalysis, possibly through interaction with factor VIIIa. Of note, thrombin cleavage of FIX is inhibited by physiologic calcium binding.[377] The catalytic domain's high affinity calcium binding site is in a loop involving Glu^{235} [c70] and Glu^{245} [c80] carboxyl groups (FIGURE 14.9B), adjacent to the autolysis loop containing a potential proteolysis site at Arg^{318}–Ser^{319}.[378] Cleavage of FIXa in the autolysis loop generates a molecule, FIXaγ, impaired in catalytic activity toward small synthetic substrates as well as in FX activation.[378,379] Thus, Ca^{2+} most likely protects the autolysis loop from cleavage by thrombin or FXIa. When Glu^{235} [c70] was replaced by Lys, 75% of activity remained as predicted from the structure of thrombin, <10% FIX reactivity remained in a recombinant species in which both Glu's were mutated to Lys.[374]

Molecular modeling studies suggest that the FIXa protease domain possesses a sodium binding site that is similar to that in factor Xa and APC but not to IIa.[155,380] However, Na^+ site was not observed in the crystal structures of mutant FIXa molecules[149,369] and in FIXa bound to antithrombin.[150] Notably, Na^+ site was also not observed in FXa bound to antithrombin.[381] Thus, the FIXa loops involved in Na^+ binding could be modulated by mutations, and Vadivel et al.[382] observed the Na^+ site involving the predicted loops in the crystal structure of wild-type FIXa at 1.64 Å resolution.

Although the specificity of the FIXa active site is restricted, it is rapidly inactivated by AT-III with heparin.[383,384] Heparin binding increases the reactivity of FIXa toward both peptidyl substrates and bovine pancreatic trypsin inhibitor (BPTI) inhibition through induced conformational changes from the exosite to the active site.[385] Residues Lys^{293} [c126], Arg^{333} [c165], Arg^{338} [c170], Lys^{390} [c230], and Arg^{393} [c233] in FIXa are part of an exosite that binds to heparin.[386] Further, the affinity of full-length FIXa for heparin is increased approximately 10-fold by Ca^{2+} indicating that Ca^{2+} promotes FIXa inhibition by the template mechanism.[387]

FIXa activation of FX is enhanced several orders of magnitude by PL and FVIIIa.[388] FVIIIa binding increases the catalytic activity of FX activation approximately 10^6-fold with no effect on synthetic substrate hydrolysis.[389,390] Strong evidence exists for the involvement of the 293-helix [c126-helix], 330-helix [c162-helix], and Asn^{346} [c178] in binding to FVIIIa.[289,368,391] Thus, it appears that heparin may bind to the same exosite on FIXa that FVIIIa binds. Further, residues Phe^{342}–Asn^{346} [c174–c178] of FIXa contribute to interaction with the endocytic receptor low-density lipoprotein receptor–related protein.[392] Ethylene glycol increases the catalytic activity of FIXa toward synthetic substrates in a bell-shaped pattern with maximum activity at approximately 35% to 45% ethylene glycol concentration,[367,393] and it binds between the Glu^{224} [c60] and Pro^{255} [c90] in FIXa, which mimics the effects of FX binding on the 266-loop [c99-loop].[367] Under some conditions, FIXa can activate $FVII^{10}$ or FVIII.[394,395]

FX/FXa. The FXa protease domain contains a Ca^{2+} binding site in the c70-loop and Na^+ binding site between the c184-loop and c220-loop (FIGURE 14.9C). The side chain of Asp [c70] and Glu [c80] and the main chain carbonyl oxygen atoms of Asn [c72] and Gln [c75] are coordinated to Ca^{2+}.[153,380] The Asp [c70] mutation to Lys abolished the Ca^{2+} binding without affecting the catalytic activity of FXa.[396] The residues Tyr [c185], Asp [c185A], Arg [c222], and Lys [c224] backbone atoms serve as Na^+ ligands.[170,380] The alanine mutational studies performed in c184-loop residues Asp [c185A] and Lys [c186] result in the impairment of Na^+ binding and reduction in catalytic activity toward the macromolecular substrate prothrombin; however, saturating concentration of FVa restores the catalytic activity.[397] FXa activates FVII,[398] FVIII,[399] and FV[400] in addition to its main role in activation of prothrombin. FXa has two physiologic inhibitors, AT and TFPI.

Prothrombin/Thrombin. Human α–thrombin can be autodegraded by cleavage at Arg382 and Asn394 in the B chain to form β-thrombin and by cleavage at Arg443 and Lys474 in the B chain to form γ-thrombin.[401,402] These degraded forms of α–thrombin retain some ability to hydrolyze small synthetic substrates[403,404] and to cleave FXIII,[405] AT,[406] and FII.[407] However, β-thrombin and γ-thrombin do not cleave fibrinogen, thrombospondin, or PC.[406,408,409]

In addition to the α–, β-, and γ-thrombin forms, there are two additional forms that also have physiologic activity: meizothrombin and meizothrombin des-fragment 1.[60] Once cleaved at Arg320, meizothrombin can then cleave itself at Arg156, thus releasing PF1 and generating meizothrombin des-fragment 1.[60] The meizothrombin des-fragment 1 can then be autolytically cleaved at Arg283 to form α–thrombin. Meizothrombin is currently viewed as the first product produced by the prothrombinase complex during prothrombin activation.[62,63] Meizothrombin is a potent vasoactive agent and acts on the adrenergic receptor[410] and is five- to sevenfold more potent than α–thrombin in inducing vascular constriction.[408,411] Meizothrombin has substantially reduced activity toward fibrinogen and platelets as compared to α–thrombin,[60,412] but it does cleave other thrombin substrates. Meizothrombin and α–thrombin have similar abilities to cleave PC, FV, FXI, and small synthetic substrates.[60,412–417]

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The PF2 (K2 region of FII) noncovalently associates with thrombin with a dissociation constant of approximately 10 nM.[418,419] PF2 interacts with exosite II (heparin-binding region) of thrombin,[161,420–423] involving salt bridges between the inner loop of K2 and FII exosite II.[424] Consequently, it inhibits AT inhibition of IIa,[425] alters the IIa active site and exosite I,[426] enhances IIa activity toward small peptide substrates,[418] inhibits IIa cleavage of fibrinogen,[427] and influences PC activation.[428]

There is considerable sequence homology as well as conservation of the disulfide bonds between α–thrombin and α–chymotrypsin. However, there are key differences between human α–thrombin and α–chymotrypsin,[429] including deletion of the residue c218, addition of NH_2-terminal residues 285 to 292 (II numbering), minor insertions of 1 or 2 amino acids, a 13-residue insertion (residues 307 to 319) called the *A* loop, and the insertion of residues 367 to 375 ("c60 B loop," FIGURE 14.8) near the active site of α–thrombin.[165,429,430,431,432] The "E loop" represents an insertion composed of residues 513 to 516 and is located near the bottom of the active site pocket.[429] The position of the "C loop" or "γ loop," residues 470 to 474 (c148 loop), varies considerably between the FPR-ck-α–thrombin and the hirudin–α–thrombin structures, indicating that this segment of α–thrombin is mobile in solution.[429]

The Na^+ binding site of IIa is contained within a cylindrical cavity formed by three antiparallel β-sheets of the α–thrombin B chain (FIGURE 14.9D). The site is more than 15 Å away from the catalytic site. The cavity containing the Na^+ binding site bisects the α–thrombin molecule from the active site to the opposite surface and contains Asp [c189].[173] In the presence of Na^+, α–thrombin recognizes and cleaves fibrinogen acting as a procoagulant; when no sodium is bound, α–thrombin has increased specificity for PC. This change in specificity allows α–thrombin to act as both a procoagulant and an anticoagulant.[433,434] IIa variants cTrp215Ala/cGlu217Ala or cGlu217Lys act as anticoagulants and activate PC in a thrombomodulin dependent manner. Structural studies reveal that these mutants are in the inactive E* form and their Na^+ binding loops c186 and c220 as well as the active site are highly disordered.[435,436]

The fibrinogen binding site (exosite I) is an anion-binding electropositive site, distinct from, but acting in concert with, the active site.[433] Exosite I also binds the COOH-terminal domain of hirudin, the hirudin-like region of the thrombin receptor protease-activated receptor (PAR)-1 and PAR-4, and the fifth and sixth EGF-like domains of thrombomodulin.[433] Exosite I is comprised of insertion loops c30–c40 and c70–c80.[165,421] Hirudin demonstrates a high affinity interaction with α–thrombin to form a stable, noncovalent complex with a dissociation constant in the range of 2×10^{-14} M.[437,438] Hirudin binds to both exosite I and the active site of thrombin.[438–440] In solution, the COOH-terminal domain of hirudin is disordered.[441,442] However, in the α–thrombin–hirudin complex, the COOH-terminal of hirudin interacts with the large groove in the α–thrombin molecule and extends across the face of the molecule to interact with exosite I which is distant from the active site of α–thrombin. The interaction of α–thrombin and hirudin is stabilized by electrostatic, polar, and hydrophobic interactions that account for the very high affinity interaction between the two proteins.

Exosite II is positively charged and serves as an anion-binding site on the opposite side of α–thrombin compared to exosite I, which binds hirudin and fibrinogen. Exosite II is responsible for binding highly sulfated polysaccharides like heparin,[443] the COOH-terminal domain of the B chain of thrombin,[422] and the chondroitin sulfate moiety of thrombomodulin.[434] The fourth binding site, the active site region, interacts with substrate molecules.[444] Kunitz inhibitor BPTI reacts very poorly with thrombin, restricted by Trpc60D, which sits over the active site pocket.[445] When Glu192 is mutated to Gln, the thrombin–BPTI complex is tightened[446] sufficiently to allow structural studies of the complex.[445] In the complex, Trp60D is shifted approximately 8 Å from its normal position, consistent with previous modeling studies.[165] Kinetic analysis showed that thrombomodulin binding to thrombin does increase the on rate approximately 10-fold,[447] suggesting that it induces conformational changes that contribute to the altered substrate and inhibitor specificity. Thermodynamic linkage between exosite II, Na^+ site, and the active site arises from the interconversions of zymogen- and enzyme-like forms of IIa.[448]

APC. The active site of APC (triad residues, His^{211}[c57], Asp^{257}[c102], and Ser^{360}[c195]) is relatively open compared to that of thrombin or FXa (FIGURE 14.9E), contributing to the resistance of APC to inhibition by plasma proteinase inhibitors. APC contains substrate recognition sites on the prime side, as well as on the non-prime side that are common to other serine proteases.[154] The APC contains a Ca^{2+} binding site and a Na^+ binding site in the protease domain (FIGURE 14.9E). The c70-loop contains two Arg residues at positions c74 and c75; Ca^{2+} bind to APC and the residues Glu [c70], and Glu [c80], and the main chain carbonyls of Asp [c72] and Arg [c75] coordinated to Ca^{2+}.[154,155] Na^+ binds between the c184-loop and c220-loop similar to that of FVIIa, FIXa, and FXa and is coordinated to the backbone carbonyl oxygen atoms of Ile [c184A] and Leu [c185] from c184-loop, and Leu [c221A] and Asn [c224]. Ca^{2+} binding enhances catalytic activity of PC and Na^+ binding to APC increases catalytic efficiency approximately 100-fold for small synthetic substrates. Na^+ binding is thermodynamically linked to both Ca^{2+} and the S1 site, while the Ca^{2+} site is linked only to the Na^+ site.[155,449]

The autolysis loop is involved in binding to the PC inhibitor (PCI),[450] and the basic residues on the surface at c30, c60, and c70-loops of the protease domain are involved in protein–protein interactions for activation and binding to heparin, heparin sulfate, and α–1 antitrypsin.[451] The Lys residues at position c37, c38, and c39 in the c37-loop of PC bind to the thrombomodulin–thrombin complex[452] and provide the substrate recognition exosite for FVa that is important for cleavage of Arg506 in FVa.[453] Lys^{217} [c62] and Lys^{218} [c63] are part of a heparin-binding site that is important for heparin-mediated inhibition of APC by PCI.[454] The Lys residues at position c37, c38, and c39 form a secondary binding site for FVa, which is important for the cleavage of FVa at Arg-506 while having no effect on Arg306 cleavage. The Thr [c99] to Tyr mutant inactivated FVa twofold better than the wild-type APC.[455] The mutations observed in the catalytic domain of PC at Arg^{169} to Trp, and the Trp^{402} to Cys cause structural instability and are related to hereditary thrombophilia.[456–458]

Glu residues at positions 309 [c192] and 310 [c39] in thrombin make unfavorable interactions with Asp residues in the P3 and P3′ 311–313 positions of PC, which are largely overcome by thrombomodulin binding to thrombin. Increasing the P3-binding energetics by mutation of Glu [c217] to Lys in thrombin resulted in considerably enhanced PC activation rates[459,460] and converted thrombin into an anticoagulant

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protein. Mutation of both the P3 and P3′ residues of PC augment PC activation[461,462] and increase the rate of thrombin activation sufficiently to allow PC to serve as an anticoagulant *in vitro*.[463] Meizothrombin is a better activator of PC than α–thrombin,[413,417] an important point, given that early on during coagulation, its concentration is higher than α–thrombin.[414,417]

To be activated to APC, PC must be bound to the endothelial cell surface where thrombin/thrombomodulin can cleave it between Arg169 and Leu170 and release a 12–amino acid activation peptide. With its cofactor PS, APC cleaves FVa and FVIIIa to inactivate them.[25,82,464] APC binding to its cofactor, PS, is necessary for FVa inactivation; however, APC inactivation of FVIIIa in the tenase complex requires both PS and FVa as cofactors.[465] PS circulates both as a free form (~40%) and in a complex with C4b-binding protein.[466] FV serves as a cofactor for APC inactivation of FVa and FVIIIa.[467] Flexibility in the APC molecule may contribute to the observed topographical changes in APC, which are observed following interaction with PS.[468] Thrombomodulin binding to thrombin potentiates the activation of PC >1,000-fold, and EPCR binding of PC potentiates PC activation another approximately 20-fold.[469] Different models have been put forth to explain this enhancement. Sadler et al.[470,471] were the first to show that point mutations within anion-binding exosite 1 on thrombin, which binds both fibrinogen and thrombomodulin, disrupt fibrinogen clotting or PC activation by the thrombin/thrombomodulin complex differentially.

Sodium bound to IIa enhances fibrinogen clotting activity approximately eightfold[472,473] and decreases PC activation rates by the thrombin/thrombomodulin complex by 30%.[474] Mutation of this site has been utilized to generate thrombin mutants with increased anticoagulant activity.[475] On the basis of detailed kinetic analysis, residues Glu [c39], Glu [c192], and Trp [c60D] are linked to the Na^+ transition.[476] Mutation of Lys [c37] to [c39] to acidic or neutral residues had no influence on the activation rates of PC by thrombin or the Ca^{2+}-dependent inhibition of this process; however, the mutants were activated approximately 10-fold slower by the thrombin/thrombomodulin complex at all Ca^{2+} concentrations tested.[452] Thus, it is clear that thrombomodulin binding to PC influences PC activation. Thrombomodulin binding to IIa mediates conformational changes, making it a better activator of PC. The EGF3 domain of thrombomodulin is important in thrombin-activatible fibrinolysis inhibitor activation, whereas the EGF4 domain of thrombomodulin has been implicated in PC activation.[477] Data supporting the thrombomodulin-induced conformational changes in thrombin includes changes in fluorescence of covalently attached dyes,[478,479] selective changes in the electron spin resonance of covalently attached probes,[480] and changes in synthetic substrate hydrolysis.[428,478,481] PC may be able to function as an antithrombotic agent, and several studies have structurally altered PC to enhance thrombin activation. Mutations of both the P3 and P3′ residues of PC augment PC activation[461,462] and increase the rate of IIa activation sufficiently to allow PC to serve as a plasma anticoagulant *in vitro*.[463]

PS circulates in free form and bound to C4b-binding protein,[466] an interaction of high affinity in the presence of Ca^{2+}.[482,483] The steroid hormone binding globulin-like region (2 LamG domains) of PS is important for binding to the β-subunit of C4b-binding protein. Specifically, the β-chain complement control protein (CCP) 1 domain binds to PS *via* a hydrophobic patch on CCP1 comprised of Ile16, Val18, Val31, and Ile33.[483,484]

APC cleaves FVa at Arg306, Arg506, and Arg679,[485,486] but an FV mutation (Arg506 to Gln), known as factor V Leiden, is resistant to APC inactivation[465]; individuals carrying this mutation are at increased risk for venous thrombosis. Notably, APC cleavage at of FVa at Arg506 is kinetically favored over cleavage at Arg306, and cleavage at Arg506 is less dependent on the presence of PS than cleavage at Arg306.[26,27,465,486,487] Modeling of APC with FVa shows that basic residues in the c60-loop, c37-loop, c70-loop, and c148-loop of the protease domain of APC likely interact with negatively charged regions surrounding the Arg506 cleavage site in FVa.[477]

FVIIIa inactivation by APC requires both FV and PS as cofactors,[465,467,488] the laminin G2 domain being important in this synergistic cofactor activity.[482,489] APC cleaves FVIIIa at Arg336 and Arg562.[490,491] APC cleavage of FV at Arg506 is important for it to serve as a cofactor.[465,488,492,493] FV's anticoagulant cofactor activity is lost when thrombin cleaves after Arg1545 between the B domain and A3 domain.[492] PS can inhibit thrombin generation in plasma in the absence of APC,[494] most prominently at picomolar concentrations of TF.[495] PS serves as a cofactor for TFPI in its inhibition of FXa,[496] decreasing the Ki for TFPI/Xa complex formation from 4.5 to 0.5 nM.[496] It is likely that PS binds *via* its Gla domain to the Kunitz domain 3 and *C*-terminal tail of TFPI.[497–499]

ENZYME AND COFACTOR INTERACTIONS

The cofactor for FVIIa is TF, that for FIXa is FVIIIIa, that for FXa is FVa, that for APC is PS, and that for PZ is PZI[24] (Table 14.2). Thrombin does not require a cofactor in performing coagulant function but requires thrombomodulin to perform its anticoagulant function. The only enzyme–cofactor complex that has been crystallized to date is FVIIa/TF.

Interaction of FVIIa/TF with FXa or FIXa

The FVIIa/TF complex activates both FIX[500] and FX,[501] which depends on Ca^{2+}/Mg^{2+} and the PL membrane. In a reciprocal fashion, FXa and FIXa both activate FVII/TF.[12,13,51,52] FX chimeras show that the affinity of FX–Gla and FX–EGF1 domains toward FVIIa/TF is stronger than the FIX–Gla and FIX–EGF1 domains and that activation is slower for FX chimeras.[227] The FIX and FX chimeras with PC EGF domains also confirm the role of EGF1 in activation.[502] The experimental evidence confirms that Gla and EGF1 domains of FIX and FX bind to FVIIa/TF and the differences in the activation process arise from the variations in their affinity toward the FVIIa/TF complex.[184,205,227,337]

Computational models have been proposed for the ternary complexes of FVIIa/sTF–FXa, FVIIa/sTF:FIXa, and FVIIa/sTF:$FIX_α$.[185,186,206,227] In the absence of experimentally determined structures, these models serve as reasonable basis for understanding how Gla and EGF1 domains of FIX/IXa and FX/Xa interact with FVIIa/TF. In these models, the Gla domains of FXa[184–186,502] and FIXa/FIXα[205,206] interact with the TF domain as well as with the Gla domain of FVIIa. Although the structures of FXa and FIXa are similar, the variations in their sequences at the interface regions with sTF and with the Gla domain of FVIIa make associations different from each other. The residues between 9 and 12 in the Gla domain of FX and FX interact with FVIIa/TF. This region is basic in FXa, whereas hydrophobic and neutral in FIXa. The sequence variations in this region

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make interactions weak for FIXa and provide an explanation for why FIX binding is weaker as compared to FX. The *C*-terminal helix of the Gla domain in FX/Xa and of FIX/IXa interacts with *C*-terminal region of sTF.[203,205,206,223,227,337,503,504] Mutagenesis studies support the role of TF residues Arg200, Lys201, Thr167, and Lys165 in its interaction with the Gla domain of FXa/FIXa.[337,503] Comparable interactions exist between the *C*-terminal helix of the Gla domains of FXa or FIXa with TF.

Arg36 in the Gla domain of FVIIa is one of the key residues in substrate recognition for FX.[183] However, the recent FVIIa/sTF crystal structure at 1.72 Å reveals that Arg36 in FVIIa is involved in network of hydrogen bonds with TF.[179] The TF region containing residues Lys165 and Lys166 are well defined, and it appears that only one of these residues can interact with FXa. The *N*-terminal regions of EGF1 in FXa/FIXa domains interact with sTF in a similar manner; however, this region does not interact with FVIIa. Very little information is available about the EGF2 domain as well as the regions other than the activation peptide that are involved in ternary complex formation. The residue Arg^{290} [c147] in FVIIa protease domain is implicated to be important in FX activation.[505] The residues involved in the ternary complexes of FVIIa/TF with FXa or FIX/FIXα are described elsewhere.[186] Experimentally determined structures of the ternary complexes are needed to precisely define the interactive regions and to precisely understand the activation process for conversion of FX and of FIX to their respective enzymes.

Interaction of FIXa with FVIIIa

The recent crystal structure of FVIII reveals its domain organization and the regions which could possibly interact with FIXa.[506,507] FIXa:FVIIIa models[508,509] show that the protease domain of FIXa interacts with the A2 subunit of FVIIIa, part(s) of the EGF2/EGF1 domains interact with the A3 subunit of FVIIIa, and the Gla domain interacts with the C2 subunit of FVIIIa (FIGURE 14.11A,B).

Binding of FVIIIa to the FIXa protease domain was suggested by anti-FIX monoclonal antibody inhibition activity.[510] This antibody binds to a conformational epitope in the amino-terminal half of the catalytic domain.[511] Mutation of charged surface residues to the homologous residue in FX, however, failed to identify a species that had reduced FVIIIa binding.[512] Strong evidence supports the involvement of residues from the 293-helix [c126-helix], the 330-helix [c162-helix], and Asn^{346} [c178] in FIXa in binding to FVIIIa.[368,389,391,513] The 330-helix [c162-helix] binds to the 558-565 region of the A2 subunit of FVIIIa.[513] Isolated A2 domain enhances FIXa activation of FX approximately 200-fold,[514] although this is very much less than the effect observed with intact FVIIIa. Plasma-derived, recombinant wild type, and two protease domain Ca^{2+}-site mutants Glu^{235} [c70] to Lys and Glu^{245} [c80] to Val were compared in terms of the effect of PL and apparent FVIIIa binding.[378] Results allow one to infer that the Ca^{2+} binding loop is not directly involved in binding to FVIIIa but instead imparts a structural change in conformation that favors binding to FVIIIa.[512] Proteolysis in the autolysis loop, residues 310 to 323 [c141–c155] of FIXa, decreases binding to FVIIIa[378,379]; further, mutation of Lys^{316} [c148] to Ala or Glu,[366] mutation of Arg^{312} [c143] to Ala,[515] and proteolysis at the Lys^{316}–Gly^{317} [c148–c149] peptide bond[379] substantially reduce the rate of FX activation. Thus, the autolysis loop of FIXa is most likely involved in FX binding. Proteolysis of the autolysis loop is hypothesized to affect FVIIIa binding because the two helices important in binding to FVIIIa, 293-helix [c126-helix] and the 330-helix [c162-helix] are each linked to the autolysis loop *via* a single β-strand.[516]

The FIXa protease domain contains a Na^+ site[516] that coordinates to the carbonyl groups of residues 353 [c184A], 354 [c185], 391 [c221A], and 394 [c224]. The two Na^+ binding loops are held together by H-bond between Tyr^{395} [c225] and His^{354} [c185], and Tyr^{395} [c225] makes van der Walls contacts with the 330-helix [c162-helix] that binds FVIIIa.[516] A hemophilia B mutation in which Tyr^{395} [c225] is changed to His has been reported,[100] which could be due to impaired Na^+ and FVIIIa binding.

There is also evidence for a specific interaction between the FIXa EGF2 domain and FVIIIa. The EGF2 domain (residues 88 to 109) is important in assembly of the tenase complex.[290] Replacement of the Asn^{89}–Lys^{91} loop region only marginally

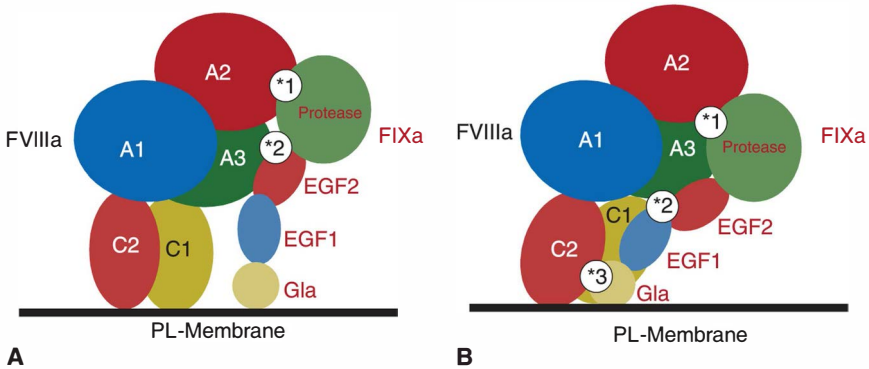

FIGURE 14.11 Schematics of the proposed models of the FVIIIa/FIXa complex. **A**: The protease domain of FIXa binding sites are localized in the A2 and A3 domains of FVIIIa and are indicated by 1* and 2*. The EGF2 domain binding sites in FIXa are localized in the A3 domain of FVIIIa and are indicated by 2*. **B**: The protease domain of FIXa binding sites are localized in the A2 and A3 domains of FVIIIa, but the EGF1 and EGF2 domains of FIXa interact with the A3 domain of FVIIIa. Further, the Gla domain of FIXa interacts with C2 domain of FVIIIa. These binding regions are indicated by 1*, 2*, and 3*.

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decreased the activation of FX in the presence of FVIIIa; however, replacement of the EGF1/EGF2 linker region Leu[84]–Thr[87] greatly decreased the activation of FX in the presence of FVIIIa (less effect with the A2 subunit).[291] Chang et al.[293] mutated every residue in the EGF2 domain to Ala and concluded that residues 89 to 94 are important in binding to FVIIIa, and residues 102 to 108 are involved in binding to FX. Thus, there is some discrepancy between these studies and results that remain to be resolved. It is possible that the EGF2 domain binds to either the A2 subunit, the A3 subunit, or to both the A2 and A3 subunits of FVIIIa.

The EGF1 domain may also influence FVIIIa binding, although this may not be due to a direct interaction. Several studies have emphasized the importance of Ca^{2+} binding to the EGF1 domain of FIXa in FVIIIa binding on PL surface,[111,294] and Gln[50] to Pro mutation only reduces affinity for FVIIIa in the presence of PL and not in its absence.[289] Studies with monoclonal antibody to the EGF1 domain indicate that conformational changes in the protease domain of factor IXa that occur during activation are allosterically transmitted to the EGF2 and EGF1 domains.[284] Thus, it appears that the EGF1 domain does not directly bind to FVIIIa but rather correctly positions the EGF2 and protease domains for proper interactions.

The Gla domain may also be involved in FVIIIa binding. Using synthesized Gla domains with Phe[9], Phe[25], or Val[46] replaced with benzoylphenylalanine, Gla25BPA and Gla47BPA constructs were cross-linked to FVIIIa, and immunoprecipitation experiments suggest that the Gla domain of FIXa interacts with the C2 domain of FVIIIa.[207,208,517] Mutation of Gly[12] to Arg has also been reported to disrupt FVIIIa binding[209]; however, this is most likely due to a structural impairment rather than from a direct binding effect. Thus, more studies are needed to elucidate the exosites on FIXa important in binding to FVIIIa, particularly with respect to the EGF2, EGF1, and Gla domains.

Interaction of FXa with FVa

The association of FXa with its cofactor FVa increases the conversion rate of prothrombin to thrombin by 10^6-fold (FIGURE 14.12). Structural models[518] have been proposed for the prothrombinase complex based on the crystal structure of inactivated bovine FVa.[519] In the FXa/FVa complex, the protease domain of FXa interacts with A2 and A3 domains of FVa, whereas EGF2 domain interacts with A2 domain of FVa. The experiments performed with FX chimera containing FIX Gla and EGF1 domains suggest that the EGF2 and protease domains in FXa are sufficient for FVa binding, whereas the Gla and EGF1 domains are not directly involved in FVa binding.[281,299,502] Alanine mutagenesis at the conserved basic residues in FXa show that the heparin-binding sites and the FVa sites overlap with each other and the residues in 344-loop [c162-loop] are involved in FVa binding.[520] Mutational experiments involving autolysis loop indicate that the activation loop is not part of the FVa binding site.[521] The *C*-terminal helical region is also shown to be involved in FVa binding.[522] The alanine mutations in c184-loop and c220-loop of FXa impair the Na^+ binding and show reduction in its activity toward both synthetic and macromolecular substrates; however, binding of the cofactor FVa restores the FXa catalytic activity.[299,523] These results support the hypothesis that the Na^+ and FVa-binding sites in FXa are energetically linked to each other and the binding of FVa to FXa induces conformational change in the Na^+ binding loops and keep them in a Na^+ bound active conformation.[381,520,523]

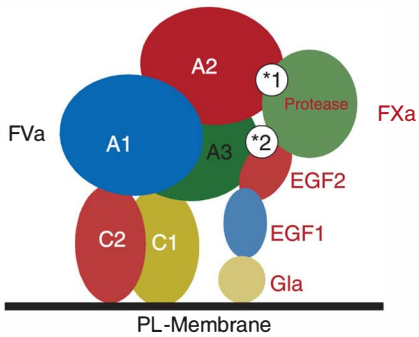

FIGURE 14.12 Schematics of the proposed model of FVa/FXa complex. The protease domain binding sites in FXa are localized in the A2 and A3 domains of FVa and are indicated by 1* and 2*, while the EGF2 domain binding sites in FXa are localized in the A3 domain of FVa and are indicated by 2*.

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CHAPTER

15A Factor XI

PETER N. WALSH AND DAVID GAILANI

Coagulation factor XI (FXI), previously known as plasma thromboplastin antecedent, was first identified by Rosenthal et al.[1] as a deficiency in the plasma of patients with abnormal bleeding and, subsequently, was found to be particularly common in patients of Ashkenazi Jewish descent.[2,3] FXI was first purified from bovine[4] and human[5] plasma as a 160-kDa homodimeric protein that participates in interactions with coagulation proteins of the contact phase of blood coagulation.[6–9] Factor XI is the zymogen of a trypsin-like serine protease that is activated by FXIIa and by both thrombin and FXIa,[10,11] and it can then recognize FIX as its normal macromolecular substrate in plasma.[12–14]

GENE STRUCTURE AND REGULATION

The approximately 23 kb human FXI gene contains 15 exons and is located on the distal end of the long arm of chromosome 4 (4q35).[15,16] The FXI cDNA derived from human liver mRNA is 2,097 nucleotides long.[17] The first exon is untranslated, while the second encodes the signal peptide. The factor XI polypeptide in plasma contains 607 amino acids encoded by exons 3 to 15 (see FIGURE 15A.1).[15,17,18] Exons 3 to 10 encode four repeats called *apple domains* (A1 to A4 from the *N*-terminus).[18] The intron–exon boundaries are located at similar positions within each apple domain, indicating duplication from a common ancestral sequence.[15] Apple domains are members of the PAN (*P*lasminogen, *A*pple, *N*ematode) module family, with homology to the *N*-terminal domains of hepatocyte growth factor and plasminogen[19] and similar core topology to PAN domains from leech antiplatelet protein,[20] the protozoan microneme antigen EtMIC5,[21] and apical membrane antigen 1 from *Plasmodium falciparum*.[22]

Exons 11 to 15 of the FXI gene encode the trypsin-like catalytic domain and are organized similarly to genes for tissue plasminogen activator and urokinase.[15] The proteases thrombin and factors VIIa, IXa, and Xa have tyrosine or phenylalanine in their catalytic domains at the position corresponding to residue 225 in chymotrypsin. This confers Na^+-induced allosteric regulation on the catalytic domain and is associated with an AGY codon for the active site serine residue (Ser195, chymotrypsin numbering), consistent with these proteases having evolved from a common thrombin-like protease.[23,24] In contrast, FXIa and the contact proteases α-kallikrein and FXIIa have proline at position 225, indicating they are not regulated by sodium. Pro225 is associated with a TCN codon for Ser195, suggesting these proteases evolved from a trypsin-like protease distinct from the ancestor of the vitamin K–dependent proteases.[23]

The amino acid sequence of human FXI is 58% identical to plasma prekallikrein (PK, the zymogen of α-kallikrein),[17] which also consists of four apple domains and a trypsin-like catalytic domain.[18,25] The PK gene contains 15 exons, with intron–exon boundaries in identical positions to those of the FXI gene,[26] indicating the genes are products of a duplication event. Consistent with this, exon 15 of the PK gene is only approximately 8 kb from exon 1 of the FXI gene in humans.[27] Analyses of vertebrate genomes reveal that fish do not have homologs for FXI or PK.[28,29] A gene encoding a serine protease with four apple domains that is clearly ancestral to both proteins is found in amphibians, birds, reptiles, and the primitive egg-laying mammal (monotreme) the duck-billed platypus.[29,30] The proteins encoded by the ancestral genes have features that are more similar to PK than FXI.[30] Distinct genes for FXI and PK are present in the opossum, a marsupial, and in placental mammals, indicating duplication of the ancestral gene occurred after the common lineage leading to these two groups diverged from monotremes.[30]

The normal site of synthesis of plasma FXI is the liver. FXI levels decrease in liver disease, and patients have acquired,[31,32] or been cured of,[33,34] FXI deficiency by liver transplantation. Northern blot analysis confirms that the liver is the major, if not exclusive, site of FXI mRNA synthesis in mice.[35] In humans, FXI mRNA has been found by northern blot[35] and reverse-transcriptase polymerase chain reaction (RT-PCR)[36] in liver, pancreas, and kidney; and by RT-PCR in platelets.[37–39] Immunohistochemistry studies confirmed that FXI protein is present in human hepatocytes, pancreatic islet cells, and the proximal and distal tubules in the kidney.[40] The sequence between base pairs −385 and −350 of the human FXI gene promoter contains a binding site for hepatocyte nuclear factor-4α (HNF-4α), a transcription factor that appears to be required for hepatocyte-specific FXI expression.[27] Mice with a liver-specific deletion of HNF-4α have reduced plasma FXI levels.[41] HNF-4α is expressed at high levels in human pancreatic islets and kidney,[42,43] explaining the presence of FXI in these tissues. There is a small amount of FXI activity associated with platelets (~0.5% of the activity in plasma),[44–48] but it is not clear that FXI mRNA found in platelets is responsible for this. Platelet FXI is discussed in detail below.

Large epidemiologic studies have demonstrated an association between plasma FXI level and venous[49] and arterial thrombotic disease[50]; however, genetic factors controlling FXI gene expression are not well understood. Common single nucleotide polymorphisms (SNPs) are present in the human FXI gene promoter at base pair −403 (G/T) and −273 (C/G), the latter of which clearly alters transcription factor binding in gel mobility shift assays.[51] However, it is not clear that there is a relationship between these SNPs and plasma FXI level. Two common SNPs within the FXI gene have recently been associated with deep

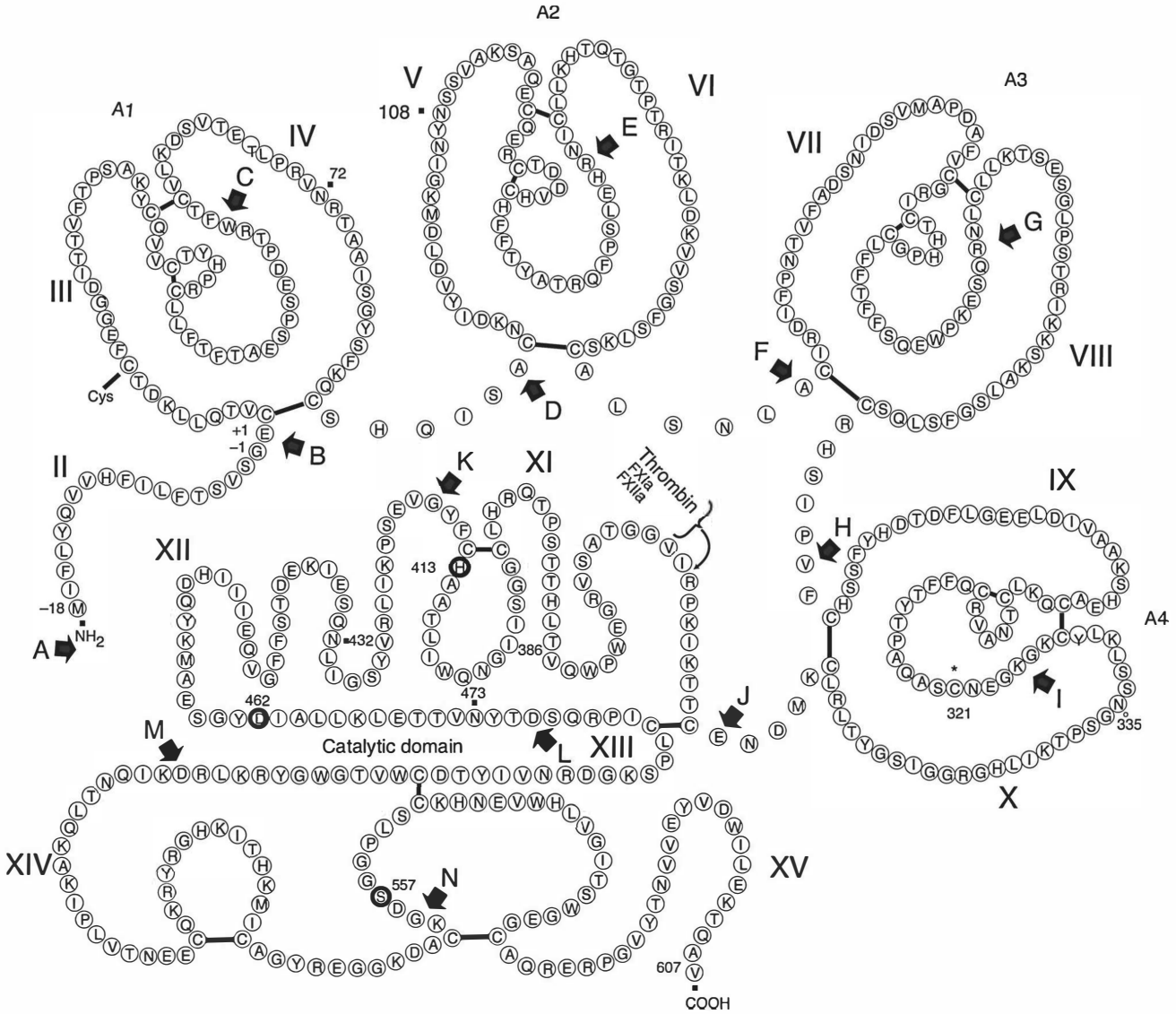

FIGURE 15A.1 Amino acid sequence and primary structure of human FXI. The signal sequence (numbered −18 to −1) is removed during biosynthesis by signal peptidase by the cleavage of the G–E bond between −1 and −2. Factor XI circulates in plasma as a homodimer connected by a single disulfide bond linking C321 in both of the fourth apple domains. The 14 introns (A to N) are shown by *solid arrows*. The exons are denoted by II to XV amino- to carboxy-terminus, with exon II representing the propeptide and exon I (not shown) representing the 5′ untranslated region. The four apple domains (consisting of 90 to 91 amino acids) are labeled as A1, A2, A3, and A4. The site of cleavage catalyzed by thrombin, factor XIa, or factor XIIa during the conversion of factor XI to factor XIa is shown with a *small, curved arrow*. The three members of the catalytic triad (H413, D462, and S557) are *circled* in *bold*. The four *N*-linked carbohydrate chains (i.e., N72, N108, N432, and N473) are shown by solid squares. (Figure redrawn and modified from McMullen BA, Fujikawa K, Davie EW. Location of the disulfide bonds in human coagulation factor XI: the presence of tandem apple domains. *Biochemistry* 1991;30:2056–2060.)

venous thrombosis (DVT) and high plasma levels of FXI.[52,53] Interestingly, the SNPs remained associated with risk of DVT even after adjustment for FXI level,[53] but the mechanisms by which these polymorphisms contribute to thrombosis risk is not known.

Full-length and partial FXI cDNA sequences are now available for more than a dozen mammalian species. The best characterized of these are the murine[35] and rabbit[54] cDNAs, which encode proteins of 624 amino acids (18 amino signal peptides and 606 amino acid mature polypeptides) with 78% and 87% amino acid sequence homology to human FXI. In most species, the two polypeptides of the FXI homodimer are covalently linked by a disulfide bond involving Cys321 in the A4 domain.[55,56] The single known exception is rabbit FXI, which migrates as a monomer during denaturing gel electrophoresis due to histidine replacing Cys321.[54] Western blot analysis using a monoclonal antibody that recognizes a conserved epitope on the apple 2 (A2) domain confirms that FXI from species

other than the rabbit can form disulfide bond-linked dimers.[57] Despite lacking the Cys321-Cys321 bond, rabbit FXI exists in plasma as a noncovalently associated homodimer that is functionally identical to human FXI.[54]

There is little detailed information regarding intracellular processing and posttranslational modification of FXI. The protein undergoes N-linked glycosylation at Asn72, Asn108, Asn432, and Asn473, but not at the potential glycosylation site at Asn335.[17] Dimer formation is an intracellular event that was initially thought to be required for normal secretion of the protein, based on observations that naturally occurring mutations in the fourth apple domain that interfere with dimerization are associated with poor secretion.[58,59] However, it is now apparent that some forms of monomeric FXI with mutations that disrupt the dimer interface are synthesized and secreted similarly to wild-type protein in culture.[56] Failure to secrete some FXI variants that have defects in dimer formation, therefore, are likely related to structural perturbations distinct from the defect in dimerization.

PROTEIN STRUCTURE

Human FXI (M_r ~ 143,000) is a disulfide-linked homodimer containing approximately 5% carbohydrate (i.e., 0.6% hexose, 2.7% *N*-acetylhexosamine, and 1.7% *N*-acetylneuraminic acid), and is present in human plasma at a concentration of 4 to 6 μg/mL (~30 nM) in the form of a zymogen that requires proteolytic activation to develop serine protease activity.[5,8,60–62] The zymogen form of the protein is unique among coagulation factors because it is a disulfide-linked homodimer consisting of two identical polypeptide chains. Each FXI polypeptide contains 607 amino acids, and each can be proteolytically activated by FXIIa, thrombin, or FXIa[5,8,9,10,11] at an internal R369–I370 bond to yield a heavy chain (i.e., 369 amino acids) and a light chain (i.e., 238 amino acids) that contain a typical trypsin-like catalytic triad, consisting of H413, D464, and S557, with a typical trypsin-like substrate-binding pocket.[17,18] After cleavage at R369–I360 bond, the catalytic domain contains an amino-terminal sequence of I-V-G-G characteristic of most serine proteases. The M_r of the homodimer is 135,979 without carbohydrate, and 143,000 with four carbohydrate chains at N72 and N108 within the heavy chain and at N432 and N473 within the catalytic domain, whereas no carbohydrate is present on N335, another potential *N*-glycosylation site.[17]

The primary sequence and domain structure of FXI, including its disulfide linkages, are shown in FIGURE 15A.1. The amino-terminal 369 residues of each FXI monomer after propeptide cleavage comprises the heavy-chain region of FXIa, which consists of four tandem repeat sequences (Apple domains) of 90 to 91 amino acids, each containing six or seven cysteine residues that form three internal disulfide bonds. The amino acid sequences of each apple domain are 23% to 34% identical to the other apple domains of FXI and 58% identical to the corresponding apple domains of plasma PK. In both plasma FXI[17,18] and plasma PK, a characteristic disulfide-bonding pattern links the first and sixth, second and fifth, and third and fourth cysteines in each of the four apple domains.[18] An additional cysteine is present at position 11 in the A1 domain of FXI and forms a disulfide bond with another cysteine residue, whereas C321 in the A4 domain is disulfide-linked to the corresponding C321 to form the homodimer.[18]

Crystal structures have recently been reported for full-length FXI[63] (see FIGURE 15A.2), and for the catalytic domain of FXIa in complex with both synthetic and natural inhibitors including

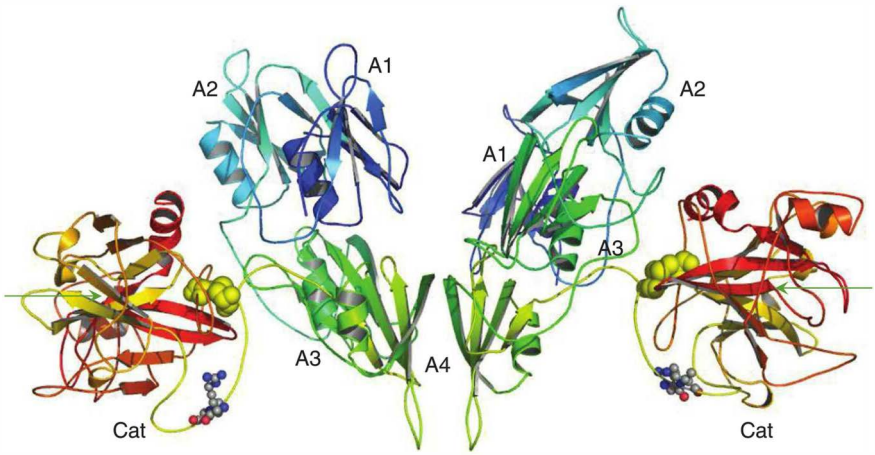

FIGURE 15A.2 X-ray crystal structure of human factor XI zymogen. The FXI dimer is shown with the two monomeric subunits interacting with noncovalent interactions mediated by residues within the two Apple 4 (A4) domains and stabilized by a disulfide bond (not shown) between the two Cys321 residues in each of the two A4 domains. The four apple domains constituting the heavy chain region of each monomer are designated A1, A2, A3, and A4 and the catalytic domains (light chains of FXIa) are designated Cat. The disulfide bonds between Cys362 in the A4 domain and Cys482 in the catalytic domain are designated by the *yellow space-filling ball structures*. The site (Arg369-Ile370) cleaved by FXIIa, thrombin, or FXIa is designated by the small space-filling balls in the connecting loops between the A4 domains and the catalytic domains. The *fine arrows* point at the active site Ser557 residues within each of the catalytic domains. (Figure modified from Papagrigoriou E, McEwan P, Walsh PN, et al. Crystal structure of the factor XI zymogen reveals a pathway for transactivation. *Nat Struct Mol Biol* 2006;13:557–558.)

the Kunitz protease inhibitor (KPI) domain of protease nexin-2 (PN2)[64,65,66]. The structural features of FXI and their implications for molecular function have recently been discussed in detail[67]. In addition, the solution structure of the FXI A4 domain has been reported[68] that predicts a major conformational change in the structure of FXI when it is activated to FXIa.

PLATELET FACTOR XI

There are differing opinions regarding the possibility that human platelets contain FXI activity and that the activity is associated with a form of FXI encoded by an alternatively spliced mRNA from the FXI gene. FXI coagulant activity and antigen have been described in washed platelet suspensions, and it has been estimated that the activity constitutes up to 0.5% of the FXI activity in normal plasma.[44–47,69] Polyclonal, but not monoclonal, antibodies to human plasma FXI cross-react with a platelet protein with an apparent M_r of 220,000 on nonreducing SDS-PAGE which decreases to 55,000 after reduction. For comparison, plasma FXI has an M_r of 160,000, and each subunit has an M_r of 80,000.[44–46] Thus, it has been suggested that platelet FXI could represent a disulfide-linked tetramer of identical 55,000-M_r subunits. Alternatively, the 55,000-M_r protein could be disulfide linked to a platelet membrane protein.[37,45,70] A candidate for this putative platelet FXI-binding protein is glycoprotein Ib (GP Ib) (M_r ~ 170,000). This possibility is supported by studies demonstrating that platelets from patients with Bernard-Soulier syndrome who lack GP Ib contained no detectable FXI activity in washed platelet suspensions.[71] It is important to point out, however, that the 55,000-M_r species has not been shown to have FXI activity (i.e. the capacity to activate factor IX) in functional assays.

If the 55,000-M_r platelet protein is encoded by an mRNA transcribed from the FXI gene, the mRNA would need to be spliced differently than FXI mRNA in hepatocytes. FXI mRNA is present in platelet RNA; however, there are conflicting reports on its nucleotide sequence.[37–39,72] The FXI mRNA in hepatocytes is 2.1 kb in size. A 1.9 kb mRNA transcript was isolated from a megakaryocytic leukemia cell line (MEG-01) that had an identical open reading frame to the hepatocyte species, with the exception that it lacked nucleotides corresponding to exon V.[37] This would result in deletion of amino acids A91 through R144 within the A2 domain. By contrast, two other groups have reported that the FXI mRNA in platelets and megakaryocyte cell lines (including MEG-01) is identical in sequence to FXI mRNA in liver.[38,39] Furthermore, attempts to express a FXI cDNA lacking exon V in a fibroblast line resulted in a poorly expressed protein of 73,000-M_r that was not secreted.[73] Thus, the data linking the platelet protein detected with the polyclonal anti-FXI antibody or the FXI mRNA species detected in platelet mRNA to FXI activity are not compelling, and it is not currently possible to conclude that the FXI activity associated with platelets is distinct from plasma FXI of hepatocyte origin.

STRUCTURE–FUNCTION RELATIONSHIPS

Factor XI Activation

Plasma FXI circulates as a zymogen in human plasma as a complex with high molecular weight kininogen (HK),[7] which is required for binding of FXI to negatively charged surfaces[74] (see Chapter 15B). FXI can be activated by four biologically relevant proteases: FXIIa, FXIa, thrombin, and meizothrombin.[5,10,11,75,76] It can participate in the contact phase of blood coagulation in a reaction that requires the presence of anionic surfaces for optimal surface-mediated *in vitro* activation by FXIIa.[6,7,74,77,78] However, because deficiencies of FXII, PK, and HK are not associated with hemostatic abnormalities but deficiency of FXI produces abnormal bleeding complications in at least 50% of affected individuals,[2,3,79–81] it has been suggested that the more relevant *in vivo* pathway for activation of plasma FXI might be by the feedback activation by thrombin or, possibly, by autoactivation by FXIa.[10,11,82] It has been shown that β_2-glycoprotein I (β_2-GPI) binds FXI and inhibits its activation by thrombin and FXIIa, whereas cleaved β_2-GPI binds FXI but fails to inhibit FXI activation.[83] All four proteases (i.e., thrombin, FXIIa, FXIa, and meizothrombin) cleave each monomer of FXI at the R369–I370 bond, generating the new amino-terminal sequence of the catalytic domain, I-V-G-G, which then activates the catalytic triad of the serine protease. It has been postulated that on anionic surfaces, such as in extracorporeal circulation, a ternary complex is formed when FXI, in stoichiometric complex with HK in plasma, is adsorbed to the negatively charged surface, where FXIIa recognizes it as a substrate.[6,7,74,77,78,84–87] The FXI homodimer is thereby cleaved to generate two disulfide-linked heavy chains (M_r ~ 50,000) and two active-site–containing light chains (M_r ~ 30,000).[5,8] Recent observations demonstrate that FXI activation by thrombin or FXIIa proceeds through the generation of an intermediate consisting of one cleaved and one uncleaved subunit, referred to as ½-FXIa, which can be detected in plasma undergoing contact activation.[88]

An important role for the dimeric structure of FXI in zymogen activation has been demonstrated. Thus, the A4 domain of FXI mediates dimer formation between the two identical polypeptide chains comprising FXI through disulfide bond formation at C321,[17,18,55,56,58,89–91] and the two identical subunits of FXI utilize a substantial interface (886 Å^2) between two adjacent A4 domains to form the dimeric structure of the mature protein.[63,68] Utilizing FXI with substitutions for Cys321, which still form stable dimers, it has been demonstrated that hydrophobic interactions involving Leu284, Ile290, and Tyr329, and salt bridges between Lys331 of one subunit and Glu287 of the other[56] are required for dimer formation, and that FXI dimer formation is required for efficient FXI activation by thrombin, FXIIa, and FXIa.[55,56,63,68,89–92]

Functional Domains of Factor XI

Prior to the availability of FXI structures from x-ray crystallography and NMR and based on the use of molecular models of the four FXI apple domains,[93–97] monoclonal antibodies, conformationally constrained synthetic peptides, and recombinant proteins[98] with site-directed mutations, the binding sites within FXI for a number of physiologically relevant plasma or cellular ligands have been identified and characterized.

Thrombin,[99,100] HK,[73,93,95,101] and the kringle 2 domain of prothrombin[103] have all been shown to bind to contiguous subdomains within the carboxy-terminal half of the A1 domain (E1–S90). Thus, HK binds to a subdomain within the A1 domain comprising residues F56–S86, whereas thrombin binds to a partially overlapping and contiguous site comprising subdomain A45–R70. Different amino acid side chains appear to be essential for these interactions because E51 and E66 are important for thrombin interactions with FXI but not for HK binding, whereas V64 and I77 are important for HK binding but not

for thrombin binding[101]. The kringle 2 domain of prothrombin can bind to a subdomain comprising residues A45–S86, but fine mapping of this site has not yet been accomplished.[102] Occupancy of these sites by their respective ligands is likely to be important in promoting the binding of FXI the GP Ibα subunit of the GP Ib/IX/V complex on the platelet surface[100] either in the presence of HK and Zn^{2+} ions or prothrombin and Ca^{2+} ions.

The A3 domain of FXI has been identified as the locus of binding sites for both platelets[97,103–108] and heparin.[109,110] Thus, activated platelets expose specific reversible, high-affinity binding sites for FXI that require the presence of HK and $ZnCl_2$,[111] or prothrombin and $CaCl_2$.[102] Binding of FXI to activated platelets through the A3 domain[97,111] is required for optimal rates of FXI activation by FXIIa.[112] The sequence of residues within the A3 domain of FXI (i.e., N235–R266) that comprises a contact surface for interaction with a platelet receptor consisting of the GP Ibα subunit of the GP Ib/IX/V complex[107,108] contains amino acids that mediate this binding, including N248, R250, K255, F260, and Q263.[104,106] The A3 domain of FXI also contains all the binding energy required for interaction of FXI with heparin in a heparin-binding consensus sequence (i.e., T249–F260) that contains amino acids K252 and K253, and possibly K255, which at least in part mediate the binding of FXI to heparin.[109,110]

A major role of the A4 domain of FXI is to mediate dimer formation, the functional significance of which is discussed in detail above in the section on FXI activation. Furthermore, a natural mutation in FXI (F283L), resulting in diminished intracellular dimerization and secretion of the protein,[58] also markedly affects dimer dissociation and the unfolding mechanism of FXI, indicating that F283 is important for noncovalent dimer formation.[91] In addition, a sequence of residues (i.e., A317–G350) within the A4 domain of FXI appears to contain three peptide structures, possibly consisting of three antiparallel β-strands that together comprise a contact surface for interaction with FXIIa. This site may participate in the activation of FXI by FXIIa.[96,103]

CELLULAR INTERACTIONS

Early observations suggesting that platelets participate in the contact phase of blood coagulation[113–117] were subsequently confirmed by the evidence that isolated human platelets activated with either adenosine diphosphate or collagen could promote the proteolytic activation of FXII by kallikrein and, subsequently, the activation of FXI by FXIIa in the presence of HK.[47,112,118,119] The mechanism underlying the role of activated platelets in the activation of FXI involves the generation of high-affinity (dissociation constant, K_Φ ~10 nM), specific, saturable binding sites (~1,500 sites per platelet) on the surface of activated platelets that require the presence of HK and Zn^{2+} ions.[111] The observation that HK also binds to platelets under similar conditions originally suggested that the two proteins bind to the platelet surface as a complex.[120,121] However, it has subsequently been shown that HK interacts with FXI through a surface-exposed site encompassing residues F56–S86 within the A1 domain[93,95,122], resulting in the exposure of a platelet binding site encompassing residues N235–R266 within the A3 domain of FXI.[97] It has also been demonstrated that prothrombin can substitute for HK as a cofactor for the binding of FXI to the surface of activated platelets.[102] In this way, prothrombin appears to bind to a site in the A1 domain of factor XI contiguous to the site used by HK for binding to the A1 domain.[102] The platelet receptor that binds factor XI and colocalizes it with thrombin for efficient activation has recently been shown to consist of the GP Ibα subunit of the GP Ib/IX/V complex that is localized within platelet membrane microdomains referred to as *lipid rafts*.[107,108] This interaction of FXI with surface membranes of activated platelets has been shown to be mediated by binding of residues (i.e., R250, K252, K253, K255, F260, and Q263) within the A3 domain of FXI[104] to the leucine-rich repeat motifs of GP Ibα.[123] The binding of FXI to activated platelets and activation by thrombin are mediated by the interaction of FXI and platelet GP Ibα with thrombin anion-binding exosites I and II, respectively.[100,124,125]

Endothelial cells can expose specific, saturable, high-affinity binding sites for both FXI and HK, as well as other coagulation proteins, which raises the possibility that endothelium as well as platelets can participate in the activation of FXI by FXIIa.[126–132] However, it has recently been shown that what was interpreted as a specific interaction of FXI with the endothelial cell surface is an artifact of binding to microtiter plates, suggesting that endothelial cells do not participate in the initiation of the consolidation phase of blood coagulation.[133,134] Moreover, the interaction of FXIa with activated platelets, but not with endothelial cells, promotes the activation of FIX.[133,134] Therefore, the endothelial cell surface is generally regarded as anticoagulant and nonthrombogenic (see Chapter 42), and it has been suggested that the binding of HK to endothelial cells may be more important for the regulation of bradykinin production[135–137] and for the activation of prourokinase[138] and plasminogen[135,138] than for the procoagulant functions promoted by FXI.[133,134,136,137]

FXIa Enzymatic Activity

After activation on artificial surfaces, FXIa remains surface bound and recognizes FIX as its normal macromolecular substrate.[12–14,139] In the presence of calcium ions, FXIa cleaves FIX, a single-chain 57,000-M_r glycoprotein with 17% carbohydrate, at an internal R145–A146 bond, forming an intermediate that is subsequently hydrolyzed at an R180–V181 bond to generate active FIXa and to form a 10,000-M_r activation peptide, 50% of which is composed of carbohydrate.[12–14,139–144] The light chain of FXIa or catalytic domain (i.e., C362–V607) contains a typical trypsin-like catalytic triad comprising residues H413, D462, and S557 (H57, D102, and S195, chymotrypsin numbering) that are activated when FXI is cleaved at R369–I370 bond to expose a new amino-terminal tetrapeptide, I-V-G-G, typical of trypsin-like serine proteases. The active site of FXIa can cleave and activate two major macromolecular substrates, including FIX[12–14] and FXI.[10,11] In addition, both FXIa and plasma kallikrein have been shown to activate prohepatocyte growth factor, raising the possibility that they may regulate processes that involve the HGF/c-Met pathway, such as tissue repair and angiogenesis.[145]

The interaction of FXIa with its preferred macromolecular substrate, FIX, involves Ca^{2+}-dependent binding of FIX to a substrate-binding site within the heavy chain of factor XIa.[14,146,147] The location and characterization of this substrate-binding site have been studied in two laboratories with conflicting results.[94,148,149] One of these studies used molecular modeling and conformationally constrained synthetic peptides to identify a sequence of residues (i.e., A134–L172) within the A2 domain of FXIa that contains three antiparallel β-strands connected by

β-turns that have the characteristics of a substrate-binding site for FIX because they act as competitive inhibitors of FIX activation by FXIa.[94] In contrast, recombinant FXI proteins, in which each of the four apple domains is individually replaced with the corresponding domain from the homologous but functionally distinct protein PK, revealed that when the A3 domain of PK was inserted into FXIa, the Michaelis constant (K_m) for activation of FIX was 35-fold higher than the K_m for activation by wild-type FXIa or by the other FXI/PK chimeras.[148,149] Mapping of the A3 domain of FXIa by alanine-scanning mutagenesis has identified residues in the *N*-terminus (i.e., I183–V191, and possibly S195–I197) and the *C*-terminus (i.e., F260–S265) of the A3 domain that are required for FIX activation.[149,150] The interaction of FXIa with FIX is also dependent on the γ-carboxyglutamic acid (Gla) domain of FIX.[151] Therefore, the putative substrate-binding site for FIX resides either within the A2 or A3 domain (or both) of FXIa. A possible resolution of these two apparently conflicting studies is that intimate interdomain interactions between A2 and A3 may be required for maintaining an intact substrate-binding site that interacts with the Gla domain of FIX. Evidence has also been published to indicate that the Gla domain of FIXa binds to the A3 domain of FXIa,[152] and that loss of the A3 domain has a more deleterious effect on cleavage of FIX at Arg180-Val181 than at Arg145-Ala146, suggesting that the A3 domain exosite facilitates cleavage of FIXα to FIXaβ.[88,153] In addition to the FIX substrate-binding exosite within the heavy chain of FXIa, recent studies demonstrate the presence of an additional exosite for FIX binding, remote from the active site, within the catalytic domain, that is required to mediate the formation of the Michaelis complex and efficient cleavages of the two spatially separated scissile bonds of FIX.[153]

Cellular Interactions and Regulation of Factor XIa

The regulation of FXIa depends on its localization (i.e., whether it appears on the platelet surface, in the proximity of endothelium, or in the plasmatic compartment of the blood). Within the plasma, four separate serine protease inhibitors (serpins) have been demonstrated to inactivate FXIa: antithrombin III, α_1-protease inhibitor, C1 inhibitor, and α_2-antiplasmin.[8,60,154–163] It has also been shown that another serpin, protease nexin-1, which is not present at physiologically significant concentrations in plasma but which is secreted in low concentrations from platelet α-granules, inhibits FXIa in the presence of heparin with potency greater than that of C1 inhibitor.[164] FXIa also has been reported to be inhibited by plasminogen activator inhibitor 1[165] and protein C inhibitor.[166] The inhibition of FXIa within the plasma compartment has been postulated to occur predominantly through the action of α_1-protease inhibitor,[156] although studies on the modulation of contact system proteases by glycosaminoglycans indicate a selective enhancement of the inhibition of FXIa by C1 inhibitor[167] and by protease nexin-1.[164]

Because FXIa can be formed on the platelet surface[47,111,112,119] and it can bind to high-affinity (K_d ~ 800 pM), specific, saturable receptors (130 to 500 sites per platelet) on activated platelets in the presence of HK, the binding being mediated by a catalytic domain exosite within the enzyme and distinct from those for FXI,[133,168,169] it has been suggested that FIX activation by FXIa might also occur on the platelet surface.[170] Although the binding of FXIa to platelets does not appear to enhance rates of FIX activation,[133,170,171] both FXIa[133,168] and FIX[172] can bind to high-affinity, saturable receptors on activated platelets. Within the environment of activated platelets, FXIa is regulated by different mechanisms from those operative in plasma because platelet-bound FXIa is protected from inactivation by α_1-protease inhibitor.[159]

In addition, platelets are known to secrete inhibitors of FXIa[173–177] including PN2, a truncated form of the transmembrane Alzheimer β-amyloid protein precursor that contains a Kunitz-type serine protease inhibitor (KPI) domain. PN2 is a potent inhibitor of FXIa (inhibition constant, K_i 2.9 to 4.5×10^{-10} M) that has considerably enhanced inhibitory activity in the presence of heparin (K_i 2.5 to 4.5×10^{-11} M),[65,175–181] which provides a template for the assembly of FXIa/PN2 complexes.[178] Although the release of PN2 from activated platelets inhibits plasma FXIa activity in the environment of the hemostatic thrombus, platelet-bound dimeric (but not monomeric) FXIa is protected from inactivation by PN2.[133,171,180] In contrast, endothelial cells have no such protective effect, suggesting that whereas FXIa is protected from inactivation by PN2 when bound to the platelet surface, inactivation of FXIa by PN2 might be enhanced on the surface of endothelial cells that contain heparan sulfate glycosaminoglycans. Therefore, it has been suggested that the colocalization of dimeric FXIa and FIX to the activated platelet surface may serve to localize FIX activation and subsequent coagulation reactions to the activated platelet surface that contains specific, high-affinity, saturable binding sites for the enzyme FIXa. In the presence of FVIII, the occupancy of these sites can increase the catalytic efficiency (k_{cat}/K_m) of FX activation by more than 20 millionfold.[172,182–184]

MOLECULAR GENETICS OF FACTOR XI DEFICIENCY

Hereditary FXI deficiency is an autosomal disorder characterized by trauma-related hemorrhage, and only rarely by the "spontaneous" bleeding typical of FVIII or FIX deficiency.[185–187] Clinical manifestations and treatment are discussed elsewhere (see Chapter 53). Bleeding is highly variable, although it tends to be more prevalent with severe reductions in plasma FXI (<0.2 U/mL).[80,185,186,188] However, patients with mild deficiency (0.2 to 0.5 U/mL) can experience posttraumatic bleeding, while those with very severe deficiency may not bleed excessively.[2,3,80,185,187–189] This situation has contributed to difficulties in establishing inheritance patterns. First described by Rosenthal et al.[1] in 1953, FXI deficiency was initially considered a dominant disorder with variable penetrance,[79,190,191] in contrast to deficiencies of other coagulation proteases, which are usually symptomatic only in the homozygous or compound heterozygous states. Rapaport et al.[2] suggested that FXI deficiency exists in major (<1% to 20% of normal plasma activity) and minor (20% to 65% normal activity) forms, more consistent with a recessive mode of inheritance. Recessive inheritance is generally accepted for the more common forms of the disorder caused by the Glu117Stop and Leu283Phe mutations (discussed below).[185,186] Brenner et al.[192] used a logistic regression model to assess bleeding in 45 families with FXI deficiency caused primarily by these mutations, and determined the odds ratios for bleeding were 13.0 and 2.6 for homozygotes and heterozygotes, respectively.

The incidence of severe FXI deficiency is estimated at one per million in the general population[193,194]; however, the disorder is common in Ashkenazi Jews in whom the carrier (heterozygous)

frequency for a FXI gene mutation is approximately 5%, with severe deficiency (<15% of normal level in this population) in 0.1% to 0.3%.[185–187,194,195,196] The Glu117Stop (type II) and Leu283Phe (type III) mutations account for >90% of abnormal FXI alleles in Jews.[185,195,196,197] The type II mutation codes for a premature termination codon in the A2 domain; however, the mRNA for this allele is unstable[198] and homozygotes lack plasma FXI antigen. The type III mutation causes an amino acid substitution in the A4 domain that partially interferes with dimer formation.[58,59] In Ashkenazi Jews, the allele frequencies for the type II and III mutations are 0.0217 and 0.0254, respectively.[196] The type II allele is also found in Iraqi, Sephardic/North African, and other Middle Eastern Jews, and in Arabs, consistent with an ancient origin (>2,500 years ago).[195,196] The type III mutation is found predominantly in Ashkenazi Jews and appears to be of more recent European origin.[195] Other relatively common FXI gene mutations include Cys38Arg, with an allele frequency of approximately 0.5% in French Basques[199]; Cys128Stop, which accounts for approximately 10% of mutant alleles in Great Britain[193,200]; and Gln88Stop, which has been observed in several families in Nantes, France.[201]

Since the description of the type II and III mutations,[197] nearly 200 FXI gene mutations have been identified in factor XI deficient patients (www.med.unc.edu/isth/mutations-databases/Factor_XI.htm; www.factorxi.org), including more than 100 missense mutations.[202,203] Congenital deficiency of a vitamin K–dependent coagulation protease (factors II, VII, IX, or X) is frequently associated with a circulating variant protein (cross-reactive material positive [CRM+] deficiency).[194] In contrast, CRM+ factor XI deficiency is rare,[80,202,204] with only seven missense mutations proven, or suspected, of causing CRM+ deficiency. Gly155Glu is in the A2 domain,[205] and recombinant protein carrying this substitution binds HK with approximately 10-fold lower affinity than wild type protein.[67] Arg184Gly replaces a residue in the A3 domain that is critical for the interaction of FXIa with FIX.[149,206] Ser248Asn, also in the A3 domain, is located within a binding site for the platelet glycoprotein 1b receptor, and interferes with FXI binding to platelets.[207,208] Val371Ile is located in proximity to the FXI activation cleavage site.[209] The substitution slows protease activation, and likely interferes with formation of a salt bridge required for catalytic domain activation.[67] Pro520Leu,[210] Gly555Glu,[211,212] and Thr575Met[213] involve substitutions in the catalytic domain that compromise substrate catalysis. A possible explanation for the rarity of CRM+ variants lies in the compact FXI structure.[63,67,205] The extensive interfaces between individual apple domains, between the disk formed by the apple domains and the protease domain, and between subunits in the dimer may make the protein sensitive to structural perturbations from amino acid substitutions.

While the preponderance of evidence indicates that the type II and type III mutations are associated with a syndrome that is inherited in a recessive manner, the dimeric structure of FXI could allow the product of a single mutant allele to exert a dominant negative effect on the product of a normal allele through formation of heterodimers. Gene mutations that result in CRM-FXI deficiency appear to fall into three mechanistic categories[214]: (a) mutations that interfere with or prevent synthesis of a polypeptide, including premature stop codons such as Glu117Stop,[185,201,215–221] frameshifts,[199,219,222] deletions,[223] intronic splicing defects,[199,222,224–226] and substitutions that cause protein instability or severe structural perturbations[67,205]; (b) mutations that interfere with protein dimer formation (i.e., Phe283Leu and Gly350Glu),[59] with associated retention of mutant FXI monomer in the cell; and (c) mutations that result in a poorly secreted polypeptide that can form homodimers or heterodimers with normal FXI polypeptide (Ser225Phe, Cys398Tyr, Gly400Val, and Trp569Ser).[59,214] When fibroblasts are cotransfected with expression constructs for one of these latter mutations and wild type FXI, secretion of wild type FXI is reduced, consistent with the mutant exerting a dominant negative effect on the wild type polypeptide.[59,214] This mechanism could account for families in which FXI deficiency is inherited as a dominant condition.[59,190,227] A dominant negative effect on wild type FXI release is not seen with cotransfection with constructs for the type II or type III mutations, consistent with the apparent recessive mode of inheritance for these mutations.[59]

PHYSIOLOGY AND PATHOPHYSIOLOGY OF FXI

A great deal of information has been acquired on the physiologic role of FXI through the study of patients with FXI deficiency and through the definition of the associated genetic defects. However, in many patients, the reported lack of correlation between the plasma level of FXI and the severity of clinical bleeding manifestations[2,3,70,80,185,188,189] emphasizes the incomplete nature of the understanding of how FXI participates in hemostasis. A study utilizing flow cytometry has shown that a small number of selected patients with severe plasma FXI deficiency and minimal or absent hemostatic defects have a platelet protein that is recognized by anti-FXI antibodies.[70] This raises the possibility that a platelet form of FXI can participate in hemostasis and may even substitute for plasma FXI.[44,45,70,71,80] However, additional work is required to determine the biochemical relation between plasma and platelet FXI, including the mechanisms and enzymes that activate these two proteins and the substrate specificity of each of the activated proteases.

An important tool to facilitate investigations of the physiologic roles of plasma FXI (and possibly of platelet FXI) is the development of a murine model of severe plasma FXI deficiency using the technique of homologous recombination in embryonic stem cells.[228] In this model, the murine FXI gene[35] was disrupted by the introduction of a neomycin phosphotransferase gene into exon V, resulting in the absence of FXI activity in plasma and of FXI mRNA from liver. No evidence of hemostatic deficiency was detected in homozygous null mice, and the genotypes of progeny from mating of mice heterozygous for the FXI null allele showed the expected Mendelian ratio of wild-type, heterozygous, and homozygous null animals, suggesting that increased intrauterine mortality did not result from severe FXI deficiency. It is not known whether normal murine platelets contain platelet FXI, but if they do, it is possible that disruption of exon V with a neomycin phosphotransferase gene would not prevent the expression of platelet FXI that lacks exon V. Future studies with murine FXI knockout models may provide important insights into the relative contributions of plasma FXI and platelet FXI to physiologic and pathologic processes.

The details of the clinical manifestations and management of FXI deficiency are presented in Chapter 53. However, insights into normal physiologic function can be gleaned from certain clinical facts. For example, responses to the infusion of plasma in patients with FXI deficiency provide evidence that the recovery of FXI is >90% after infusion and that the half-life of the plasma protein is 52 ± 22 (mean ± SD) hours.[229,230] Another clinical fact worthy of note is that patients with severe plasma FXI deficiency are especially at risk for bleeding from tissues with high local fibrinolytic activity, including the urinary tract, the nose, the oral cavity, and the tonsils.[185,231] Moreover, FXI can be activated by thrombin[10,11] and appears to play a role in the protection of fibrin clots against fibrinolysis.[82] These facts suggest that FXI, in addition to its procoagulant activity, promotes an antifibrinolytic activity. This FXI-dependent downregulation of the activation of fibrinolysis[82] has been postulated to occur as a consequence of a FXI-dependent burst in thrombin generation, resulting in the activation of thrombin-activatable fibrinolysis inhibitor (TAFI) or procarboxypeptidase B,[232–235] which inhibits the activation of plasminogen by removing carboxy-terminal lysines from fibrin that are essential for plasminogen binding and activation.[234–236]

In addition to its demonstrable role in hemostasis, published evidence has focused attention on the possible contributions of high levels of FXI as a risk factor for venous thrombosis[49] and cardiovascular disease in women,[237] although inherited FXI deficiency apparently confers no protection against acute myocardial infarction.[238] However, there appears to be a reduced incidence of ischemic stroke in patients with severe FXI deficiency.[239] One study followed 600 patients with a first episode of venous thromboembolism and found an increased risk of recurrence, especially among those with increased levels of TAFI and FXI,[240] whereas another study showed that increased levels of TAFI, but not FXI, increased the risk of venous thromboembolism in patients with factor V_{Leiden} carrier status.[241] These studies are consistent with the results of an investigation in rabbits demonstrating increased rabbit jugular vein thrombolysis by antibody neutralization of FXI because of diminished indirect activation of TAFI.[242] Two studies in mice with targeted FXI gene deletion, either alone[243] or in combination with targeted deficiency of protein C,[244] showed protection from $FeCl_3$-induced carotid thrombosis[243] or from early lethality caused by thrombosis in protein-C–deficient animals.[244] Another study reported the important role of FXI in the growth of arterial thrombi using a rabbit model of arterial thrombosis,[245] whereas another demonstrated the dependence of surface- and tissue factor–initiated thrombus propagation on FXI in primates.[244] Therefore, FXI levels may comprise a significant risk factor for thrombosis, and the FXI molecule may constitute a reasonable target for the development of antithrombotic drugs.[246]

The physiologic pathways by which FXI is activated and the mechanisms by which it participates in normal blood coagulation and hemostasis are important aspects of a revised theory of blood coagulation that focuses on the assembly of coagulation protein complexes on the platelet surface. The original theories of blood coagulation[247,248] envisioned a series of enzymatic reactions initiated by activation of intrinsic blood coagulation by the assembly of the contact proteins on a foreign surface. However, the absence of bleeding complications in patients with deficiencies of FXII, PK, and HK, and the presence of hemostatic defects in patients with FXI deficiency, has led to a search for alternative pathways of FXI activation. Although the demonstration[249,250] that the TF–FVIIa complex can directly activate not only FX but also FIX has been used to suggest an explanation for the absence of bleeding manifestations in patients with contact phase deficiencies[251]; this observation does not account for the presence of abnormal bleeding in some patients with FXI deficiency. Nonetheless, evidence exists that the initiation of blood coagulation *in vivo* is a consequence of the formation of the catalytic complex that occurs when FVII or FVIIa is assembled on the transmembrane protein TF found in many cell types.[252] Furthermore, the discovery of the tissue factor pathway inhibitor (TFPI) and the elucidation of its mechanism of action provides some of the information necessary to understand why both FXI and FVII appear to be necessary for normal hemostasis.[253–259] The complex of TF and FVIIa is almost immediately inhibited when a complex is formed between FXa and TFPI.[260,261] Therefore, the generation of FXa results in the immediate inactivation of the enzyme complex that initially forms it (i.e., TF–FVIIa). Furthermore, it has been observed that the formation of thrombin through the extrinsic pathway is transient and that the continuous generation of thrombin by the intrinsic pathway is required to assure normal hemostasis.[261] All of these observations suggest that FXI is required for normal functioning of the intrinsic pathway and that an alternative mechanism for the activation of FXI exists independent of the contact proteins.

The mechanism of FXI activation and the expression of FXIa enzymatic activity on the platelet surface are discussed in detail in Chapter 35 (The Role of Platelets in Blood Coagulation). Both Naito and Fujikawa[10] and Gailani and Broze[11] have independently demonstrated that human FXI can be activated either by thrombin, by FXIIa, or by FXIa (i.e., autoactivation of FXI). Although the kinetics of FXI activation by thrombin in solution are quite unfavorable, the presence of dextran sulfate as a negatively charged surface enhances the rate of FXI activation by thrombin by approximately 2,000-fold.[10,11,262] However, the presence of either HK[10,11,263] or fibrinogen[263] has major inhibitory effects on the autoactivation and the thrombin-mediated activation of FXI in the presence of dextran sulfate. The physiologic surface upon which FXI activation by thrombin occurs remains elusive.

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CHAPTER

15B The Factor XII–Driven Plasma Contact System

THOMAS RENNÉ

SUMMARY

The contact system is a protease cascade that is initiated by coagulation factor XII activation and operates on cardiovascular cells. The plasma proteins trigger procoagulant and proinflammatory reactions, *via* the intrinsic pathway of coagulation and the kallikrein–kinin system, respectively. The biochemistry of the contact system is well understood; however, its *in vivo* functions are just beginning to emerge. Recent data in animal models have shown that deficiency in contact system proteins largely abolishes thrombus formation but does not interfere with hemostatic mechanisms. Here we present an overview about the biochemistry and cell biology of the plasma contact system and its contribution to thromboembolic and inflammatory diseases.

PROTEINS OF THE PLASMA CONTACT SYSTEM

In the original waterfall cascade model, fibrin formation may be initiated by two distinct pathways, triggered by exposure of blood to a damaged vessel wall (extrinsic) or to blood-borne (intrinsic) factors.[1,2] The intrinsic pathway of coagulation is initiated by factor XII (FXII, Hageman factor), in a reaction involving high molecular weight kininogen (HK) and plasma kallikrein (PK), collectively referred to as plasma contact system.[3,4–7,8,9] Contact with negatively charged surfaces induces a conformational change in zymogen FXII resulting in a small amount of active FXII (FXIIa).[10] FXIIa cleaves PK to generate active kallikrein, which in turn reciprocally activates FXII zymogen.[11] FXIIa initiates fibrin formation through its principal substrate of the intrinsic coagulation pathway, factor XI (FXI), and also triggers liberation of the inflammatory mediator bradykinin (BK) by PK-mediated HK cleavage.[5] Binding of BK to the kinin B2 receptor (B2R) activates various proinflammatory signaling pathways that dilate vessels, induce chemotaxis of neutrophils, and increase vascular permeability and fluid efflux[12] (FIGURE 15B.1). The serpin C1 esterase inhibitor (C1INH) is the major plasma inhibitor of FXII and PK and controls proteolytic activity of the system.[13]

Factor XII

Factor XII (FXII, Hageman factor, EC = 3.4.21.38) is the 596–amino acid single-chain zymogen[14] of the plasma serine protease α-factor XIIa.[15] The single-chain glycoprotein migrates with an apparent molecular weight of 80 kDa in SDS-polyacrylamide gel electrophoresis (PAGE). FXII has a plasma concentration of 375 nM (30 μg/mL; normal range 15 to 45 μg/mL) and circulates in plasma with halftime of 50 to 70 hours.[16] The human FXII gene spans approximately 12 kb on chromosome 5 (q33-qter) and has 14 exons.[17,18] The protein is primarily expressed by hepatocytes; however, low levels of extrahepatic FXII mRNA synthesis have been detected in other tissues such as the placenta.[19] Hepatocyte-specific expression of FXII in mice depends on the transcription factor HNF4α.[20] Whether HNFa accounts for liver-specific expression of the coagulation factor in humans is not entirely clear; however, HNF4α antagonizes estrogen-induced upregulation of FXII gene expression in humans.[21] Estrogens increase expression of FXII, and elevated FXII plasma levels are found in woman on hormonal contraception and during pregnancy. Indeed, the FXII gene promotor harbors a consensus motive (5′-G^{-43}GGCANNNTGACC^{-31}-3′) for an estrogen-responsive element.[22]

Protein Biochemistry of Factor XII

FXII is a multidomain plasma protein. The clotting factor is composed of a *N*-terminal fibronectin type II domain (encoded by exons 3 and 4), an epidermal growth factor (EGF) domain (exon 5), a fibronectin type 1 domain (exon 6), a second EGF domain (exon 7), a kringle domain (exons 8 and 9), a 55–amino acid long "proline-rich" hinge region (exon 9), and a trypsin-like protease domain (exons 10 to 14).[18] The signal peptide that mediated secretion of the protein is coded by exon 2, whereas the first exon is noncoding. FXII binds to negatively charged surfaces *via* its heavy chain (*N*-terminal 353 residues) with implications for contact-mediated autoactivation of the zymogen (see below).

The FXII surface-binding site is not precisely known, and several regions of the heavy chain appear to contribute to a discontinuous binding pocket. Studies using recombinant FXII deletion variants or antibodies that interfere with surface-mediated FXII activation identified nonoverlapping portions in the proximal *N*-terminus (residues 1 to 28), the fibronectin type II domain (residues 39 to 47), the fibronectin type 1 domain (residues 134 to 153), the second EGF domain, the kringle domain, and the proline-rich region as potential FXII surface-binding sites.[23–26] The monoclonal anti-FXII antibody (F1) binds to the kringle domain and enhances proteolytic activation.[27] Within the first fibronectin type II domain, two histidine-containing Zn^{2+} ion-binding sites have been identified (residues 40 to 44 and 78 to 82[28]). Binding to zinc ions stabilized intermediate structures of the activation process and largely accelerates contact-induced formation of the active serine protease.[29] FXII is activated by cleavage of a single peptide bond (Arg353-Val354). Limited proteolysis is either a result of autoactivation[30] or mediated by another protease such as α-kallikrein, factor XIa,

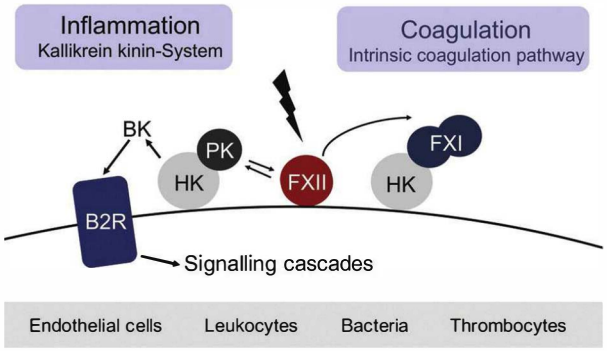


FIGURE 15B.1 The factor XII (FXII)-driven contact system. Contact with negatively charged surfaces activates coagulation factor XII (FXII) on leukocytes, bacteria, endothelial cells, and thrombocytes and initiates procoagulant and proinflammatory protease cascades. FXII triggers fibrin formation through the factor XI (FXI)-driven intrinsic pathway of blood coagulation. Activated FXII initiates the kallikrein–kinin system that generates the proinflammatory peptide hormone BK by PK-mediated cleavage of HK.

factor XIIa, plasmin, or trypsin.[13] Among these FXII processing enzymes, α-kallikrein is by far the most potent activator.[11] The product of activation, α-FXIIa, consists of a 52 kDa *N*-terminal heavy chain and 28 kDa *C*-terminal light chain (protease domain), connected by a disulfide bond between residues Cys340 and Cys367.[31] Both binding to negatively charged surfaces and binding to zinc ions largely accelerates FXII activation. Plasma prokallikrein (PPK) and factor XI are the principal substrates of α-FXIIa during contact activation.[32,33] FXIIa can activate plasminogen and factor VII; however, the *in vivo* relevance of these steps remains to be established. α-FXIIa gets further cleavages at Arg334 and Arg343 resulting in β-FXIIa. The latter form lacks most of the heavy chain, binds poorly to surfaces, and does not support surface-mediated activation of PPK or factor XI.[34,35] β-FXIIa can activate PPK in the fluid phase and also activates complement factor C1 in plasma.[36] FXII and FXIIa stimulate the proliferation of cultured human hepatoma cells (HepG2) through a mitogenic activity that may reside in the EGF-like domains.[37] Consistently, FXII stimulates ERK1/2 and Akt through uPAR, integrins, and the EGFR to initiate angiogenesis.[38] The major plasma inhibitor of both α- and β-FXIIa is C1INH, with antithrombin III (ATIII) and plasminogen activator inhibitor-1 (PAI-1) having FXIIa-blocking activity.[39]

Plasmakallikrein

PPK is the zymogen of the trypsin-like serine protease α-PK.[40,41] PPK circulates in plasma at a concentration of approximately 580 nM (50 μg/mL), 75% to 90% in a noncovalent 1:1 complex with HK.[42] PPK is primarily expressed by hepatocytes; however, PPK mRNA is found in numerous tissues, and small amounts of PPK are secreted from non-liver cells including pancreatic islets and kidney tubules.[43] PK is a paralog of factor XI and both genes have evolved from a common ancestor.[44] The human PK gene covers approximately 30 kb on chromosome 4q34–35,[45] and is approximately 8 kb distant from the factor XI gene locus, consistent with a gene duplication event as underlying mechanisms for the two distinct proteins.[46] Exon 1 of the PK gene is not translated, exon 2 encodes the signal peptide, exons 3 to 10 the four apple domains, and exons 11 to 15 the protease domain.

Plasma Kallikrein Functions

The principal mechanism of PK is to cleave the high molecular weight bradykinin precursor (HK) that liberates BK.[47,48] Despite similar names, PK is not related to the large group of kininogenases called tissue-type (urinary or glandular) kallikreins. Mature human PK is a 619–amino acid single-chain polypeptide. Similarly to factor XI, PK is composed of four 90– to 91–amino acid apple domains (Apple 1 to 4) that form the heavy chain of the molecule and a *C*-terminal protease domain.[49] The high degree of homology between PK and factor XI is supported by the findings that apple domains of PK and factor XI can be exchanged without compromising the capacity of the protein to be synthesized and secreted.[50] In contrast to factor XI, PK is a monomer. PK migrates as a doublet with 86 and 88 kDa on nonreducing SDS-polyacrylamide gels. The two bands represent differentially glycosylated proteins. The residue Cys321 that forms the interchain disulfide bond in factor XI (connecting the two polypeptides of the dimeric protein) forms an intrachain bond with Cys326 in Apple 4 domain. PPK is a substrate of α-FXIIa, β-FXIIa, and trypsin.[51] PPK is converted by these proteases to active α-kallikrein by proteolytic cleavage of the Arg371–Ile372 bond.[52] Limited proteolysis of the single peptide bond results in heavy and light chain fragments (50 and 35 kDa) that remain connected by a disulfide bond connecting Cys364 in the Apple 4 domain and Cys484 within the protease domain.[41] Similarly to FXII, a surface-dependent PPK autoactivation has been shown, but the physiologic importance of that mechanism is disputed. Following long-term incubation in buffer, α-kallikrein undergoes further conversion to β-kallikrein. Cleavage of the Lys140–Ala141 bond within Apple 2 domain abolished β-kallikrein binding to HK.[50,53] So far the β-kallikrein form has not been identified *in vivo*. PPK may also be activated independent of FXII activity involving the prolylcarboxypeptidase (PRCP).[54] PRCP has been originally recognized as a lysosomal exopeptidase exopeptidase. The physiologic relevance of PRCP as potential PPK activator *in vivo* and the molecular mechanisms by an exopeptidase that could function as an endopeptidase mediating limited proteolysis of PPK are subject to ongoing research. Gene targeting of PRCP accelerates thrombosis in mice.[55] In contrast, targeting PPK expression interferers with thrombosis,[56] arguing against PRCP-mediated PPK activation in thrombotic diseases. Additionally, extracellular carbonic anhydrase may activate PPK with implications for control of retinal permeability.[57] PK functions as a kininogenase and cleaves HK resulting in release of BK. In a positive feedback loop, α-kallikrein also activates surface-bound FXII to α- and β-XIIa.[58,59] Furthermore, PK has the capacity to convert prourokinase to urokinase[60] and plasminogen to plasmin[61] and for triggering the renin–angiotensin system by conversion of prorenin to renin.[62] Due to its BK forming capacity, α-kallikrein has potent *in vitro* effects on leukocyte migration, stimulates monocytes, and induces neutrophils aggregation,[63] chemotaxis, and elastase release.[64] α-Kallikrein bound to mast cell surfaces has been identified as an activator of plasminogen during epithelial cell apoptosis, adipocyte differentiation, and stromal remodeling during mammary gland involution. Recent studies have suggested a role of PK for control of intracerebral hemorrhage in hyperglycemia.[65] In contrast, hereditary deficiency in PK (Fletcher trait) and its substrate HK

(Fitzgerald trait) is not associated with increased bleeding risk or hemostatic abnormalities.[66] The enzymatic activity of PK is primarily regulated by C1INH and α_2-macroglobulin, with some contribution of protein C inhibitor, PAI-1, and ATIII.[67,68]

High Molecular Weight Kininogen

HK is a 120-kDa β-globulin glycoprotein that circulates in plasma at a concentration of 670 nM (80 μg/mL) and is a member of the cystatin family of cysteine protease inhibitors. The human kininogen gene that is 27 kb in size contains 11 exons, and is located on chromosome 3 (3q-26qter). It is part of a cluster of related genes including α_2-HS glycoprotein (α_2-HS; also termed fetuin-A) and histidine-rich glycoprotein (HRG).[8,69,70] Through alternative mRNA splicing, the kininogen gene produces transcripts for HK (3.5 kb) and its splice variant low molecular weight kininogen (LK, 1.7 kb), a 68 kDa glycoprotein with a plasma concentration of 1.3 μM (90 μg/mL).[71] Alternative splicing of the primary transcript is controlled by the direct interaction of U1 small nuclear ribonucleoprotein ("snurp") and the complementary sequences of the kininogen hnRNA. Exons 1 to 9 and a portion of exon 10 that codes for BK and its *C*-terminal 12 amino acids are common to the mRNAs for HK and LK, and the proteins share identical heavy chain regions (domains D1 to D3) and a BK-containing domain (D4) coded by these exons. The 5′-terminal portion of exon 10 encodes the unique 56-kDa *C*-terminal light chain of HK. This part of exon 10 and its flanking 90-nucleotide sequence are spliced out in the LK mRNA. The 4 kDa light chain of LK is coded by exon 11.[72]

In rats a pseudogene resembling the "canonical" kininogen gene as well as two other functional kininogen ("T-kininogen") genes have been identified that are each transcribed as a single mRNA species. The T-kininogen mRNAs produce two closely related isoforms of T-kininogen (TK-I and TK-II), the precursors of T-kinin (TK, ISRPPGFSPFR).[73] In contrast, mice have two functional highly homologous genes for kininogens (*Kng1, Kng2*) that result from a gene duplication event. Each *Kng* gene account for about half of the concentration of H-kininogen 1 and 2 and L-kininogen 1 and 2, respectively, in mouse plasma.[74]

In humans and rodents, HK and LK are predominantly expressed in the liver but can also be found in endothelial cells, granulocytes, renal tubular cells, and α-granules of platelets.[75–77] It is not clear whether these cells synthesize kininogens or whether the proteins are taken up from plasma or cell culture supernatant containing serum components. Kininogen secretion into the plasma is mediated by amino-terminal signal peptides of 18 residues. The mature forms of LK (409 amino acids) and HK (626 amino acids) contain multiple posttranslational modifications such as hydroxylation and glycosylation.[78] The detailed functions of the various carbohydrates are unknown, but they most likely affect solubility, half-life, and secretion of the kininogens. LK and HK contain 18 cysteine residues each, which form 9 disulfide bridges.[79] Eight loops are present in the *N*-terminal portions, while a single disulfide bridge spans the extreme amino- and carboxy-terminal portions of LK and HK. The kinin-free form of HK (HK_a) migrates with largely different apparent molecular weight than the 120 kDa single-chain form. The discrepancy indicates that HK undergoes major structural changes upon BK liberation. Under reducing conditions on SDS-PAGE, HK_a has a heavy chain of 63 kDa and a light chain of 58 kDa, the latter of which is rapidly degraded by α-kallikrein and FXIa to smaller-sized fragments. HK is a typical multidomain plasma protein. HK domains D1, D2, and D3 of the heavy chain are homologous to cystatins, with D2 and D3 retaining cysteine protease inhibitory activity.[80] The D4 domain harbors the kinin peptide that is releasable from both HK and LK through limited proteolysis mediated by kininogenases. HK is the preferred substrate for α-kallikrein, and its cleavage at residues Lys362 and Arg371 generates the nonapeptide BK (NH_4-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg).[81] In contrast, LK is cleaved predominantly by tissue kallikreins resulting in formation of Lys-BK (kallidin).[82,83] Kininogens are also cleaved by factor XIIa, factor XIa, and plasmin in reconstituted systems, but the physiologic relevance of these reactions is not clear. Release of BK and kallidin differs in kinetics and possible mechanism. BK is liberated locally and in response to stimuli on cell surfaces by the contact activation mechanism. There is a constant turnover of LK and basal static kallidin release.[84] The two-chain form of LK that is the result of kallidin release is composed of a heavy chain (residues 1 to 362) and a light chain (residues 372 to 409). A single disulfide bridge spanning Cys10 with Cys389 connects both fragments. BK-free HK contains the same heavy chain as LK (domains D1 to D3, residues 1 to 362), and a much larger light chain composed of domains D5H and D6H (residues 372 to 626) is connected to the heavy chain by a single disulfide bond (Cys10–Cys596).[8,85]

A total deficiency of kininogens is rarely found in humans. In the case of Williams trait, a single C to T base transition at position 587 changes a CGA codon (Arg) to a TGA codon (stop) in exon 5, affecting the synthesis of both LK and HK.[86] HK and LK deficiency in Brown-Norway Katholiek rats results from a single base pair substitution leading to a Tyr for Ala exchange at amino acid 163. This subtle change in kininogen sequence does not affect biosynthesis but leads to defective posttranslational modification and/or secretion of the kininogens such that their plasma levels are <1% of normal.[87] T-Kininogen genes and, therefore, T-kininogen biosynthesis are unaffected in these rats.

Bradykinin

BK and kallidin are ligands of kinin B2 receptors and bind to the G-protein–coupled receptor with binding affinities in the lower nanomolar range.[88,89] Kinins in plasma have short half-lives of <20 seconds and are rapidly degraded to inactive peptides by angiotensin converting enzyme, neutral endopeptidases, and aminopeptidases. BK and kallidin are also cleaved by carboxypeptidases, collectively referred to as kininase I (carboxypeptidase N, EC 3.4.17.3), to yield des-Arg9-BK and des-Arg10-Kallidin, which have longer half-lives and activate the kinin B1 receptor. Kininase II, identical to the angiotensin-converting enzyme (ACE), and neutral endopeptidase (NEP24.11, EC 3.4.24.11) inactivate BK or kallidin by removing a dipeptide, Phe–Arg, from the *C*-terminus.[90,91] Kinins are potent proinflammatory mediators. The peptide hormones initiate an increase in intracellular Ca^{2+} that activates endothelial nitric oxide synthase–mediated nitric oxide formation.[92] Endothelial denudation of large arteries abrogates the vasodilatory response to kinins. Kinins also stimulate the production and release of autacoids such as prostaglandin PGI2 and prostacyclin PGE_2.[93] Kinins induce vasodilatation, regulate local tissue perfusion, and importantly increased vascular permeability.[94] Excessive BK production is the cause for a life-threatening swelling disease, hereditary angioedema (HAE) (see below). The importance of

the blood pressure lowering effects of kinins is highlighted in the clinical use of ACE inhibitors such as captopril and ramiprilate for the treatment of hypertension and cardiac failure.[95,96]

Kinin-Independent HK Functions

In addition to the kinin sequence, other portions of HK have been associated with biologic function. The histidine/glycine-rich region in the HK D5H domain mediates binding of the protein to anionic surfaces such as heparin.[97] A peptide spanning the region in D5H between His479 and His498 of HK (HKH20) has been identified as the major cell-binding site of HK. Residues in the D3 domain contribute to HK binding to a broad variety of cells with 1,000 to 10^7 binding sites per cell.[98] Heparan and chondroitin sulfate type proteoglycans have been identified as major HK cell surface binding site.[99,100] The glycosaminoglycan chains of proteoglycans such as syndecans and glypicans bind the plasma protein to cardiovascular cells. Consistent with the interaction of the histidine-rich polypeptide in D5H with negatively charged glycosaminoglycans, zinc ions critically promote HK cell binding—presumably by an intercalating mechanism. Binding to glycosaminoglycans protects HK from cleavage, and detaching the hormone precursor seems to be a prerequisite for BK formation.[101] Alternatively, HK has been shown to interact with p33/gC1qr (a mitochondrial protein[102]), urokinase receptor, and the cytoskeleton compound cytokeratin-1.[103,104] The physiologic relevance of the proposed ternary complex for providing cell-surface HK-docking sites on intact cells is currently not precisely known.

Peptides from the D5H domain have antimicrobial activity and potently kill pathogens such as *Escherichia coli, Pseudomonas aeruginosa*, and *Enterococcus faecalis* by disrupting bacterial membranes. Similarly, an antibacterial fragment of the HK D3 domain has antibacterial activity versus *Streptococcus pyogenes, Staphylococcus aureus*, and *Salmonella.*[105] A synthetic 26–amino acid peptide had similar properties. A protease from *P. aeruginosa* generates antimicrobial peptides from the D5H domain, demonstrating that products of HK processing other than BK provide part of the first line of defense against microbial invasion *in vivo*. D5H fragments have also been shown to have antiangiogenetic[106] and leukocyte-recruiting[107] activities with potential therapeutic implication.[108] Cleaved HK or peptides from HK's domain D5H blocks FXII-induced ERK1/2 and Akt phosphorylation.[38] The D6H domain in the HK light chain region contains binding sites for factor XI and PK, and most of these proteins circulate in plasma bound in a complex with HK. Both PK and factor XI bind with high affinity (9 nM) to HK. The factor XI and PK binding sites on HK overlap, therefore, one molecule of HK can bind either a molecule of factor XI or a molecule of PK. Consistent with a high degree of homology PK and factor XI, apple domains A1, A2, and A4 form a discontinuous binding site for HK. Domain swapping experiments between PK and factor XI support a similarity in binding site among the zymogens and indicate that the Apple 2 domain contains the core region of the discontinuous HK-binding site.[50] The contact induced procoagulant activity of HK as measured in the activated partial thrombin time (PTT) assay requires both D5H-mediated association of HK with artificial anionic surfaces and D6H-mediated binding of factor XI and PK to HK. Since LK is not binding to PK or factor XI, the protein lacks procoagulant activity in the PTT assay (FIGURE 15B.2A,B).

CONTACT ACTIVATION

Mechanism of Contact Activation

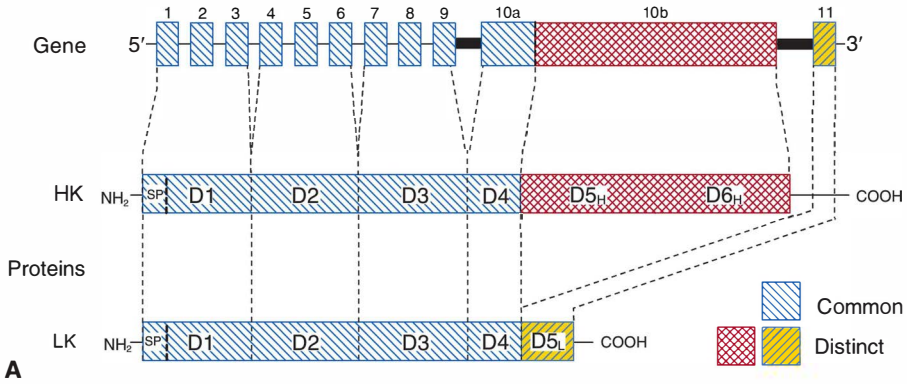
Contact activation is the mechanism of contact-induced FXII zymogen activation. Contact activation is initiated by activation of FXII on a surface, and activated FXII (α-FXIIa) catalyzes activation of additional FXII. Alternatively, α-FXIIa catalyzes conversion of PK to α-kallikrein, which in turn amplifies the process through reciprocal activation of FXII.[11] The relative importance of FXII autoactivation on surfaces versus reciprocal activation of FXII and PK remains to be analyzed and may depend on the nature of the contact system activating surface. Contact activation can propagate from the activating surface into the plasma fluid phase. α-Kallikrein cleaves HK and liberates BK, activates the renin–angiotensin system by converting prorenin to renin, and initiates the alternative complement pathway by activation of C3. Surface-bound α-FXIIa is concerted to β-FXIIa, which does not bind to surfaces but potently activates PK, and the classical pathway of complement *via* its substrate C1. The importance of contact system–driven activation of the complement system for host-defense reactions *in vivo* is not precisely known; however, some individuals with severe deficiency in contact system proteins appear to suffer from increased susceptibility for bacterial infections.[109]

Contact activation provides the mechanistic basis for one of the most commonly used diagnostic clotting test, the activated partial thromboplastin time (aPTT). The contact surface used in aPTT assays is typically a negatively charged nonphysiologic substance such as kaolin (aluminum silicate), celite (diatomaceous earth), or ellagic acid. The aPTT is widely used in clinical practice for preoperative screening and monitoring of anticoagulation therapy. Despite its indispensible role in fibrin formation in the aPTT assay, FXII initiated coagulation was believed to have no important function *in vivo*. This notion is based on the clinical observation that FXII deficiency in humans and animals is not associated with abnormal bleeding despite causing a marked prolongation of the aPTT.[110,111] In contrast, deficiency of the extrinsic pathway trigger factor VII results in severe bleeding in humans, whereas complete tissue factor (TF) deficiency in mice is not compatible with life due to intrauterine bleeding.[112] Similar to FXII-deficient individuals, humans lacking the other contact proteins, PK or HK, do not have impaired hemostasis and do not show signs of any symptoms of pathologic spontaneous or injury-related bleeding. Contact factors can assemble and activate *ex vivo* when blood is exposed to bioincompatible surfaces, contributing to the systemic inflammatory response that occurs when blood is passed through an extracorporeal circulation system, such as during cardiopulmonary bypass surgery.

Contact activation-independent pathways for FXII activation have been suggested. Several proteinases including elastase, trypsin, bacterial proteases, and plasmin, convert FXII to FXIIa (aFXIIa form). Activation of the contact proteases by bacterial surface proteases is important for tissue invasion by the organism. The pathogens utilize the host contact system to form BK on their surfaces. Local BK activity increases vascular permeability and facilitates the invasion of bacteria from the blood into the surrounding tissues.[113]

Contact Activators

FXII can be activated by macromolecular constituents of the subendothelial matrix such as glycosaminoglycans and collagens, nucleotides, sulfatides, soluble polyanions, or nonphysiologic



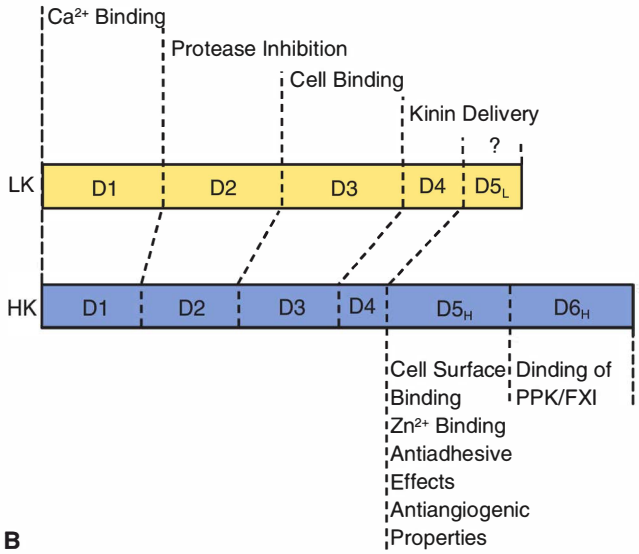


FIGURE 15B.2 Kininogen gene and structure–function relationships. **Left**: LK and HK are products of a single gene of 11 exons (E1 to E11). E1 to E9 encode domains D1 to D3 preceded by a signal peptide. Domain D4 contains the kinin moiety and is encoded by the 5′-terminal portion of exon 10 (E10a). The mRNAs for LK and HK are identical in their 5′-parts including exons 1 to 10a. They differ in their 3′-portions. The 3′-segment of exon 10 (E10b) encodes HK domains $D5_H$ and $D6_H$, and exon 11 codes for LK domain $D5_L$. Abbreviations: SP, signal peptide; LK consists of five domains (D1 to $D5_L$), and HK contains six domains (D1 to $D6_H$). **Right**: The domain structures of human kininogens are shown (not drawn to scale). Their common domains D1 to D4 bind Ca^{2+} (D1), inhibit cysteine proteinases (D2, D3), mediate cell binding (D3), and contain the kinin segment (D4). The biologic function, if any, of LK-specific domain $D5_L$ is unknown: HK-specific domain D5H contains a major cell binding site, Zn^{2+} binding site(s), and mediates antiadhesive effects of kininogens: Domain $D6_H$ exposes overlapping binding sites for FXI and PPK.

materials such as glass or polymers (reviewed by[9]), but the physiologically relevant mechanism for FXII activation has remained enigmatic for decades. Just recently, physiologic FXII contact activators have been identified (see below).

Glass was one of the first substances identified to activate FXII. The white clay minerals kaolin and celite, two other silica-rich compounds, are the most commonly used FXII activators for coagulation diagnostics. Kaolin is also used to terminate blood loss after serious injury in combat victims.[114] Although the idea of employing the contact system to seal injuries is interesting, exposing flowing blood to kaolin triggers thromboembolic events[115] and produces excessive hydratization energy causing burn necrosis at the wound site. Another classical FXII activator is ellagic acid (2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde] chromene-5,10-dione). Ellagic acid triggers FXII-driven coagulation in plasma,[116] and the substance is commonly used as the starter for the aPTT assay. Infusion of ellagic acid into humans is prothrombotic.[117] However, it remains unclear whether the hypercoagulant response is a direct result of FXII activation or ellagic acid effects on platelet aggregation, or a combination of both. Ellagic acid also activates the fibrinolytic system possibly involving PK activity[118] and when administered *in vivo* reduces blood pressure presumably involving BK activity.[119]

A well-characterized FXII contact activator is high molecular weight dextran sulfate (DX500.000). Dextran sulfate is a polysulfated polysaccharide of glucose moieties. DX-mediated FXII activation is critically dependent on the chain length and degree of sulfation of the polyanion.[120] High molecular weight DXS (500 kDa) is a potent stimulator of FXII, whereas shorter DXS polymers fail to activate FXII. When injected in large animals, long-chain DXS induces BK-mediated hypotension,[121] but does not trigger intravascular coagulation. Polysaccharide-stimulated BK generation may have fatal effects. Starting November 2007, there was a dramatic increase in heparin-induced adverse reactions such as lethal acute hypersensitivity reactions in the United States and Germany in patients intravenously receiving commercial heparin of specific lots from a single manufacturer (http://www.fda.gov/cder/drug/infopage/heparin/adverse_events.htm). Greater than 150 patients deceased from anaphylactic hypotension, associated with intravenous heparin treatment. Comprehensive analyses identified a nonnatural contaminant occurring in suspect preparations of heparin that was characterized as oversulfated chondroitin sulfate (OSCS).[122] OSCS-contaminated heparin has greatly increased potency for activating FXII and triggering PK-mediated BK formation in human plasma and in a model of

experimental hypotonic shock *in vivo*.[123] Additionally, infusions of OSCS-contaminated heparin were associated with complement activation indicated by elevated plasma levels of C3a and C5a. These catastrophic reactions in patients are reminiscent of experimental shock models induced by DX-stimulated BK formation in pigs. Infusion of DX500.000[120] induced transient systemic hypotension, and icatibant (a B2R antagonist) blocked the drop in blood pressure.[121] BK-forming activity seems to be dependent on negative charge density of the polysaccharide rather than on a defined structure. OSCS activates the kallikrein–kinin and complement systems as potently as does DXS.

Charge-dependent contact system activation is not restricted to polysaccharides. It is known for decades that FXII binds to insoluble collagen, enhancing coagulation.[124] The interaction is dependent on a repetitious presentation of negative charges in the collagen fiber.[124] However, later reports argued against a direct activation of FXII by collagen. Recent studies have reevaluated the importance of collagen for FXII activation.[125] FXII was shown to bind to collagen fibrils of various origins. When added to plasma (equine), type I collagen promoted thrombin formation and plasma clotting in an FXII-dependent manner. FXII activity is critical for collagen-stimulated thrombus formation in flow chambers.[126] Additionally, collagens may stimulate the contact system indirectly.[127]

Dextran sulfate may have physiologic analogues. It was believed that mast cell-triggered leakage is predominantly if not exclusively mediated by histamine release, and targeting histamine signaling is widely used therapeutically to treat edema formation associated with aberrant mast cell activity.[128] Mast cell secretory granules also contain highly sulfated polysaccharides, with heparin as a major constituent. Addition of mast cell-derived heparin initiated BK formation in plasma in an FXII-dependent manner and triggered the FXII/PK cascade resulting in HK procession. Consistent with *ex vivo* data,[129] topical heparin application increased vascular permeability in an FXII-dependent manner in mice. Intravital microscopy and tracer extravasation experiments identified BK as the active mediator for increased leakage in heparin-driven plasma extravasation in mice.[130] Under these conditions the FXII substrate FXI was not activated. The detailed mechanism for selective activation of the FXIIa substrate PK over FXI is not entirely clear but may reflect a higher plasma concentration of PK *versus* FXI and higher specificities of heparin-dependent plasma inhibitors such as AT III for activated FXI *versus* PK. Similarly mast cell–derived chondroitin sulfates have been proposed as activators of the kallikrein–kinin system *in vitro*.[129] However, the physiologic relevance of this mechanism has not been elaborated so far.

Based on the experimental data, targeting heparin-initiated BK formation may represent a promising strategy to protect against defective barrier function attributed to mast cell activation in a broad variety of diseases. Indeed, FXII gets activated on particulate matter that is a component of air pollution.[131] BK formed by the FXII-driven contact pathway may contribute to intestinal pulmonary edema in allergic responses to air pollution. A similar mechanism contributes to bacterial infections. Contact system proteins assembled on the surface of *E. coli* and *Salmonella* through their specific interactions with fibrous bacterial surface proteins, curli and fimbriae, induce proinflammatory reactions *via* BK release.[113] Pharmacologic inhibitors of contact system–driven BK formation interfere with Salmonella-mediated pneumonia and pulmonary leakage in rodent models.[132] BK liberation from HK contributes to the host defense response to infection; however, high BK levels can contribute to hypotension and vascular leak associated with septic shock. Furthermore, BK generated on the bacterial membrane contributes to tissue invasion of the pathogen by opening endothelial cell–cell contacts. Plasma levels of contact factors are reduced in septic shock, and low factor XII and HK levels are predictors of fatal outcome during the systemic inflammatory response syndrome that is a feature of sepsis. Contact factor activation has been noted during meningococcal sepsis and in toxic shock syndrome. The importance of contact activation to the sepsis syndrome is shown by studies of *E. coli* sepsis in baboons, where FXII inhibition prevented hypotension (Table 15B.1).

Contact Activation in Vivo

A series of recent studies have identified candidates for physiologic or pathologic initiators of contact activation that include collagen, nucleotides, polyphosphate, and several proteoglycans.[5,125,134,135] Polyphosphates, inorganic nonbranched polymers of 60 to 100 orthophosphate residues that are released from platelet dense granules have procoagulant and proinflammatory effects that are mediated by FXII. Factor XII binding to polyphosphates initiates fibrin formation on procoagulant platelets with critical importance for thrombus formation. The biologic function of polyphosphate-triggered contact activation is discussed below. FXII activation may also be triggered by RNA in plasma,[136] and infusion of RNAse (an enzyme that degrades RNA) is thromboprotective in a mouse arterial thrombosis model.[135] Consistently, a synthetic RNA homologue (polyIC) that is not degradable by plasma RNAses is prothrombotic in a FXII-dependent manner. Misfolded protein aggregates, such as those found in plasmas of patients with amyloidosis, have been shown to trigger FXII activation.[137] Similarly to heparin, misfolded protein aggregates specifically initiate BK formation *via* activation of the kallikrein–kinin system and do not trigger activation of the intrinsic pathway of coagulation.[130]

One possible explanation for the selective activation of PK could be that local concentrations of PK molecules exceed those of FXI.[138] However, both zymogens are surface-bound *via* HK and share a conserved HK-binding site.[50,139,140] Moreover, negatively charged surfaces like kaolin do support FXII-dependent FXI activation under the same circumstances. Factor XII differs in binding to negatively charged surfaces and misfolded protein aggregates with possible implications for substrate preferences. Surface binding is mediated through the fibronectin type 2, second EGF, and kringle domains,[25,26] whereas interaction to aggregates is mediated by the fibronectin type 1 domain.[137] Differences in FXII binding may also modulate the conversion of α-FXIIa to β-FXIIa by kallikrein and shift FXII activity toward kinin formation.

Activated (procoagulant) platelets support FXII activation, dependent on integrin α_{IIb}-β_3 signaling,[141] and enhance activation of the kallikrein–kinin system in plasma.[142] Activated platelets promote fibrin formation in an FXII-dependent manner *in vitro*.[143] However, based on normal hemostasis in FXII deficient individuals, platelet-driven FXII activation was neglected. Cumulatively, the classical data suggest that FXII is activated on or by procoagulant platelets. Moreover, platelets

Table 15B.1

Factor XII activators and their effects on coagulation or inflammation

	In Vitro Effect		*In Vivo* Effect	
Compound	**Intrinsic Coagulation Pathway**	**Kallikrein–Kinin System**	**Procoagulant**	**Proinflammatory**
Nonphysiologic Materials				
Glass	+	+	+	+
Kaolin	+	+	+	+
Ellagic acid	+	+	–	+
Dextran sulfate (high molecular weight)	+	+	–	+
Ellagic acid	+	+	–	+
OSCS	–	+	–	+
Poly I:C	+	+	+	?
Biomaterials				
Dialysis membranes (e.g., Cuprophane/ Polyacrylonite/AN69)	+	+	+	+
Vascular grafts (Dacron/PTFE/ PDMS)	+	+	?	?
Metal (Steel/titanium/aluminium)	+	+	?	?
Therapeutical Compounds				
Aptamers	+	+	?	?
Iron oxide nanoparticles	?	+	?	+
Endogenous Substances				
Glycosaminoglycans	–	+	?	?
Lipopolysaccardide	–	+	?	?
Nucleic acids (RNA, DNA)	+	+	?	?
Sulfatide	+	+	+	+
Urate Crystals	+	+	?	+
Misfolded protein aggregates	–	+	–	+
Polyphosphates	+	+	+	+
Amyloid β peptide	–	+	–	+

Adapted from Maas C, Oschatz C, Renné T. The plasma contact system 2.0. *Semin Thromb Hemost* 2011;37(4):375–381, Ref.[133]
? represent "unknown effect."

support the activation of FXII and factor XI in the presence of thrombin (feedback) and TF inhibition,[142,144] showing that platelets specifically trigger the intrinsic coagulation pathway.[141,143,145] Recent studies identified the platelet-derived FXII activator: polyphosphate, an inorganic polymer of 60 to 100 phosphate residues. Polyphosphate was originally identified in nonmammalian cells[146] but was later shown to be enriched in platelet dense granules.[147] Synthetic polyP is used as water softener in technical processes and has been shown to be a potent modulator of plasma clotting, affecting the intrinsic pathway, the fibrinolytic system, factor V activation, and the fibrin structure.[134,148] Platelet polyphosphate activates FXII in reconstituted systems and in plasma[134] *via* directly binding.[127] Polyphosphate-stimulated FXIIa initiates thrombin and BK formation in an FXI- and PK-dependent manner, respectively. In mice, platelet-secreted endogenous polyP induces pathologic thrombus formation (platelet-driven pulmonary embolism) and BK-driven edema. Targeting polyphosphate with phosphatases, which degrade polyphosphate, almost completely abolished procoagulant platelet activity and largely protected animals from activated-platelet driven thrombosis *in vivo*. Together, the data highlight the importance of polyphosphate as endogenous activator of the FXIIa-driven contact system on platelets *in vivo*. Synthetic polyphosphate restored defective clot formation in platelet-rich plasma from Hermansky-Pudlack patients, who suffer from hereditary polyphosphate deficiency[127] suggesting that polyphosphate may be utilized as hemostatic agents.[149] In contrast to heparin[130] and misfolded protein aggregates,[137] polyphosphate initiates FXII-dependent clotting and potently activates the kallikrein–kinin pathway

generating BK. Bacterial polyP initiates lethal hypotonic reactions in a FXII-dependent manner[127] and platelet-derived polymers initiate skin edema *via* BK signaling. The inorganic polymer presents the long sought link of primary and secondary hemostasis.[150,151] Interrupting polyphosphate-driven activation of the FXII-driven contact system efficiently and selectively interferes with thrombosis and edema formation *in vivo* and thus may present a new paradigm to fight thromboembolic and proinflammatory disease.[152]

THE CONTACT SYSTEM IN THROMBOSIS AND HEMOSTASIS

FXII in Thrombus Formation

Injury to a blood vessel triggers activation of blood platelets and the plasma coagulation system, leading to formation of a blood clot consisting of platelets and fibrin. Formation of fibrin is mediated by a group of tightly regulated plasma proteases and cofactors, the blood coagulation cascade. In the original waterfall models, fibrin formation is initiated by two distinct pathways, triggered by exposure of blood to a damaged vessel wall (extrinsic) or to blood-borne (intrinsic) factors.[1] Fibrin formation *via* the intrinsic pathway of coagulation is triggered by FXII and contact system activation. Despite its function for initiating coagulation *in vitro*, numerous patients lacking FXII have undergone surgery with no apparent excess bleeding, despite abnormal results on *in vitro* clotting assays.[153] Since deficiency in FXII is not associated with increased spontaneous or injury-related bleeding tendency, FXII was considered to play no role in blood coagulation *in vivo*, and subsequent revisions of the coagulation cascade usually exclude FXII.[154] The absence of a bleeding phenotype in FXII deficiency, in contrast to deficiencies of other components of the extrinsic coagulation cascade such as factor VII, TF, and factor IX (deficiency causes the bleeding disorder Hemophilia B), has lead to the reasonable hypothesis that fibrin formation *in vivo* is initiated largely, if not exclusively, through the extrinsic pathway of coagulation by factor VIIa/TF.[112] The factor VIIa/TF-driven model for hemostasis is supported by the observation that FXI, a major substrate for activated FXII (FXIIa) during contact-initiated clotting, can be activated by thrombin independently of FXII. The FXII-independent "revised model of coagulation" had been widely accepted.[154]

FXII-deficient ($FXII^{-/-}$) mice were generated and phenotyped to study the function of coagulation FXII *in vivo*.[155,156] Similar to FXII deficient humans, $FXII^{-/-}$ mice have a normal hemostatic capacity as assessed by a tail-bleeding assay.[157] Completely unexpected, intravital fluorescence microscopy and blood flow measurements in three distinct arterial beds revealed a severe defect in FXII-deficient mice in thrombus formation induced by different methods of injuries.[157] The data with FXII null mice challenge the "revised model of coagulation" and demonstrate a crucial role of FXII for fibrin formation *in vivo* (FIGURE 15B.3).

Reconstitution of FXII null mice with human FXII restored the prolonged aPTT found in untreated animals and fully restored the capacity of infused animals for thrombus formation. The reconstitution experiments suggest that FXII operates similarly in mice and humans. Indeed, the contact system is highly conserved among mammalian species.[158] Thrombus formation in FXII heterozygous mice—having 50% of normal plasma levels—was similar to wild type control mice (100% FXII), indicating that half of normal plasma concentration is sufficient for vessel occlusive clot formation. *Vice versa*, drugs that target FXII activity need to substantially reduce activity of the protease to provide thromboprotection. The essential role of FXII for thrombus formation has implications for thromboembolic disease. FXII gene–deficient mice are protected from the effects of cerebral ischemia in an experimental stroke model.[159] This protective effect is related to impaired fibrin formation in the microvasculature of the ischemic tissue. Mice lacking FXI are similarly protected from vessel occlusive fibrin formation, suggesting that FXII impacts only on pathologic clotting *via* the intrinsic pathway.[160,161] Despite the striking protective effect in these models, FXII-deficient mice, like their

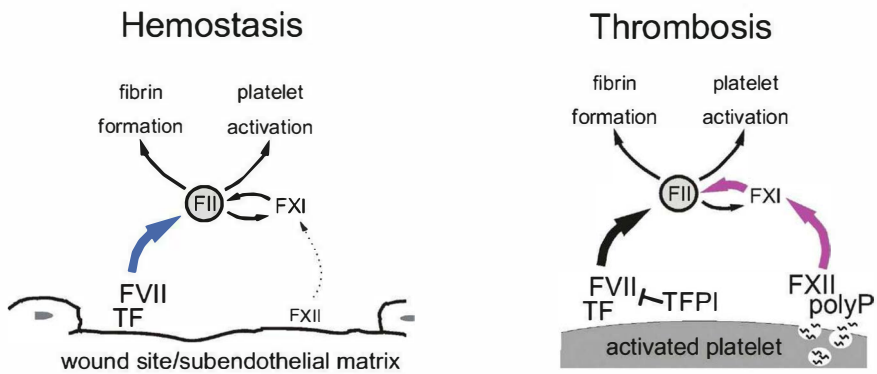


FIGURE 15B.3 Role of FXII in coagulation. *Hemostasis*: Thrombin (FII) formation at sites of lesions is predominantly due to TF exposed in the subendothelial matrix. TF in complex with factor VII (FVII) initiates thrombin formation, which promotes fibrin formation and platelet activation. The contribution of factor XII (FXII) for hemostasis is minor. *Thrombosis*: Additional fibrin forming activity is necessary to form a thrombus when TF/FVII-driven coagulation is blocked by TF pathway inhibitor. FXII is activated by platelet polyphosphate on activated platelet surfaces contributes to thrombin generation and additional platelet activation, propagating thrombus growth. Accordingly, FXII as well as factor XI (FXI) deficiency severely impairs thrombus formation, but have no function for hemostasis. (Reprinted with permission from Maas C, Renné T. Regulatory mechanisms of the plasma contact system. *Thromb Res* 2012;129(Suppl 2):S73–S76.)

human counterparts, do not have spontaneous or injury-related hemorrhage. Pharmacologic inhibition of FXII activation using the peptide-based inhibitor PCK in wild type mice also provides protection from cerebral ischemia, without causing excessive bleeding at the surgical injury site.[159,162] Consistently, a recombinant infestin-4–based inhibitor that specifically targets FXII activity provided protection from cerebral ischemia in experimental stroke models albeit did not affect the hemostatic capacity in inhibitor-treated mice.[163] These results demonstrate that FXII-driven fibrin formation is specifically important for pathologic thrombus formation but has no function for fibrin formation during normal hemostasis. This raises the possibility that targeting FXII may offer a strategy for prevention or treatment of pathologic thrombosis that is not associated with the high rate of hemorrhage that accompanies currently used anticoagulants.[153,164,165] Furthermore, given the results found in mouse models, the concept that pathologic thrombus formation represents disequilibrium of the processes that normally produce a clot at a site of injury likely needs revision (FIGURE 15B.3). The FXII-driven intrinsic pathway seems to be specifically important on activated platelet surfaces propagating thrombus formation when the initial procoagulant activity provided by TF/FVII is not sufficient.

The importance of the contact system for thrombosis is not restricted to animal models. Proteins of the contact system are highly conserved between mice and humans, and results from animal models can easily be transferred to human disease states; for example, FXI appears to participate in thrombosis in humans similarly to its role in mouse models.[166] Together the new data from contact system deficient mice and patients challenge the concept that pathologic thrombus formation represents a dysregulation of normal hemostatic mechanisms and suggest that the dogma of the coagulation balance is in need for revision.[167]

In test tubes, plasma FXI can be activated both by FXIIa and by thrombin according to the classical or revised model of coagulation, respectively. A comparison of mice with deficiency in FXI, FXII, or combined deficiency in the two clotting factors did not reveal additional thromboprotection in FXI/FXII-double deficient mice in a model of lethal pulmonary embolism.[127] The data indicate that FXII is predominantly, if not exclusively, operating *via* its substrate FXI in pathologic thrombus-forming mechanisms in mice. Consistently, antibodies that specifically block FXIIa-mediated FXI activation interfere with thrombus formation in baboons.[168] However, FXI may also be activated by thrombin independently of FXII.[169] The latter study strongly supports the existence of FXI feedback activation *in vivo*.[170] The relative importance of FXIIa versus feedback activation for generating active FXI *in vivo* are not completely understood and may have different importance depending on injury type, vascular bed, and site of activation.

Functions of the Contact System for Thromboembolic Disease in Humans

As today, there is a lack of clinical case controlled studies that systematically compare the incidence or severity of thromboembolic events such as stroke, myocardial infarction, or pulmonary embolism in humans with severe FXII deficiency and normal individuals. The first reported FXII deficient individual, John Hageman, died from a pulmonary embolism,[171] and several subsequent small clinical studies suggested an increased risk of thrombosis in FXII deficient humans.[172–174] However, as noted by Mr. Hageman's physician, Dr. Oscar Ratnoff, the patient had sustained a hip fracture a few days prior to his death,[175] and the trauma and subsequent immobilization were clear FXII-independent risk factors for venous thromboembolism.[109] The observation that deficiency in FXII interfered with polyphosphate-driven fibrin meshwork formation in the thrombus[127] suggests that severe FXII deficiency might increase risk for secondary embolic disease. Consistently, studies showed that thromboses in FXII-deficient patients were probably related to other risk factors, and not to FXII deficiency.[176] However, the detailed association of FXII deficiency and risk for thrombosis is probably more complex. Defective thrombus stability in FXII$^{-/-}$ mice revealed by intravital microscopy,[157] reports of pulmonary emboli in humans with congenital FXII deficiency,[177] and reduced clot firmness in FXII inhibitor–treated blood[127] raise an intriguing hypothesis. FXII deficiency interferes with thrombus propagation apart from the vessel wall and provides thromboprotection. However, FXII-deficiency may also have adverse effects. As in the case of John Hageman, thrombi may develop under some circumstances by FXII-independent mechanisms, especially when thrombosis is driven by massive TF exposure. These thrombi are unstable,[127] and easily embolize causing secondary embolic events. An interesting study from Austria supports the Janus-sided function of FXII for thrombosis in humans. Endler et al. analyzed the correlation between FXII levels and mortality in almost 10,000 patients and found a peculiar inverted U-shaped association. Overall mortality and mortality from cardiovascular disease increase as FXII levels decrease; however, the mortality of patients with severe FXII deficiency (<10% of normal) was similar to mortality for individuals with normal FXII levels (70% to 130%).[178] A C/T polymorphism at residue 46 of the human FXII gene promoter modulates expression levels of the protein. Homozygosity for 46T is associated with lower plasma FXII levels,[179] with an average plasma factor XII level of 53 U/dL (42 to 67) for 46TT, 87 U/dL (77 to 99) for 46CT, and 132 U/dL (97 to 151) for 46CC.[173] A clear association of the 46TT genotype and arterial disease remains to be demonstrated,[180] however, various studies associated the 46 C/T SNP with increased thromboembolic risk.[181–183]

In contrast, large clinical studies in Switzerland and the Netherlands clearly failed to demonstrate any correlation between severe FXII deficiency and an increased risk of thrombosis.[184,185] None of these studies analyzed thromboprotection conferred by FXII deficiency. In contrast, a recent clinical study has analyzed incidence of ischemic stroke in humans with severe FXI deficiency. Similarly to factor XI-null mice, FXI deficient humans are protected from cerebral ischemia[186] supporting the decisive role of the intrinsic pathway for arterial thrombosis in humans.

The data suggest a fundamental difference between individuals with severe or with mild to moderate deficiency in FXII and indicate that FXII may trigger different pathways depending on its activity. These findings are consistent with results from the SMILE project, involving patient from the Leiden thrombophilia study. In the SMILE project, an inverse relationship between plasma FXII and risk of myocardial infraction, with an odds ratio for the highest quintile of FXII levels of 0.4 compared to the lowest quintile, was seen.[187] In contrast, other groups have shown that levels of FXIIa activity were increased in studies of patients with myocardial infarction[188] or

unstable angina.[189] The discrepancies in the literature may be due to the fact that FXII behaves as a negative acute phase protein, with FXII levels decreasing following a thromboembolic event.[190] Additionally, age and ethnic background may influence FXII levels. For example, Orientals have lower plasma levels of FXII than whites.[191] In the second Northwick Park Heart Study, FXIIa was found to be an independent risk factor for coronary heart disease in middle-aged men. Men in the upper third of the study population for FXIIa level had a higher risk compared to those in the middle third. In a second analysis of this data, an increased incidence of coronary events was only found for men with high FXIIa.[192] In contrast, in the West of Scotland Coronary Prevention Study, factor FXIIa levels were not associated with an increased risk of coronary events.[182,193] When evaluating the clinical data on FXII, it is important to note that antibodies to FXII and reduced FXII levels have been associated with antiphospholipid antibodies (APA) and the APA syndrome, a known prothrombotic risk factor. Thus, 50 years after the first description of hereditary FXII deficiency in humans,[111] FXII and the contribution of the contact phase system to thrombosis in patients remain a topic of research.[184] Clearly, patients with deficiency of other contact system proteins such as PK or HK, like FXII deficient patients, do not suffer from abnormal bleeding despite significantly prolonged aPPTs.[194] So far there are no large clinical studies that analyze thromboembolic events in these individuals; however, some case reports associate PK deficiency and thrombosis.[195]

Deficiency in Contact Factors Protects from Thrombosis

Formation of an occlusive arterial thrombus can be divided into two phases: (a) the initiation phase, which results in formation of a "hemostatic" thrombus covering a site of endothelial injury or atherosclerotic plaque rupture, and (b) the propagation phase, which culminates in formation of a three-dimensional occlusive thrombus.[196] Both FXI and FXII deficient animals were largely protected as compared to wild type animals from arterial thrombus formation induced by chemical ($FeCl_3$-induced vascular injury), mechanical damage, or ligation types of vessel injury.[157,160,197] Infusion of human FXI or FXI into FXI or FXI deficient mice, respectively, restored the prolonged aPTT found in plasma of the animals and completely restored thrombus formation in arterial and venous pulmonary embolism models. The reconstitution experiments support the premise that FXI and FXII function similarly in mice and humans. The findings in genetically altered mice data strongly indicate that FXII is essential for thrombus propagation, and that the FVIIa/TF complex is not sufficient for formation of stable occlusive thrombi. The conundrum of normal hemostasis and abnormal pathologic thrombus formation is not restricted to FXII deficient mice. Mice lacking FXI also have relatively normal hemostasis and are protected from thrombus formation.[197] A comparison of FXI null mice and FIX-null (hemophiliac) mice revealed similar levels of protection from carotid artery[161] and *vena cava* thrombosis,[160] in stark contrast to the corresponding impacts of FXI and FIX deficiency on hemostasis. FXI null mice have normal hemostasis following injury to the tail, while FIX-null animals (a model of hemophilia B) bleed to death when challenged in the same manner.[161]

HK deficient mice also have an antithrombotic phenotype, although a different vascular injury model was used other than for the FXI and FXII deficient mice. The mouse has two kininogen genes (kininogen 1 and 2), which account for about 50% of kininogen plasma concentration each.[74] Targeted inactivation of kininogen 1 was sufficient to prolong the aPTT and to impair thrombus formation in the carotic artery induced by the Rose Bengal photothrombosis model.[198] Using the same experimental system, FXII deficiency in mice did not confer protection from cerebral ischemia.[199] The data suggest that Rose Bengal-induced fibrin formation is rather initiated by the "extrinsic" TF /factor VII pathway than the FXII-triggered contact system and indicate the need for experimental thrombosis model in mice that are more indicative for human disease.

HEREDITARY ANGIOEDEMA

The contact system has the capacity of activating the classical pathway of the complement system,[200] and simultaneous activation of the contact and complement system may often occur under pathologic conditions. HAE (MIM #106100) is a rare autosomal dominant disorder, life threatening swelling disorder, that develops in individuals, who are deficient in C1INH (HAE type I) or have a dysfunctional C1INH protein (HAE type II). Urticaria (itching) is not a feature, indicating that histamine is not important to the pathology of the disease. C1INH is the major inhibitor of the classical complement pathway proteases C1r and C1s and regulates generation of the inflammatory mediator BK by the plasma contact system.[5] Deficiency in functional C1INH facilitates excessive activation of the complement and contact system cascades and the development of edema in HAE type I and II patients.[201,202] In addition to these two classical HAE types, a third variant exists that almost exclusively affects women. HAE type III (C1INH HAE type) patients have normal biochemical C1INH functions and plasma concentration, but similarly to other patients suffer from edema.[203] Clinically, all types of HAE are characterized by recurrent episodes of acute swelling involving the skin or the oropharyngeal, laryngeal, or gastrointestinal mucosa. The pathophysiology of increased vascular permeability in HAE has remained controversial. Elegant studies with genetically modified mice demonstrated that edema formation in C1INH-dependent HAE forms is due to pathologic contact system activation. In mice, inactivation of C1INH using a "gene-trap" approach increases vascular leakage. Crossings of C1INH null mice with kinin B2 receptor deficient animals "rescued" the edema phenotype suggesting that vascular leak in HAE is predominantly if not exclusively mediated by BK. Consistently, pharmacologic inhibition of B2R blocked edema formation in C1INH deficient animals.[204] Inactive C1INH increased FXII and PK activity and resulted in excessive PK-mediated release of BK in humans[205] and mice.[204] BK has been shown to be elevated in acute swelling attacks strongly suggesting that increased BK activity mediated elevated vascular leakage.[206] Hence, HAE types I and II are treated by infusion of plasmatic or recombinant C1INH[207] or B2R-receptor antagonists (Icatibant).[208] Alternatively, recombinant PK inhibitors (Ecallantide) may be used to interfere with acute swelling attacks in HAE patients.[209] In contrast to C1INH-dependent HAE forms, the pathomechanism and therapy of HAE type III have remained enigmatic. Using genome-wide linkage analyses in families from France, Switzerland, and Germany, HAE type

III was shown to be an autosomal dominant disease and association with a single missense mutation (c.1032CrA) in the gene of coagulation FXII.[210] A later study involving other families confirmed the original findings and showed that HAE type III could also be associated with a different mutation that affects the same nucleotide of the FXII gene, c.1032CrG.[211] Both point mutations translate into amino acid exchanges Thr328Lys and Thr328Arg, respectively, on protein level. In plasma of HAE type III patient, the enzymatic activity of FXII is elevated as compared to healthy controls.[210] Of note, the aPTT assay results in normal values in HAE type III patients and fails to detect affected individuals.[212] Indeed, the aPTT assay starts fibrin production by preactivated FXII. FXII is activated by exposure to strong contact activators such as kaolin for about 120 to 180 seconds. This time period is sufficient for generating maximal FXII activity independently on presence or absence of the endogenous inhibitor C1INH or FXII-activating conformations (forms of FXII promoting its active potential) such as found in the FXII-Thr328 mutants.[213] Recently, a FXII deletion (residues 324 to 340) was identified in a family with HAE.[214] It remains to be demonstrated, by which mechanism a deletion in the proline rich region of FXII increases BK levels.

Estrogens regulate FXII expression,[215] which might be responsible for the women's specificity of HAE type III.[216] However, FXII plasma levels in HAE type III patients are in the normal range,[210] suggesting that yet unknown mechanism trigger edema predominantly in women. Diagnosis of HAE is difficult as the clinical symptoms are highly variable regarding localization, frequency, triggering factors, and severity of swellings. In contrast to HAE types I and II that are diagnosed by analysis of C1INH plasma levels and function, diagnosis for HAE type III is elaborate and needs FXII gene sequencing that is available in only few diagnostic laboratories. The clinical symptoms suggest that edema in HAE type III is due to aberrant BK formation; however, other FXII-driven pathways such as the intrinsic pathway of coagulation, the complement system, or the fibrinolytic system may contribute.[153] HAE patients experience recurrent attacks of swelling, but the stimuli that trigger these periodic episodes of excessive vascular leakage are poorly defined.[201,202] BK is generated in allergic disease and contributes to increased vascular permeability.[209,217,218] *In vitro*, the mast cell–derived polysaccharide, heparin liberates BK by triggering contact system activation.[129,219,220] The data obtained in plasma samples suggest a role of heparin for triggering swelling episodes in HAE patients *in vivo*. Consistently using a mouse model for HAE type I, mast cell derived heparin as identified as potential trigger for edema attacks *in vivo*.[130]

TARGETING CONTACT SYSTEM PROTEINS FOR ANTICOAGULATION

Current anticoagulation therapy is based primarily on heparins, vitamin K antagonists, or indirect inhibitors of FXa like the synthetic pentasaccharide Fondaparinux. The need for regular monitoring and dose adjustments for some of these drugs (unfractionated heparin and warfarin) or for parenteral administration (heparins, fondaparinux) are inconvenient. Furthermore, use of these drugs is associated with an increased risk of life-threatening bleeding. New oral anticoagulations with lower, but still significant, bleeding risk that include direct inhibitors of thrombin (e.g., Dabigatran etexilate) or FXa (e.g., Rivaroxaban) are entering the market. The antithrombotic effects and bleeding risks for these drugs have been assessed in several clinical trials.[221] Bleeding is an expected consequence with these compounds, because they all target proteins that are critical components of the hemostatic mechanism. In contrast, the limited roles of FXI and FXII in hemostasis[164] suggest that agents targeting these proteases should not increase the incidence of major bleeds.

Wild type mice treated with the peptidergic FXIIa inhibitor D-Pro-Phe-Arg chloromethylketone (PCK), similar to FXII$^{-/-}$ deficient mice, are protected from pulmonary embolism and ischemic stroke without increased bleeding.[127,159] While PCK is not highly selective for FXIIa, recombinant FXIIa inhibitors based on the fourth domain of infestin (infestin-4, a nonclassic Kazal-type serine protease inhibitor protein from *Tryatoma infestans*)[222] fused to human albumin (rHA-infestin-4) have been developed. rHA-infestin-4 is a potent FXIIa inhibitor in human, mouse, and rat plasmas, but does not prolong bleeding times in rodents, even after infusion of large doses. Pretreatment of mice with rHA-infestin-4 resulted in markedly reduced cerebral infarction in the tMCAO model.[163] Similarly, the recombinant contact phase inhibitor Ir-CPI, a Kunitz-type protein from the salivary glands of the tick *Ixodes ricinus*, blocks FXIIa, FXIa, and α-kallikrein and protects mice from venous thromboembolism.[223] A nonpeptidic 3-carboxamide-coumarin (COU254) inhibitor selectively blocked FXII activity in plasma, but was not effective in the tMCAO model in mice.[224] Other protein inhibitors with some degree of selectivity for FXIIa, including cabbage seed protease inhibitor,[225] the pumpkin seed inhibitor CMTI-V,[226,227] trypsin inhibitor from corn (CTI),[228] inhibitors from hematophagous insects,[229] and Ecotin (an inhibitor found in the periplasm of *E.coli*),[230] are effective FXIIa inhibitors in plasma, but also inhibit other coagulation proteases including thrombin, FXIa, and α-kallikrein. A monoclonal antibody against FXIIa that interferes with the FXIIa–FXI interaction has antithrombotic properties and might be useful to prevent thrombosis in patients (United States Patent Application 20090304685, Pritchard D.).

A new and exciting method to inhibit FXII is based on antisense technology.[56] Highly specific antisense oligonucleotides (ASOs) bind complementary sequences on the mRNA of a protein of interest by base pair hybridization. Subsequent selective cleavage and degradation of the target mRNA leads to a reduction in target protein level. In a phase III trial to treat hypercholesterolemia, ASOs directed against the mRNA of apolipoprotein B were potent, selective, and safe[231] (Table 15B.2).

EVOLUTION OF THE CONTACT SYSTEM

The contact system is a young protease cascade in terms of evolution. Orthologs for FXII and PK are not found in fish and birds (or other "primitive" organisms). An ortholog for the FXII gene first appears in amphibians, and is found in mammals, but is missing from the chicken genome.[233,234] An analysis of the region surrounding the FXII homologous protein, hepatocyte growth factor activator (HGFA), and the FXII gene locus sheds some light on this situation. In frogs and mammals, the genes flanking HGFA are paralogs of those flanking FXII, demonstrating that the FXII gene is the result of duplication of the chromosomal segment containing the HGFA gene. The duplication events were followed by translocation to a different

Table 15B.2

Thromboprotective effects of contact system inhibitors *in vivo*

Target	Inhibitor	Arterial Thrombosis	Venous Thrombosis	Ischemic Stroke
FXIIa	Infestin 4 from *Triatoma infestans* fused to recombinant human albumin (rHA-Infestin-4)[163]	x	x	x
	Nonpeptidic inhibitor (3-carboxamide-coumarin, COU254)[224]			—
FXIIa/PK	Peptide-based inhibitor (D-Pro-Phe-Arg chloromethylketone, PCK)[159,127]	x	x	x
FXIIa/FXIa/PK	Recombinant *I. ricinus* inhibitor (Ir-CPI)[223]	x	x	
FXII/PK	ASOs[232]	x	x	

chromosome. Of note, the duplicated region is similar in chicken as compared to mammalians, except that the FXII gene is missing in that specific gene cluster. Comparison of whole genomes shows that the FXII gene was lost during evolution along the lineage leading to birds.[235] This is consistent with the observation that plasma from birds lacks FXII activity and does not clot in aPTT assays.[236] Factor XII gene expression has also been lost in *Cetaceans* (whales and porpoises) due to mutations that converted the gene into a pseudogene in a common ancestor of this order.[233,234] In contrast, kininogens and kinin receptors are ubiquitous in vertebrates.[237] Kininogen genes in amphibians appear to be related to an original kininogen gene in early vertebrates, which has been lost in other modern vertebrate groups. Kininogens in fish, birds, and mammals are related to this original gene that has been transformed into a pseudogene in amphibians.[158]

In summary, FXII predecessor first appeared in tetrapods and may represent an adaptation to a terrestrial environment. While FXIIa may have been an activator of the PK/factor XI predecessor, and subsequently PK and factor XI, the absence of FXII (and contact activation) in birds and *Cetaceans* suggests alternative mechanisms for activation of the contact system that await further analysis.

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CHAPTER

16 Fibrinogen Structure and Function

JOHN W. WEISEL AND CARL-ERIK H. DEMPFLE

Fibrinogen, originally factor I (FI) in the clotting cascade, is a fibrous adhesive glycoprotein, present in the plasma at a concentration of approximately 2.5 g/L, which is essential for platelet aggregation and for fibrin gel formation in hemostasis, wound healing, inflammation, angiogenesis, and other biologic processes. This chapter reviews basic and clinical aspects of fibrinogen's structure and functions, focusing on fibrinogen's role in hemostasis and thrombosis; other roles are covered in other chapters.

AMINO ACID SEQUENCE AND DISULFIDE BONDING

Fibrinogen is a large complex glycoprotein made up of three pairs of polypeptide chains, designated as Aα, Bβ, and γ, with molecular masses of 66.2, 54.5, and 48.4 kDa, respectively, for a total mass of about 340 kDa, including the posttranslational addition of asparagine-linked carbohydrate to the Bβ- and γ-chains.[1–3] The unusual nomenclature for fibrinogen—(Aα, Bβ, γ)$_2$—arises from the inclusion of "A" and "B," representing the small peptides that are cleaved from fibrinogen by thrombin to yield fibrin monomer, which is designated as (α, β, γ)$_2$.

In the most common forms of the polypeptide chains, there are 610, 461, and 411 amino acids in the Aα-, Bβ-, and γ-chains, respectively (FIGURE 16.1). The numbering for the amino acid sequence has been based on the mature product, but the numbering from the primary translation product is now used frequently. The amino acid sequences of the three chains are homologous, but there are also important differences that are discussed at the appropriate sections of this review. The evolutionary implications of these homologies are also be summarized.

Fibrinogen has 8, 11, and 10 cysteines in each Aα-, Bβ-, and γ-chain, respectively, for a total of 58, all of which participate in disulfide bonds.[1,2] The *N*-termini of all six chains are held together by disulfide bonds, with five interchain bonds linking the two halves of the molecule, including one between the Aα-chains, two between the Aα- and Bβ-chains, and two between the γ-chains (FIGURE 16.1). There are two pairs of disulfide rings linking all three chains at either end of the coiled-coil regions. There are also several intrachain disulfides.

THREE-DIMENSIONAL STRUCTURE

Overall Molecular Organization

Rather than describing the historical steps in the elucidation of the structure of fibrinogen, the most important aspects of the structure itself will be outlined, starting with general features. On the other hand, some of the early work will be mentioned where it is still important for current studies and understanding functional aspects of this protein.

Fibrinogen is a fibrous protein about 45 nm in length and consists of a central nodule linked to two end nodules by rod-like α-helical coiled coils (FIGURE 16.2).[3] The central nodule contains the disulfide bonds mentioned above that hold the two halves of the molecule together. There are at least two domains in the central nodule: *N*-terminal portions of both γ-chains meet at the center to form the γ*N*-domain, and portions of two Aα- and Bβ-chains that meet to form what is called the funnel-shaped domain.[4,5] In addition, the *N*-terminal Bβ-chain, which is longer than the others, has been called the Bβ-domain. Each end nodule consists of a β-module and a γ-module, which are each made up of the *C*-termini of β- and γ-chains, respectively, and are homologous to each other. The β-module and the γ-module each consist of three domains, the *N*-terminal A-domain, central B-domain, and *C*-terminal P-domain. The *C*-terminal Aα-chain, which is not homologous to the other two chains, is called the αC region and is made up of a compact *C*-terminal portion called the αC-domain and a flexible tether called the αC-connector that is variable in different species of fibrinogen. The two α-helical coiled coils are made up of all three polypeptide chains, and the distal portions are four stranded, with the addition of a part of the Aα-chain that runs antiparallel to the other three strands.

Visualization of Individual Molecules by Electron Microscopy

Aspects of the overall shape of fibrinogen have been deduced from a variety of physicochemical and structural methods. A diverse array of structures were proposed over many years, and it should be remembered that crystallographic structures do not capture the molecular flexibility and other potential structures that may be present in solution.

One of the first proposals for fibrinogen's shape arose from transmission electron microscopy of rotary shadowed molecules.[6–10] Fibrinogen appeared to have an elongated, 46 nm long trinodular shape (FIGURE 16.3A). A variety of artifactual images of negatively contrasted fibrinogen were then published, in retrospect suggesting that the protein reacts adversely to the low pH of most common heavy metal stains. Subsequent studies revealed that fibrinogen carefully prepared by this method shows the same basic shape.[7,10] Electron microscopy continues to be useful for observations of binding interactions, aspects of fibrin polymerization, and visualization of parts of the molecule that are not seen in crystal structures.

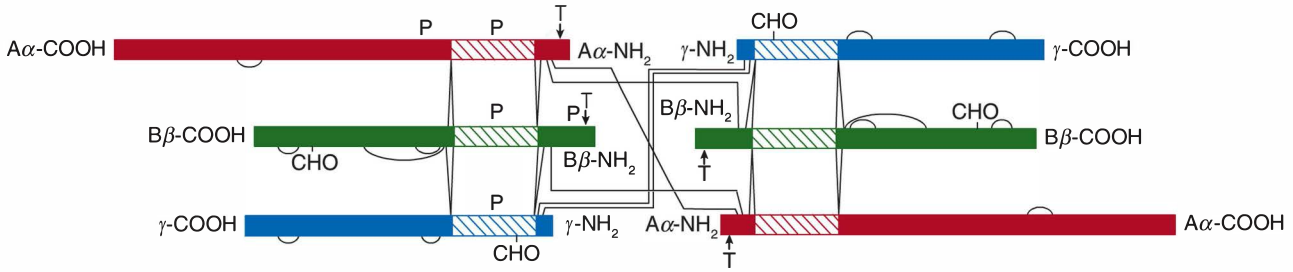

FIGURE 16.1 Schematic diagram of the three pairs of polypeptide chains and disulfide bonding. The Aα-, Bβ-, and γ-chains are represented by bars with lengths approximately proportional to the number of amino acids in each chain. The *N*- and *C*-terminal ends of the chains are labeled and disulfide bonds are shown. The coiled-coil regions are shown as diagonally striped boxes. The carbohydrate locations are shown as CHO. The sites of cleavage of Fps by thrombin are indicated by T and the major sites of plasmin cleavage by P. (From Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem* 2005;70:247–299.)

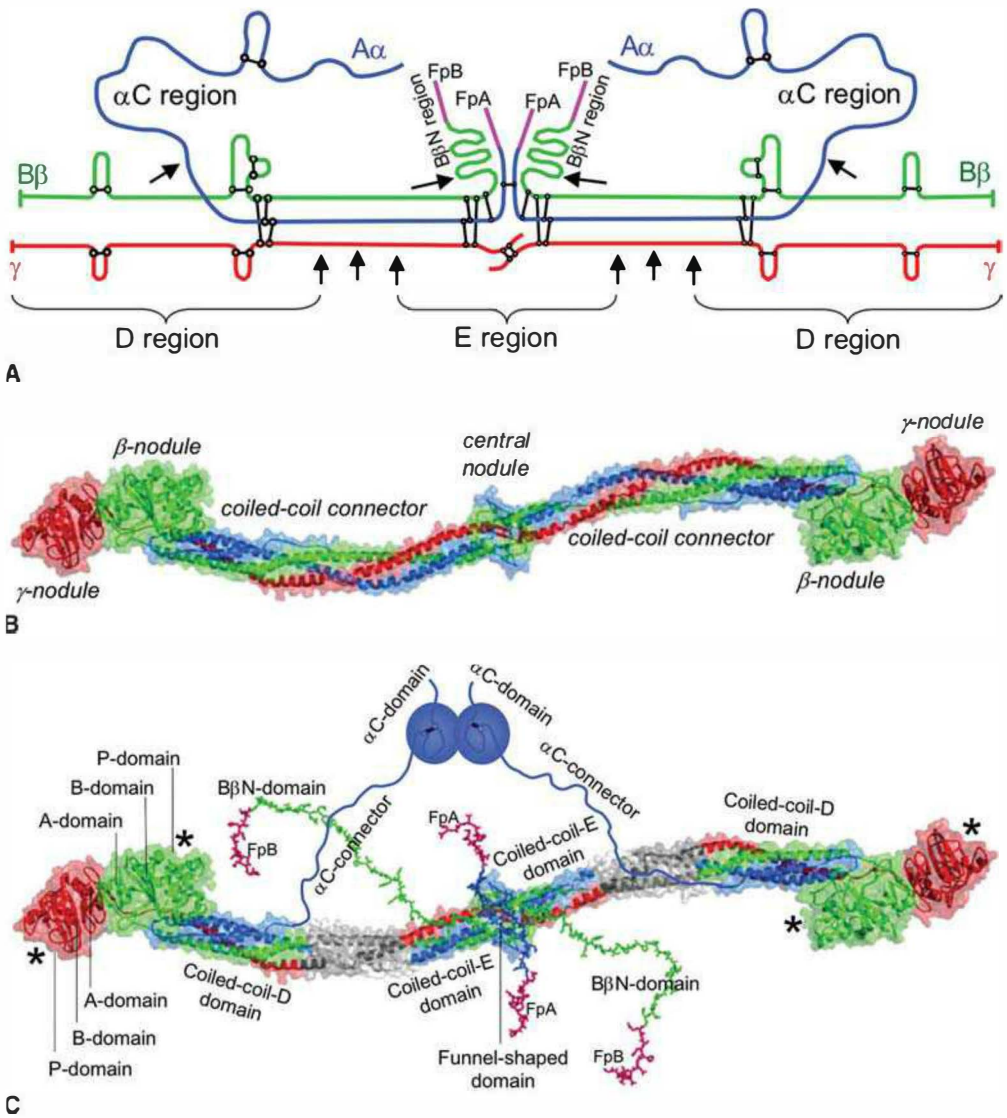

FIGURE 16.2 Structure of fibrinogen. The Aα-chains are *blue*, the Bβ-chains are *green*, and the γ-chains are *red*. **A:** Schematic diagram to show locations of the disulfide bonds and the major regions of fibrinogen, D, E, and αC regions. Major plasmin cleavage sites are indicated by *arrows*. **B:** X-ray crystallographic structure of fibrinogen, with some regions indicated. **C:** The same crystallographic structure with the parts of the molecule that are missing added and labeled. (From Medved L, Weisel JW. Recommendations for nomenclature on fibrinogen and fibrin. *J Thromb Haemost* 2009;7:355–359.)

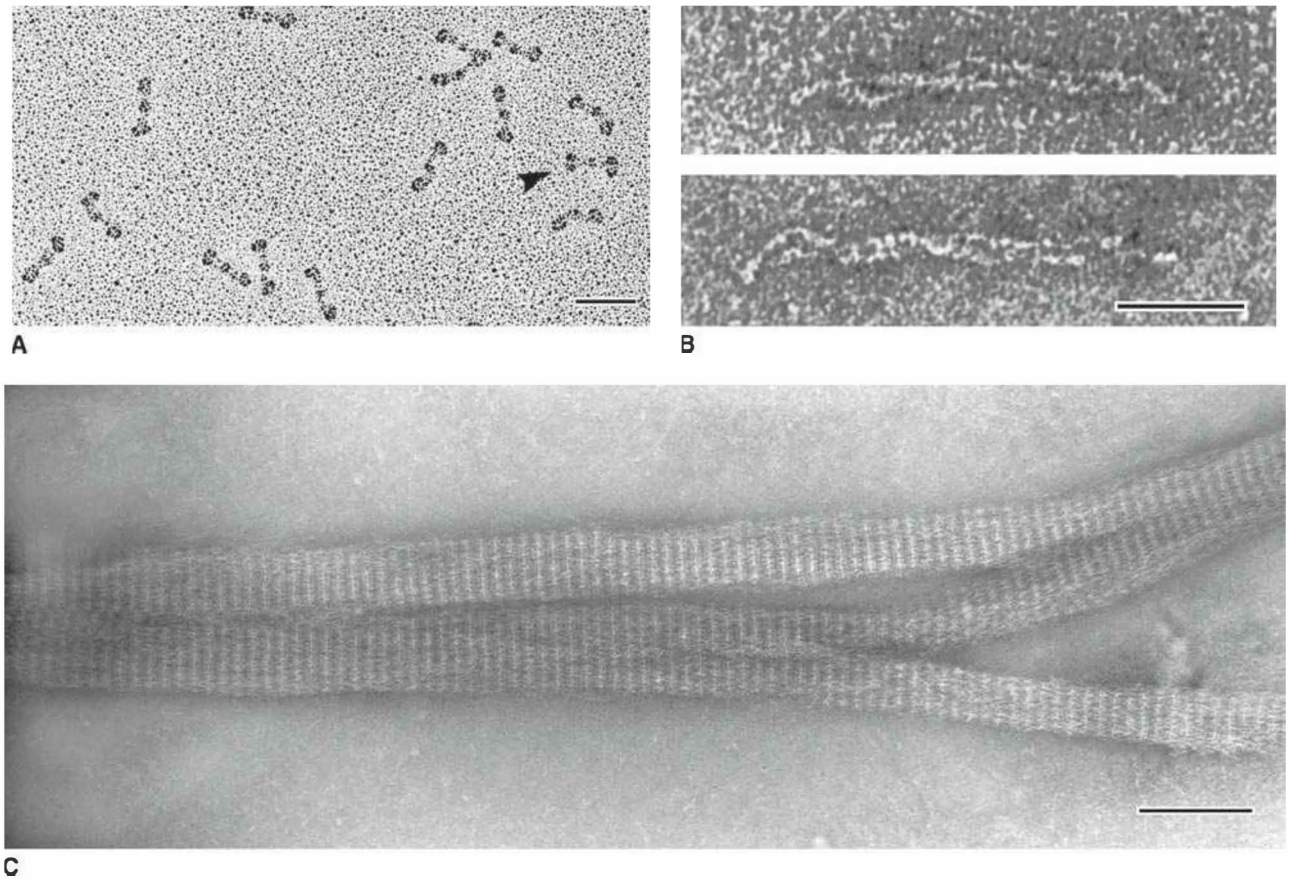

FIGURE 16.3 Transmission electron microscope images of fibrinogen molecules, protofibrils, and fibers. **A:** Individual fibrinogen molecule rotary shadowed with a thin layer of tungsten, showing the 45-nm-long elongated protein molecules with a central region and two nodules at each end, connected by thin rods. Magnification bar, 50 nm. **B:** Negatively contrasted fibrin protofibrils, showing the half-staggering of fibrin monomers and the twisting of the two filaments of the protofibril. **C:** Negatively contrasted fibrin fibers, which have a 22.5-nm repeat, and a distinctive band pattern that arises because areas with high protein density exclude stain and appear bright, while areas with less protein allow stain to penetrate and appear darker. Magnification bar, 200 nm. (From Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem* 2005;70:247–299.)

Structural Insights from Limited Proteolysis of Fibrinogen

Before the crystallographic structure of fibrinogen was determined, the enzymatic degradation products of fibrin(ogen) were extensively analyzed to decipher the structure of the molecule.[11] The nomenclature originally developed has persisted, with the main parts of fibrinogen that remain significant being fragments X, Y, D, and E. These proteolytic fragments are still extensively used in research, are important to understand fibrinolysis (see Chapter 20), and are used in some clinical assays.

Fibrinogen is first converted by plasmin to a clottable derivative, called fragment X, which is missing the *N*-terminal end of the Bβ-chains 1 to 42 and the *C*-terminal ends of the Aα-chains. Fragment X is then cleaved asymmetrically into fragment D and fragment Y, the latter in turn cleaved into another fragment D and fragment E. Fragment D is made up of Bβ-modules and a γ-module and approximately the distal half portion of the coiled-coil region. D-dimer (or double D) consists of two D fragments covalently cross-linked by FXIIIa. Fragment E is made up of the central domain and the proximal portion of both coiled coils. It is important to note that each of these fragments is made up of multiple domains, so the older practice of calling them domains is inappropriate, and they should be called D and E regions.

Carbohydrate

Each fibrinogen molecule contains two pairs of biantennary oligosaccharides attached *via* *N*-glycosyl bonds at Bβ364 and γ52. The amino acid motifs at these two sites are NXT and NXS, respectively, typical of *N*-glycosylation. The Aα-chains contain two NXS sequences but no attached carbohydrate, although extended AαE-chains do. Circulating fibrinogen is variably desialated, leading to heterogeneity, with about equal amounts of mono- and disialyated chains, but generally no asialo chains.[12,13]

The presence of carbohydrate on fibrinogen has important functional consequences. The most striking example comes from patients with cirrhosis and other liver disease who have hypersialated fibrinogen that forms clots with thin fibers and many branch points.[14] Conversely, if all sialic acid on fibrinogen is removed with neuraminidase, fibrinogen makes clots consisting of thicker fibers with fewer branch points.[15] Further, complete

removal of all carbohydrate has more extreme effects, leading to clots with extremely thick fibers and even fewer branch points.[16] These results indicate that the carbohydrate on fibrinogens modulate clot structure through effects of both charge and mass on polymerization, probably particularly lateral aggregation.

Circulating fibrinogen also contains variable amounts of nonenzymatic glycation. Patients with uncontrolled diabetes have increased levels of fibrinogen glycation due to the high concentrations of sugar in their blood, but even nondiabetics have several sugar residues attached. The effects of nonenzymatic glycation have not been fully elucidated, but higher levels of glycation appear to result in clots made up of thinner fibers and more branch points.[17–24]

Structure of the End Regions of Fibrinogen

The homologous β- and γ-modules are each made up of three domains, the *N*-terminal A-domain, central B-domain, and *C*-terminal P-domain,[25] but the two modules are oriented quite differently (FIGURE 16.4).[26] The β-module is folded back about 130 degrees with respect to the γ-module, as predicted from analysis of the molecular packing in fibrinogen crystals.[9]

The P-domain is named because it contains a major polymerization pocket. Before structure determinations of this part of fibrinogen, short peptides containing the Gly-Pro-Arg sequence, the *N*-terminal end of the α-chain of fibrin, were found to bind to fibrinogen and to be effective polymerization inhibitors.[27] Such peptides were incorporated into crystals of the γC-module and fragment D and were identified in the binding pocket of the P-domain.[28] There are only subtle changes in the conformation of the P-domain upon peptide binding.[29] The binding sites for the peptide are present on three extended loops that form a cavity with many negatively charged side chains complementary to the peptide amino acids. Residues in contact with the peptide are Gln329, Asp330, His340, and Asp364.

Peptides with the sequence Gly-His-Arg, corresponding to the *N*-terminus of the β-chain, also inhibit polymerization, but to a lesser extent.[27] In contrast to Gly-Pro-Arg binding to the γ-module, binding of Gly-His-Arg to the β-module results in a major conformational change.[29,30] Specifically, two carboxylates from BβAsp398 and BβGlu397 rotate inward to complete formation of the β-module P-domain–binding pocket.

There is one strand of β-sheet near the *C*-terminal end of the γ-chain that is inserted into the central B-domain. This strand can be pulled out of the γ-module without destroying its compact structure, which could be significant for FXIIIa-induced cross-linking and for the mechanical properties of fibrin.[31,32]

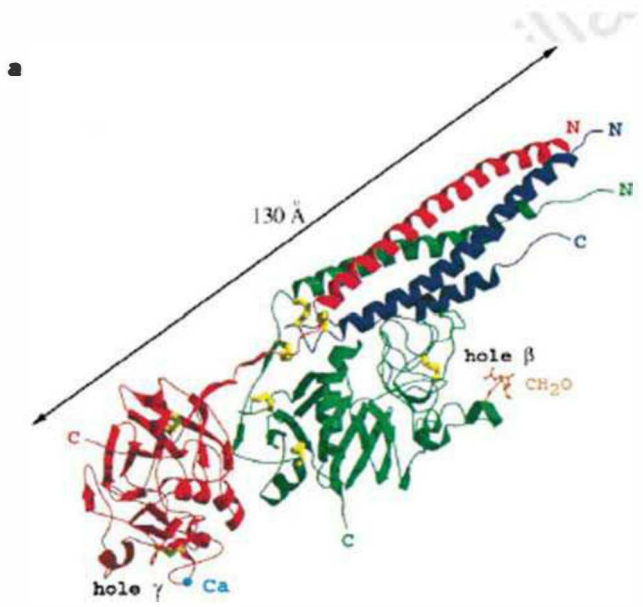

FIGURE 16.4 Crystal structure of fragment D. The Aα-chains are *blue*, the Bβ-chains are *green*, and the γ-chains are *red*. The four-stranded α-helical coiled coil is visible and the γ- and β-modules, with corresponding holes "a" (labeled hole γ) and holes "b" (labeled hole β). The *N*- and *C*-terminal ends of the polypeptide chains are indicated as is the carbohydrate-binding site (labeled CH_2O). (From Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its cross-linked counterpart from fibrin. *Nature* 1997;389:455–462.)

Structure of the Central Region of Fibrinogen

The central region contains portions of all six chains tightly folded into a compact structure held together by disulfide bonds.[4] In addition, there is an extensive "handshake" interface with many noncovalent interactions (FIGURE 16.5). Neither the A nor B fibrinopeptides (Fps) are visible in the crystal structure nor is the larger *N*-terminal end of the Bβ-chain. On one face of the central region, the Aα- and Bβ-chains from the two half molecules form a funnel-shaped domain with a hydrophobic core, while on the opposite face, the *N*-terminal γ-chains form a separate domain. Although the two halves of the molecule are identical in amino acid sequence, a pair of adjacent disulfide bonds constrain the two γ-chains to adopt different conformations. The resulting asymmetry of the molecule may be responsible for the twisting of oligomers and protofibrils.[33,34]

Coiled Coils

The central and end regions of fibrinogen are joined by α-helical coiled coils that are principally responsible for fibrinogen's elongated shape. Although the coiled coils represent only 30% of its molecular mass, fibrinogen was one of first proteins in which

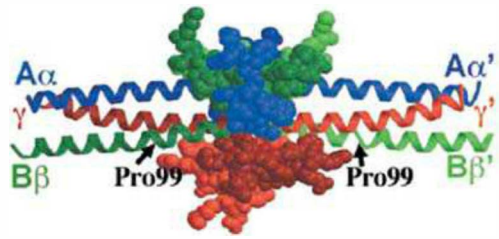

FIGURE 16.5 Crystal structure of fragment D. The Aα-chains are *blue*, the Bβ-chains are *green*, and the γ-chains are *red*. The central region is shown as a space-filling model while the three-stranded coiled coils are shown as ribbons. (From Madrazo J, Brown JH, Litvinovich, et al. Crystal structure of the central region of bovine fibrinogen E5 fragment at 1.4 Å resolution. *Proc Natl Acad Sci U S A* 2001;98:11867–11972.)

this structural element was identified from wide-angle x-ray diffraction patterns.[35] There are 111 or 112 residues in each of the three chains that form the coiled coils that are bounded at each end by disulfide rings. The amino acid sequences are largely consistent with those of typical coiled coils with nonpolar residues pointing inward forming a hydrophobic core shielded from solvent and charged residues exposed to solvent on the outside; there are specific interactions between the chains.[36,37] Near the middle of the coiled coil, there are residues less consistent with the canonical sequence, giving rise to a kink in the molecule.[38] This region is susceptible to proteolytic attack and is a major site of cleavage during fibrinolysis. The distal portion of the coiled coil has a fourth strand of α-helix formed from the *C*-terminal part of the Aα-chain.[39]

The coiled coils provide the length needed for fibrin polymerization and for the platelet cross-linking responsible for platelet aggregation. The coiled coils are stable and relatively rigid structures whose mechanical properties are likely critical for the overall viscoelastic behavior of fibrin clots. Although force spectroscopy experiments have suggested that the coiled coils may unfold,[40,41] the situation now appears to be much more complex. The γ-modules appear to unfold first, with the coiled coils acting as molecular capacitors, reversibly stretching to buffer the unfolding transitions of the γ-module.[32] The coiled coils also provide a surface containing some specific motifs for binding of other proteins.

Structure of the αC Region

Each αC region is made up of a relatively compact or globular region, the *C*-terminal portion of which is called the αC-domain and the extended *N*-terminal region, called the αC-connector. Together, the two αC regions comprise about 25% of the total mass of fibrinogen. The αC-domain has a β-hairpin turn and another loose β-hairpin turn, yielding a mixed parallel/antiparallel β-sheet structure.[42–44] The αC-connector is highly variable in different species of fibrinogen and is partially made up of 10 tandem repeats of 13 amino acids, each containing a characteristic Trp residue and rich in Gly, Ser, Thr, and Pro residues. Such sequences are found in extended poly-L-proline type II helices, which are left-handed with three residues per turn.[45] It is possible that the αC-connector also has such a structure, since these helices are highly hydrated, are stabilized by hydrogen bonds with water molecules, and tend to form interdomain links or "sticky arms."

In fibrinogen, the two αC regions interact with each other intramolecularly.[46–48] Following cleavage of the Fps, particularly FpB, the αC regions dissociate and interact intermolecularly. The αC regions interact specifically to form long, branching polymers, closely related to structures formed in fibrin clots.[46,49] In addition, these conformational changes on conversion of fibrinogen to fibrin expose binding sites for various ligands that are otherwise cryptic[49,50] (Tsurupa, 2009 #2483).

Structure of the AαE-Domain

A less common variant of the Aα-chain, called AαE, has an additional 236 amino acids at its *C*-terminal end.[51,52] Unlike the more common *C*-terminus of the Aα-chain, the AαE-domain does show significant homology to the β-module and the γ-module. In contrast to these structures, however, the binding cleft of the AαE-domain is essentially neutral and is not likely to bind Gly-Pro-Arg or Gly-His-Arg, suggesting that it does not participate directly in polymerization.[53] It has been proposed that this domain may act as a lectin-like carbohydrate-binding receptor.[53]

BIOSYNTHESIS AND METABOLISM

Gene Regulation

The three fibrinogen genes, *FGA*, *FGB*, and *FGG*, are expressed coordinately, such that the relative proportion of mRNA for the three chains is nearly equal.[54] They share common regulatory mechanisms, with cis-acting regulatory elements upstream of sites of transcription initiation.[55] However, a TATA-like sequence, a CAAT-like sequence, and a USF-binding site control liver-specific expression of FGG. In contrast, liver-specific expression of FGA and FGB is controlled by other transcription factors, such as hepatic-specific nuclear receptor.

Fibrinogen is an acute-phase protein whose levels increase in response to injury and the accompanying release of cytokines.[54] Upregulation of fibrinogen expression is controlled by interleukin 6 (IL6) through type II IL6-response elements in the 5'-flanking regions of all three fibrinogen genes. Fibrin(ogen) degradation products stimulate monocytes/macrophages to produce IL6 and hence upregulate fibrinogen synthesis. A glucocorticoid response element also upregulates fibrinogen expression.

There are also negative regulators of transcription, including the cytokines IL4, IL10, IL13, and transforming growth factor-β (TGF-β). In addition, TGF-β antagonizes IL6 induction of fibrinogen mRNA at late, but not early times, after IL6 treatment. The combined early and late effects of these cytokines may be responsible for the coordinate regulation of fibrinogen synthesis during inflammation.

Synthesis and Secretion

The polypeptide chains of fibrinogen are assembled in an ordered fashion in the rough endoplasmic reticulum of the liver.[56] Newly synthesized human Bβ-chains are secreted and used more rapidly than the other two chains that accumulate in intracellular pools. The interchain disulfide bonds at the ends of the coiled coil, the coiled coils themselves, and some disulfides between the two halves of the molecule are important for proper assembly of the molecule.

Tissue-Specific Synthesis

Most fibrinogen is synthesized in the liver, with a steady-state rate of production of 1.7 to 5.0 g per day and a large reserve capacity of up to 20-fold.[57] The half-life of circulating fibrinogen is 3 to 5 days, with the mechanism of turnover largely unknown. Coagulation and lysis accounts for only 2% to 3% of the total catabolism, and the classic catabolism pathways for plasma proteins do not apply.[58] Fibrinogen, but not the γ-chain variant, is present in platelet α-granules and appears to be taken up by the granules *via* receptor-mediated endocytosis. Fibrinogen synthesis, albeit in lower amounts, has been detected in tissues other than the liver, suggesting that it may function in structural or adhesive capacities in the extracellular matrix.[59]

FIBRIN POLYMERIZATION

Fibrinopeptide Release

Fibrin polymerization is initiated by the enzymatic cleavage of the Fps from fibrinogen by thrombin, yielding fibrin monomer. Specific Arg-Gly bonds are hydrolyzed in the Aα- and Bβ-chains, in part arising from a set of hydrophobic residues in FpA that bind to a complementary apolar region on thrombin, but other distinct regions of FpA are also involved in binding and catalysis.[60] FpB is released at a considerably slower rate than FpA. The ordered release of the Fps is dictated by the specificity of thrombin for its substrate[61] since replacement of FpB with a FpA-like sequence results in nearly equal rates of Fps release. As polymerization proceeds, the rate of FpB release increases greatly, suggesting that either it is preferentially released from polymers[62] or that conformational changes resulting from polymerization facilitate its release.[63,64]

Oligomer Formation

Although the Fps are small, their release has profound effects on the solubility of the molecule as a result of exposure of previously buried binding sites. The Fps mask knobs "A" that are complementary to holes "a" that are always exposed (FIGURE 16.6),[1,65] although the Fps and knobs are not visible in x-ray crystallographic structures of fragment E.[4] On the other hand, crystal structures of fragment D with peptides representing the *N*-terminal sequences of fibrin's α- and β-chains have demonstrated peptide-binding pockets in the γC- and βC-modules,[26,29,66,67] providing strong evidence that these are holes "a" and "b" respectively. It is not yet known if each peptide represents an entire knob, but it is likely that there are more extensive regions involved. Thus, two fibrin monomers thus interact with each other in a half-staggered manner, so that knob "A" fits into hole "a."[68,69] Because of the dimeric nature of the molecule, there are two "A"–"a" knob–hole interactions holding the two monomers together (FIGURE 16.6). However, it should be noted that fibrinogen can bind to fibrin monomer because fibrinogen has holes that can accept the exposed knobs on fibrin monomer. These fibrinogen–fibrin complexes are present most of the time and represent an important buffer mechanism to prevent the polymerization of small amounts of fibrin that is generated in the blood.[70,71] Additional monomers add longitudinally to the fibrin dimer forming larger oligomers (FIGURE 16.3B), which lengthen into protofibrils, defined as oligomers that are long enough to aggregate (~0.5 μm).[62,72] The "A"–"a" knob–hole interactions are sufficient to make fibers and a branched clot network.[73,74]

FpB cleavage exposes the knobs "B," but the functional consequences of this event are not clear. FpA cleavage alone produces clots made up of thinner fibers than those initiated by cleavage of both Fps. This led to the hypothesis that knob "A"–hole "a" interactions are responsible for the interactions within a protofibril, while knob "B"–hole "b" interactions are responsible for lateral aggregation.[75] However, lateral aggregation of protofibrils and fiber formation can occur without FpB cleavage, which is not consistent with this hypothesis.[73,74] Moreover, knob "B"–hole "b" interactions are potentially problematic because holes "b" are not at the ends of the molecule but 8 to 9 nm away. Although conformational changes accompanying conversion to fibrin could potentially shorten that distance, this effect is not likely to be substantial because the band pattern of fibrin indicates that the β-nodule has nearly the same spatial relationship to the γ-nodule in fibrin as in fibrinogen (FIGURE 16.3C).[9] On the other hand, the *N*-termini of the β-chain could be highly extended and act as "crab claws" to reach the holes.[76,77] Evidence that knob "B"–hole "b" interactions can actually occur in fibrin come from the dysfibrinogenemias Metz and Frankfurt XIII in which all fibrinogen molecules are homodimers from which FpB, but not FpA, can be cleaved yielding clots with thin fibers at low temperature.[74,78] In addition, recombinant mutants of γ364 that have no functional holes "a" form clots slowly with thrombin but not with reptilase.[79] Thus, it appears that B:b bonds can occur at least when A:a interactions are compromised, but their role under physiologic circumstances remains unclear. There is no evidence for A:b or B:a interactions in fibrin polymerization.[80,81]

Addition of a third molecule to a half-staggered fibrin dimer produces an end-to-end junction where the ends of two molecules abut each other. This D–D interface is an important feature of most of the known crystal and microcrystal forms.[26,38,82] At this interface, γArg275 makes contact with γTyr280 and γSer300. These interactions produce a broad interface that is likely to be similar to that at the end-to-end junction between monomers in protofibrils.

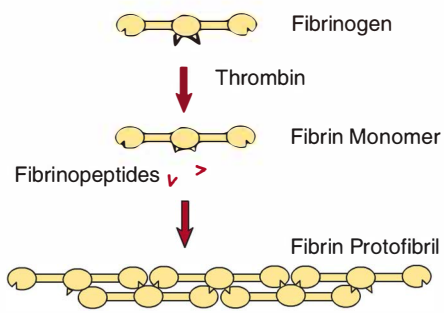

FIGURE 16.6 General schematic diagram of complementary binding sites or knob–hole interactions in fibrin polymerization. Fps in the central region cover knobs that are complementary to holes that are always exposed at the ends of the molecule. When the knobs are removed by fibrin, knob–hole interactions occur, giving rise to the two-stranded protofibril made up of half-staggered fibrin monomers. (From Weisel JW. Fibrinogen and Fibrin. *Adv Protein Chem* 2005;70:247–299.)

Protofibril Growth by Lateral Aggregation

The existence of a structure, called the protofibril, was based on the observation that oligomers must reach a certain length before they can aggregate laterally, so protofibrils are essentially longer oligomers.[68] Structurally they are two-stranded structures made up of half-staggered fibrin monomers. Protofibrils are twisted structures, with a long pitch.[34,69]

Protofibrils associate with each other laterally to make fibers (FIGURE 16.7). Because protofibrils do not associate until they reach a length of at least 0.5 μm,[62,72] it is likely that the interactions responsible for lateral aggregation are weak and additive over the length of the protofibril. However, little is known about the nature of these interactions or their localization. Potential clues come from the analysis of interactions between D fragments in crystals (FIGURE 16.8).[76] Although there is as yet no evidence that the crystallographic contacts are present in fibrin, structural proteins that self-assemble do tend to use similar

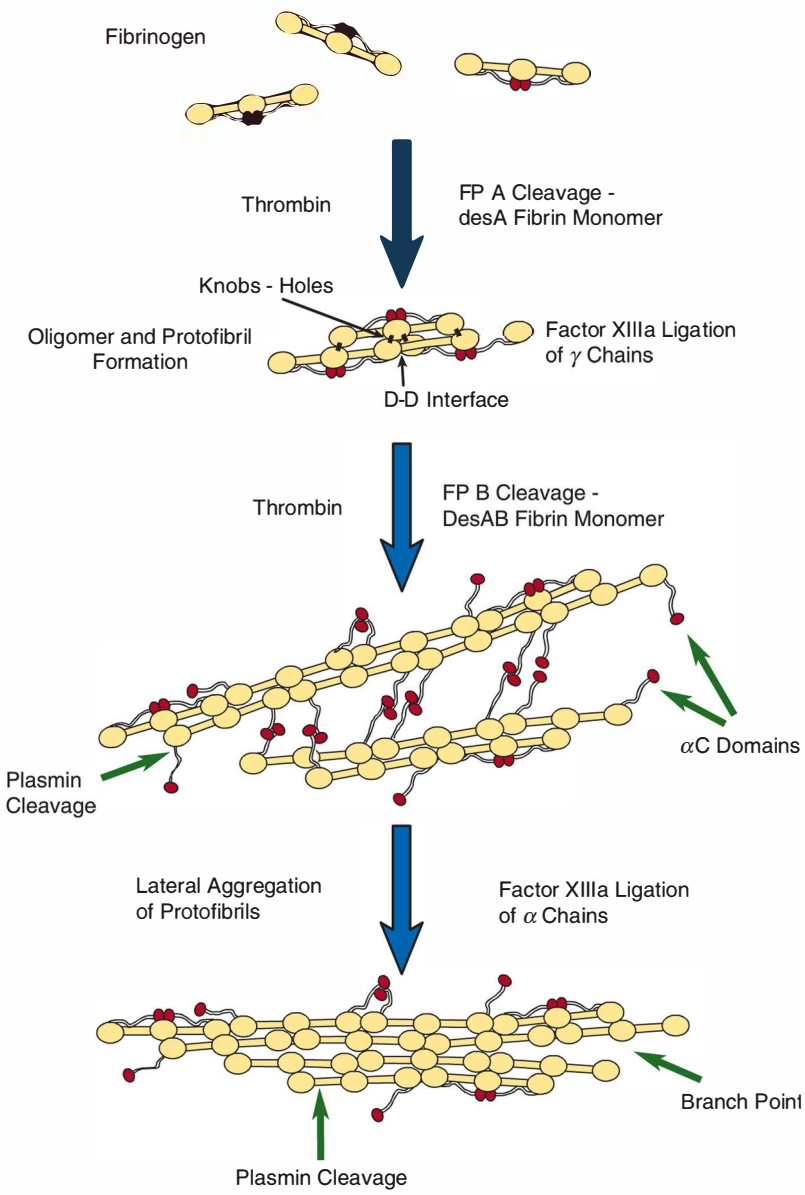

FIGURE 16.7 Schematic diagram of fibrin polymerization. FpA is cleaved first, producing desA fibrin monomers, which aggregate *via* knob–hole interactions to make oligomers. The D–D interface between the γ-modules of two fibrin monomers is shown. The oligomers elongate to make protofibrils. FpB is cleaved primarily from polymeric structures. Protofibrils aggregate laterally to make fibers, enhanced by interactions between αC regions. Factor XIIIa cross-links γ-chains more rapidly than α-chains. Plasmin cleaves the αC regions and at β42 and then cuts across fibrin in the middle of the coiled coil. At the bottom of this figure, a branch point has been initiated by the divergence of two protofibrils. (From Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem* 2005;70:247–299.)

binding interactions *in vitro*.[82,83] In this scheme, the D regions of two laterally aligned protofibrils interact, such that residues γ350-360 and γ370-380 are shielded from water. In addition, the αC regions of fibrin interact with each other intermolecularly and enhance lateral aggregation (FIGURE 16.7).[47–49,84] Although they are not required for lateral aggregation, clots made from fibrinogen missing the αC regions are made up of thinner fibers.

Although such binding interactions may be present in fibrin, packing in fibrin is not crystalline, but paracrystalline.[85,86,87] Consequently, fibrin molecules are in good register in the longitudinal direction but are less well ordered across the fibers.[88] In addition, the twist present in protofibrils leads them to twist around each other to make twisted fibers (FIGURE 16.3B).[34] Because the periodicity of 22.5 nm is maintained as new protofibrils are added (FIGURE 16.3C), they must be stretched as their path length increases as fibers grow thicker. It has been proposed that fibers stop growing laterally when the energy necessary to stretch an added protofibril exceeds the energy of bonding.

Fibrin Branching

Fiber branching is necessary to produce a three-dimensional network. Although little is known about mechanisms of branching, one clue comes from the fact that fiber ends are seldom observed in an undamaged clot. Thus, it is likely that protofibrils do not add to a growing fiber with their ends in register but with random longitudinal spacing and that protofibrils of different lengths and smaller oligomers add to the growing fiber.

Nearly all branch points in fibrin clots are trifunctional, in other words, a junction of three fibers.[89] Observations of branching by either scanning electron microscopy or transmission electron microscopy of negatively contrasted specimens show that the diameters of all three fibers at a branch point are often, but not invariably, similar (FIGURE 16.9). There

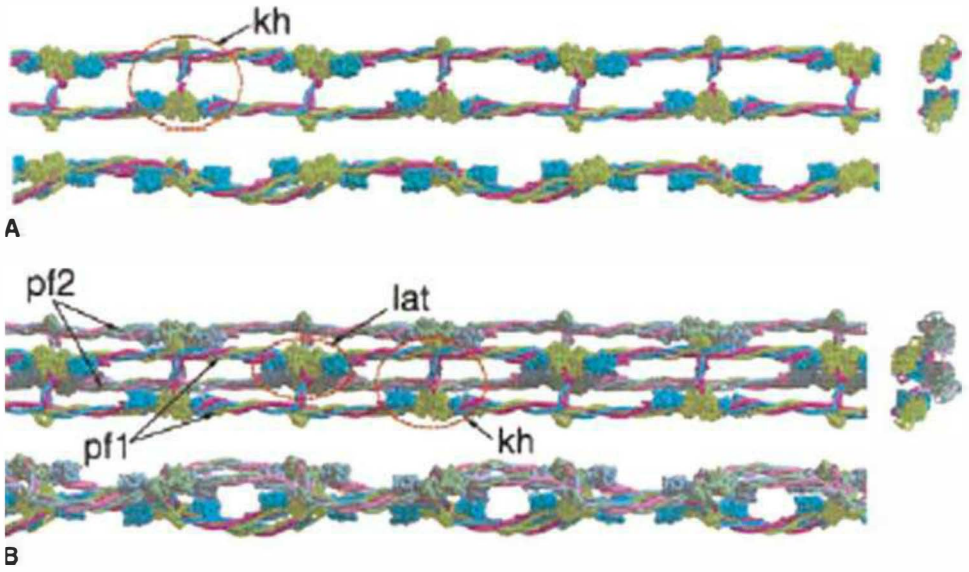

FIGURE 16.8 Potential model of protofibril structure and lateral aggregation from crystal structure packing. Space-filling models of the x-ray crystallographic structure of fibrinogen (missing some parts) were used together with knowledge about fibrin interactions to make a theoretical model for a protofibril and lateral aggregation between protofibrils. This model also uses contacts between fragments D that have been observed in the crystals, assuming that such interactions may also be present in fibrin. **A:** Top, side, and end-on views of a two-stranded protofibril, with the *red oval* outlining the knob "A"–hole "a" (kh) interactions that are responsible for fibrin polymerization. Note that the structure of the holes is known from the crystallographic data but not the knobs. **B:** Top, side, and end-on views of two protofibrils (pf1 and pf2) with presumed interactions between them. One *red oval* outlines the knob–hole interactions (kh) within a protofibril and the other *red oval* outlines lateral (lat) interactions between protofibrils. (From Yang Z, Mochalkin I, Doolittle RF. A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. *Proc Natl Acad Sci U S A* 2000;97:14156–14161.)

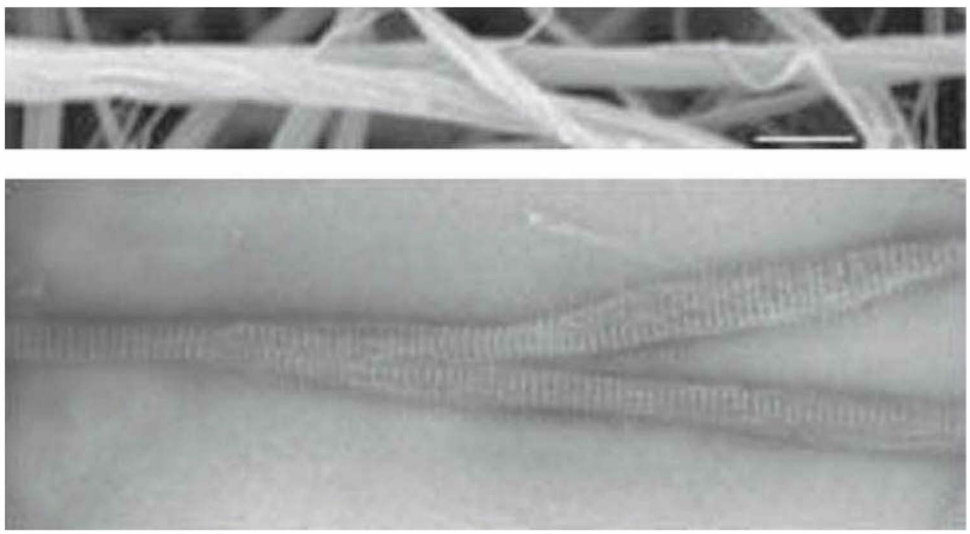

FIGURE 16.9 Electron microscope images of fibrin branch points. Nearly all branch points observed by electron or light microscopy are trifunctional, that is, they are formed by the junction of three fibers. The fibers can be of different or similar diameters. **Top:** Scanning electron micrograph of branch point. Note that the images show that fibers are made up of protofibrils that twist around each other. **Bottom:** Transmission electron micrograph of negatively contrasted fibrin fibers at a branch point. Note that the band patterns of the three fibers are aligned with each other. (From Weisel JW. The mechanical properties of fibrin for basic scientists and clinicians. *Biophys Chem* 2004;112:267–276.)

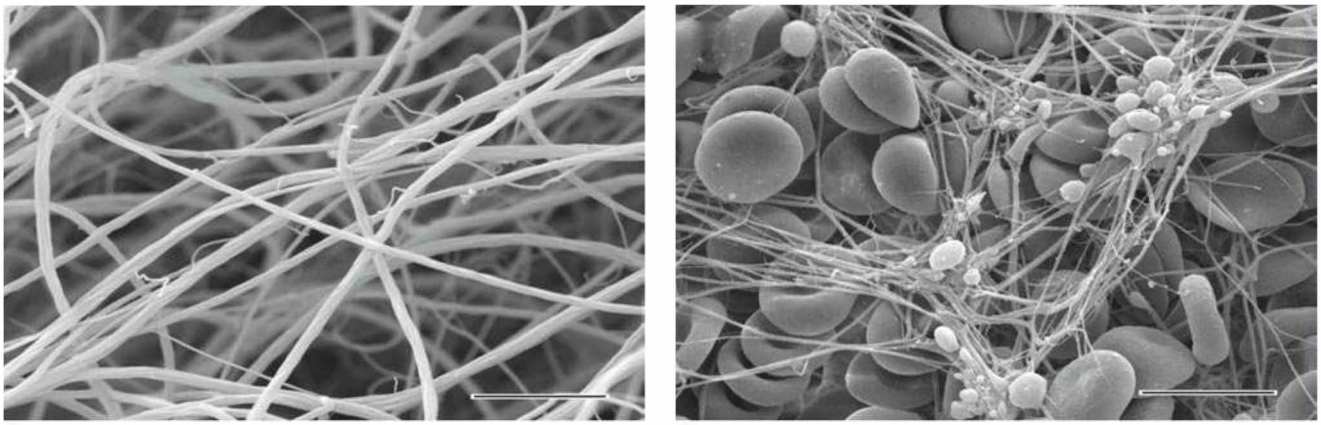

FIGURE 16.10 Scanning electron microscope images of clots. **A:** Clot formed by the addition of thrombin to purified fibrinogen. Note that in such images it is sometimes difficult to distinguish between true branch points and fibers crossing at different depths in the clot. Magnification bar, 5 μm. **B:** Whole blood clot made from freshly drawn blood with no additions. Fibrin fibers commonly originate from platelet aggregates, and red and white blood cells are trapped in the meshwork. Magnification bar, 10 μm. (From Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem* 2005;70:247–299.)

is a general correspondence between numbers of branch points and fiber diameter; as the number of branch points increase, the fiber diameters decrease.[89] Together, these observations suggest that branching and lateral aggregation are opposite sides of the same coin, that is, conditions that favor lateral aggregation tend to produce clots with thick fibers and few branch points, while conditions that inhibit lateral aggregation tend to yield clots made up of thin fibers with many branch points.

At the molecular level, branches could result from two protofibrils that associate over part of their lengths and then diverge from each other or from the addition of two fibrin monomers that diverge from each other at the end of an individual protofibril.[90]

Fibrin Structure and the Gel Point or Clotting Time

Once the branching fibers form a space-filling, three-dimensional network, a clot or gel exists (FIGURES 16.10 and 16.11). The gel point or clotting time has been used as an indication of altered or pathologic coagulation. The gel point occurs relatively early in polymerization, perhaps when only 10% of the fibrinogen has been incorporated into the gel. Under standard conditions, however, it is a sensitive measure of the overall kinetics of the clotting cascade, and hence an indicator of abnormalities. Although there are correlations between gel time and final clot structure,[91] the complete and final network is generally not established at the gel point, with new fibers and branch points still being established after the gel point has been reached.[92]

The structure of a clot can be characterized by fiber diameter, density, number and nature of the branch points, distances between branch points, and the size of the pores (FIGURE 16.10). Although diffusion of proteins is rarely affected by the fiber network, perfusion of fluid through the gel is a sensitive measure of pore size and hence clot structure.[93]

Role of Calcium in Fibrinogen Structure and Function

Calcium binding to fibrinogen is important for its structural stability and function. X-ray crystallography has revealed one calcium ion in the γ-module and two in the β-module.[25,26] Specific residues in the calcium-binding loop, γ311-336 (as

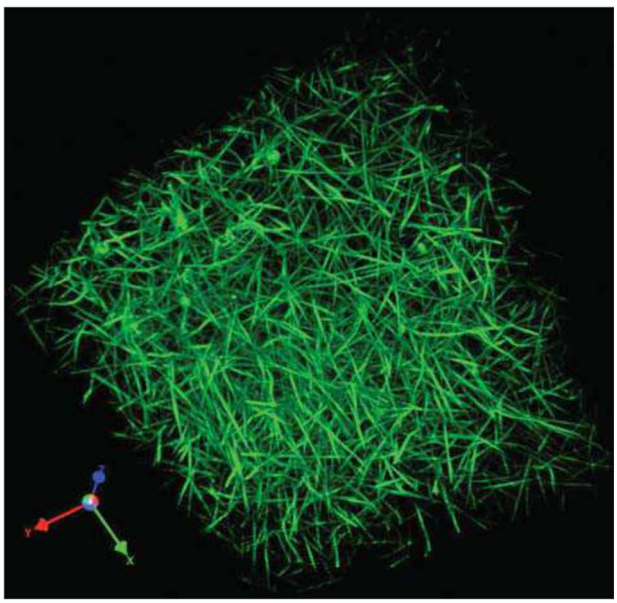

FIGURE 16.11 Three-dimensional structure of a hydrated fibrin clot. Three-dimensional reconstruction of optical sections by confocal microscopy of a fibrin clot made by the addition of thrombin to plasma, spiked with fluorescently labeled fibrinogen. (From supplementary online materials of Brown AE, Litvinov, RI, Discher, et al. Multiscale mechanics of fibrin polymer: gel stretching with protein unfolding and loss of water. *Science* 2009;325:741–744.)

well as corresponding residues in the βC-module), together with two strongly bound water molecules, form a high-affinity calcium-binding site that differs from a classic EF hand. Since the dissociation constant for calcium ion binding to these sites is much lower than the free calcium ion concentration in plasma, the sites should normally be fully occupied in fibrinogen. Physiologic concentrations of calcium are important for maintaining the stability of fibrin(ogen), protecting against plasmin digestion and denaturation by heat and high temperature, as well as the extent of reduction of disulfide bonds.[94–96]

There are also lower affinity calcium-binding sites that may have regulatory roles, some of which may bind to sialic acid.[15] Several lower affinity calcium-binding sites are associated with interactions of the βC-module with the coiled coil.[29,67,97] A movable flap made up of β397Glu and β398Asp is pinned back to the coiled coil with a calcium bridge until the GHR peptide occupies the βC-module–binding pocket.

There is no effect of calcium on thrombin-catalyzed Fp release, but it does have effects on subsequent polymerization step(s). Key calcium-binding residues in the γC-module appear to be necessary for protofibril formation.[98] Calcium increases the rate and extent of lateral aggregation, such that thicker fibers are formed at higher calcium ion concentrations. There is a calcium-dependent conformational change in fibrin that is correlated with the release of FpB,[99] and simultaneously, two calcium-binding sites shift from low to high affinity,[100] which may be related to the structural changes observed by x-ray crystallography.[29,67]

The peptide representing the knobs "B," GHRP, binds 10 times more strongly to hole "b" in the presence of 2 mM calcium than in its absence. Correspondingly, the βC-modules have low-affinity binding sites,[66] and mutation of one of them resulted in increased lateral aggregation, consistent with a role for knob "B"–hole "b" interaction involvement in lateral aggregation.[97]

Factor XIIIa–Catalyzed Cross-Linking of Fibrin

FXIIIa facilitates clot stability by catalyzing the formation of covalent by Nε-(γ glutamyl) lysine bonds between fibrin molecules. FXIIIa also cross-links other proteins, including α_2-antiplasmin, plasminogen activator inhibitor-2 (PAI-2), and fibronectin, to fibrin. FXIII is discussed in detail in Chapter 17, but several aspects of FXIII relevant to fibrin properties are discussed here.

γ-Chain Cross-Linking

Fibrinogen γ-chains are cross-linked more rapidly than α-chains and γ-chains are cross-linked more rapidly in fibrin than in fibrinogen. The latter results from the physical proximity of the acceptor and donor sites in fibrin.[101] Residues γGln398/399 and γLys406, located near the *C*-terminal end of the γ-chain, are cross-linked.[102] The three-dimensional structure of the cross-links is yet unknown, since the crystal structure of cross-linked human D-dimer does not show the region where the cross-links are present, likely because of the flexibility of the end of the γ-chains. However, half of one cross-link is visible in the crystal structure of lamprey D-dimer.[103] There is still controversy about whether the γ-chains of fibrin are cross-linked longitudinally or transversely; evidence for both viewpoints is summarized elsewhere.[104–107] The slow formation of γ-chain multimers confers greater resistance to fibrinolysis.

α-Chain Cross-Linking

Cross-linking *via* α-chains occurs more slowly, with multiple bonds per molecule. The α-chains contain potential glutamine acceptor sites at residues 221, 237, 328, and 366 and lysine donor sites at residues 508, 539, 556, 580, and 601.[102,108] The noncovalent association of αC regions brings acceptor and donor sites in proximity, facilitating the formation of the isopeptide bonds catalyzed by FXIIIa. The result is a network of αC regions, called αC polymers.

MECHANICAL PROPERTIES OF FIBRIN

The unique mechanical properties of fibrin are essential for its functions in hemostasis and wound healing since fibrin must be rigid enough to stop bleeding and yet open for perfusion of liquid and penetration of cells. The mechanical properties of fibrin in a thrombus will determine how it responds to flowing blood.[109] Reversible elastic deformation, irreversible plastic deformation, and embolization are all responses that can have serious clinical outcomes. Clots of low stiffness may lead to bleeding, while stiff clots are associated with premature coronary thrombosis.[110] Moreover, the effects of nearly all therapeutic regimes involving clots or thrombi, including thrombolysis, percutaneous coronary interventions, and surgery, are dependent on these mechanical properties.

Fibrin forms a branched three-dimensional network that is a viscoelastic polymer.[109] Finite stiffness is first established when a continuous network is established at the gel point and the stiffness increases over time as polymerization proceeds. Stiffness is also increased by the formation of covalent bonds introduced by FXIIIa, making the gel more stable mechanically and more resistant to proteolytic digestion. The viscoelastic properties determine how the clot responds to the forces to which it is subjected, such as flowing blood or contraction of muscle. A stiff clot will deform less than a softer one with the same applied stress. A clot with a larger inelastic or viscous component will incur more irreversible deformation than one with a smaller inelastic component.

Clots are highly extensible, meaning that they can be greatly stretched before breaking.[111] At least part of the reason for this behavior is the great extensibility of individual fibers making up the clot,[112,113] but the structural rearrangements that occur with stretching also play a role.

For small strains over short times, many clots are highly elastic with little inelastic component,[114] meaning that stiffness remains the same under a variety of conditions during the application of force, which can be quite different depending on whether the clot is arterial or venous. The origins of the inelastic component of fibrin are unknown, but it has been suggested that protofibrils may slide past each other, or knob–hole bonds may be broken and reformed, or certain domains of the fibrin may unfold.[109] In contrast to most nonbiologic polymers, fibrin exhibits strain stiffening or hardening, which means that the stiffness increases with increasing strain.[115–117] This nonlinear elasticity is probably important to prevent damage to clot structures at high strains.

The viscoelastic properties of clots can only be understood by integration of their material properties over all levels of structure, including macroscopic, network, fiber, and molecular. The imposition of stress on a clot causes distinctive changes at each level of structure. Initially, fibers align

along the direction of strain, bundling fibers into higher order structures. Then, fibers become aligned as a result of the strain, stretching, which causes unfolding of some domains, exposing buried hydrophobic residues, which aggregate and expel water.[111]

The bundling of protofibrils into fibers also makes their response to large strains unique with a built-in stress response, which has been used in a hierarchical model for clot mechanical properties.[118] There is also evidence that the αC region of fibrinogen is the origin of the low modulus, high extensibility, and strain stiffening that characterizes fibrin fibers.[119,120]

Variation in Fibrin Clot Structure and Properties

The complexity of fibrin polymerization and the ordered sequences of steps results in the possibility of a variety of clot structures and properties that are unequaled by any other biologic polymer. The factors that affect clotting can be divided into four general categories: (a) variations in protein structure; (b) environmental conditions of polymerization; (c) the presence of cells; and (d) fluid flow.

Splice variants, single-nucleotide polymorphisms, and an array of modifications to various parts of the molecule, including carbohydrate, proteolytic digestion, phosphorylation, tyrosine sulfation, oxidation, nitration, proline hydroxylation, asparagine or glutamine deamidation, glutamine cyclization, and acetylation, result in clots of different structure. Nearly all dysfibrinogenemias have effects on fibrin polymerization.

Ionic conditions, including pH and ionic strength, as well as albumin and other proteins present in plasma, have effects on clot structure. Thrombin concentration has a profound effect, with high concentrations producing clots made up of many thin fibers, many branch points, and small pores and lower concentrations producing clots composed of fewer thick fibers, fewer branch points, and large pores. Many proteins bind specifically to fibrin(ogen) and affect polymerization. Most of these observations are accounted for by the effects on the kinetics of fibrin polymerization and the effects on individual steps of polymerization.[121]

Physiologic clots contain variable numbers of platelets, which have a profound effect on clot structure. Clots formed in platelet-rich plasma contain platelet aggregates surrounded by dense networks of fibers that are thinner than other fibers in the clot; many radiate out from the platelets (FIGURE 10B).[122] Other intravascular and extravascular cells also have effects on the fibrin formed in their vicinity.[123,124] The effects of erythrocytes are complex. Depending on the number incorporated into a clot, they affect fiber thickness, alter mechanical properties, and increase heterogeneity (FIGURE 10B).[125]

Clots or thrombi formed in the vasculature experience the effects of blood flow. Clotting *in vitro* ceases when all fibrinogen is converted to fibrin and polymerizes. In normal plasma containing about 2.5 g/L of fibrinogen, such clots will contain only 0.25 g/100 mL, or 0.25% protein. In contrast, flow *in vivo* continually brings more fibrinogen to the forming clot, which can as a consequence be much more dense. In addition, the dynamics of flow results in heterogeneity throughout the thrombus, with a platelet-rich head upstream and fibrin-rich tail downstream. Flow can also cause orientation of the fibrin fibers.[126,127]

INTERACTION OF FIBRIN(OGEN) WITH OTHER PLASMA PROTEINS

Thrombin

Fibrin contains two classes of thrombin-binding sites that differ in location and affinity. The anion-binding exosite I of thrombin interacts with a negatively charged surface in the central region of fibrinogen such that its catalytic triad can engage FpA.[128,129] Two thrombin molecules can bind simultaneously to one fibrinogen molecule. The exosite II of thrombin recognizes sulfated tyrosines at γ418 and γ427.[60,130,131] Optimum high-affinity binding requires both exosites.[132]

Thrombin binds strongly enough to fibrin that most thrombin is sequestered in clots or thrombi. Thus, what had been called antithrombin I is actually fibrin.[133,134] There are important clinical consequences for thrombin binding to fibrin, in that it limits the extent of clotting. For example, individuals with fibrinogen mutations that impair thrombin binding tend to have serious thromboembolic disease.[135,136]

Factor XIII

The affinity of FXIII for fibrinogen (K_d 10 nM) is such that nearly all circulating FXIII zymogen circulates as a FXIII–fibrinogen complex. Interactions of fibrin(ogen) with FXIII, thrombin, and calcium generate active FXIIIa *via* thrombin cleavage of FXIII, followed by calcium-dependent release of active α_2-subunits. FXIII binds strongly to the γ form of fibrinogen and is most likely the carrier of the zymogen in blood.[137,138,139] In the ternary complex of fibrin–thrombin–FXIII, FpA is cleaved more than 40× faster than FXIII activation, likely reflecting the difference in concentration of the two thrombin substrates.[140]

Fibronectin

The adhesive protein fibronectin not only binds to cells *via* integrins but also to a number of proteins, including fibrinogen, collagen, and gelatin, that play important roles in cell adhesion and wound healing.[141] Fibronectin binds to fibrinogen and is covalently cross-linked by FXIIIa.[142] Fibronectin binding affects fiber diameter and density in clots, with the magnitude determined by the concentration of fibronectin and whether or not it cross-linked to fibrin.

Thrombospondin

Thrombospondin stabilizes platelet aggregates by binding to fibrinogen and platelet integrins.[143,144] Thrombospondin binds to fibrinogen[145] and causes a decrease in fiber diameter and increase in the number of fibers in clots.[121,146] The thrombospondin-binding regions of fibrinogen are the αC region[147] and/or in the middle of the Bβ- and γ-chains.[144]

von Willebrand Factor

von Willebrand factor (vWF) binds noncovalently to fibrinogen, but can be cross-linked to it by FXIIIa.[148,149] High molecular weight multimers of vWF released from endothelial cells bind to fibrin. vWF primary function is to anchor platelets to fibrin under high shear conditions and also in the formation of the platelet–fibrin thrombus.

Fibulin

The extracellular matrix protein, fibulin-1, was first isolated as a complex with fibrinogen, can bind to the *C*-terminal end of the Bβ-chain with high affinity, and is incorporated into clots and thrombi-containing fibrin.[150] Fibulin-1 supports platelet adhesion to the extracellular matrix under flow *via* fibrinogen bridges.[151]

Heparin

Heparin binding to fibrinogen may modulate the procoagulant activity of thrombin or the fibrinogen binding to cell surfaces. The heparin-binding site has been shown to be β15 to 57. However, the intrinsic dimeric structure of fibrin(ogen) is essential for high-affinity heparin binding.[152]

Growth Factors

The provisional fibrin matrix localizes growth factors to the site of injury, thereby enhancing cellular processes for repair and regeneration. Thus, fibroblast growth factor-2 binds to fibrin at two sites,[153,154] whereas IL-1β, but not IL-1α, binds to fibrinogen and fibrin at the same or similar sites.[155] Similarly, vascular endothelial growth factor binds to two classes of sites on either fibrinogen or fibrin.[156]

INTERACTION OF FIBRINOGEN AND FIBRIN WITH PROTEINS OF THE FIBRINOLYTIC SYSTEM

Plasmin(ogen) and t-PA

Both fibrinogen and fibrin are degraded by plasmin. In this context, fibrin acts as cofactor in tissue-type plasminogen activator (tPA)-induced plasminogen activation, thus linking fibrin formation and fibrin proteolysis.[157] However, the role of fibrinogen and fibrin as substrates for plasmin is independent of the cofactor function of fibrin in plasminogen activation.[157]

tPA is produced as a fully active enzyme and is constantly released into circulation by endothelial cells,[158] although its release is strongly increased by venous stasis[159] or other causes of endothelial activation. Enhancement of tPA-induced plasminogen activation requires binding of both tPA and plasminogen to specific binding sites on fibrin that are generated by conformational changes that occur when fibrinogen is converted to fibrin.[160,161] Fibrinogen is largely inert, limiting the action of tPA to fibrin, where it is dependent upon the interaction of two D regions of adjacent fibrin units with a fibrin E region within the polymer.[162] Fibrin formation results in an approximately 1,000-fold enhancement of plasminogen activation rate[163] and has been utilized for development of an assay system for the measurement of soluble fibrin.[164]

Two fibrin(ogen)-binding sites for plasminogen and tPA have been identified: γ312 to 324, and Aα148 to 160.[165] These sites are cryptic in fibrinogen and fibrin monomers,[50] and their exposure is linked to fibrin polymerization.[166] The γ312-324–binding site is located adjacent to parts of hole "a," whereas Aα148-160 is buried by the β-module and the fourth helix of the Aα166-195 region.[50] The conformational changes leading to exposure of both sites are induced by the binding of knobs "A" to holes "a."[167]

Additional binding sites for tPA and plasminogen are present in the αC region of fibrinogen.[168] More binding sites are formed in the course of fibrin degradation by exposure of *C*-terminal lysine residues at plasmin cleavage sites, further enhancing plasminogen activation and fibrin degradation. The stimulatory effect of soluble fibrin complexes on tPA-induced plasminogen activation is lost upon fibrin cross-linking.[169] tPA binding to fibrin is inhibited by lysine.[170]

Fibrin proteolysis is not limited to particulate fibrin clots, but also applies to soluble fibrin complexes. At high levels of plasmin, proteolysis may extend to fibrinogen, resulting in fibrinogenolysis and the formation of fibrinogen degradation products. Such high levels of plasmin are observed during infusions of streptokinase[158] and during treatment with thrombin-like snake venom enzymes, such as ancrod.[171,172] It can also be seen with trauma[173] and in some cases of disseminated intravascular coagulation.

During fibrin proteolysis, thin fibers are dissolved more rapidly than thick fibers,[174,175] although in fibrin clots, plasmin appears to move laterally across fibers, the rate of lysis being faster for clots made up of thicker, rather than thinner, fibers.[175,176] Plasmin proteolysis releases large fibrin fragments from fibrin clots.[175,177] Further degradation of these fragments is dependent upon the availability of active plasmin. Consequently, a considerable proportion of the fibrin degradation products found in clinical blood samples have a higher molecular weight than the terminal fibrin degradation products generated during *in vitro* fibrin proteolysis.[178] Therapeutic fibrinolysis caused by infusion of profibrinolytic agents proceeds from the surface of the clot.[176]

PAI-1

PAI-1 is the primary inhibitor of tPA and is produced by many cell types, including endothelial cells and hepatocytes. It is released from platelets following platelet activation, resulting in high PAI-1 concentrations in platelet-rich clots.[179] Binding to fibrin protects tPA from PAI-1 inhibition.[176]

TAFI

TAFIa (thrombin-activated fibrinolysis inhibitor a) is an antifibrinolytic enzyme that is converted from a proenzyme by thrombin bound to thrombomodulin[180,181]; it can also be activated by plasmin.[182] TAFIa is a carboxypeptidase that cleaves lysine residues exposed by plasmin proteolysis, reducing the cofactor effect of fibrin on tPA-induced plasminogen activation, thereby inhibiting further proteolysis by plasmin.[183] Conversion of TAFI (the procarboxypeptidase) to TAFIa (the active carboxypeptidase) requires a high concentration of thrombin. TAFI is a FXIIIa substrate and is covalently bound to fibrin.[184]

α_2-Antiplasmin

α_2-antiplasmin, the main inhibitor of plasmin in blood,[179,185] is covalently linked to fibrin,[162,186–188] as well as to fibrinogen, by FXIIIa.[189] Cross-linking to fibrin is strongly enhanced by cleavage of an *N*-terminal 12-residue peptide from Met-α_2-antiplasmin secreted from the liver cells by antiplasmin-cleaving enzyme.[190] The resulting Asn-α_2-antiplasmin is cross-linked to fibrin 12-fold faster than Met-α_2-antiplasmin. Fibrin-bound α_2-antiplasmin renders the fibrin more resistant to plasmin proteolysis.[189] Thus,

the antifibrinolytic effect of FXIIIa is fully explained by the covalent incorporation of α_2-antiplasmin into the clot.[189]

Prior to this covalent incorporation, α_2-antiplasmin is noncovalently adsorbed to polymeric fibrin, as well as surface-adsorbed fibrinogen and fibrin.[191] Binding occurs to the D and the αC regions of fibrinogen and fibrin molecules with plasminogen enhancing binding to the D region. The binding sites are cryptic in fibrinogen, but become accessible upon formation of polymeric fibrin and upon surface adsorption of fibrinogen (both mechanisms, fibrin polymerization and surface adsorption, evidently leading to similar conformational changes).[191]

α_2-antiplasmin is cross-linked to lysine Aα303 in the *C*-terminal part of the Aα-chain of fibrinogen and fibrin.[192] It has been hypothesized that under certain circumstances, such as low α_2-antiplasmin levels, covalent binding of α_2-antiplasmin to fibrin clots may be reversible[193] with FXIIIa-dependent release of free α_2-antiplasmin. It also has been shown that FXIII may covalently link α_2-antiplasmin to fibrinogen.[194]

PAI-2

PAI-2 is also covalently attached to fibrin by FXIIIa.[195] PAI-2 is produced by monocytes and placental trophoblast cells and is released in response to stimulation by thrombin.[195] PAI-2 is attached to lysine residues within the Aα-chain of fibrin(ogen).[196]

PLASMIN DEGRADATION

Fibrinogen

Plasmin has trypsin-like specificity, with an affinity for the hydrolysis of lysyl and arginyl bonds, although only 50 to 60 of the potential 362 lysine and arginine residues of fibrinogen are cleaved, 10% rapidly and the remainder more slowly.[197] Degraded fibrinogen can be separated by ion-exchange chromatography into five fractions (A, B, C, D, and E), of which fragments D and E are the major end products of the original molecule.[198] The identification and characterization of the intermediate fragments X and Y[199,200] provided the insight into the asymmetric scheme of fibrinogen degradation[201] (FIGURE 16.12, **left**). The cleavages responsible for these fragments and their composition have already been described above ("*Structural Insights from Limited Proteolysis of Fibrinogen*" section). Fragment X is slowly coagulable by thrombin, but fragments Y and D have potent antipolymerizing effects, mostly a result of disruption of the proper alignment of fibrin protofibrils

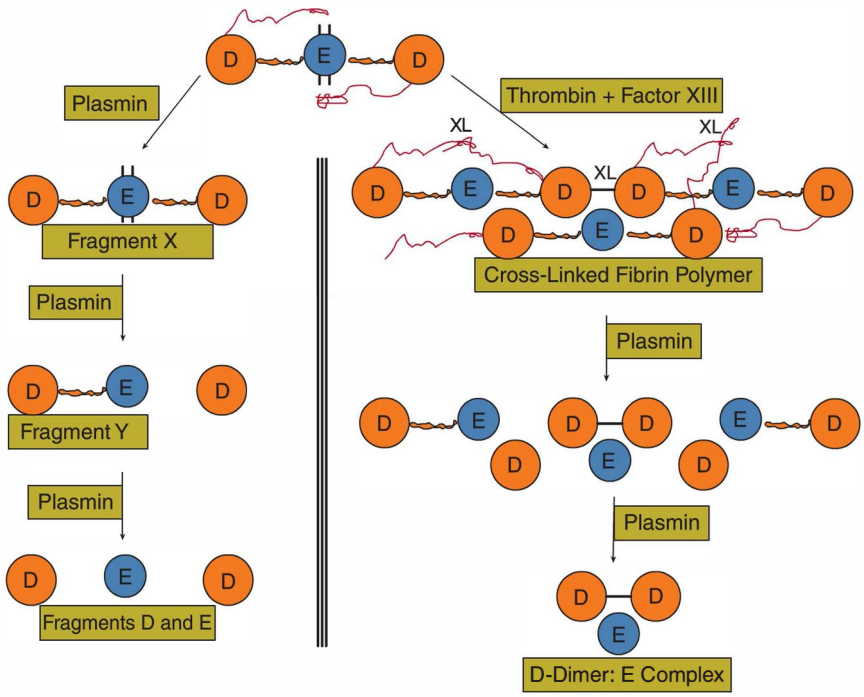

FIGURE 16.12 Plasmic degradation of fibrinogen and of cross-linked fibrin by plasmin. The principal structures of fibrinogen **(top)** are three globular regions from which degradation fragments D and E derive, the α-helical coiled coils that connect them, and Aα-chain extensions from the carboxy-terminal region of the D regions. Shown on the **(left)**, fibrinogen is degraded asymmetrically[201] by cleavage of the coiled coils. Intermediate degradation fragment X consists of all three nodules connected by coiled coils, but lacks the Aα-chain extensions and the Bβ1-42 sequence. Fragment Y consists of the central E region connected by a coiled coil to a fragment D region. Formation of cross-linked fibrin from fibrinogen is shown schematically on the right, by enzymatic actions of thrombin and factor XIIIa. Thrombin liberates Fps from the E region, and factor XIII cross-links (XL) fibrin longitudinally between D regions and in a less well-defined manner between the α-chain extensions. Plasmic cleavage of the two-stranded protofibrils removes the cross-linked α-chains first and then cleaves the coiled coils to liberate a series of complexes, the smallest of which is DD/E. Complexes that are larger than DD/E (such as DY/YD) also are liberated from cross-linked fibrin, but they are subsequently degraded to the DD/E moiety.

through competing for fibrin knobs.[200–202] Fragment E contains the amino-terminal portion of all six polypeptide chains.[200] In the presence of physiologic concentrations of calcium,[203–205] the molecular weight of fragments D and E varies from M_r 85,000 to 100,000 and M_r 35,000 to 50,000, respectively.[201,206–208] Under denaturing conditions[209] or with limited calcium content, fragment D can be further degraded to M_r 46,000. The late cleavages of fragment E disrupt binding sites involved in fibrin monomer polymerization, bonds that are probably critical for complex formation with D-dimer as well[210,211] (FIGURE 16.12, **right**).

Fibrin

With plasmin digestion of fibrin, initially very large pieces of fibers are released and then they are subsequently broken down into smaller fragments.[212] However, plasmic degradation of non–cross-linked fibrin results in a pattern of bands on gel electrophoresis under reducing conditions identical to that of fibrinogen, supporting the view that there is little structural change during polymerization.[213,214] On the other hand, cross-linked fibrin is degraded by plasmin more slowly, and the FXIIIa-mediated cross-link isopeptide bonds result in significantly different degradation products.[215–217] Prolonged exposure of fibrin to plasmin *in vitro* produces a unique fragment (D-dimer), consisting of two fragment D moieties from adjacent fibrin monomers, covalently bound by cross-links between their γ-chain remnants[11,218,219] (FIGURE 16.12, **right**). These D-dimers are very important clinically in the diagnosis of a variety of conditions as described below. Fragment E exists in both free and noncovalent complex with D-dimer (DD/E complex).[220,221] The derivation of this complex from degraded fibrin can be envisioned on the basis of the two-stranded, half-staggered overlap structure of the fibrin protofibril, and these structures have been confirmed by electron microscopy studies.[212] A variety of large degradation products are released by plasmin from cross-linked fibrin.[75,215,216,222–224] These noncovalently bound complexes represent ever-longer stretches of the two-stranded, half-staggered fibrin protofibril,[216] each of which is held in apposition with the same noncovalent bonds that influenced fibrin polymer formation. The smallest complex is DD/E, with larger forms being longitudinal strings of the DD/E complex held together by uncleaved coiled coils.[216] The structure of the DD/E complex provided evidence for the half-staggered organization and knob–hole interactions of fibrin monomers in the clot. The entire sequence of derivatives, such as DY, YY, DXD, and YXD, arises directly from the specific cleavages in the middle of the coiled-coil regions and the half-staggered arrangement in fibrin protofibrils and has been identified in the blood of patients with thrombotic and thrombolytic disorders.

INTERACTIONS OF FIBRIN WITH PLATELET, ENDOTHELIAL CELL, AND LEUKOCYTE RECEPTORS

Fibrin(ogen) binds specifically to integrins and other receptors on platelets, endothelial cells, and other cells. These binding events are important for platelet aggregation and wound healing where fibrin(ogen) serves as a bridge between cells and as a scaffold for cell migration. Fibrin also promotes physiologic inflammation and angiogenesis through interactions with leukocytes and endothelial cells.

Fibrinogen is the primary adhesive protein necessary for platelet aggregation. Fibrinogen binds to the activated conformation of the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) *via* residues located in the *C*-terminus of the γ-chains, bridging platelets because of the dimeric nature of fibrinogen molecule (FIGURE 16.13). Human fibrinogen also has two pairs of RGD sequences, one in the coiled-coil region and a second in the *C*-terminal regions of the Aα-chains. However, these do not appear necessary for fibrinogen-mediated platelet aggregation.[225,226] However, these RGD sequences do appear necessary for clot retraction and hence may be involved in the interaction of $\alpha_{IIb}\beta_3$ with fibrin.[227,228] Other sequences in fibrin(ogen) have also been identified as sites of potential interaction with $\alpha_{IIb}\beta_3$.[229,230]

Endothelial cells adhere to fibrin or surface-immobilized fibrinogen *via* the integrin $\alpha_v\beta_3$, ICAM-1 (intracellular adhesion

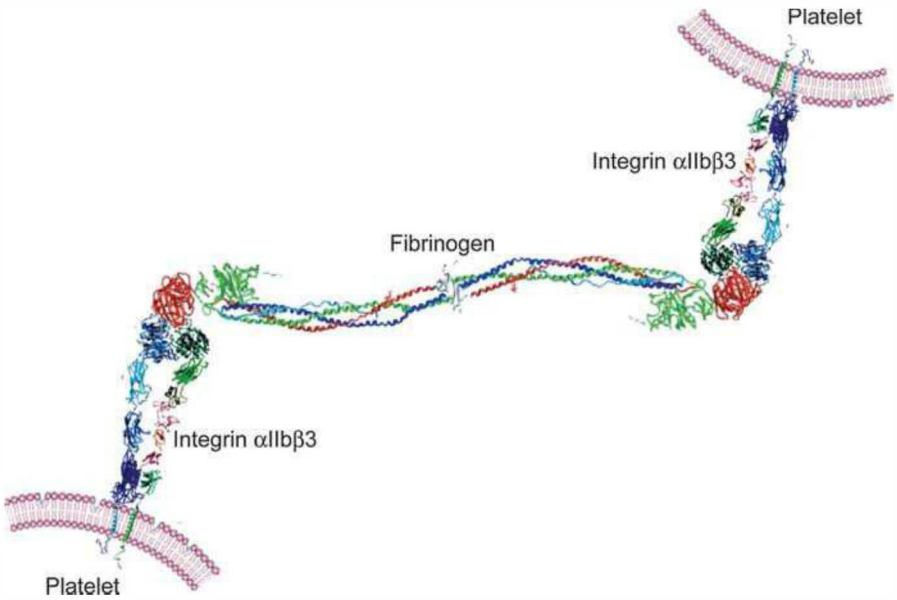

FIGURE 16.13 Binding of fibrinogen to the platelet integrin $\alpha_{IIb}\beta_3$. Ribbon diagrams of the structures of fibrinogen and the ectodomain of $\alpha_{IIb}\beta_3$ were combined in the orientation shown by electron microscopy of the rotary shadowed complexes. (The specific representation of fibrinogen was taken from Brown AEX, Litvinov RI, Discher DE, et al. Forced unfolding of coiled coils in fibrinogen by single molecule AFM. *Biophys J* 2007;92:L39–L41 and that of $\alpha_{IIb}\beta_3$ was from Rocco M, Rosano C, Weisel JW, et al. Integrin conformational regulation: uncoupling extension/tail separation from changes in the head region by a multiresolution approach. *Structure* 2008;16:954–964.)

molecule 1), and VE-cadherin (vascular endothelial cadherin). Integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, present on platelets and endothelial cells, bind fibrinogen in part through the RGD sequences in the *C*-terminal region of the α-chain of fibrin(ogen)[231–233] and can be covalently cross-linked, possibly by endothelial cell transglutaminase.[234] Additional binding sites for $\alpha_v\beta_3$ are located in the region of γ190 to γ202 and γ346 to γ358, both in the γ–module. A binding site for $\alpha_5\beta_1$ is located in the region of γ370 to γ383. Fibrin promotes the formation of capillary tubes by interactions of two β*N*-domains of fibrin with the third extracellular domain of VE-cadherin.[235] β_1 and β_3 integrins on smooth muscle cells also mediate interactions with fibrin and contraction of fibrin clots.

Fibrinogen binds to leukocytes *via* the integrin $\alpha_M\beta_2$. This interaction is important, attracting leukocytes to sites of injury. The I-domain of the α_M–subunit of $\alpha_M\beta_2$ binds to the γ-module with a complex binding site made up of the P1- and P2-domains including γ228 to 253.[236,237,238] Binding only occurs when fibrinogen is bound to a surface or to fibrin.

EVOLUTION OF FIBRINOGEN

Fibrin polymerization catalyzed by thrombin cleavage of Fps is common to all extant vertebrates; the fibrinolysis system is present as well. The amino acid sequences of large portions of the Aα-, Bβ-, and γ-chains are homologous to each other, indicating that they evolved from a common ancestor.[239,240] The homologies extend to the genomic sequences, with two intron/exon boundaries being conserved in all three chains and another one being common to the Bβ- and γ-chains.[241] Evidence suggests that there were a series of gene duplications and inversions starting about a billion years ago. The current hypothesis is that an ancestral gene duplicated at around that time to produce the α-chain and a pre-β–γ-gene, and then the pre-β–γ-gene duplicated about 500 million years ago to make the β-gene and the γ-gene.[239,240]

Protochordates do not have clotting systems like vertebrates, but whole genome searches have revealed sequences of several related proteins. There are sequences in *C. intestinales* that are homologous to fibrinogen's γC- and βC-modules and that are flanked by sequences predicted to form coiled coils of about the same length, with disulfide rings at either end similar to those of fibrinogen.[242] Finally, the location of many introns in the *C. intestinales* protein is the same as that in fibrinogen's β- and γ-genes, suggesting the possibility of alternative splicing to yield a shorter polypeptide chain similar to that of the most common variant of the γ-chain. An intriguing possibility is that the middle portion of this gene was either deleted in the lineage on the way to vertebrates or inserted on the way to tunicates.

Studies of invertebrate genomes have identified high numbers of fibrinogen-related loci, suggesting important and perhaps diverse unknown functions. The functions of vertebrate fibrinogen may provide some clues to potential roles in invertebrates. Invertebrates do not have coagulation systems that use fibrinogen or a homologous protein. However, invertebrates do have many proteins containing fibrinogen-related domains. Many of the proteins have been linked to defense, self/nonself recognition, and innate immunity. The fibrinogen-related protein (FREP) gene family, also known as fibrinogen domain immunolectin, is a common pattern receptor gene family in invertebrates. Invertebrates use FREPs in defense against bacteria and other parasites.[243] Many of these proteins contain one or more Ig superfamily domains combined with a domain homologous to the fibrinogen β-module or γ-module. The fibrinogen-related domains bind to the parasite or to excretory products of the parasite, targeting them for elimination. In some cases, FREPs are specifically expressed and greatly diversified in response to infections.

In evolutionary terms, we may generalize the function of FREPs. The knob "A"–hole "a" strong binding may be considered a prototypical example of specific and tightly controlled interactions. Fibrinogen is also a fibrous adhesive protein with specific binding to cells.

SURFACE-ADSORBED FIBRINOGEN

Fibrinogen adsorbs to various foreign surfaces. Surface adsorption, for example, to polystyrene or glass, leads to conformational changes of the fibrinogen molecule. Depending on the fibrinogen concentration, adsorption may occur horizontally, with the whole fibrinogen molecule in contact with the surface, or vertically, with only a portion of the D region in contact with the surface.[244,245] Conversion to fibrin enhances surface binding.[246] Adsorbed fibrinogen develops functional properties otherwise unique to fibrin, such as the promotion of platelet adhesion.[247–249] In addition, adsorbed fibrinogen may provide a surface for FXII-related contact phase activation.[250]

FIBRINOGEN THERAPY

Fibrinogen is present in a variety of therapeutics, including whole plasma (fresh frozen plasma), various virus-inactivated whole plasma products, cryoprecipitate, and platelet-rich plasma. However, the most efficient means for therapeutic application of fibrinogen are purified fibrinogen concentrates. In patients with severe trauma, fibrinogen levels have been identified as a major predictor of blood loss and death,[251] and application of fibrinogen concentrate may be sufficient to restore hemostasis.[252] Therapeutic success is directly proportional to the fibrinogen levels achieved.

A logical indication for fibrinogen concentrate is treatment of patients with congenital afibrinogenemia, especially for perioperative management or in case of trauma or severe hemorrhage.[253] Some cases of dysfibrinogenemia are also associated with bleeding and may require treatment with fibrinogen concentrate.

Low levels of fibrinogen are observed in patients with massive blood loss, for example, in trauma or obstetrical complications, as well as in patients with hyperfibrinolysis.[254] In trauma patients, treatment with fibrinogen concentrate or other fibrinogen-containing blood products has been found to be highly efficient. Current protocols for massive transfusion combine erythrocyte concentrates with plasma or other fibrinogen-containing preparations.[251] Relevant amounts of fibrinogen are found in plasma, platelet concentrates, and cryoprecipitate. Fibrinogen concentrates (currently, only plasma-derived fibrinogen concentrates are available) allow rapid fibrinogen replacement without the risk of volume overload anticipated when using plasma. In cardiopulmonary bypass surgery, intraoperative treatment with fibrinogen concentrate aiming at a fibrinogen level in the high normal range leads to significantly reduced blood loss.[255]

Target fibrinogen levels in replacement therapy appear to be above 2 g/L, as lower levels lead to impaired clot properties.[256] Higher levels further improve the mechanical stability of

clots.[257] Levels below 1 g/L lead to the prolongation of clotting time in prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays.[257]

Fibrinogen also is a component of various pharmaceutical products used in surgery. Current fibrin adhesives consist of a fibrinogen solution and a thrombin solution, plus calcium and other components.[258,259] Methods have been developed for generation of fibrin sealants from autologous plasma. Novel technologies include use of recombinant fibrinogen and thrombin. In addition, dry preparations are available or under development, which are directly applied to wounds and do not require reconstitution with a buffer solution or water.

FIBRINOGEN-RELATED LABORATORY DIAGNOSTICS

Fibrinogen Assay Systems

Fibrinogen levels are measured using a variety of assays, including functional assays and assays detecting the total amount of fibrinogen by immunologic or precipitation procedures. The earliest assays were based on precipitation by heat or various chemical agents.[260,261]

Measurement of the total clottable protein involves incubation of a fibrinogen-containing sample with thrombin or a thrombin-like enzyme, extraction of the resulting fibrin clot, washing the clot to remove nonfibrin(ogen) protein, and measurement of the amount of protein incorporated into the clot by clot weight,[262] by photometric methods, using UV adsorption or other methods for quantitating proteins.[262,263] Such assays will detect all functionally intact fibrinogen, including slow-clotting variants of fibrinogen, but not nonclottable fibrinogen, as observed in some dysfibrinogens. Also, some fibrin derivatives, such as high molecular weight soluble fibrin complexes, might become incorporated into a clot, resulting in overestimation of the fibrinogen level.

Immunoassays using polyclonal antisera detect the total amount of fibrinogen antigen, including fibrinogen degradation products, fibrin complexes, and fibrin degradation products.[264] Assays available are based on various technologies, including radial immunodiffusion, microtiter plate ELISAs, as well as optical assays based on turbidity generated by aggregation of fibrinogen by free or microbead-bound antibodies. In healthy subjects, the amounts of fibrin(ogen) derivatives are low; therefore the total fibrinogen antigen approximates the amount of fibrinogen detected by total clottable protein or other functional assays. Patients with nonclotting abnormal fibrinogens display higher levels of fibrinogen antigen, compared to the functional levels. Similarly, discrepant values are found in subjects with thrombosis, disseminated intravascular coagulation, trauma, hyperfibrinolysis, or other conditions, leading to high plasma levels of fibrinogen or fibrin degradation products. Assays for intact fibrinogen have been developed, based on monoclonal antibodies against epitopes removed from the fibrinogen molecule early in the process of fibrin formation and plasmin proteolysis.[265–267]

In the clinical setting, fibrinogen levels are, in most cases, measured by clotting time assays, and by the PT-derived fibrinogen method.[268] For clotting assays, biologic samples are mixed with a high concentration of thrombin or a thrombin-like enzyme.[269] If samples are diluted with buffer, clotting time is mainly dependent on the fibrinogen concentration of the sample, with lower fibrinogen concentration leading to slower clotting.[270] Fibrinogen concentration is determined using a reference curve based on serial dilutions of a sample of known fibrinogen concentration.

Clot formation can be detected by mechanical or optical methods. A threshold is used for turbidity in optical clot detection. Clot formation may also be continuously monitored in optical assays.[271] In a modification of the kinetic method, thrombin is replaced by a thrombin-like snake venom enzyme to eliminate the influence of heparin and other thrombin inhibitors.[272] As some earlier automated coagulation analyzers were not capable of adequately diluting samples, an alternative method was developed that uses undiluted samples, but prolongs the time to clotting by adding an inhibitor of fibrin polymerization. In this assay, the effect of anticoagulants and other compounds present in the samples is expected to be considerably greater than in the original method involving sample dilution.

Clotting time assays and kinetic methods are potentially influenced by the presence of substances that impair fibrin polymerization and lead to underestimation of fibrin concentration.[273] Nonclotting or slow-clotting fibrinogen variants (including a large proportion of known dysfibrinogens) also result in low fibrinogen levels in clotting assays. A large discrepancy between the clotting assay and immunologic fibrinogen assay results is a strong indicator for a dysfibrinogen.[274] In contrast, low fibrinogen levels in both clotting and the immunologic assays indicate hypo- (or a-) fibrinogenemia. The influence of fibrinogen degradation products on the clotting time assays appears to be small. An overestimation of fibrinogen levels may occur in patients with high concentrations of soluble fibrin complexes. Soluble fibrin complexes may increase the rate of fibrin formation, leading to shorter clotting time (and faster increase of turbidity in assays involving optical detection). There is some evidence that high levels of high molecular weight fibrinogen, as observed during inflammatory conditions as part of the acute-phase response, result in a moderate overestimation of fibrinogen concentration due to more rapid clotting of high molecular weight fibrinogen compared to the lower molecular weight variants.[275]

The PT-derived fibrinogen assay is based on the observation that the final turbidity of the clot generated during measurement of the PT correlates with the amount of fibrinogen present in the original plasma sample. Fibrinogen levels are generated as a by-product of PT measurement at no additional cost.[268,276] The assay can only be performed with platelet-poor plasma using optical detection systems. As an endpoint method, the assay does not result in an underestimation of fibrinogen levels by circumstances leading to slow clotting. Recent results indicate that slow-clotting dysfibrinogens actually lead to an overestimation of fibrinogen levels in PT-derived fibrinogen.[277–279] Similarly, anticoagulant drugs that result in lower levels of active thrombin lead to the generation of fibrin clots with higher turbidity and an overestimation of fibrinogen levels. Inhibitors of fibrin polymerization also may lead to increased clot turbidity and overestimation of fibrinogen concentration.[280] Samples are not diluted for measurement of the PT. Anticoagulants, fibrin polymerization inhibitors, and other compounds have greater influence in PT-derived fibrinogen than in the majority of clotting time assays. If PT-derived fibrinogen is used as screening method, a large proportion of dysfibrinogens may escape detection because clot turbidity may be normal or increased (despite or because of the delayed fibrin formation).[280] Treatment with

hydroxyethyl starch and other plasma expanders may lead to overestimation of fibrinogen levels in PT-derived fibrinogen assays, probably by volume exclusion mechanisms.[280,281] PT-derived fibrinogen assays display a rather small measuring range that cannot be extended by dilution.

Various coagulation assays that are influenced by fibrinogen concentration include the PT, aPTT, thrombin time, batroxobin time, thromboelastography, and rotational thromboelastometry, as well as clotting-based assays for monitoring of anticoagulants. Prolongation of the PT occurs at fibrinogen levels below approximately 1 g/L.[257] At very low fibrinogen levels (below 0.25 g/L), clot formation may be below threshold for detection of the clotting time, resulting in "infinite" PT or aPTT. In thrombelastography, fibrinogen deficiency leads to reduced maximum amplitude of the tracing, indicating reduced clot stability. Thrombelastography and related methods may serve as an alternative for the detection of fibrinogen deficiency under certain circumstances.[282–285]

ASSAY SYSTEMS FOR FIBRINOPEPTIDE A AND SOLUBLE FIBRIN

Apart from fibrinogen, clinical blood samples contain a variety of fibrinogen derivatives, including fibrin complexes, fibrin degradation products, and fibrinogen degradation products. The presence of fibrin complexes, as well as fibrin degradation products, indicates *in vivo* fibrin formation. Whereas fibrin complexes generally are derived from intravascular coagulation processes, fibrin degradation products (and especially lower molecular weight fibrin degradation products) may stem from extravascular sources, such as sites of tissue injury. Fibrin degradation products are generated by a combination of coagulation activation (leading to the production of thrombin) and activation of fibrinolysis. Fibrinogen degradation products are generated by proteolysis of fibrinogen by plasmin (or other enzymes) without prior action of thrombin.

Fps may be detected by various methods, including immunoassays.[286,287] Current immunoassays available for measurement of FpA require removal of fibrinogen from the plasma samples by precipitation or adsorption, for example, to bentonite.

Various assay systems have been developed for the detection of soluble fibrin.[288] The earliest systems were based on precipitation or "paracoagulation" of fibrin complexes upon addition of ethanol[289] or protamine.[290,291] These assays were found to be quite specific, but not very sensitive in the diagnosis of disseminated intravascular coagulation.

Fibrin monomers may bind to insolubilized fibrinogen attached to microbeads, chromatography media, or other media such as sheep erythrocytes. In the assay using fibrinogen adsorbed to sheep erythrocytes, fibrin complexes are detected by observing aggregation of the modified erythrocytes.[292]

Fibrin complexes act as cofactors in tPA-induced plasminogen activation. If tPA and plasminogen are added to a diluted plasma sample, the amount of plasmin formed is primarily dependent upon the amount of fibrin present in the sample. The resulting plasmin may be detected photometrically, by adding a chromogenic substrate.[164] The sensitivity of the assay may be increased by the addition of mini-plasmin rather than full-size plasmin, or by the addition of an antiserum neutralizing the α_2-antiplasmin present in the sample.[293]

Release of Fps leads to the generation of neoepitopes, directly related either to Fp release or to conformational changes induced by Fp release or fibrin polymerization. The earliest assays used monoclonal antibodies against the neo-*N*-terminus of the fibrin α-chain.[294–296] Detection of this epitope is difficult due to the fact that it is part of the fibrin polymerization site knob "A" and therefore participates in the interaction between the fibrin monomer units, making the epitope inaccessible for antibodies.[295,296] Incubation of samples with denaturing agents such as thiocyanate strongly enhances detection due to disaggregation of the fibrin complexes, but also expands assay reactivity to include various fibrin degradation products. The enzyme immunoassay using monoclonal antibody 2B5 directed against the peptide sequence NH2-GPRVVER-X as capture and tag antibody and potassium thiocyanate sample treatment was therefore found to be a global assay for fibrin.

New assays are based upon latex-enhanced photometric immunoassay technology, using fibrin-specific monoclonal antibodies attached to latex microbeads. The monoclonal antibody IF-43 reacts with fibrin monomer units within a fibrin complex.[297,298] The epitope is located within the *N*-terminal α-chain (α 17 to 78), but reactivity of the antibody requires fibrin complex formation. MAb F405 is specific for a neoepitope at the neo-*N*-terminus of the fibrin α-chain NH2-GPRVVERHQ-X, but in contrast to MAb 2B5, binding of the antibody does not seem to be impaired by fibrin polymerization, presumably because the epitope does not involve the *N*-terminal three amino acids.[299] A different approach was selected in the development of MAb J2-23. This monoclonal antibody reacts with an epitope within the *C*-terminal Aα-chain of fibrinogen (Aα502 to 521), which is not accessible in fibrinogen, but becomes accessible after FpA release due to conformational changes related to the "handshake domain" of the fibrinogen molecule.[297,298] In native fibrinogen, the *C*-terminal Aα-chain region positioned close to the central region of the fibrinogen molecule. After FpA cleavage, the "handshake domains" dissociate from the central region and from each other. As the *C*-terminal Aα-chains are degraded early in the course of plasmin proteolysis, thus eliminating the antigenic site detected by MAb J2-23. Therefore, the assay is highly specific for non–plasmin-degraded fibrin complexes and displays no reactivity with fibrin degradation products or fibrinogen degradation products. Covalent linkage of α-chains by FXIIIa also appears to eliminate reactivity in the assay, restricting reactivity to non–cross-linked and nondegraded fibrin complexes.

Assays for soluble fibrin complexes may be used for diagnosis of intravascular coagulation processes, but due to heterogeneity of assay systems and lack of clinical trials, these assays have not yet gained widespread clinical use. Another reason might be the ubiquitous availability of D-dimer assays.

ASSAY SYSTEMS FOR D-DIMER ANTIGEN

Immunization of laboratory animals with the fibrin fragment D-dimer can yield antibodies specific for the dimerized D regions.[300,301] These antibodies are fibrin specific, since the epitope is not found in fibrinogen or in proteolytic fragments of fibrinogen.[300] The antibodies react with conformational epitopes induced by covalent linkage of the D regions.[302]

D-dimer assays not only react with the D-dimer fragment, but also with larger molecules containing the dimerized D regions, including freshly formed cross-linked fibrin complexes, as well as larger proteolytic fragments of cross-linked fibrin (D-dimer antigen).[303,304] These proteolytic fragments may be

derived from fibrin clots, but also from "soluble" fibrin formed in the flowing blood. As formation of D-dimer antigen involves at least two enzymatic processes (thrombin releasing FpA and FXIIIa inducing covalent bonds between adjacent D regions of fibrin monomer units within a fibrin polymer), it seems to be more robust toward preanalytical errors than other assays for fibrin-related markers. Assay systems for D-dimer include enzyme-linked immunoassays, latex-enhanced photometric immunoassays, fluorogenic assays, membrane-based assays, and other assay formats including point-of-care assays performed with whole blood.[305]

Many D-dimer assays display a high degree of sensitivity for the exclusion of acute deep vein thrombosis and pulmonary embolism.[306–310] In patients with high clinical probability, further diagnostic procedures should be performed even if D-dimer levels are normal.[311,312]

Apart from venous thrombosis, D-dimer antigen may reflect other diseases associated with fibrin formation, such as disseminated intravascular coagulation, trauma, pregnancy complications, malignant disease, or vascular abnormalities including aortic aneurysm or hemangiomas. Therefore, elevated D-dimer antigen levels have to be interpreted in the context of possible underlying diseases and clinical symptoms. Negative or low D-dimer antigen levels may be used for exclusion of clinical disease states typically associated with fibrin formation. For example, acute aortic dissection causes intense chest pain, which may be quite similar to the chest pain of patients with acute coronary syndromes. The pain is caused by rupturing of the vascular intima of the aorta, often in the context of preexisting atherosclerotic lesions. Both aortic dissection and aortic aneurysms cause local formation of fibrin, but some fibrin appears in the flowing blood and may be detected in the venous blood sample as D-dimer antigen. In contrast to patients with acute coronary syndromes, which generally display normal range D-dimer levels initially,[313] patients with aortic dissections or aortic aneurysms display massively elevated D-dimer antigen levels.[314,315]

ASSAY SYSTEMS FOR FIBRINOGEN AND FIBRIN DEGRADATION PRODUCTS

Specific assays for fibrinogen degradation products are currently not in widespread clinical use. Peptides released from the *N*-terminus of the Bβ-chain, including peptide Bβ1-42,[316] may serve as specific markers for plasmin proteolysis of fibrinogen. Fragment D may stem from fibrinogen, as well as from fibrin, as not all D regions within fibrin are cross-linked by FXIIIa. Fragment E also is generated both by proteolysis of fibrinogen and fibrin, but currently no assays specific for fibrinogen fragment E are commercially available.

There are assay systems for fibrinogen/fibrin degradation products in general, based on monoclonal antibodies specific for plasmin action, but independent of fibrin cross-linking, which may distinguish between fibrinogen degradation products and fibrin degradation products.[317,318] The advantage of such assays over D-dimer assays has not been proven.

Other enzymes, including leukocyte elastase, may degrade fibrinogen and fibrin. Differences in substrate specificity result in formation of specific fibrin derivatives not generated by plasmin proteolysis. These products may be detected with specific monoclonal antibodies.[319–321] Assay systems for elastase-related fibrinogen/fibrin degradation products may be helpful in sepsis and other inflammatory conditions, but additional clinical trials are necessary.

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Full Reference Lists can be found online

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CHAPTER

17 Factor XIII

CÉDRIC DUVAL, HELEN PHILIPPOU, AND ROBERT A.S. ARIËNS

Factor XIII (FXIII) is an unusual blood coagulation factor, circulating as a heterotetramer composed of two catalytic A-subunits and two noncatalytic B-subunits. It is a plasma transglutaminase (TG), mostly associated with fibrinogen, which is activated by thrombin in the presence of calcium in the final stages of the coagulation cascade. It acts by stabilizing the clot through the covalent cross-linking of polymerized fibrin chains and the incorporation of fibrinolysis inhibitors into the fibrin clot. Genetic polymorphisms in FXIII are associated with cardiovascular diseases (CVD), including coronary artery disease (CAD), stroke, and deep vein thrombosis (DVT). FXIII deficiency leads to severe bleeding, impaired wound healing, and recurrent miscarriages.

In this chapter, we review the basic functions of FXIII and focus on the most recent findings highlighting the role of FXIII in cardiovascular disease, in particular an emerging link to thrombosis. We use the detailed recommended FXIII nomenclature published in 2006 by the Scientific and Standardization sub-Committee of the International Society on Thrombosis and Haemostasis.[1]

DISCOVERY OF FACTOR XIII

The original discovery of a blood protein that makes fibrin chemically resistant to dissolution was made in the 1940s by two independent scientists. In the United States, Robbins[2] described a serum factor that stabilizes fibrin during his PhD studies at the University of Illinois. At the same time, in Hungary and later in the UK, Lorand made similar discoveries of a blood protein involved in fibrin formation[3,4] and discovered that the protein involved was from plasma rather than serum.[5] Over the years, several names have been given to this factor including fibrin-stabilizing factor, Laki-Lorand factor, fibrinoligase or fibrinase. During meetings of the International Society on Haemostasis and Thrombosis in the 1950s and early 1960s, it was decided that the name FXIII should be used, based on its main function, that is the stabilization of fibrin, and its position in the coagulation cascade.

FXIII was first purified from blood plasma by Lorand[5] and later to further homogeneity by Loewy[6–8] and Schwartz. It was discovered that FXIII is composed of two A-subunits and two B-subunits, arranged in a heterologous tetrameric structure.[9–11] Discoveries of the first patient with FXIII deficiency and the fact that lack of FXIII leads to a serious bleeding disorder were made in Switzerland in 1960 by Duckert et al.[12] The primary amino acid sequences for both the A- and B-subunits and the structure of the A-subunit gene have been elucidated by Ichinose and others,[13,14,15,16–18] and, more recently, the crystal structure of the FXIII A-subunit (FXIII-A) dimer has been resolved at high resolution by Yee et al.[19] and Weiss et al.[20] These studies have paved the way for a profound understanding to be developed of the biology, structure, and function of this intriguing coagulation factor.

FACTOR XIII TETRAMER STRUCTURE

Cellular FXIII is composed of a dimer of A-subunits. FXIII in plasma is, however, composed of two A- and two B-subunits[9–10] (FIGURE 17.1). Binding to the B-subunits is necessary to provide stability to the hydrophobic A-subunit in the aqueous environment of the blood; without FXIII B-subunit (FXIII-B) the A-subunit is unstable as demonstrated by the absence of FXIII-A in blood plasma in patients with congenital B-subunit deficiency.[21] The structure of FXIII-A_2B_2 is that of a heterologous tetramer, with a molecular weight of 325.8 kDa.[22] FXIII-A forms a dimer with a central axis of symmetry, and the two monomers aligned in opposite *N*- to *C*-terminal orientation as characterized by x-ray crystallography[19,20] (FIGURES 17.1 and 17.2). The tertiary structure of FXIII-B and that of the tetramer remains, however, unknown. The closest information we get on the structure of the FXIII-A_2B_2 tetramer is that obtained by rotary-shadowed FXIII analyzed by electron microscopy (EM).[23] This study showed that FXIII-A_2 demonstrates a globular structure, whereas FXIII-B in isolation is a monomer and demonstrates a flexible strand-like structure. It has been suggested that the two B-subunits wrap around the globular A-subunit dimer in a protecting manner.[23] However, the resolution is too low to conclude with confidence on the structure for the FXIII-A_2B_2 tetramer, and future studies of higher resolution will be needed to determine the structural requirements for interaction between the A- and B-subunits. FIGURE 17.1 shows a schematic representation of the FXIII-A_2B_2 tetramer for illustration purposes; the conformation of FXIII-B in this structure in particular is hypothetical.

FXIII-A is produced as a typical cytoplasmic protein by monocytes, macrophages, and megakaryocytes.[24–26] The protein has no leader sequence for the classical secretion pathway.[14,17] Recent evidence suggests the existence of nonclassical secretion mechanisms for FXIII-A from macrophages.[27] FXIII-B on the other hand is synthesized in the liver and secreted via the classical secretion pathway from hepatocytes.[28] At which site the FXIII-A_2B_2 tetramer is assembled, and by which mechanism, remains unknown. As FXIII-B is normally secreted and circulates in excess, one line of thought is that FXIII-B could be delivered to the site of synthesis for FXIII-A and that it might be required to aid the release of FXIII-A from the cell. Phenotype changes for FXIII-A after bone marrow transplantation, and for FXIII-B after liver transplantation, suggest that these two organs are the main sites of synthesis for the FXIII subunits.[29]

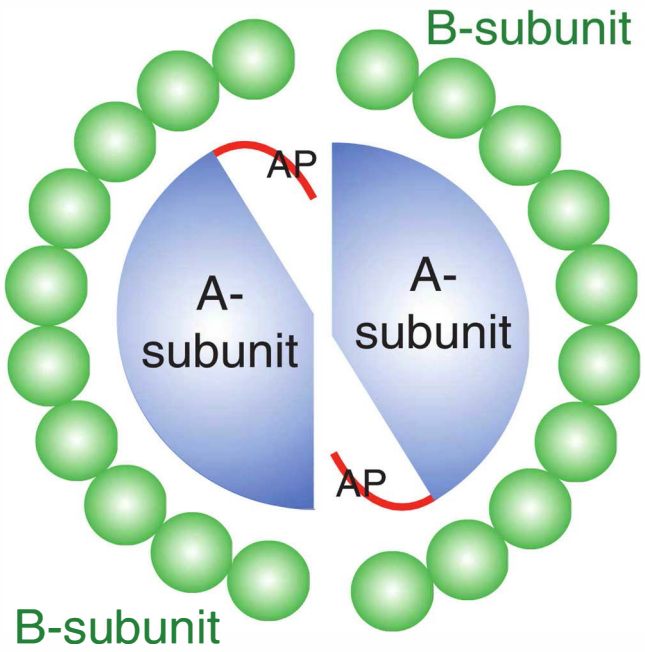

FIGURE 17.1 Schematic representation of FXIII A_2B_2 tetrameric structure. Two FXIII-A molecules (*blue*) are aligned in opposite *N*- to *C*-terminal orientation around a central axis of symmetry. The flexible, strand-like B-subunits (*green*) are thought to wrap around the hydrophobic A-subunits. The conformation of the B-subunits in this representation is hypothetical and based on previous EM studies.[23] The FXIII activation peptides (APs) are depicted in *red*.

FACTOR XIII A-SUBUNIT, CATALYTIC TRIAD, AND CALCIUM BINDING

The gene for the FXIII-A is located on chromosome 6 bands p24–25.[30,31] It contains 15 exons that code for a 731–amino acid mature protein.[17] The FXIII-A gene shows significant sequence homology with those of the other TGs, in particular keratinocyte TG 1 and tissue TG 2.[32–34] However, while most TG genes are <30 to 40 kb in size, the FXIII-A gene spans more than 160 kb, due to the large size of the introns in this gene. The FXIII-A gene shows linkage with other genes on chromosome 6 including the human leukocyte antigen genes of the major histocompatibility complex[31] and the plasminogen gene at bands q26–27.[35]

The mature FXIII-A protein has a molecular weight of 83.2 kDa[22] and is composed of five structurally and functionally distinct regions, which from *N*- to *C*-terminus include an activation peptide (AP) (residues 1 to 37), a β-sandwich domain (38 to 184), the catalytic core (185 to 515), a β-barrel domain 1 (516 to 628), and a β-barrel domain 2 (629 to 727)[19] (FIGURE 17.2). The catalytic triad involved in transamidation is composed of Cys314, His373, and Asp396.[19] FXIII-A_2 is a dimer of two subunits in antiparallel orientation. The AP from one subunit crosses the interface and interacts with the second subunit (FIGURES 17.1 and 17.2). FXIII-A shares its basic domain structure with many other members of the TG enzyme family. However, most other TGs lack an AP and do not form a dimer.[36,37] It has been suggested that dimerization of FXIII-A enhances substrate specificity of this enzyme by increasing the surface available for substrate binding.[36]

FXIII-A contains nine cysteine residues, none of which are disulfide bonded. Oxidation of the active site Cys314 leads to loss of enzyme activity. In the catalytic core of the zymogen, Cys314 is sandwiched between another cysteine residue (374) and an aromatic tyrosine residue (560) to protect it from oxidation.[19,20] Tyr560 is located in β-barrel domain 1, and hence removal of this residue from the active site by a conformational change of β-barrel 1 could play an important role in the exposure of Cys314 for interaction with the substrate. A conformational change that leads to β-barrel 1 separating from the catalytic core is exactly what happens when tissue TG is activated,[37] and it is therefore possible that a similar conformational change is involved in FXIII activation. Evidence for large conformational changes during the activation of FXIII-A is still scant. An x-ray crystal structure of activated FXIII-A did not show any major conformational changes compared with

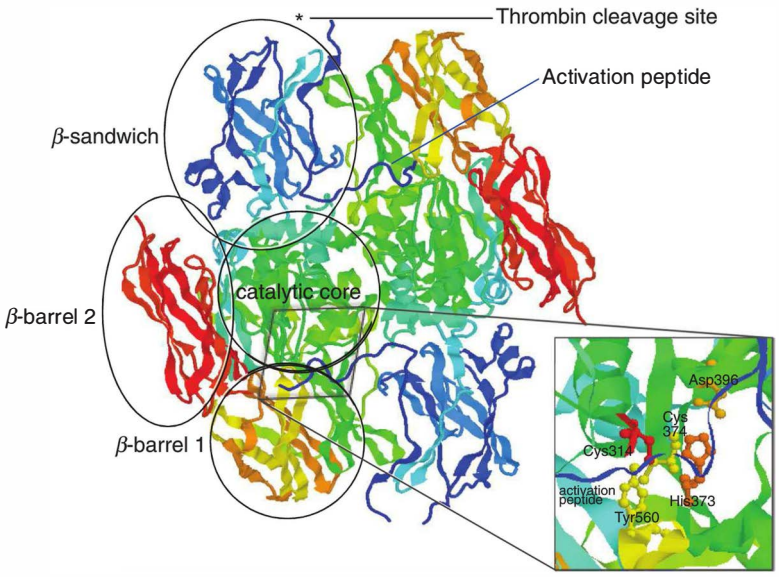

FIGURE 17.2 Tertiary structure of the FXIII A_2 dimer. FXIII-A forms a dimer of two subunits in antiparallel orientation. Each A-subunit is composed of (from *N*- to *C*-terminus) an AP that is cleaved by thrombin at peptide bond Arg37–Gly38 (indicated by an *asterisk*, not resolved on the crystal structure), a β-sandwich domain, a catalytic core, a β-barrel 1 domain, and a β-barrel 2 domain. The **inset** shows a zoom in on the catalytic core, with the catalytic triad, Cys314, His373, and Asp396 highlighted in *red*, *orange*, and *ocher*, respectively. Residues Tyr560 from the β-barrel 1 domain and residue Cys374, which protect the active site Cys314 from oxidation, are highlighted in *yellow*. Image was drawn using RasMol 2.7.5 and the crystal structure coordinates from Weiss MS, Metzner HJ, Hilgenfeld R. Two non-proline *cis* peptide bonds may be important for factor XIII function. *FEBS Lett* 1998;423(3):291–296 (PDB file 1F13).

FXIII-A zymogen.[38] However, studies of solvent accessibility of residues in FXIII-A by chemical labeling of surface exposed cysteines and lysines did indicate that conformational changes of FXIII-A are likely to play an important role in activation.[39] Feasible conformational changes in FXIII-A during activation have recently been modeled based on the x-ray crystal structure of active tissue TG.[40]

Calcium plays important roles both in FXIII activation and in the catalytic mechanism (see below). The main calcium binding site in FXIII-A is composed of residues Asn436, Asp438, Ala457, Glu485, and Glu490 of the catalytic core region.[41] Mutations at Glu485 and Glu490 significantly reduced catalytic activity of activated FXIII (FXIIIa), in agreement with a major role of these residues in calcium binding.[42] This calcium binding site can be found at the dimer interface and is located very close (<10 Å) to β-barrel domain 1. Its location close to β-barrel domain 1 suggests that calcium binding may be involved in the conformational change that removes β-barrel domain 1 and Tyr560 away from the catalytic core during activation.[41] Location of the calcium binding site at the dimer interface may also account for the allosteric effects of calcium binding on the exposure of the catalytic site for substrate interaction in both subunits, even if only one of them has been cleaved by thrombin, as reported by Hornyak and Shafer.[43]

Another structurally important characteristic of FXIII-A, which could be involved in conformational changes of the catalytic domain during activation, involves the presence of two nonproline *cis*-peptide bonds in FXIII-A at Arg310–Tyr311 and Gln425–Phe426, an uncommon feature in protein structures.[20] These nonproline *cis*-bonds, together with the Gly410–Pro411 *cis*-bond, could be involved in a major conformational "switch" in the catalytic core when it is transformed into an energetically favorable transconformation.[20] In agreement with this, mutations in either Arg310 or Tyr311 have been shown to result in an inactive FXIII molecule, indicating the potential importance of one of the two nonproline *cis*-bonds in FXIII activation.[44]

FACTOR XIII B-SUBUNIT STRUCTURE AND FUNCTION

The gene for FXIII-B is located on chromosome 1 bands q32–32.1 and is 28 kb in size.[45] It contains 12 exons separated by 11 introns and codes for a 641–amino acid mature protein.[46] The FXIII-B gene is part of a cluster of homologous genes located at bands q31–32 on chromosome 1, including the genes of five complement regulating proteins, factor H,[47,48] C4 binding protein,[49] C3b/C4b receptor,[50] decay accelerating factor,[51] and membrane cofactor protein.[52] The products of the genes at this locus contain a varying number of short consensus repeats called Sushi domains.[15,53,54] Sushi domains are typically involved in protein–protein interactions, and the FXIII-B protein is entirely composed of 10 Sushi domains with a 20–amino acid leader sequence for secretion and a short 11–amino acid *C*-terminal sequence.[15] Each Sushi domain contains four cysteine residues that participate in two disulfide bonds that provide stability to the structure of the domain. In total, there are therefore 20 disulfide bonds in FXIII-B. The tertiary structure of FXIII-B is hitherto unknown; however, FXIII-B appears as a long filament, or as "beads on a string," when imaged by EM.[23] Using recombinant FXIII-B and truncations of the molecule, it has been suggested that the first Sushi domain is responsible for binding FXIII-A and that the fourth and ninth Sushi domains are responsible for binding to another B-subunit.[55]

The B-subunit plays a number of roles in FXIII physiology. First, two B-subunits bind two A-subunits to form the heterologous tetramer that provides stability to the protransglutaminase in blood plasma. Whether FXIII-B also somehow aids the secretion of FXIII-A from its source of production is unknown, but it has been suggested that some hepatoma cell lines (PLC/PRF/5) are capable of expressing both FXIII-A and FXIII-B and that they secrete intact FXIII-A_2B_2.[56] Second, the dissociation of the B-subunit from FXIII-A is a critical event in the activation of FXIII (see below). FXIII-B may also play an important role in the interaction of FXIII with fibrinogen γ' (see below), thereby directing the cross-linking activity of FXIIIa to one of its major substrates. It is not known if FXIII-B is involved in other protein interactions.

FACTOR XIII ACTIVATION

FXIII-A_2B_2 is converted into its active form by a two-stage process (FIGURE 17.3). The first step involves the cleavage of the Arg37–Gly38 peptide bond by thrombin that results in the release of an *N*-terminal AP of 37 amino acids to generate FXIII-A′, followed by the second step of calcium-dependent dissociation of the B-subunits from the cleaved A-subunits.

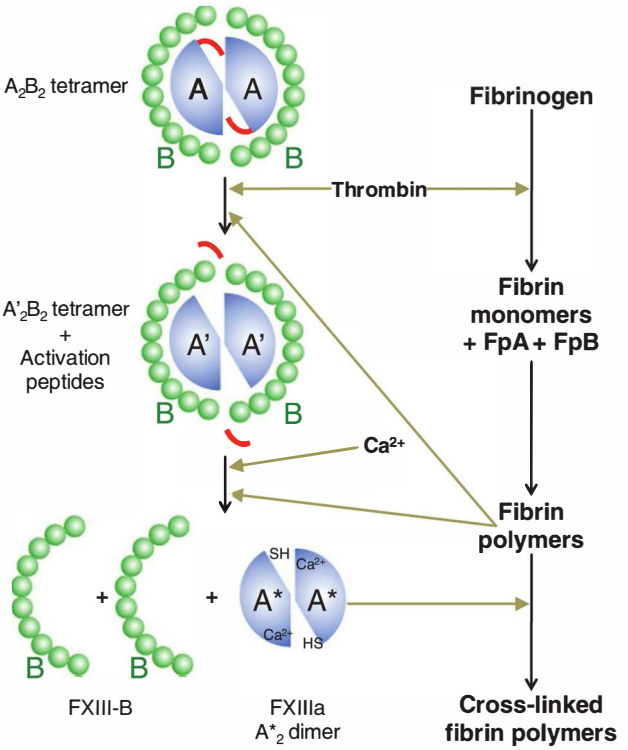

FIGURE 17.3 Activation of FXIII. FXIII is activated in a two-step process: in a first step thrombin cleaves the AP from FXIII-A, and in a second step the B-subunits dissociate from the active A-subunit dimer in the presence of calcium. Both steps are enhanced by the presence of the main substrate of FXIIIa in plasma, fibrin. Polymerizing fibrin increases cleavage of FXIII-A by thrombin as well as subunit dissociation. Fibrin formation and FXIII activation are therefore closely regulated to occur at the same time.

The cleaved A'-subunits in the absence of the B-subunits adopt an active conformation in the presence of calcium, FXIIIa.[57,58] As a consequence of FXIII activation, the active site cysteine, which is usually buried and inaccessible, is unmasked allowing access to potential substrates. Under physiologic circumstances thrombin is not able to cleave FXIII without the presence of the cofactor, fibrin.[59] Fibrin has been shown to enhance both steps of FXIII activation (FIGURE 17.3). Polymeric fibrin I and II (des-A- and des-A, B-fibrinogen) enhance the rate of thrombin cleavage of the A-subunits by approximately 80-fold. Fibrin I and II reduce the Michaelis constant (K_m) for thrombin during the activation of FXIII by forming a ternary complex with thrombin and FXIII, enabling activation to occur under physiologic conditions of clot formation.[59,60,61] The fibrin enhancement effect on thrombin cleavage only occurs with FXIII-A_2B_2 and not cellular FXIII-A_2.[62,63] Fibrin also accelerates the rate of A- and B-subunit dissociation following thrombin cleavage and it has been reported that the α-chains of fibrin play a role in this.[64] Calcium is essential for enabling the dissociation of FXIII-A from FXIII-B by modulating the conformation of FXIII-A´.[39,65] Fibrin enhancement of FXIII activation ensures that the formation of FXIIIa only occurs at the site of a forming fibrin clot, where FXIIIa cross-linking activity is required.

A second thrombin cleavage site (Lys513–Ser514) has been identified in the FXIII-A.[16] This site is not considered to play an important role *in vivo* because its cleavage is only observed *in vitro* when thrombin is incubated with FXIII in the absence of calcium, but not in its presence.[66]

TRANSGLUTAMINASE REACTION

Unlike the other eight members of the TG family mainly found in tissues, FXIIIa is a plasma-specific endo-γ-glutamine:ε-lysine transferase enzyme that plays an important role in the formation of fibrin gels.[7] It catalyzes protein cross-linking reactions by specifically replacing the ammonia of a glutamine residue[67,68] with an amino group from a lysine residue,[69,70] therefore creating a covalent isopeptide bond between these two amino acids. Upon activation by thrombin, FXIIIa cross-links adjacent fibrin molecules and induces stabilization of the fibrin clot.

The first step of the most common fibrin γ-chain cross-linking process is the reaction of the thiol group of factor XIIIa active site Cys314[71] with the carbonyl of the γ-glutamine residues (398/399) of a fibrin molecule (Table 17.1, FIGURE 17.4), resulting in the generation of an acyl enzyme intermediate and release of ammonia (FIGURE 17.5). The second step requires the recognition of a closely located Lys406 from an adjacent fibrin molecule (Table 17.1) aligned during fibrin polymerization, and the subsequent formation of an isopeptide bond between the ε-amino group of the lysine and the γ-glutamine residue, resulting in the cross-linking of γ-chain fibrin and the release of FXIIIa (FIGURE 17.5). If the appropriate lysine substrate is not available, the glutamine residue can then be deaminated (Fibrin-$(CH_2)_2$-CO-S-F_{XIII} + H_2O ↔ F_{XIII}-SH + Fibrin-$(CH_2)_2$-COOH), or linked to a substrate primary amine, the latter resulting in a peptide bond (Fibrin-$(CH_2)_2$-CO-S-F_{XIII} + H_2N-Amine ↔ F_{XIII}-SH + Fibrin-$(CH_2)_2$-CONH-Amine).[72]

The amino acid sequences surrounding both glutamine and lysine residues play some role in substrate recognition. This peptide sequence specificity is particularly important for recognition of glutamine by FXIIIa. Studies have shown that FXIIIa only recognizes specific glutamine residues,[73] and that the peptide sequence surrounding this amino acid plays a role in substrate recognition.[74] The latter study, using amino acid substitution, highlighted the requirement of leucine (pos−4), lysine (pos+2), valine (pos+3), and leucine (pos+4) residues in the vicinity in order for the glutamine residue (pos0) to be recognized by FXIIIa. The nature of the amino acids preceding the lysine residue is less influential on the cross-linking reaction. A comprehensive study has shown that on one hand, valine, arginine, and phenylalanine have a favorable effect to substrate reactivity, with serine, alanine, leucine, tyrosine, and asparagine also promoting cross-linking. On the other hand, glycine, aspartic acid, or to a lesser extent proline, histidine, and tryptophane have an adverse effect on creating an isopeptide bond between lysine and glutamine.[75] It is possible that tertiary structure surrounding the glutamine and lysine residues plays a more important role in substrate recognition for FXIIIa than primary structure. Future structural studies on the interaction of FXIIIa with different substrates will be required to elucidate this further.

Although fibrin γ-chain dimerization is the major product of FXIIIa enzymatic activity, fibrin α-chains cross-linking putatively occurs at Gln221, 237, 328, and 366, and Lys208, 219, 224, 418, 427, 429, 448, 508, 539, 556, 580, 601, and 606 (Table 17.1, FIGURE 17.4) at a much slower rate and results in highly cross-linked α-chain multimers.[76–78] Fibrin α–γ hybrid heterodimers and multimers[79] and γ-chain trimers/tetramers[80] have also been documented but are normally not a major product of FXIIIa-induced cross-linking.

To date, no human isopeptidase has been identified that is capable of disrupting the covalent ε(γ-glutamyl)lysyl bond created during fibrin cross-linking, but FXIIIa itself has been reported to display effective isopeptidase activity *in vitro*, with K_m values of 10^{-4}–10^{-5}.[81] However, the physiologic relevance of this reaction and the mechanisms by which it occurs remain unclear.

HOMOLOGY WITH OTHER TRANSGLUTAMINASES

TGs are a family of ubiquitous enzymes, with presence in bacteria, plants, and animals. They are part of a superfamily of enzymes that are classified as *R*-glutaminyl-peptide:amine γ-glutamyltransferases (EC 2.3.2.13).[82] The reactions catalyzed by the EC 2.3.2.13 enzyme family include transamidation or cross-linking, deamidation, and amine incorporation.[83] The biologic functions of TGs span a plethora of different activities, from involvement in egg fertilization, semen coagulation, cell death, and epidermal and hair structure to blood coagulation.

In humans, besides FXIII, there are at least eight other TGs (Table 17.2). These include keratinocyte TG or TG1 involved in epidermis formation,[84] tissue TG or TG2, a ubiquitous enzyme involved in a variety of cell functions including cell death,[85–87] TG3 involved in hair follicle stabilization,[88] TG4 with unknown function in the human prostate,[89] TG5 involved in epidermal differentiation,[90] TG6 and TG7 with unknown functions,[91] and erythrocyte protein 4.2 that plays a role in erythrocyte membrane integrity.[92] The genes of the TG family demonstrate significant sequence homology, and the proteins show remarkable structural similarity with a basic domain structure that includes a β-sandwich domain, and a catalytic core followed by two β-barrel domains. The catalytic core regions of

Table 17.1

Factor XIII substrates

Substrate	Cross-linking Site	Cross-linked with	Known or Potential Function
Fibrin(ogen) γ-chain[109,110]	Gln398, 399 and Lys406	Itself and α-chain	Clot stabilization
Fibrin(ogen) α-chain[379–381]	Gln221, 237, 328, 366, and Lys208, 219, 224, 418, 427, 429, 448, 508, 539, 556, 580, 601, 606	Itself and γ-chain	Clot stabilization
α_2-Antiplasmin[148–151]	Gln2/14[a]	Lys303 fibrin α-chain	Resistance to fibrinolysis
TAFI[175]	Gln2, 5, 292	Fibrin, itself	Resistance to fibrinolysis
PAI-2[166–168]	Gln83, 86	Lys148, 176, 183, 230, 413, 457 fibrin α-chain	Resistance to fibrinolysis
Fibronectin[382,383]	Gln3	Itself, fibrin, collagen	Migration of cells into the clot; wound healing
Collagen[382,384]	—	Fibronectin, fibrin	Stabilization of extracellular matrix
Von Willebrand factor[385,386]	—	Fibrin, collagen	Platelet adhesion to the clot
Vitronectin[387,388]	Gln93	—	—
Thrombospondin[389]	—	Fibrin	—
Factor V[390,391]	—	Fibrin, platelets	Increased thrombin generation at the clot surface
Actin[216,217]	—	Fibrin	Clot retraction, stabilization of the platelet cytoskeleton
Myosin[218]	—	Itself	Clot retraction, stabilization of the platelet cytoskeleton
Vinculin[219]	—	Fibrin	Clot retraction, stabilization of the platelet cytoskeleton
Hsp27[220]	—	—	Clot retraction, stabilization of the platelet cytoskeleton
$\alpha_{IIb}\beta_3$[226,228,230]	—	Fibrin	Stabilization of the platelet—fibrin clot
$\alpha_V\beta_3$[229,230]	—	—	Adhesion of platelets to endothelial cells

[a]Gln2 and Gln 14 relate to the same glutamine residue from the short and long forms of α_2-AP respectively.

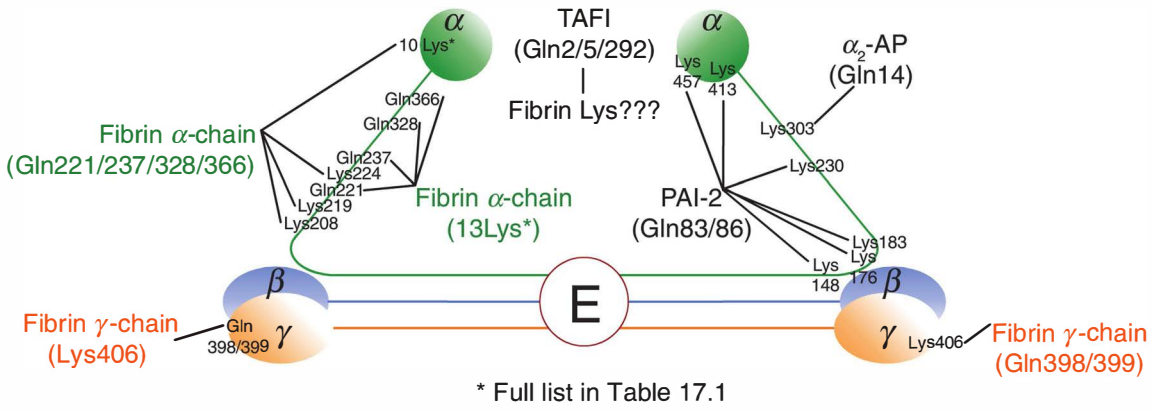

FIGURE 17.4 Fibrin cross-linking sites. Schematic of the fibrinogen molecule, with the central E-region, coiled coil region, and two distal D-regions. The sites where FXIIIa introduces cross-links are indicated in the fibrinogen molecule. The γ-chain (*orange*) of fibrin is cross-linked at residues Lys406 and either Gln398 or 399. The α-chain (*green*) has known cross-linking sites for fibrinolysis inhibitors such as α_2-AP and PAI-2, and many sites have been reported to be involved in α–α cross-linking.

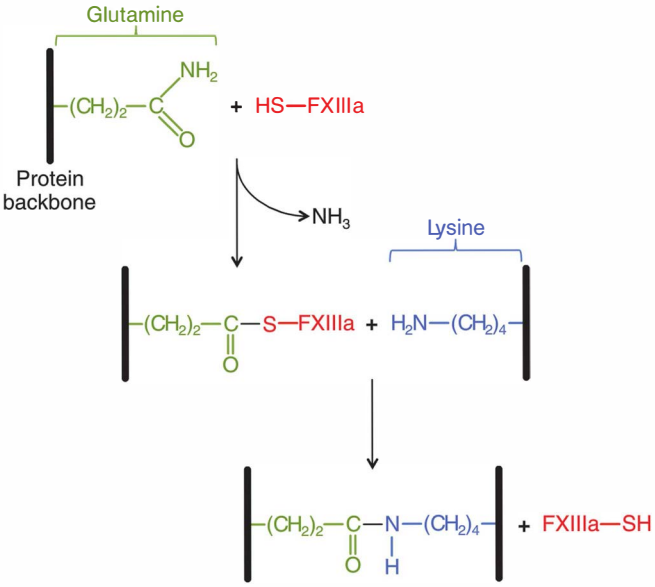

FIGURE 17.5 Transglutaminase reaction by FXIII. FXIIIa active Cys314 reacts with a substrate glutamine to generate an acyl enzyme intermediate and release a molecule of ammonia. The acyl enzyme then reacts with a lysine substrate, resulting in the formation of covalent ε(γ-glutamyl)lysyl bond and release of FXIIIa.

TGs are significantly conserved, with a 75% sequence homology between the active site of TG2 and FXIII-A for example.[82] The only member of the TG superfamily that does not contain a functional active site is erythrocyte protein 4.2.[93] This protein, although not active as a TG, is very abundant in the human red blood cell membrane where it plays a significant role in membrane stability through interactions with CD47, band three, and ankyrin, as demonstrated by hereditary spherocytosis (a form of hemolytic anemia) caused by protein 4.2 deficiency.[94]

FXIII has several unusual characteristics compared with other TGs. Whereas FXIII-A (the TG part of the proenzyme) forms a heterologous tetramer with FXIII-B, most other TGs exist in the form of a monomer. The only other TG that is not a monomer is rat TG4 that has been reported to form a homodimer, similar to FXIII-A.[83] Another uncommon feature of FXIII is that its activation involves proteolytic cleavage by thrombin in the presence of calcium. Most other TGs are activated by the presence of calcium alone, but there are two other exceptions to this. TG3 is activated by proteolytic cleavage by dispase that leads to a 50 kDa *N*-terminal fragment containing the catalytic core and a 27 kDa *C*-terminal fragment.[95,96] The activation of TG1 is also regulated by an additional mechanism, but unlike FXIII-A and TG3 not by limited proteolysis. Activation of TG1 in the absence of extracellular calcium is regulated by conformational changes induced by interaction with tazarotene-induced gene 3 (TIG3).[97,98] TIG3 is also a substrate for TG1, hence it is interesting to compare its stimulatory effect on TG1 activation with the enhancing effect that fibrin has on FXIII activation (see above).

Most TGs are intracellular, cytoplasmic proteins, apart from FXIII-A and TG2 both of which are secreted. TG1 is anchored to the cell membrane via palmitate and myristate linkages at a cluster of five cysteine residues in the *N*-terminus of the protein.[99] TG2 demonstrates guanosine triphosphate (GTP) hydrolizing activity in addition to its cross-linking activity.[100,101] This GTPase activity competes with cross-linking activity in TG2, as binding of GTP inhibits the cross-linking function of TG2.[102] Cross-linking by FXIIIa is however unaffected by GTP,[102] indicating that FXIII does not support GTPase activity.

INTERACTIONS WITH FIBRIN(OGEN) AND ROLE OF FIBRIN α-CHAIN

As mentioned above, the fibrin α-chain contains several potential cross-linking sites, and its cross-linking by FXIIIa leads to high molecular weight polymers. All the α-chain cross-linking sites are located in the *C*-terminal half of the chain (αC), which contains many protein binding sites and plays an important role in lateral aggregation of fibrin.[103] The cross-linking of this chain by FXIIIa therefore contributes to the stability of the laterally aggregated fibrin fiber.

Several studies have been performed to elucidate the interactions of various forms of FXIII with fibrinogen and fibrin. Greenberg's group showed that nonactivated FXIII-A_2B_2 bound specifically to fibrinogen via the FXIII-A_2 subunits with an equilibrium constant (K_D) of 10 nM[104] and identified that FXIII-A_2B_2 binding also occurred with fibrinogen polymerized with protamine sulfate, fibrin I produced with reptilase and fibrin II, indicating that binding of FXIII is not dependent on thrombin or the release of fibrinopeptides.[105] The authors went on to propose a model wherein FXIII zymogen reversibly binds to fibrinogen (via the A-subunits), remaining there after cleavage of fibrinopeptide A, which is cleaved from the fibrinogen α-chain by thrombin during the conversion of fibrinogen to fibrin. At this point the AP of the A-subunits of FXIII is cleaved by thrombin during the formation of fibrin II polymers. Once the A-subunits of FXIII are cleaved, the B-subunits are able to dissociate in the presence of calcium to form FXIIIa.[105] Hornyak and Shafer[63] compared thrombin-cleaved, nonactivated platelet FXIII-A and nonactivated FXIII-A_2B_2 for binding to fibrin. The thrombin-cleaved FXIII-A′ bound with a higher affinity to fibrin (K_D = 2.1 μM) compared to noncleaved FXIII-A subunit (K_D = 14 μM). However, the tetramer FXIII zymogen (A_2B_2) bound to fibrin with a much greater affinity (K_D = 0.2 μM) than either form of the A-subunit preparations. These authors saturated fibrin with plasma FXIII zymogen, and this did not modify the interaction of thrombin-cleaved FXIII-A subunit, suggesting FXIII zymogen and FXIIIa bind to separate sites on fibrin.

Lorand's group established that residues 242 to 424 of the fibrin α-chain play a role in enhancing the dissociation of the A- and B-subunits of FXIII-A_2B_2 following thrombin cleavage.[64] This work therefore indirectly infers that these residues of the α-chain are involved in the binding of thrombin-cleaved FXIII-A_2B_2, which is likely to involve the A-subunit. Subsequent work by Greenberg's group[106] showed, using immunoblotting techniques, that binding of FXIII-A took place on the carboxy-termini of the α- and β-chains, but not on the E fragment (which is the central fragment of the molecule that contains the *N*-termini of all the chains) of fibrinogen (see FIGURE 17.4 for diagram of fibrinogen structure). Procyk et al.[107] found that thrombin-activated FXIII-A subunit bound to residues 389 to 402 of the α-chain, by competing the binding of FXIIIa to fibrin columns with antibodies directed against this

Table 17.2

The transglutaminase family

Name	Location	Tissue Distribution	Main Function(s)	References
Factor XIII	Intra- and extracellular	Blood plasma fraction, platelets, monocytes, macrophages, dendritic cells, chondrocytes, osteocytes, osteoblasts, placenta	Clot stabilization Resistance to fibrinolysis Platelet adhesion to fibrin and stabilization of the platelet—fibrin clot Clot retraction, stabilization of the platelet cytoskeleton Increased thrombin generation at the clot surface Migration of cells into the clot; wound healing Adhesion of platelets to endothelial cells Stabilization of extracellular matrix	109,110,379,380,381 148–151,166–168,175 226,228,230, 385,386 216–220 390,391 382,383 229,230 382,384
TG1 (keratinocyte TG)	Intracellular and membrane	Keratinocytes, brain	Epidermis formation	84
TG2 (tissue TG)	Intra- and extracellular	Ubiquitous	Cell death, multiple other cellular functions	85–87
TG3	Intracellular	Squamous epithelium, brain	Hair follicle stabilization	88
TG4	Intracellular	Prostate	Copulatory plug in rodents, human function unknown	89
TG5	Intracellular	Mainly (but not exclusively) epidermis	Epidermal differentiation	90
TG6	Intracellular?	Unknown	Unknown	91
TG7	Intracellular?	Unknown	Unknown	91
Erythrocyte protein 4.2	Intracellular and membrane	Red blood cells, bone marrow, spleen	Erythrocyte membrane integrity	92

sequence of the fibrin α-chain. More recently, taking a direct approach of recombinant expression of truncated fibrinogen α-chains and using surface plasmon resonance to study the interaction of FXIIIa to these α-chain fragments, FXIII-A was found to bind to residues 233 to 425 in the αC with a K_D of 2.4 μM and this interaction was further localized to αC residues 389 to 403. Using a site-directed mutagenesis approach, it was found that mutation of the single residue Glu396 of the αC-chain abolished the binding to FXIIIa.[108]

ROLE OF FIBRIN(OGEN) γ CHAIN

The fibrin γ-chain is one of the preferred substrates for FXIIIa; its cross-linking is rapidly completed (5 to 10 minutes) after clot formation. FXIIIa cross-links the γ-chains between Lys406 and one of two tandem glutamine residues situated at position 398 and 399.[109,110] Cross-linking occurs when two γ-chains are lined up, the D-regions (which contain the *C*-terminal domains of both the γ- and the β-chains of fibrin) facing each other, in the half-staggered D-E-D fibrin protofibril arrangement of fibrin (see FIGURE 17.4 for diagram of fibrinogen structure). Two γ-chain cross-links are introduced in this assembly across the D-dimer interface in an antiparallel orientation.[111]

In agreement with the structural orientation of the γ-chain at the D–D interface, fibrin γ-chain cross-linking does not normally lead to higher molecular weight complexes, such as those formed during cross-linking of the α-chains, but leads to the formation of γ-chain dimers.[112] When cross-linked fibrin is digested by plasmin, proteolysis of the coiled coils on either side of the cross-linked D-regions leads to the formation of D-dimers.[113–115] Mutation of Lys406 in the fibrinogen γ-chain modifies the topology of fibrin cross-linking by FXIIIa with the production of γ-α hybrid cross-links.[116] Similar γ-α hybrid cross-links are also found when fibrin is cross-linked by

TG2,[117,118] suggesting that Lys406 is a specific acyl-acceptor for FXIIIa and not for TG2.

Some studies have suggested that the γ-chain cross-links introduced by FXIIIa show a different configuration than the longitudinal orientation of antiparallel cross-links across the D–D interface. Mosesson suggested that γ-chain cross-links show a transverse orientation, spanning from one γ-chain in one protofibril across to a second in another protofibril, suggesting that this agrees with fiber extensibility upon stretching of the fiber, which has lead to some debate on this topic in the literature.[119,120] Recent data from atomic force microscopy studies of fibrin fibers at stretch, however, has provided further evidence for the γ-chain longitudinal cross-linking model[121] in agreement with previous x-ray crystallography[122] and EM studies.[123]

Cross-linking of the fibrin γ-chains is inhibited by a monoclonal antibody against the *C*-terminal region of the γ-chain[124] and by clumping factor A from *Staphylococcus aureus* that binds to the same region.[125] Residue Ile387 of the fibrin γ-chain seems to be important for the interaction of the γ-chain with FXIIIa, as mutation of this amino acid impairs γ-chain cross-linking.[126]

It has been suggested that FXIIIa binds to the fibrinogen γ-chain *C*-terminal region. In particular, a splice variant of the γ-chain, fibrinogen γ′, has been reported to interact with FXIIIa. Studies using zymogen FXIII-A_2B_2 suggested that the γ′-chain of fibrinogen was responsible for binding FXIII-A_2B_2, as FXIII-A_2B_2 copurifies with γA/γ′ but not γA/γA fibrinogen. Contrary to this work, studies by Lord's group, using recombinant expression of γA/γA and γA/γ′ fibrinogen, found similar binding affinities for FXIII-A_2B_2, regardless of the presence of γ′.[127] Fibrinogen γ′ is caused by alternative polyadenylation,[128] which leads to two messengers differing at the *C*-terminus. For the γ′-chain, the last 4 residues are replaced by 20 different residues.[129] FXIII copurifies with the fibrinogen γ′-chain, and this effect is due to the B-subunit, as A-subunit on its own does not coelute.[130,131] These findings suggest that FXIII-B may bind to the γ′ sequence, which is negatively charged and also contains a thrombin-binding site.[132–134] FXIII also binds to a γ′ peptide, which competitively inhibits binding of FXIII to fibrinogen.[135] Fibrinogen γ′ has been shown to associate with increased activation of FXIII.[136] The mechanisms behind the γ′/FXIII interaction remain largely unknown and further studies will be needed to characterize the physiologic relevance of these findings.

CROSS-LINKING OF INHIBITORS OF FIBRINOLYSIS

FXIIIa cross-links several protease inhibitors to fibrin, in order to protect the clot from breakdown (fibrinolysis) (Table 17.1, FIGURE 17.4). Plasmin is the main protease responsible for fibrinolysis, and its major inhibitor is the serine protease inhibitor α_2-antiplasmin (α_2-AP). Deficiencies and abnormalities in plasma α_2-AP result in severe hemorrhagic disorders,[137–146] characterized by increased susceptibility to fibrinolysis, which can be reverted *in vitro* by addition of purified α_2-AP to plasma from deficient patients.[147] FXIIIa is able to cross-link Gln2/14, 21, 419, 447 of α_2-AP[148–150] to Lys303 of α-chain fibrin.[151] Circulating free α_2-AP inhibits tissue plasminogen activator (tPA)-induced fibrinogenolysis,[152] whereas FXIIIa-mediated cross-linking of α_2-AP to fibrin results in inhibition of fibrinolysis.[153] Plasmin bound to fibrin is protected from inhibition by α_2-AP.[154,155] There are two forms of α_2-AP circulating in human plasma. The native α_2-AP is secreted as a 464–amino acid protein with amino-terminal methionine (Met–α_2-AP) that can be cleaved between Pro12–Asn13 in the circulation to form a 452–amino acid protein with asparagine at the amino-terminus (Asn–α_2-AP).[156,157] The enzyme responsible for this cleavage has been characterized as antiplasmin cleaving enzyme (APCE).[158,159] Both forms of α_2-AP have similar inhibitory activity toward plasmin,[158] but the major form (70%) Asn–α_2-AP is more rapidly cross-linked to fibrin than the Met–α_2-AP form (30%), and the plasma clot lysing time increases with Asn–α_2-AP/Met–α_2-AP ratio.[158] It appears therefore that the *N*-terminal 12–amino acid sequence is inhibitory toward the cross-linking of α_2-AP by FXIIIa. Recently, a single nucleotide polymorphism (SNP) has been shown to affect the sixth amino acid of α_2-AP, Arg6Trp.[160–162] The arginine and tryptophane alleles have a frequency of 79.5% and 20.5%, respectively.[163] Also, this Arg6Trp SNP results in an altered ability of the Met–α_2-AP(Trp6) to be cleaved by APCE, compared to Met–α_2-AP(Arg6), with potential effects on fibrinolysis.

Plasminogen activator inhibitor-2 (PAI-2), which inhibits the conversion of plasminogen into plasmin by urokinase plasminogen activator and tPA,[164,165] is also a substrate of FXIIIa for cross-linking to fibrin. PAI-2 reacts with FXIIIa through putative Gln83 and 86,[166] and is cross-linked to Lys148, 176, 230, 183, 413, and 457 of the fibrin α-chain.[167,168] The inhibitory effects of PAI-2 are not affected by cross-linking,[166] and PAI-2 cross-links with fibrin at sites different to that of α_2-AP, resulting in an increased stabilization of the fibrin clot. Although α_2-AP circulates in the plasma, PAI-2 is not detected in normal plasma; it is mainly secreted into the matrix of tissues,[169,170] and could play an important role in reducing fibrinolysis during wound/tissue repair.[171] Its incorporation into fibrin may also play a role during pregnancy, as circulating PAI-2 levels are elevated throughout pregnancy, and during the first few days postdelivery, before returning to undetectable levels.[172] Unlike α_2-AP, no polymorphism in the PAI-2 gene that affects cross-linking to fibrin has been reported to date.

Another inhibitory protein of the fibrinolytic pathway, thrombin-activatable fibrinolysis inhibitor (TAFI),[173,174] was also identified as a FXIIIa substrate, with residues Gln2, 5, and 292 participating in the cross-linking to fibrin,[175] although the reactive lysine residues have yet to be identified. Pro-TAFI is activated through cleavage by thrombin at Arg92,[173,174] resulting in the release of the AP and the loss of two major cross-linking sites (Gln2 and 5). Activation of TAFI may therefore result in the release of the inhibitor from its cross-linked position in fibrin, leading to fibrin-localized antifibrinolytic activity. TAFI is a plasma carboxypeptidase B,[173,174] of which the antifibrinolytic activity is associated with the removal of *C*-terminal arginine and lysine from fibrin, resulting in a decreased binding of plasminogen to fibrin, and therefore a reduced conversion of plasminogen to plasmin on the fibrin clot.[176] In the last decade, SNPs have been shown to play a role on TAFI-related inhibition of fibrinolysis; Ala147Thr and Thr325Ile polymorphisms play a role on TAFI level in plasma,[177,178] and the Ile325 variant shows greater activity than the Thr325 variant.[179] The Ala147Thr polymorphism is in linkage disequilibrium with a promoter-C1 542G polymorphism that strongly affects the level of TAFI expression,[180] and C1542G and Thr325Ile are related to different types of acute coronary syndrome.[181] The Thr325Ile polymorphism is also strongly associated with risks of stroke,[182] coronary

heart disease,[183] venous thromboembolism,[184] and restenosis.[178] Despite its link with TAFI levels, the Ala147Thr polymorphism did not show any association with stroke,[182] acute coronary syndrome,[181] or aneurysmal subarachnoid hemorrhage.[185]

ROLE OF CROSS-LINKING BY FACTOR XIII IN FIBRIN STRUCTURE AND ELASTICITY

One of the main functions of cross-linking of fibrin by FXIIIa is to increase the stiffness of the blood clot. Fibrin clots demonstrate both elastic and viscous properties. Fibrin has unique properties amongst biologic polymers in that it can be stretched several times without damage to its structure.[186] The viscoelastic properties of fibrin can be attributed to its properties at the molecular level, with unfolding of domains providing a basis for stretching of the molecule,[121,187] and at the level of individual fibers,[188] as well as to structural characteristics at the level of the whole clot.[189]

Cross-linking by FXIIIa increases the storage modulus (stiffness) of fibrin, and decreases the loss tangent or amount of energy dissipated during deformation.[190] This occurs in purified systems as well as in plasma.[191] The elastic modulus of individual fibrin fibers is increased significantly by cross-linking as analyzed by optical tweezers and atomic force microscopy.[121,188,192] The strength of branch points is also increased by cross-linking, resulting in a reduction of ruptured branch points from 70% to 40% when branched fibers are stretched using the atomic force microscope.[193] The stiffness of fibrin increases when the clot is stretched, a phenomenon that is called strain hardening.[194] Cross-linking by FXIIIa has been shown to reduce strain hardening by around 50%.[121] Permanent deformation of fibrin structure at strain is reduced by cross-linking; therefore, the elastic properties of cross-linked fibrin gels are maintained at higher strain than those that are not cross-linked.[195]

It has been described that the timing of the increase of fibrin stiffness during clot formation correlates more closely with the formation of α-chain cross-links, rather than with the earlier formation of γ-chain cross-links, suggesting that the increase in stiffness is largely due to α-chain cross-linking.[196] Studies using an autoantibody that inhibited primarily α-chain cross-linking, appeared to confirm that the increase of fibrin stiffness is largely due to α-chain cross-linking and the formation of highly cross-linked polymers.[197] However, more recent studies of fibrinogen with mutations in the γ-chain cross-linking sites showed that γ-chain cross-linking also significantly contributes to clot stiffness, with a 40% increase in the storage modulus due to γ-chain dimerization by FXIIIa.[116] The same study also showed that α–γ hybrid cross-links were least effective in enhancing fibrin stiffness.[116] It has been suggested that the αC-region, which extends between protofibrils, mediates the extensibility of fibrin.[192] Cross-linking in this region by FXIIIa may reduce the effective length of the αC-region link between protofibrils and hence reduces the length by which fibrin can be stretched.[121]

Besides its role in fibrin elastic properties, FXIIIa has been suggested to modulate the structure of the fibrin clot. One study indicated that cross-linking by FXIIIa did not introduce gross changes in fibrin clot architecture, but that it reduced fiber diameter by around 10% to 20%.[198] Other studies on FXIII-A Val34Leu polymorphism found that the Leu34 allele associates with thinner fibrin fibers and smaller clot pores,[199] and that these changes are dependent on fibrinogen concentration.[200] Fibrin formation is a dynamic process, as fibrin fibers continue to grow and rearrange themselves long after the initial gel point.[201] FXIII activation is regulated by fibrin formation as described above. Hence, the architecture of the clot is determined by the conditions that regulate the kinetics of fibrin formation and those of FXIII activation.

FACTOR XIII AND PLATELETS

Before forming the circulating heterotetramer form of FXIII, both subunits A and B are synthesized and secreted by different cell types. The B-subunits are synthesized by hepatocytes,[29,202] whereas macrophages and monocytes[25,203,204,205,206] are a source of catalytic A-subunits. FXIII-A is mainly synthesized by megakaryocytes[207] and stocked in the cytosol of nascent platelets,[208–211] where it reaches concentrations estimated at 100- to 150-fold higher than in the blood plasma.[212] Some heterodimeric form of FXIII is also taken up into the platelet α-granules by endocytosis, but its amount is negligible when compared with that of cytosolic FXIII-A.[213] The role of this platelet FXIII-A pool in contributing to plasma FXIII levels remains to be clarified. An initial study showed that plasma FXIII-A levels correlated to platelet counts but not monocyte counts,[214] and the authors concluded that platelets contribute significantly to plasma FXIII-A levels. However, other factors could contribute to this correlation and FXIII-A plasma levels. In a more recent study, platelet counts were not altered in two transgenic mice lines displaying thrombocytopenia through decreased platelet life span and megakaryocytes generation.[27] Although these latter findings cannot exclude the existence of compensatory mechanisms, they indicate that platelets are not the major source for plasma FXIII-A. Also, unlike FXIII-B, the A-subunit does not contain an endoplasmic reticulum signal sequence,[14,17] crucial for integration in the ER–Golgi secretion pathway, and therefore cannot be secreted. Further studies will be required to unequivocally identify the source of plasma FXIII-A, and the mechanism by which its levels are maintained. FXIII-A appears to be associated with peripheral Golgi proteins,[27] highlighting a possible release of FXIII-A through a nonclassical secretion pathway identified for the secretion of other proteins.[215]

In addition to the documented extracellular role of FXIIIa in fibrin clot stabilization and tissue repair, intracellular FXIII-A plays a role in cytoskeletal remodeling. Upon activation of platelets, cytoplasmic calcium concentration increases, leading to intracellular FXIII-A activation through a nonproteolytic process.[212] FXIII-A plays a role in cytoskeletal reorganization, associated with platelet activation, through the cross-linking of various substrates (Table 17.1). Actin[216,217] and myosin[218], playing a major role in cell migration, are cross-linked to each other by FXIII-A, resulting in stabilization of the cytoskeleton. Furthermore, vinculin[219] that plays a role in linking the cytoskeleton to the plasma membrane, and heat-shock protein 27[220] that regulates actin polymerization, are also FXIII-A substrates. Since it plays a role in cytoskeleton remodeling and platelet contraction plays a role in clot retraction,[221] a recent study investigated the role of FXIII-A on this process *in vivo*.[222] FXIII-A knock-out mice were found to have impaired clot retraction, but the relevance of cross-linking of cytoskeletal proteins by FXIIIa in this process remains to be fully explored.

FXIII is also present on the surface of activated but not resting platelets,[223–226] through binding to glycoprotein (GP) IIb/IIIa (integrin $\alpha_{IIb}\beta_3$)[226,227] (Table 17.1). Platelets activated by a mixture of collagen and thrombin, known as COAT platelets,

secrete many procoagulant proteins, such as fibrinogen/fibrin and α_2-AP, from the α-granules,[228] which are then bound to the cell surface in a FXIII-dependent manner.[228] In this study, the authors also highlighted the necessity of serotonin cross-linking to these proteins for cell surface binding. Although serotonin is a TG substrate,[228] further studies are required to assess the role of FXIII in the serotonylation of the α-granule proteins, and to identify the serotonin receptor. In addition to binding platelets, FXIII-A mediates the binding of these platelets to endothelial cells, through the dual binding of FXIII to platelet GP IIb/IIIa and endothelial cell integrin $\alpha_V\beta_3$.[229,230] This FXIII-dependent adhesion of platelets to endothelial cells could play a role during tissue repair following vascular injury.

HETEROGENEITY OF FACTOR XIII A-SUBUNIT

The gene coding for FXIII-A is highly polymorphic, as first established by agarose gel electrophoresis.[231] Two common alleles have been described by isoelectric focusing, FXIIIA*1 and FXIIIA*2. Further isoelectric focusing analysis showed that these two isoforms could be split into four subtypes, FXIIIA*1A, FXIIIA*1B, FXIIIA*2A, and FXIIIA*2B.[232] The four allele subtypes were later identified as product of two SNPs, A614T and C1894T,[233] resulting from four combinations of the two sets of different amino acids coded by these SNPs.

The most studied SNP in FXIII-A is a prevalent G103T mutation resulting in amino acid change Val34Leu.[234] In white populations, the frequency of the Leu34 allele ranges from 24.5% to 28.8%.[235,236,237–242] This frequency is lower in black[238,241] and Asian Indian[237] communities, and extremely rare in these Japanese population.[241,243]

The FXIII Leu34 variant is more quickly activated by thrombin,[199,240,244] and the resulting clots have thinner fibers and smaller pores.[199] The higher activation rate of this variant is related to the fact that the SNP is located only four amino acids away from the cleavage site (Arg37–Gly38) by thrombin. The substitution of valine with leucine at residue 34 leads to improvements in both the K_m and catalytic constant (k_{cat}) during thrombin cleavage of Arg37–Gly38.[245] Once fully activated, there is no influence of this polymorphism on FXIIIa specific activity.[244]

FXIII Val34Leu has been associated with a wide range of CVD. Despite contradicting individual studies, meta-analysis studies have concluded that Leu34 allele is associated with a protective effect on CADs,[246] myocardial infarction (MI),[247] and DVT,[248] but studies of the role of FXIII Val34Leu in stroke has produced inconsistent data,[242,249–253] and no effect was observed on peripheral artery disease (PAD).[250] FXIII Val34Leu has also been associated with increased risk of aneurysmal subarachnoid hemorrhage[185] and thrombotic microangiopathies,[254] and worse efficacy of thrombolytic therapy.[255–257] Interestingly, an association between Val34Leu polymorphism and Alzheimer disease has been documented.[258] Although the mechanisms linking FXIII to Alzheimer disease are still unclear, TG activity is documented to be responsible for cross-linking of amyloid β-peptides,[259] and FXIIIa has been detected in reactive microglia and macrophages of various neurologic disorders, including Alzheimer disease.[260]

Two other common SNPs in FXIII-A, an A614T substitution and a C1894T point mutation resulting in Tyr204Phe[243] and Pro564Leu[233] respectively, have been studied to a lesser extent. The Phe204 allele is associated with decreased plasma FXIII level and activity, whereas the Leu564 allele is associated with lower level and higher activity.[261,262] Only a few studies have linked these polymorphisms to CVD. Variant Phe204 may be a marker of susceptibility to ischemic stroke (IS),[263,264] but does not seem to affect risks of MI.[250,265] Leu564 allele is associated with slightly increased risk of MI in younger women[265] and smaller venous ulcer surface.[266] Although this variant did not influence the risk of IS,[250,264] concomitant presence of the Leu34 allele was associated with an increased risk of IS.[267] Two recent studies are in contradiction regarding a link between Leu564 and aneurysmal subarachnoid hemorrhage.[185,268]

Other SNPs, such as Val650Ile and Glu651Gln,[13,14] have been described (FIGURE 17.6), but their roles in CVD are unknown. A study using 201 dizygotic twin pairs showed that Val34Leu, Tyr204Phe, and Pro564Leu, but not Val650Ile or Glu651Gln, polymorphisms are associated to levels of FXIII activation.[269] In this study, Val34Leu was found to be the main functional SNP influencing FXIII activation.

HETEROGENEITY OF FACTOR XIII B-SUBUNIT

The noncatalytic FXIII-B is a highly polymorphic blood protein, like FXIII-A. Three main isoforms were identified by agarose gel electrophoresis and isoelectric focusing experiments.[270–272] These alleles, FXIIIB*1, FXIIIB*2, and FXIIIB*3, are related to European, African, and Asian populations respectively, with a clear segregation of the alleles between the continents.[273] Other less frequent isoforms include FXIIIB*4, FXIIIB*6, and FXIIIB*13 to 15.[274–276] Hitherto, two major SNPs have been identified (FIGURE 17.6), His95Arg (FXIIIB*2),[277] and C29756G ([IVS11]+144C>G) change in intron 11 (FXIIIB*3), generating a new splice variant.[278,279]

There have been a few studies on the functional characteristics of FXIII-B His95Arg and its associations with thrombosis. In the first study, plasma FXIII-B dissociation from the tetrameric form was found to be increased for Arg95 variants, which could be associated with increased FXIIIa enzymatic activity.[277] In this study, *in vitro* steady-state binding experiments did not confirm these results, so further investigations are required to fully understand the effects of His95Arg polymorphism. Although very few studies have investigated the role of FXIII-B polymorphisms in relation to CVD, carriers of the Arg95 variant of FXIII-B may have an increased risk of IS,[264] DVT,[277] and aneurysmal subarachnoid hemorrhage,[268] but this variant may not affect risks of MI.[265,280] Interestingly, combination of both FXIII-A Leu34 and FXIII-B 95Arg alleles reduced MI risk by 70% in women in estrogen therapy.[281]

The 29756G allele, mainly found in Asian population, results in a splicing variant coding for a 15–amino acid longer B-subunit at the carboxy-terminus compared to the wild-type protein.[278] Although this isoform presents a different isoelectric point,[278] the location of these extra amino acids is thought to not influence the Sushi-domain structure of the protein.[279] Together with another extremely rare Glu368Val polymorphism (FXIIIB*4),[278], the biochemical and clinical properties of this splicing variant remain to be further investigated.

FACTOR XIII DEFICIENCIES

Congenital FXIII deficiency is a rare autosomal recessive disorder,[282–288] first documented in 1960 in Switzerland,[12] with a frequency of 1 per 2 million and a higher prevalence in areas of high rate consanguinity.[289] The disease was initially classified as one of two subtypes, type 1 and type 2, which were characterized

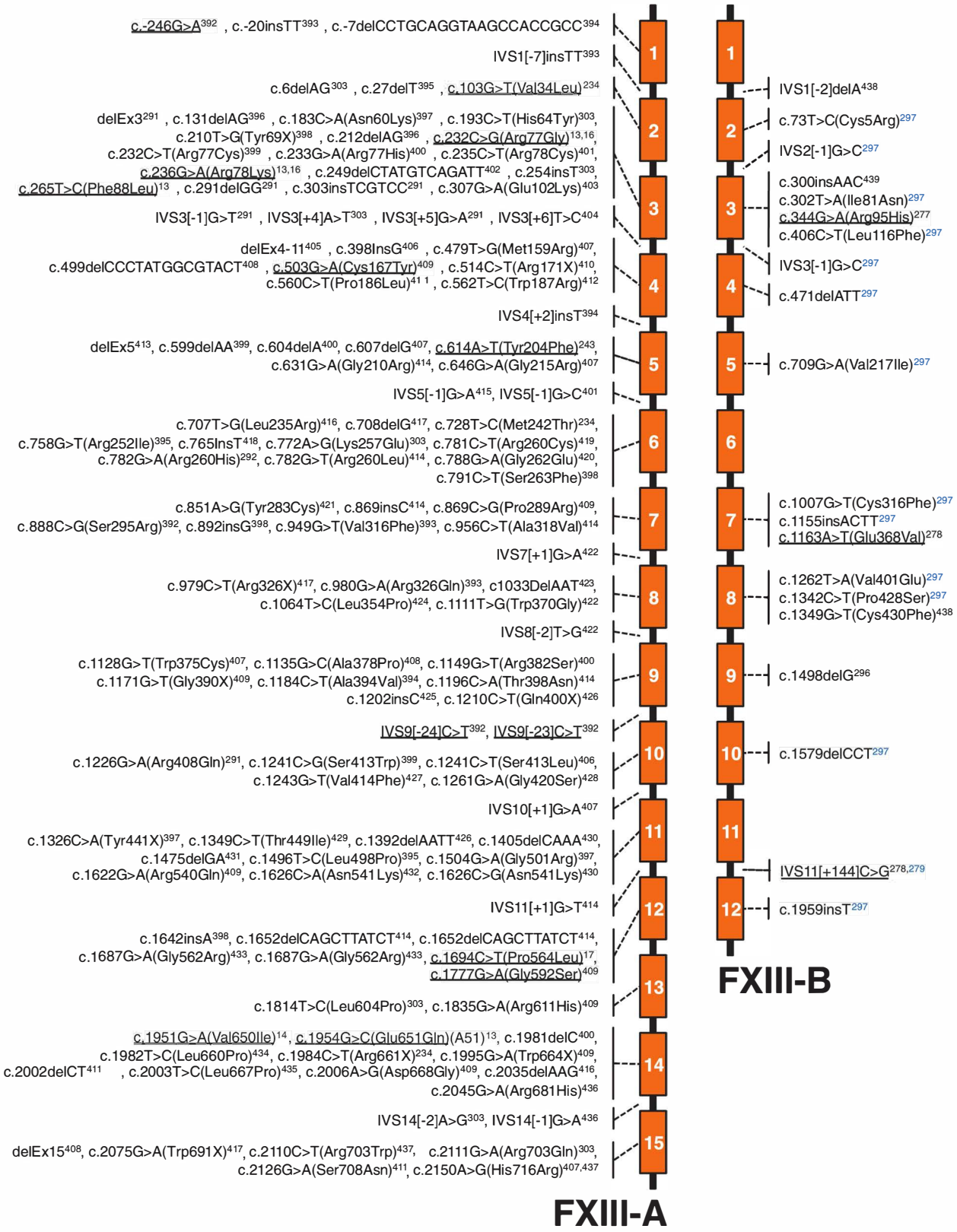

FIGURE 17.6 Genetic mutations of FXIII A- and B-subunits. Common polymorphisms in FXIII are underlined, and the remaining mutations have been identified in FXIII deficient patients. Nucleotides are numbered from the published cDNA sequences of FXIII-A[17] and FXIII-B,[46] with nucleotide one corresponding to the A of methionine-coding ATG translation initiation codon. (Modified and updated from Karimi M, Bereczky Z, Cohan N, et al. Factor XIII deficiency. *Semin Thromb Hemost* 2009;35(4):426–438.)

by the absence in the circulation of both FXIII-A and -B or the absence of FXIII-A alone, corresponding to FXIII-B deficiency (type 1) and FXIII-A deficiency (type 2) respectively.[290]

Genetics of Factor XIII-A Deficiency

FXIII-A deficiency is the most frequently described (>95% of cases) of the two subtypes, with 54 missense, 9 nonsense, 14 splice-site, 23 deletion, and 9 insertion mutations leading to this type of deficiency reported up to December 2010 (listed in FIGURE 17.6). Mutations in FXIII-A associated with deficiency are mainly located in exons 3 to 15. So far, none has been reported in the catalytic triad (Cys314, His373, and Asp396), but mutations within the active domain, such as Arg408Gln,[291] have been reported and could grossly affect the activity of the protein. In many studies, FXIII-A mRNA levels were unchanged in patients suffering from this deficiency, but intracellular proteins could not be detected, indicating that these mutations lead to the formation of unstable proteins that are degraded within the cell.[292–294]

Genetics of Factor XIII-B Deficiency

FXIII-B deficiency is the less common type (<5% of cases). Up to December 2010, eight missense, three splice site, three deletion, and three insertion mutations have been reported to be responsible to this disease (FIGURE 17.6). Similarly to FXIII-A deficiencies, FXIII-B mutations result in decreased ability of the protein to be secreted from cells,[295,296] and decreased protein stability.[297] Despite FXIII-A synthesis being unaffected in this particular subtype, the lack of B-subunit results in an unstable plasma A-subunit, which will affect its functions.[298] Similarly, FXIII-$B^{-/-}$ mice exhibit decreased FXIII activity and A-subunit levels. Injection of recombinant B-subunit in the FXIII-$B^{-/-}$ mice resulted in an increase in both cross-linking activity and FXIII-A antigen levels, highlighting the importance of the B-subunit in stabilizing the A-subunit in the plasma.[299]

Clinical Phenotypes

The main clinical symptoms of FXIII-A deficiency are severe bleeding, recurrent miscarriages, and impaired wound healing. In 70% of cases, umbilical cord bleeding is reported, and most patients will exhibit ecchymoses, hematomas, and prolonged bleeding.[300–303] Intracranial hemorrhage is observed in 30% of FXIII-A deficient patients.[300–303] The other main sites of bleeding are mouth and gums, muscles and joints, mucous membranes, peritoneum, and sites of surgery.[300–303] FXIII-$A^{-/-}$ mice were also described to present subcutaneous, intraperitoneal, and intrathoracic hemorrhage.[304] Since the first case of recurrent miscarriage in a FXIII deficient patient was reported,[305] all subsequent studies reported that women with this deficiency suffer from recurrent pregnancy loss, although the causes are still unknown.[306] The level of FXIII required for carrying pregnancy to term is around 10%.[307] FXIII-$A^{-/-}$ mice exhibited vaginal bleeding, placental hemorrhage, detachment, and necrosis.[308] Impaired wound healing has been reported in up to 30% of FXIII-A deficient patients,[300,301,303] and FXIII-$A^{-/-}$ mice also showed impaired wound and cardiac healing.[309–311] All cases of FXIII-B deficiency reported to date showed milder bleeding symptoms, which are mostly caused by trauma, surgery, and delivery,[297] but successful pregnancies can be observed.[297,298] FXIII-$B^{-/-}$ mice also exhibit milder phenotype of FXIII deficiency.[311] A likely reason for the milder phenotype of FXIII-B deficiency is the normal presence of intracellular FXIII-A, which, through the contribution of platelet FXIII-A at the site of injury, may contribute to fibrin cross-linking and local wound healing.

Diagnosis

Because of the high level of mortality and morbidity in FXIII deficiency when left untreated, it is important that the deficiency of this coagulation factor is correctly diagnosed. Diagnosis is complicated by the fact that routine coagulation test (activated partial thromboplastin time, prothrombin time, thrombin time) parameters are unaffected by FXIII deficiency. For a long time, it was common practice to base the diagnosis of FXIII deficiency on a qualitative test of clot solubility in strong urea or acid.[312] However, a recent study showed that this test may only detect very severe FXIII deficiencies, is characterized by poor reproducibility, interlaboratory variability, and poor sensitivity, and it was therefore not recommended as a screening test for this deficiency.[313] The correct diagnosis of FXIII deficiency requires a quantitative activity assay followed by antigen assays, in order to classify the type of deficiency. Several quantitative activity assays have been reported that are commonly based on the measurement of ammonia released in the first step of cross-linking, or on the measurement of the cross-linking of a biotinylated amine substrate into a protein substrate, the latter usually coated on a microtiter plate. In ammonia release assays,[314–316] the amount of free ammonia released by FXIII-dependent cross-linking of substrates is proportional to the amount of NAD(P)H consumed by glutamate dehydrogenase (NH_3 + α-ketoglutarate + NAD(P)H → glutamate + NAD(P)). The decrease in NAD(P)H is then measured spectrophotometrically by absorbance at 340 nm. Automated assays are available commercially, showing similar sensitivities.[317] In amine incorporation assays, the amount of labeled amine substrate bound to glutamine substrate,[318–325] in the presence of FXIII, is measured by fluorescence, radioactivity, or colorimetry.[318–325] Automated microtiter plate tests are available using the following reaction: fibrinogen + 5-(biotinamido)pentylamine (BAPA) → fibrinogen-BAPA + NH_3. Bound BAPA is then chromogenically measured. This type of assay has been shown to efficiently detect FXIII deficiency.[326] Research and development is still ongoing in order to increase the sensitivity of these techniques.[327–329] ELISA-based antigen assays have been developed,[261,330–333] which may be used to quantify FXIII A-subunits, B-subunits and A_2B_2 heterodimers, determining the type of FXIII deficiency.

Treatment

Patients suffering from FXIII deficiency are currently treated with a number of sources for FXIII supplementation. The three main types of supplementation are fresh-frozen plasma, cryoprecipitate, and pasteurized plasma FXIII concentrate. Lifelong prophylactic therapies are commonly used in FXIII deficient patients due to high risks of spontaneous hemorrhage. Since fresh-frozen plasma and cryoprecipitate present high risks of transmission of blood-borne diseases, viruses-inactivated plasma concentrate is the most commonly used source of FXIII. This treatment does not present major inconvenience to patients due to the long half-life of plasma FXIII (around 12 days),[334] and many reports have

indicated efficient prophylaxis for 10 to 20 U/kg in 4 to 6 weeks interval over periods of 5 to 20 years.[335–337] More recently, human recombinant FXIII-A_2 homodimer concentrate has been produced from *Saccharomyces cerevisiae*.[338] In phase I trials, rFXIII-A_2 was shown to associate with endogenous B-subunit, and normalize clot solubility. The heterodimer A_2B_2 half-life was 8.5 days, and no safety issue (yeast antibodies formation, clinical parameters) was observed. A preliminary clinical report using such rFXIII-A_2 showed that 35 U/kg may be the appropriate dose for pre/postoperative replenishment of FXIII-A in cardiopulmonary bypass.[339] To prevent abortion in FXIII deficient woman, plasma FXIII level must be maintained above 10% by replacement therapies, preferentially using pasteurized plasma FXIII concentrate.[307] To maintain this 10% level, recommended doses are 250 U/kg every 7 days in early gestation and 500 U/kg every 7 days after 23 days.[307] In order to prevent delivery-triggered hemorrhage, the levels of plasma FXIII should be higher than 30%, which can be achieved with a booster dose of 1,000 U/kg prior to labor.[307]

ACQUIRED FACTOR XIII DEFICIENCY

Acquired FXIII deficiency can occur as a consequence of an autoimmune response,[340,341] or as a secondary event to an underlying disease state where there is decreased synthesis and/or increased consumption of FXIII. Examples of the latter include disseminated intravascular coagulation, certain inflammatory diseases (e.g., Crohn disease and ulcerative colitis), leukemia, myelodysplasia, myeloproliferative disorders, and liver disease.[21] Acquired FXIII deficiency also occurs perioperatively. Here, it has been shown that early substitution of FXIII levels during surgery benefits patients.[342] Phase III clinical studies are underway to investigate the benefits of recombinant FXIII-A therapy in patients undergoing heart surgery.

It is interesting that in the cases of acquired FXIII deficiency even mild levels of deficiency are able to cause bleeding problems, unlike conditions of hereditary deficiency where apparently moderate levels of deficiencies can be tolerated.[342] Therefore, it is important to be able to diagnose cases of acquired FXIII deficiency with sensitive enough assays to allow treatment of patients under appropriate circumstances.[317]

FACTOR XIII AND THROMBOSIS

Cardiovascular disease is the most common cause of morbidity and mortality globally, causing 17.1 million deaths every year, rising to an estimated 23.6 million by 2030 (World Health Organization). In these patients, thrombotic complications such as MI, IS, PAD, DVT, pulmonary thromboembolism (PE), and coronary sclerosis (CS) are the ultimate cause of death.[343]

The role of fibrin clot in relation to thrombosis and thrombolysis is still poorly understood. Sustained presence of fibrin clot could play a major role in the formation and maintenance of the thrombus, therefore highlighting a potential role of FXIII in this process. Two initial studies highlighted the potential role of FXIII in thrombosis. The first study, using a canine model of coronary thrombosis, showed that inhibition of FXIII increased endogenous thrombolysis, and decreased reperfusion time.[344] In the other study, inhibition of FXIII activity increased emboli lysis in a ferret model of PE.[345]

Clinical studies suggest that FXIII could play a role in thrombosis, although the mechanisms involved remain unclear. FXIII activity and antigen levels have been investigated in many thrombotic diseases. Patients with PAD show elevated activity and antigen levels.[346–348] Female but not male patients show increased risks of CAD, such as MI and CS, when FXIII levels are increased.[349] Patients diagnosed with IS show elevated FXIII-B, but not A-subunit, levels,[350,351] whereas patients suffering from DVT and PE show decreased FXIII-A levels.[352,353]

As described earlier in this chapter, the first step of FXIII activation consists of cleaving the AP by thrombin. A recent study showed that AP is released from FXIIIa following activation, and a specific ELISA method was developed to detect this peptide in the plasma.[354,355] The authors propose FXIII AP as a possible marker for acute thrombotic events, prior to the formation of a thrombus.

Studies of genetic polymorphisms also highlighted a potential role for FXIII in thrombosis. Both FXIII A- and B-subunit polymorphisms have been associated with various thrombotic diseases (see above). The effects of FXIII polymorphisms can be modulated by other factors influencing the risk of arterial thrombosis, such as fibrinogen concentration and insulin resistance. FXIII-A Leu34 variant was associated with increased risks of CAD when fibrinogen concentrations were low, whereas high concentrations resulted in decreased risk.[200,356] Similarly, in the Hungarian population, high fibrinogen concentration was associated with a protective role of Leu34 against CS and MI.[357] This association between the FXIII Val34Leu polymorphism and fibrinogen concentration was also reported for risk of DVT associated with Leu34 polymorphism.[358,359] In another study, the protective effects of Leu34 on CAD were lost in patients with insulin resistance.[360]

Treatment of certain thrombotic events consists of inhibiting thrombus formation with oral anticoagulants such as warfarin, however this approach does not affect fibrinolysis of already formed thrombi, and requires regular monitoring of coagulation parameters. Fibrinolytic agents may be used in certain acute situations of MI or IS, but they can induce severe complications such as fatal bleeding.[361,362] It is therefore primordial to develop techniques allowing diagnosis of early thrombus formation. Fibrin-based contrast agents have been developed for *in vivo* thrombus visualization by magnetic resonance imaging (MRI) in animal models,[363–370] and one human study using such approach indicated a potential use of this method in humans.[371] New probes have been developed based on the finding that factor XIIIa ability to cross-link α_2-AP into thrombi declines with time,[372] therefore allowing the detection of newly formed thrombi. These probes are based on the N_{13}QEQVSPLTLLK$_{24}$ sequence of α_2-AP, required for cross-linking to fibrinogen by FXIII. Two probes, A14 and A15, were developed for use in near-infrared fluorescence (NIRF) and MRI, respectively.[373] NIRF probe A15 successfully visualized newly formed intravascular thrombi in mouse models by intravital fluorescence microscopy (IVM),[374,375] although this type of probe might not be useful in human studies due to the limited penetration depth of IVM. MRI techniques using contrast agents related to A14 have now been optimized in mouse models of thrombosis.[376–378] These peptides may provide useful future tools for potential early diagnosis of thrombotic events in humans, and for the study of the relevance of FXIII to thrombosis *in vivo*.

CONCLUDING REMARKS

In more than six decades of FXIII research, a great deal has been learnt about this clotting factor. We know that FXIIIa stabilizes the fibrin clot by the formation of covalent isopeptide bonds. The gene and protein structures of the catalytic subunit have been deciphered, and we know that both FXIII subunits are highly polymorphic. FXIII deficiency is associated with severe bleeding, poor wound healing, and pregnancy loss. Yet, FXIII continues to intrigue the scientific community and many novel aspects of FXIII physiology and pathology are continuously being discovered in new studies.

We are only recently beginning to understand the molecular mechanisms involved in the interaction of FXIII with its main substrate, fibrin, which not only acts as a substrate but also regulates FXIII activation. FXIII interacts with both fibrin α- and γ-chains and recent studies have focused on elucidating the relative roles of these two interactions in FXIII activation and function. The fibrin clot has a remarkable elasticity, and the role of α- and γ-chain cross-linking in the regulation of this aspect of fibrin formation is beginning to be understood. The molecular structure of FXIII has only been partly characterized. While the detailed structure of FXIII-A is known, the structure of FXIII-B remains to be discovered at atomic level. Also the structure of the tetramer complex is poorly described and the mechanism by which the FXIII-A_2B_2 zymogen is secreted remains to be fully elucidated.

One of the main features of the FXIII subunits is their heterogeneity, which has lead to major discoveries about the role of FXIII in disease. Common genetic variants of FXIII have been discovered, which are functional and associate with atherothrombotic disease. The role of FXIII in thrombosis is however still controversial and the mechanisms that are involved remain to be fully elucidated.

It has been known for some time that congenital deficiency of FXIII, with FXIII levels of <5%, leads to a severe bleeding disorder. However, recent findings of acquired deficiency indicate that also moderately decreased levels associate with a risk for bleeding, suggesting that the association between FXIII and bleeding is not a threshold relationship but could be more gradual. While FXIII-A deficiencies have been described more extensively, recent studies have also found increasing evidence for FXIII-B deficiencies. FXIII deficiency may currently be underdiagnosed as routine coagulation screening assays do not readily detect it and specific assays need to be performed for accurate diagnosis.

While many of the features of FXIII have been detailed, others are still elusive and remain to be discovered. The intriguing possibilities of developing new diagnostic tools based on FXIII and those of designing new drugs that modulate FXIII function for the treatment of disease are a major motivator for such future studies of this remarkable clotting factor.

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CHAPTER

18 Antithrombin and the Serpin Family

SUSAN C. BOCK

ANTITHROMBIN III

Overview

Antithrombin III (ATIII) is a plasma proteinase inhibitor that inactivates thrombin and the enzymes responsible for the generation of thrombin. This combination of properties makes ATIII a very powerful and important endogenous anticoagulant molecule and explains why patients with even modest ATIII deficiencies experience clinical thrombosis. The anticoagulant activity of pharmaceutical heparins derive from their ability to potentiate the inhibitory activity of ATIII by mechanisms that are similar to physiologic activation of ATIII by vessel wall heparan sulfate proteoglycans (HSPGs). In addition to its well-known anticoagulant activity, ATIII also has anti-inflammatory, antiproliferative, antiangiogenic, and antiviral properties that are related to its ability to block noncoagulant as well as coagulant functions of thrombin and to its ability to assume a variety of protein conformations.

Biochemistry

The plasma concentration of human ATIII[1] is about 2.4 μM (i.e., 140 μg/mL).[2] It circulates as two glycoforms: the predominant ATIII-α glycoform accounts for more than 90% of the ATIII in plasma, whereas the minor ATIII-β variant represents <10% of ATIII concentration.[3] Both isoforms have an identical polypeptide backbone, for which the complete amino acid sequence is known.[4,5] Disulfide bonds link cysteines 8 with 128, 21 with 95, and 247 with 430.[5] The major α-ATIII isoform is modified with four identical sialylated, biantennary complex oligosaccharides on asparagines 96, 135, 155, and 192,[6,7] and these account for 15% of the total mass of 58,000 Da. The minor β-ATIII isoform is not glycosylated on asparagine135, and some of its properties are distinct from those of the α-isoform, as discussed in the section on β-antithrombin. Both ATIII isoforms adopt several different protein conformations, which have distinct physical and functional properties. Crystal structures are available for several conformations and have considerably advanced the understanding of ATIII mechanism and regulation.[8–14]

The enzymes that are targets of ATIII inhibitory activity mediate blood coagulation, platelet activation, protease-activated receptor (PAR) signaling, and cell proliferation (see FIGURE 18.1). The name "antithrombin" is somewhat misleading because it suggests that ATIII is primarily an inhibitor of thrombin, when in fact, it has broad inhibitory activity for many different factors in the coagulation cascade. ATIII serves as an important regulator of hemostasis and thrombosis at several levels by blocking: (a) thrombin-mediated fibrin clot formation, (b) common pathway factor Xa (FXa)-mediated thrombin generation, and (c) coagulation factors that are higher up in the intrinsic and extrinsic pathways (factors IXa, XIa, XIIa [and its fragments], and plasma kallikrein [high molecular weight kininogen] and factor VIIa–tissue factor [TF]). ATIII inhibition of thrombin also serves to regulate its noncoagulant functions that mediate platelet activation and vascular cell signaling and proliferation (FIGURE 18.1).

Serpin Inhibition Mechanism

ATIII exhibits sequence, structural, and functional homology to members of the serpin (*ser*ine *p*roteinase *in*hibitor) gene family.[5,15] Many different serpins are present in blood and contribute to the regulation of the coagulation (ATIII), vascular remodeling (heparin cofactor II [HcII]), complement (C1 inhibitor), fibrinolytic (plasminogen activator inhibitor-1 and α_2-antiplasmin), and inflammatory (α_1-antitrypsin [α_1-proteinase inhibitor]) pathways. Serpins inhibit their target enzymes *via* a suicide-substrate inhibition mechanism that requires full function of the target proteinase catalytic apparatus and involves the formation of a covalent complex between the enzyme active site serine and the serpin.[16] Serpins are globular molecules with protruding reactive center loops (RCLs) containing substrate-like sequences that serve as "bait" for their target enzymes (FIGURE 18.2A). Inhibitory complex formation begins when a target enzyme cleaves the scissile P1–P1′ bond (nomenclature of Schechter and Berger[17]) of the RCL. This generates an acyl–enzyme complex in which the P1 residue is covalently linked to the target proteinase active site serine, and in which the polypeptides generated by reactive loop cleavage become mobile and are rearranged.[18,19] The P1-containing polypeptide incorporates as strand 4A in central β-sheet A of the serpin, translocating the covalently bound target enzyme approximately 70 Å to the opposite pole of the inhibitor.[20] Structures of the canonical serpin α_1-antitrypsin in inhibitory complexes with trypsin[21] and elastase[22] (FIGURE 18.2B) show that inhibitory complex formation results from critical protein conformational changes in both the inhibitor and the target enzyme. The P1–P1′ bond of the RCL is cleaved by the target enzyme, which promotes a large-scale protein conformational change, wherein the stressed, five-stranded A-sheet metastable conformation relaxes into a hyperstable, six-stranded A-sheet conformation in which the acylated enzyme is translocated to the opposite pole of the serpin. The translocated target enzyme molecule is crushed against the hyperstable serpin molecule in such a way that distortion of catalytic triad geometry prevents deacylation and regeneration of active proteinase.

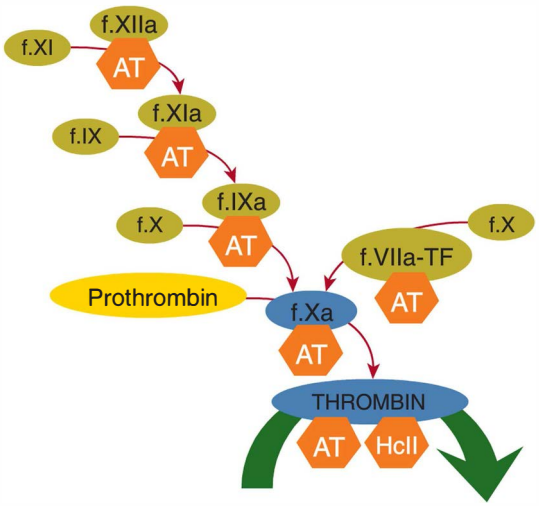

FIGURE 18.1 ATIII and HcII inhibition of coagulation pathway enzymes. ATIII inhibits coagulation factors in the intrinsic, extrinsic, and common pathways. ATIII and HcII inhibit thrombin, which has coagulant and noncoagulant functions.

Heparin Cofactor Activation of Antithrombin III

ATIII exhibits the general serpin structural and mechanical features described above, but it is somewhat unusual in being a considerably less efficient inhibitor than typical members of this family. Whereas rate constants for target proteinase inhibition by most serpins are in the 10^6 to 10^7 M^{-1} sec^{-1} range, the values for ATIII inhibition of its target enzymes are approximately three orders of magnitude lower. In contrast to other serpins, which have fully exposed RCLs, native ATIII circulates in a conformation in which the amino-terminal end of its RCL is partially inserted into its central β-sheet A[12,14] (see P14 residue S380 of *orange* RCLs of native and intermediate ATIII in panels a and b of FIGURE 18.3). RCL insertion was originally thought to reduce ATIII inhibition rates by limiting target enzyme access to the arginine393 P1–P1′ scissile bond (*blue* sphere on *orange* RCL).[23] However, it is now appreciated that native conformation presentation of an exosite (*cyan* surface under *orange* RCL in FIGURE 18.3), which comprises elements that positively and negatively influence interactions with factors Xa and IXa, contributes substantially to reduced ATIII inhibition rates in the absence of cofactor heparin.[24,25]

Heparin and HSPGs containing a very specific pentasaccharide sequence serve as pharmaceutical and physiologic cofactors for ATIII and activate it to achieve inhibition rates comparable to those that most other serpins are capable of without cofactors. The specific pentasaccharide sequence[26,27] that mediates high-affinity binding and activation of ATIII has the structure illustrated in FIGURE 18.4. This pentasaccharide is present in the approximately 30% of pharmaceutical heparin molecules having anticoagulant activity[28] and in vascular wall and matrix HSPGs, which are endogenous ATIII receptors and activators.[29,30]

ATIII thrombin, FXa, and FIXa inhibition rates are accelerated up to several thousand-fold by a combination of heparin-dependent conformational change and bridging mechanisms. The relative importance of the conformational change and approximation mechanisms varies with the target enzyme.

A bridging effect is the most important factor in the activation of thrombin inhibition. Thrombin inhibition is accelerated just twofold by pentasaccharide, whereas pentasaccharide-containing heparin chains of >18 sugar units in length accelerate the rate of inhibitory complex formation by more than 1,000-fold, using an approximation mechanism wherein different regions of the same extended heparin chain bind to the pentasaccharide-binding site on ATIII and to anion-binding exosite II on thrombin.[31,32]

In contrast, an allosteric induced fit conformational change mechanism plays an important role in the acceleration of ATIII FXa and FIXa inhibition by heparin. Pentasaccharide alone accelerates rates of FXa and FIXa inhibitory complex formation by approximately 300-fold.[28,33,34] In the presence of Ca^{2+}, full-length heparin produces further inhibition rate increases, revealing that in addition to the primary conformational change-based mechanism of FXa and FIXa activation, there are also template contributions to full-length heparin cofactor-mediated inhibition of these targets.[33–35] The FXa/FIXa exosite has been mapped to a region of β-sheet C (*cyan* in FIGURE 18.3) located under the RCL loop (*orange* in FIGURE 18.3). The protein conformation of the exosite as it is presented in native ATIII is unfavorable for interactions with the target enzymes. However, cofactor binding to the heparin-binding site of ATIII induces long-range allosteric conformational changes that expose exosite features that promote recognition and interaction with factors Xa and IXa and minimize repulsive interactions that were present in the native conformation exosite.[24,25,36,37]

As illustrated in FIGURE 18.3, the ATIII pentasaccharide-binding site is located at the junction of the *N*-terminal polypeptide (*ruby*) with helices A (*light yellow*) and D (*pink*), some 30 Å distant from the RCL (*orange*). The pentasaccharide is drawn in stick representation with *blue* carbon, *red* oxygen, *yellow* sulfur, and *dark blue* nitrogen atoms. Crystal structures of AT*H5 complexes[8,9,12] allowed direct identification of ATIII surface residues that contact the cofactor and largely confirmed heparin-binding site residue assignments previously made on the basis of early chemical modification studies and the analysis of naturally occurring, thrombosis patient ATIII mutants with heparin-binding defects. Detailed information about the roles of specific residues and their quantitative contributions to the binding of the heparins and to the stabilization of the high-affinity AT*H complexes have more recently become available from investigations of recombinant mutant ATIIIs.

Heparin binding to and activation of ATIII has been described in terms of the two-step, three-state mechanism presented in FIGURE 18.5.[38] An initial weak binding complex, AT–H, forms first. Then, in a second step, a protein conformational change converts the AT–H intermediate to the activated, high-affinity AT*H complex that is competent for rapid FXa and FIXa inhibition. The overall affinity (K_d) of antithrombin for heparin results from the combined effects of the K_1 binding and k_{+2} and k_{-2} rate constants defined in the two-step mechanism. K_1 represents the dissociation equilibrium constant for formation of the initial weak binding complex, AT–H. k_{+2} measures the rate for

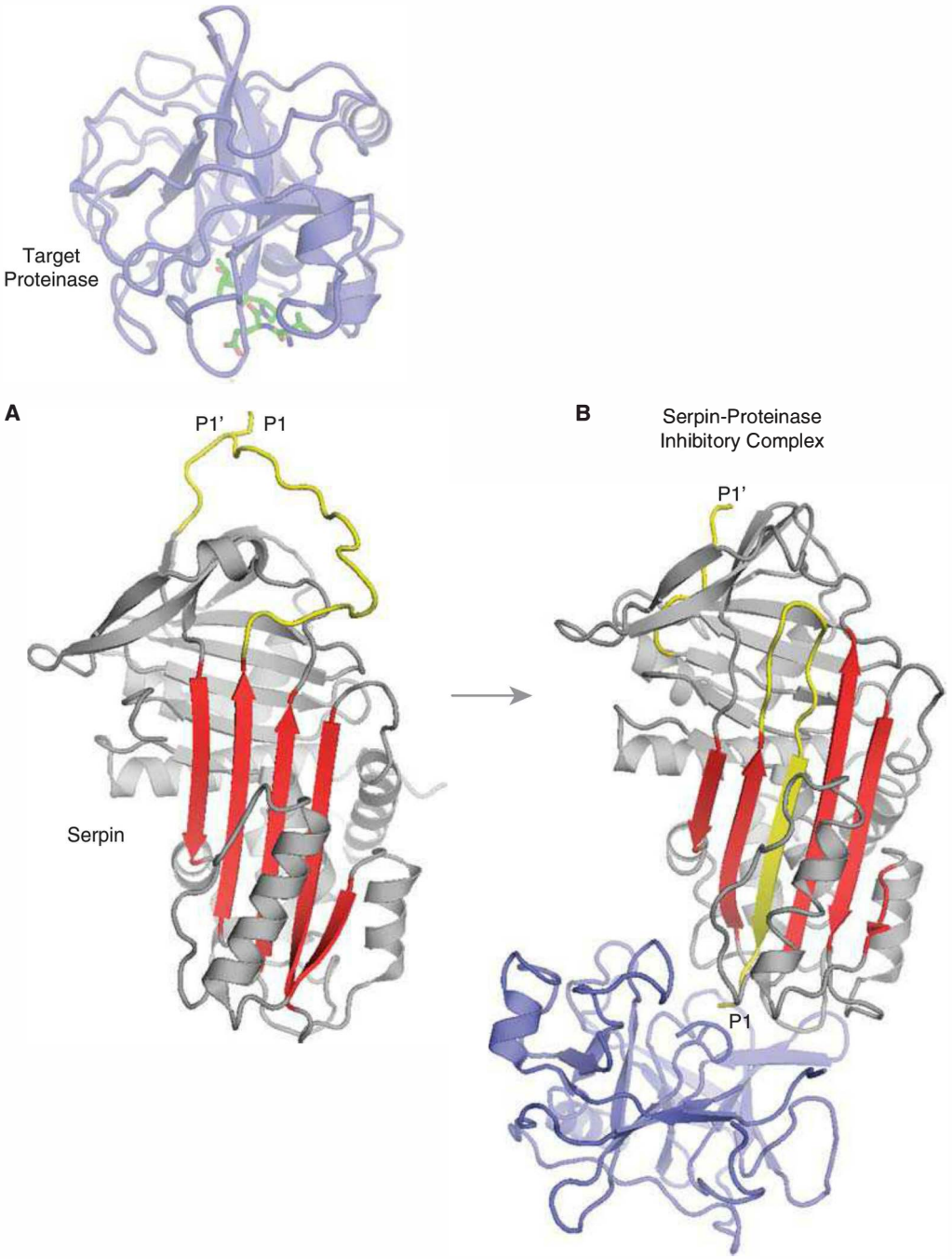

FIGURE 18.2 Serpin inhibition mechanism. **A**: The native serpin has a five-stranded central β-sheet A (*red*) and an exposed RCL (*yellow*). A substrate-like sequence in the RCL is recognized by a target enzyme (*blue*), which initiates cleavage of the P1–P1′ scissile bond. **B**: Formation of an inhibitory complex between the serpin and target proteinase involves (a) recognition and cleavage of the P1–P1′ peptide bond in the RCL polypeptide, (b) formation of a covalent acyl linkage between the serpin P1 residue and the enzyme active site serine, (c) expansion of the serpin central β-sheet by incorporation of the cleaved RCL polypeptide as a 6th strand, (d) translocation of the P1 residue and covalently bound enzyme from the north to the south pole of the serpin molecule, and (e) deformation of active site geometry in the translocated proteinase, which prevents deacylation and regeneration of active enzyme. Drawings were generated from native α1-proteinase inhibitor (1HP7.pdb), pancreatic elastase (1H9L.pdb), and covalent α1-PI–PPE covalent complex (2D26.pdb) structures using PyMol.

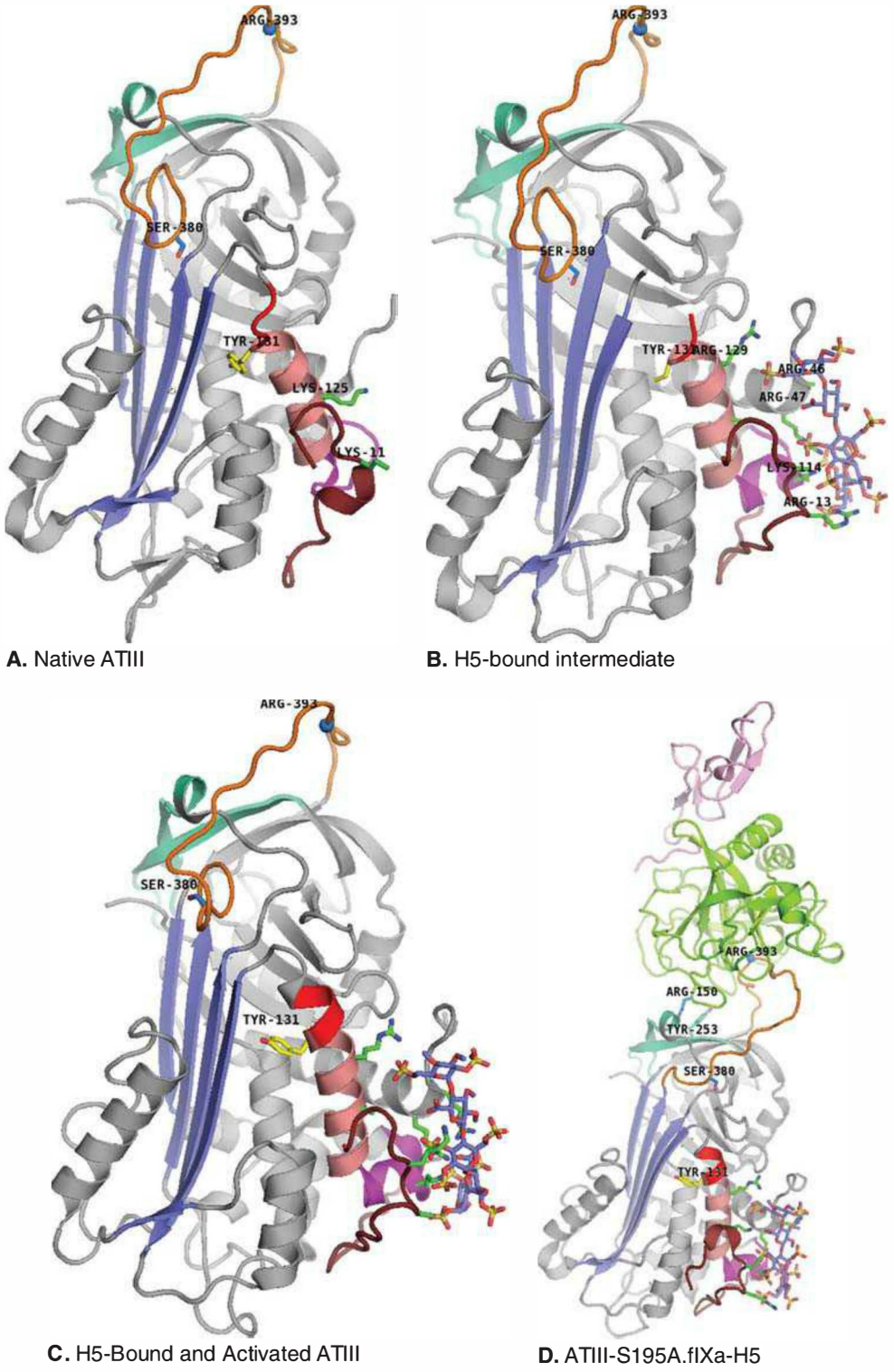

FIGURE 18.3 Heparin-mediated induced-fit allosteric activation of ATIII. (a) Native ATIII (1E04.pdb), (b) pentasaccharide-bound intermediate (1NQ9.pdb), (c) pentasaccharide-bound and activated ATIII (1E05.pdb), (d) ATIII-S195A.FIXa–pentasaccharide complex (3KCG.pdb). Drawings were generated from the indicated pdb files using PyMol. *Color coding*: β-sheet A is *slate blue* and the RCL is *orange*. P1 Arg-393 and P14 Ser-380 residues of the RCL are indicated by a *marine blue* sphere and a *marine blue* side chain, respectively. The β-sheet C region comprising the ATIII exosite for factors IXa and Xa is *cyan*. Structural elements forming the heparin-binding site are drawn in shades of reds and yellow: helix D *pink*; helix P *magenta*; *N*-terminal polypeptide *ruby*; *C*-terminal helix-D extension polypeptide *red*; helix A *light yellow*. Side chains of basic residues of the heparin-binding site are shown as *green-element* sticks. Pentasaccharide is drawn with *blue-element* sticks.

(Continued on page 290)

conformational conversion of initial weak AT–H complex to the high-affinity AT*H complex. Finally, k_{-2} represents the reverse rate of the conformational change (i.e., the stability of the AT*H complex). The extent to which individual ATIII residues and pentasaccharide components contribute to each of the above steps was determined by measuring the binding equilibrium and kinetic constant values for ATIII mutants and truncated pentasaccharides and by comparing them to those of ATIII and pentasaccharide controls.

K_1 analysis showed that initial recognition and low-affinity binding of the pentasaccharide to native ATIII is mediated by its DEF nonreducing end (see FIGURE 18.4), which interacts with K125 from helix D and K11 in the *N*-terminal polypeptide.[39–43] Side chains of the two basic residues that participate in the initial binding step are drawn and labeled in the native structure of FIGURE 18.3A.

The weak binding interaction of the first step leads to conformational adjustments in both the pentasaccharide and the antithrombin molecules. Pentasaccharide DEF complementarity with ATIII is enhanced and creates a complementary secondary site for binding a preferred conformer of the GH disaccharide.[40,41] On the antithrombin side, the protein conformational change converting AT–H to AT*H requires K114, K125, and F122,[39,44,45] which make the largest contributions to k_{+2} and mediate the formation of a new helix P (*magenta* in FIGURE 18.3) when pentasaccharide is bound. The activated AT*H complex that forms is stabilized by interactions requiring F122, R129, and, especially, K114, as evidenced by large increases in k_{-2} when these residues are mutated.[39,44,45] Side chains of basic residues that participate in the second binding step leading to further increases in heparin affinity, and ultimately long-distance conformational changes that mediate exosite presentation, are drawn and labeled in the intermediate structure of FIGURE 18.3B.[39–43]

Helix-D elongation (see *red* polypeptide at the *C*-terminal [top] end of helix D [*pink*] in FIGURE 18.3) also contributes to increased affinity for heparin and allosteric activation of FXa inhibition. ATIII mutants in which helix-D elongation is inhibited by deletion(s)[46] or by proline introduction[47] in the elongated helix-D region exhibit decreased pentasaccharide- and heparin-binding affinities and cannot be fully activated for FXa inhibition. Conversely, replacement of helix-D residue Y131 with a leucine produces 30-fold activation of FXa inhibition in the *absence* of cofactor and increased affinity for heparins.[48] Thus, the rotation of helix-D residue Y131 (compare positions of *yellow* Y131 side chain in panels a to d of FIGURE 18.3), caused by occupancy of the heparin-binding site or by Y131 phenol side chain substitution with smaller, nonphenyl groups, contributes to allosteric activation of ATIII by promoting consolidation of the hydrophobic core underlying central β-sheet A and long-range protein conformational changes that are associated with rearrangement of bonding networks formed by residues in the A-sheet, the hF arm, and the reactive loop polypeptide,[9,49] and more favorable presentation of the exosites for factors Xa and IXa recognition. FIGURE 18.3D shows the crystal structure for the encounter complex of S195A FIXa (*green* heavy chain, *pink* light chain) with H5-activated ATIII. Note that pentasaccharide binding has induced activating protein conformational changes that elongate helix-D and rotate tyrosine-131 to promote closure of central β-sheet A, P14 S380A expulsion, and favorable interactions of the exosite surface and key residue Y253[36,50] with arginine-150 of FIXa, and P1 arginine393 with the S195A-FXa "catalytic" triad.[10]

The K_1 binding and k_{+2} and k_{-2} rate constants from the two-step mechanism of FIGURE 18.5 together determine the overall affinity of the ATIII–pentasaccharide interaction. At physiologic pH and at ionic strength, the K_d for binding of the major α-ATIII isoform of human plasma to pentasaccharide and longer chain high-affinity (i.e., pentasaccharide-containing) heparin molecules are approximately 50 and 20 nM, respectively, as determined by both binding equilibrium and binding kinetic measurements.[51] Measurements of the free energy of binding with pentasaccharide for mutants of key heparin-binding site residues have determined that lysine114 and phenylalanine122 make the greatest contributions to the binding energy.[39,45] Lysine125 and arginine129 also make significant contributions,[42,44] whereas lysine11 and arginines13 and 47 make only small contributions.[43,52] Analysis of binding energy losses across the mutant series also indicates that cooperative interactions between combinations of the previously described residues contribute to high-affinity cofactor binding.[53]

The binding of pentasaccharide and ATIII is mediated by ionic as well as by nonionic interactions. From the salt dependence of the K_d, it has been determined that ionic interactions of the pentasaccharide and the six basic residues on ATIII are responsible for approximately 40% of the free energy of binding.[28,32,39,42–44,52] Nonionic interactions (i.e., hydrogen bonds and van der Waals and hydrophobic interactions) contribute approximately 60% of the binding free energy, with centrally

(Continued) Conformational changes induced by H5 binding to antithrombin can be visualized by comparing ***panels a, b,*** *and* ***c:***

Native ATIII (a) is a poor inhibitor due to suboptimal presentation of FXa/FIXa exosites (*cyan*) and the presence of repulsive sites in the surface surrounding the RCL (*orange*). Heparin allosterically activates ATIII by binding to site that is on the other side of the molecule. The cofactor initially binds to K11 and K125 of the native conformation (*green side chains* in **panel a**). Induced-fit conformational changes in *both* the H5 and ATIII molecules promote K114, R13, R46, R47, and R129 interactions with the cofactor and the formation of helix P (*magenta*) (see **panel b**). These binding interactions extend helix D at its *C*-terminal end (*red*), which causes tyrosine-131 rotation (compare positions of yellow Y131 side chain in **panels a, b,** and **c**). Exposure of buried Y131 ring leads to consolidation of the serpin core and long-distance changes in the electrostatics and H-bond network of the surface that underlies the RCL. The ATIII exosite for FXa and FIXa (*cyan*) is rearranged in a way that promotes favorable interactions and alleviates negative interactions with these target enzymes. See chapter text for greater detail.

Structure of encounter complex of S195A-FIXa with H5-activated ATIII:

FIXa heavy chain is *green* and light chain is *pink*. Pentasaccharide binding has induced activating protein conformational changes that elongate helix D and rotate tyrosine-131 to promote closure of central β-sheet A, P14 S380A expulsion, and favorable interactions of the exosite surface and key residue Y253 (*cyan* side chain) with R150 (*blue* side chain) of FIXa, and P1 arginine393 (*blue* sphere) with the S195A-FXa "catalytic" triad.

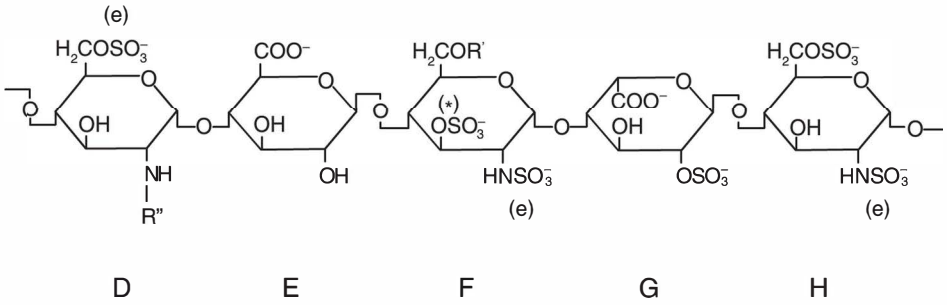

FIGURE 18.4 Structure of the heparin pentasaccharide that binds ATIII with high affinity and activates it for efficient target proteinase inhibition. Structural variants are indicated by R′ (–H or $–SO_3^-$) and R″ ($–SO_3^-$ and $–COCH_3$). The 3-*O*-sulfate group marked by an *asterisk* is unique to the antithrombin-binding region of the heparin molecule. In addition, the three sulfate groups indicated by (e) are essential to the high-affinity binding of antithrombin. (Modified from Tollefsen, DM, *Arterioscler Thromb Vasc Biol* 2007;27:456, with permission.)

located phenylalanine122 being responsible for a substantial share by positioning R47 and K114 for extensive contact with the cofactor and bridging structural elements involved in forming and stabilizing the high-affinity conformation.[45]

Endogenous Activation of Antithrombin III

As previously noted, native conformation ATIII molecules circulating in the blood have limited inhibitory activity. They become activated, however, upon binding to vessel wall $HSPG^{act}$ molecules (3-*O*-sulfated pentasaccharide-bearing anticoagulantly active HSPGs), which serve as the physiologic cofactor of ATIII. $HSPG^{act}$ is present on vascular endothelium and in the underlying matrix and serves to localize and concentrate activated antithrombin on and in the vessel wall. It is generally believed that activated ATIII molecules bound to vascular surface $HSPG^{act}$ receptors contribute substantively to the anticoagulant and antithrombotic properties of the endothelium by inhibiting free thrombin, FXa, and other activated coagulation-factor molecules that are generated by membrane-bound activation complexes and released into the diffusion layer at the blood vessel–wall interface.

Physiologic activation of ATIII by a vascular component was initially investigated in a rat hindlimb quarters model perfused with ATIII and thrombin. A tightly bound, heparinase-sensitive component accelerated the rate of thrombin–antithrombin complex formation 15- to 19-fold.[30] Further studies with normal versus mast cell–deficient mice established that the accelerating vascular component was $HSPG^{act}$ rather than a mast cell–derived heparin species.[54] This anticoagulantly active $HSPG^{act}$ species constitutes about 5% of the total HSPG associated with rat microvascular endothelial cells.[55] The integral membrane proteins ryudocan and syndecan serve as core proteins for both $HSPG^{act}$ and $HSPG^{inact}$ (anticoagulantly *in*active HSPG).[56] Ryudocan and syndecan have, respectively, three and five extracellular serine attachment sites that are posttranslationally modified with glycosaminoglycans (GAGs) composed of alternating *N*-acetylglucosamine and glucuronic acid/iduronic acid residues. The copolymer is further modified by multiple sulfation reactions to generate heparan sulfate chains with regions of varying structure. The presence of chains bearing pentasaccharide sequence(s) with essential 3-*O*-sulfation (See FIGURE 18.4) confers $HSPG^{act}$ status on a small subset of HSPG molecules.

The anatomic localization of $HSPG^{act}$ has been studied using immunohistochemistry and radiolabeled ATIII perfusion techniques. ATIII localization was investigated using immunohistochemistry techniques in biopsies of normal human hearts before transplantation.[57] Venous endothelium, but not capillary endothelium, stained positively for ATIII. ATIII was also present around smooth muscle cells and around intima of small arteries and arterioles, but not around large arteries, in apparent contradiction to the results of subsequent studies in which normal rat aorta was perfused with ^{125}I-labeled ATIII. These perfusion studies[29] demonstrated the presence of ATIII-binding $HSPG^{act}$ on and under the aortic endothelium. Small amounts of $HSPG^{act}$ (1%) were observed on the luminal side of aortic endothelial cells, whereas the subendothelial basement membrane and extracellular matrix on the abluminal side of the endothelium contained much greater amounts of ATIII-binding $HSPG^{act}$. The observed distribution suggests that luminal $HSPG^{act}$ may provide a basal level of activated ATIII for scavenger inhibition of activated coagulation enzymes exposed to the endothelium, and that these basal levels may be dramatically increased in injured regions following endothelial damage and liberation of the larger pool of abluminal $HSPG^{act}$ and its associated bound and activated ATIII.

The physiologic importance of ATIII binding to endogenous vascular surface $HSPG^{act}$ is supported by the occurrence of lethal thrombosis in mice homozygous for an ATIII mutation that reduces binding to heparin/HSPG.[58] Similarly, thrombosis was prevented in stents coated with the high-affinity, pentasaccharide-containing fraction of heparin (which binds antithrombin) but not in stents coated with low-affinity,

$$AT+H \underset{}{\overset{K_1}{\rightleftharpoons}} AT–H \underset{K_{-2}}{\overset{K_{+2}}{\rightleftharpoons}} AT^*H$$

FIGURE 18.5 Two-step mechanism for heparin binding to and activation of ATIII. AT, antithrombin III; H, heparin; AT–H, initial weak antithrombin–heparin intermediate; K_1, dissociation equilibrium constant for AT–H formation; AT*H, high-affinity antithrombin–heparin complex which is competent for rapid FXa inhibition; k_{+2}, rate of conformational conversion of the weak AT–H intermediate to the high-affinity AT*H complex; k_{-2}, reverse rate of the conformational change.

pentasaccharide-depleted heparin (which does not support ATIII binding).[59] Therefore, antithrombin–HSPG interactions play a crucial role in maintaining circulatory system patency. A previous contrary report based on the nonthrombotic phenotype of mice deficient for 3-OST-1[60] may reflect redundancy of enzymes that mediate 3-*O*-sulfation of the pentasaccharide sequence of HSPG.[61,62]

Finally, in addition to vascular HSPGs, the endothelial cell thrombin receptor thrombomodulin can bind ATIII and enhance its inhibition of receptor-bound thrombin by a factor of approximately 8.[63] This thrombin-specific effect is mediated by a highly sulfated chondroitin sulfate chain on thrombomodulin,[64,65] which interacts with both thrombin (at exosite II,[66]) and with ATIII. Chondroitin sulfate occupancy of exosite II of anticoagulant thrombin (i.e., thrombomodulin-bound thrombin) blocks heparin binding to the enzyme and precludes heparin/ATIII inhibition at the very rapid rates observed for procoagulant thrombin.[67]

Pharmacologic Activation of Antithrombin III

The clinical use of heparin as an anticoagulant began more than 60 years ago.[68] The name heparin derives from the fact that the anticoagulant was originally extracted from liver; however, commercial preparations are now usually obtained from pig intestinal mucosa or bovine lung, which are good sources of mast cells. Mast cell heparin proteoglycan is composed of a serglycin core protein modified with 60 to 100,000 Da heparin sulfate GAG chains.[69] The heparin proteoglycan polysaccharide chains are intracellularly cleaved to form smaller fragments (7,000 to 25,000 Da) that are present in pharmaceutical heparin. Standard pharmaceutical heparin is a heterogeneous mixture of polysaccharide molecules (average 15,000 to 18,000 Da). In addition to a high degree of size/length heterogeneity, there is also a significant amount of compositional heterogeneity. Typically, only one-third of the molecules in a standard pharmaceutical heparin preparation bear the ATIII-binding pentasaccharide sequence and exhibit anticoagulant activity.

Unfractionated heparin is used widely in clinical settings. It is given to prevent and treat venous thrombosis and for the management of arterial disease. Intravenous administration of heparin doses that prolong the activated partial thromboplastin time (aPTT) to 1.5 to 2.5 times control values is highly effective for the prevention and treatment of venous thrombosis.[70] Heparin is also widely utilized in extracorporeal circulation, for flushing indwelling catheters, for coating biomaterials and medical devices, and in many other hospital procedures. However, there are major drawbacks associated with the use of unfractionated heparin, including increased risk of hemorrhage and thrombocytopenia, variable patient response, and prolonged hospitalization because of the need for anticoagulant monitoring and dosing adjustments. In addition to inhibiting clotting enzyme function, heparin also interferes with platelet function, which contributes to its hemorrhagic side effects. On the basis of studies with size-fractionated heparins, which indicated that the high molecular weight fraction (average 20,000 Da) was considerably more active at inducing platelet aggregation than was the low molecular weight fraction (average 7,000 Da),[71] low molecular weight heparins (LMWHs) were suggested as a potentially advantageous alternative to standard heparin.[72]

Several LMWHs have been developed and evaluated in clinical trials and are now frequently used in hospital and outpatient settings. LMWHs are derived from unfractionated heparin by enzymatic or chemical cleavage. Their range of fragment size is from 1,800 to 12,000 Da, with an average of around 5,000 Da. As a result of their small fragment size and the cleavage into smaller pieces of most chains that were long enough (>18 sugars) to approximate (bridge) ATIII and thrombin, LMWHs preferentially inhibit factor Xa and have antifactor Xa to antithrombin ratios of 2:1 to 4:1 (vs. 1:1 for standard heparin). Compared with standard heparin, LMWHs have decreased affinity for endothelial cells[73,74] and exhibit reduced nonspecific binding to heparin-binding proteins.[75] These properties improve bioavailability and produce a more predictable dose response. The improved pharmacokinetic profile permits LMWHs to be administered subcutaneously once or twice daily on a weight-adjusted basis without laboratory monitoring. These attributes are an important advantage over standard heparin, which has a highly variable patient response that necessitates frequent laboratory monitoring, dose adjustment, and prolonged hospitalization. Clinical trials have established that for eligible patients with deep vein thrombosis, outpatient, unmonitored, subcutaneous, weight-adjusted LMWH is as effective and as safe as inpatient treatment with intravenous, unfractionated heparin and affords considerable cost reductions.[76]

A synthetic pentasaccharide, fondaparinux, has also been developed as an anticoagulant pharmaceutical.[77] Fondaparinux accelerates ATIII inhibition of FXa but has no activity against thrombin. It is believed that selective FXa inhibition may be advantageous because it targets amplification steps that generate large numbers of thrombin molecules through the common and intrinsic pathways while still permitting the trace thrombin that does form to facilitate hemostasis. The half-life of fondaparinux is approximately 17 hours and is dose independent, which allows for once-daily subcutaneous administration without monitoring of coagulation parameters. Fondaparinux has been approved for thromboprophylaxis after major orthopedic surgery. A concern with the use of fondaparinux is the lack of an antidote. Protamine sulfate is used to reverse the anticoagulant effects of unfractionated and LMWHs, but does not neutralize fondaparinux. However, a recombinant ATIII has recently been reported to reverse fondaparinux overdosing in a mouse model.[78] The antithrombin antidote to fondaparinux has (a) increased affinity for pentasaccharide in order to more effectively compete for fondaparinux binding and (b) a proline insertion in its RCL, in order to prevent formation of inhibitory complexes with coagulation enzymes, and exacerbation of bleeding.

Antithrombin III Inhibition of Target Enzymes Associated with Activating Complexes and Fibrin Clots

Freely circulating and purified thrombin and factor Xa are readily inhibited by ATIII and ATIII/heparin. However, when associated with the surface-activating complexes that generated the thrombin and FXa or with platelets or with fibrin clots, these target enzymes are resistant to ATIII and ATIII/heparin inhibition. Factor Xa in prothrombinase complexes formed by its addition to platelets or reconstitution with factor V, phospholipid, and

calcium is blocked from inhibition by ATIII.[79–82] Meizothrombin, a fragment-2–containing, fibrinogen-clotting intermediate in the generation of thrombin from prothrombin, is less susceptible to inhibition by ATIII and heparin than is thrombin. This effect is mediated by fragment-2 of prothrombin,[83] which interacts with the heparin-binding site of thrombin. The gradient of fragment-2 around a clot contributes to the reduction in ATIII inhibition of thrombin in the immediate vicinity of the clot and allows for more rapid thrombin inhibition at greater distances from the site of injury where fragment-2 concentrations are reduced.[83] Platelet-bound thrombin is protected from inactivation by ATIII/heparin by platelet GPIbα occupancy of its exosite II heparin-binding site.[84]

Clot-bound thrombin and thrombin bound to soluble fibrin-degradation peptides generated by tissue plasminogen activator–induced clot lysis are refractive to inhibition by ATIII/heparin.[85,86] Fibrin blocks ATIII/heparin thrombin inhibition by prior formation of ternary thrombin–fibrin–heparin complexes in which thrombin exosite I interacts with fibrin and thrombin exosite II binds the heparin molecule of the ternary complex (which is also connected to the ternary complex through the heparin-binding site of fibrin). Therefore, thrombin in the ternary complex is resistant to ATIII/heparin inhibition because the ATIII/heparin cannot access exosite II, which is occupied by the heparin molecule from the ternary complex.[87] Clot-associated factor Xa is also resistant to inhibition by ATIII/heparin.[88] Therefore, coagulation is difficult to regulate in the immediate microenvironment of a clot because clot-bound factor Xa is able to generate thrombin (and fragment-2—see the preceding discussion) from prothrombin, and, by binding to fibrin, the newly formed thrombin may also evade inactivation by ATIII/heparin.

In contrast to ATIII inhibition of factor Xa and thrombin, which is attenuated in activation complexes, ATIII inhibition of kallikrein and factor VIIa (FVIIa) is potentiated by the association of these intrinsic and extrinsic enzymes with activating surface-bound proteins. In the case of kallikrein, binding of high molecular weight kininogen promotes inhibition by ATIII/heparin through a bridging mechanism that involves binding of ATIII and kallikrein–kininogen to the same heparin molecule.[89] FVIIa also becomes more sensitive to ATIII inhibition when bound to its activating cofactor, TF.[90,91] In this case, increased reactivity of FVIIa with ATIII has been attributed to a conformational change in the enzyme active site that is induced by TF binding.

Antithrombin III as a Scavenger

As discussed in preceding text, native ATIII, HSPG^{act}-bound ATIII, and ATIII/heparin that are formed by administration of the pharmaceutical inhibit "free" factor Xa and "free" thrombin more efficiently than they inhibit factor Xa and thrombin located on activation surfaces and in fibrin clots. Hence, these forms of ATIII may serve as scavengers in the blood and on vascular surfaces and may neutralize thrombin and factor Xa molecules that have escaped from activation complexes and clots. In this capacity, ATIII would serve an important role in blocking coagulation and thrombin noncoagulant functions in uninjured areas, where they are less necessary.

ATIII may also play a role in regulating the low-level coagulation activation that occurs normally in the unperturbed circulation. ATIII regulation of TF-induced coagulation has been studied from several angles, and these investigations collectively suggest that there are multiple points at which ATIII may contribute to the control of coagulation initiation. ATIII inhibits FVIIa–TF complexes formed on OC2008 cells (which express TF and cell-surface GAGs constitutively) in the presence, as well as in the absence, of heparin.[91] ATIII in combination with tissue factor pathway inhibitor (TFPI) was also able to prevent the formation of prothrombinase activity in a model system containing FVIIa–TF, FVIII, FV, FIX, FX, and prothrombin reconstituted on phosphatidyl choline and phosphatidyl serine vesicles. This model procoagulant system contained physiologic concentrations of the listed components, but no heparin or HSPG. TFPI potentiated the action of ATIII by decreasing the rate of prothrombinase formation and by enabling ATIII to effectively scavenge the limited amounts of FIXa and FXa formed in the presence of TFPI.[92] Studies in a different model system reconstituted with zymogen FVII and human brain thromboplastin suggest that ATIII may also contribute to regulation of TF-induced coagulation through its ability, in the presence of heparin, to inhibit the activation of plasma FVII by FXa, thrombin, FIXa, and other proteinases.[93] Therefore, ATIII (in combination with TFPI) appears to play a crucial role in controlling low-level basal activation of the coagulation system that occurs in the normal circulation.

Antithrombin III Clearance

Different conformations and complexes of ATIII are cleared from the circulation by different pathways. High-affinity binding of the native conformation to vessel wall HSPG^{act} mediates its clearance from plasma into noncirculating vascular-associated and extravascular pools.[94,95] As will be discussed in the next section, the β-ATIII isoform clears from plasma 2.5 times faster than the α-ATIII isoform.[96] Thrombin–ATIII (TAT) inhibitory complexes (and, presumably, inhibitory complexes of ATIII with other target enzymes) are rapidly cleared from the circulation by the liver.[97] This clearance mechanism is specific for complexed ATIII, with TAT complexes being eliminated from the plasma many times more rapidly than native and cleaved ATIII.[98] Furthermore, clearance of ATIII inhibitory complexes exhibits competition with the clearance of other serpin complexes, indicating that serpin–enzyme complexes utilize a common clearance pathway.[97–99] The primary receptor for clearance of serpin–enzyme complexes is low-density lipoprotein receptor–related protein (LRP).[99] LRP is expressed by many tissues and cell types and is a prominent receptor in the liver. It interacts with numerous ligands, including serpin–enzyme complexes, and plays a key role in proteinase regulation and lipoprotein metabolism.

β-Antithrombin III

β-ATIII is a naturally occurring glycoform of ATIII that binds to heparin and HSPGs with higher affinity than the predominant ATIII isoform, α-ATIII.[3,51] β-ATIII synthesis is the result of inefficient utilization of the N-X-S–type consensus sequence specifying *N*-glycosylation of asparagine135.[100] Because of partial glycosylation at this site, plasma contains two ATIII glycoforms: α-ATIII, which is glycosylated at all four consensus sequences, and β-ATIII, which is glycosylated at only three consensus sequences (the asparagine135 N-X-S remains unmodified[101]).

β-ATIII binds heparin and pentasaccharide, respectively, six times and three times more tightly than does α-ATIII due to asparagine135 oligosaccharide effects on the formation of the initial AT–H intermediate, its conformational conversion to activated AT*H, and its stabilization of this complex.[51] The enhanced affinity of the β-isoform for heparin and its more rapid clearance from the circulation compared with α-ATIII suggest that β-ATIII, although a minor species in the plasma, may play an important role on the vessel wall.[96] β-ATIII associates 30% to 50% faster with uninjured aorta, and three times faster with deendothelialized aorta, than does α-ATIII, and approximately five times more β-ATIII than α-ATIII was recovered in detergent extracts of intima media from uninjured rabbit aortas.[102] Compared to plasma, a higher proportion of the ATIII in extracts of human organs is β-ATIII.[103] β-ATIII is also more effective than α-ATIII at inhibiting thrombin appearance on the wall of injured aorta.[104] Because it is believed that antithrombin molecules that are bound in an activated conformation to vessel wall HSPGs are crucial for efficient scavenging of activated thrombin and other coagulation factors, β-ATIII may represent a functionally important component in the physiologic regulation of coagulation enzyme activity, despite the fact that it is only a minor component in blood. Furthermore, according to this hypothesis, the apparently low content of β-ATIII (<10%) compared with α-ATIII (>90%) in plasma may actually reflect its increased binding and physiologic consumption on vascular surfaces.

Inherited Antithrombin III Deficiencies and Their Treatment

ATIII is an essential physiologic anticoagulant.[105] Its complete absence is incompatible with life, and its partial deficiency in mice[106] and humans increases the individual's risk for thrombosis. The prevalence of genetic ATIII deficiency in humans is approximately 0.02% to 0.05% (reviewed in Ref.[107]). However, this increases to approximately 5% for patients with a history of thromboembolic disease.[108]

The ATIII gene is composed of seven exons and six introns and spans 13,477 base pairs of genomic DNA[109] in the q23–q25 region of chromosome 1.[110] Genetic ATIII deficiencies, therefore, exhibit an autosomal dominant pattern of inheritance, with functional activity levels of the affected heterozygotes usually ranging from 40% to 60% of normal, and antigen levels varying according to the type of deficiency. Type I ATIII deficiencies result from failure of ATIII synthesis or from defective secretion of ATIII from hepatocytes and exhibit parallel reduction of functional activity and antigen levels to approximately 50% of normal values. Type I deficiencies are caused by a wide variety of mutations, including insertions and deletions, missense mutations, nonsense mutations, and the introduction of cryptic splice sites. Type II ATIII deficiencies are caused by missense mutations and *N*-glycosylation consensus sequence mutations that lead to the production of variant antithrombins with altered functional properties. ATIII antigen levels generally exceed ATIII functional levels in patients with type II deficiencies, and the characterization of numerous type II variant proteins has provided extensive ATIII structure–function information (reviewed in Refs.[107,111,112,113]).

There is great variability in the occurrence and severity of symptomatic thrombosis in individuals with genetic ATIII deficiencies. The onset of thrombotic episodes most often occurs in the third decade, but a considerable number of affected individuals reach old age without thrombotic problems, whereas some infants and children develop thrombosis early in life. Disease severity and age of onset are influenced by properties of the specific defective ATIII allele that is inherited, and by other factors that can decrease or enhance the thrombotic tendency caused by genetic ATIII deficiency. For example, protein C resistance secondary to the factor V_{Leiden} mutation (FV R506Q) has been shown to influence the expression of thrombosis in patients with genetic ATIII deficiencies. In a study of six families that segregated type I and type II ATIII defects and factor V_{Leiden}, the coinheritance of the factor V_{Leiden} allele with the ATIII alleles reduced the age of onset and increased the clinical severity of thrombosis.[114]

Thrombotic episodes in individuals with genetic ATIII deficiencies are mainly confined to the venous system. Common problems include recurrent episodes of deep vein thrombosis, mesenteric thrombosis, and/or pulmonary emboli. Similar to venous thrombosis occurring in persons without ATIII deficiency, precipitating factors in patients with ATIII deficiency include pregnancy, surgery, trauma, and infection. However, many episodes of thrombosis in genetically deficient persons appear to occur spontaneously, without a precipitating event. Pregnancy is a time of increased thrombotic risk in women with genetic ATIII deficiency. In a retrospective study, the incidence of thromboembolic complications during pregnancy in women with congenital ATIII deficiency has been estimated at about 70%. Prophylactic subcutaneous administration of heparin may be considered as soon as the pregnancy has been diagnosed, and supplementation with ATIII concentrates to normalize antithrombin levels during delivery and postpartum is also recommended.[115] As with any genetic disease, patient education is important because it enables the patient to avoid stimuli that are likely to induce thrombosis and to effectively recognize and to seek prompt treatment for thrombotic symptoms when they do occur.

Episodes of acute venous thromboembolism (VTE) in patients who have ATIII deficiency have been successfully managed by heparin alone or heparin in combination with antithrombin concentrates. Administration of ATIII concentrates together with heparin is considered the treatment of choice for patients with known hereditary ATIII deficiency and who present with massive and potentially life-threatening thromboembolic disease.[116] By increasing the plasma antithrombin concentration, it is believed that potential "heparin resistance" secondary to genetically low levels of ATIII can be overcome more readily, and clinical benefit can be achieved more rapidly. However, a retrospective study of Swedish patients with ATIII deficiency suggested that heparin resistance may not actually represent a substantial problem for most of these individuals because administration of heparin alone produced a more than 1.5 times prolongation of the aPTT in at least 90% of patients.[117]

Individuals with ATIII deficiency are at a high risk for postoperative and posttraumatic thrombosis, as well as thromboembolism during pregnancy and after delivery, and antithrombin concentrates have been used in combination with heparin for prophylaxis during surgery, delivery, and postpartum.[118–120] Although there are no randomized clinical trials establishing the efficacy of this approach, the preponderance of favorable outcomes reported in the literature suggests that prophylactic treatment with ATIII concentrates and heparin is helpful.

Prophylactic anticoagulation with vitamin K antagonists is sometimes prescribed for genetically deficient persons who have experienced recurring episodes of thrombosis. In contrast, prophylactic anticoagulation of asymptomatic carriers of ATIII deficiency genes is regarded as unnecessary and is not recommended.[121]

Estrogen-containing contraceptives and postmenopausal replacement therapies should be avoided by women with genetic ATIII deficiencies because estrogen usage has been associated with the development of thrombosis in normal women, as well as in ATIII-deficient females.[122,123] Progestogen-containing contraceptives, on the other hand, do not appear to lower ATIII levels.[124]

Acquired Antithrombin III Deficiencies

Acquired antithrombin deficiencies are encountered more frequently than genetic ATIII deficiencies. Acquired ATIII deficiencies occur in association with many conditions and procedures, including severe sepsis, trauma, burns, malignancies, extracorporeal circulation, and surgery. These disorders have consumptive coagulation and acute inflammatory components. Antithrombin levels decline because of stoichiometric consumption of ATIII during neutralization of activated coagulation enzymes. Additionally, in inflammatory settings, activated leukocytes release neutrophil elastase and matrix metalloproteinases that cleave the reactive loop of ATIII[125,126,127] and induce a functional activity deficiency.

Data from several studies suggest that a decrease in plasma ATIII concentration to below 60% to 70% of normal represents a crucial point in the pathogenesis of venous thrombosis. A study of patients with nephrotic syndrome reported that 8 of 11 patients with antithrombin concentrations of <70% developed thrombosis, whereas only 1 of 37 patients with ATIII concentrations of >70% experienced thrombosis.[128] Finally, in trials of antithrombin concentrate in patients undergoing hip or knee replacement, all patients with nadir ATIII concentrations of <60% developed thrombosis.[129]

ATIII levels decrease profoundly during cardiopulmonary bypass to 40% to 50% of preoperative levels and remain low even after protamine neutralization.[130] Several factors contribute to the development of ATIII deficiency during bypass including (a) hemodilution, (b) ATIII consumption by the formation of inhibitory complexes, and (c) increased systemic neutrophil elastase concentrations and the heparin-mediated elastase inactivation of ATIII.[131]

ATIII deficiency also develops in response to high-dose chemotherapy regimens used to prepare patients with hematopoietic malignancies for bone marrow transplantation (BMT). ATIII levels decline in parallel with albumin and prealbumin levels, suggesting that impaired hepatic synthesis and/or extravascular redistribution contribute to ATIII deficiency in BMT. Nadir ATIII levels typically occur at 2 to 3 weeks following the start of the preparative regimen and are correlated with the development of vascular occlusive disease of the liver, pulmonary dysfunction, and central nervous system dysfunction. Patients who developed multiorgan failure had lower ATIII levels than those who did not develop organ failure or who experienced single-organ failure.[132,133]

Localized ATIII deficiency has been observed in unstable cardiac allografts.[57] Immunohistologic studies on serial biopsies from donor and transplanted organs (for periods of up to a decade) have shown that clinically stable grafts maintain a normal pattern of vascular ATIII reactivity in contrast to grafts that fail. Interestingly, a proportion of late unstable allografts, which have progressed to the stage of losing normal venous and arterial ATIII reactivity and showing evidence of fibrin deposition, may go on to subsequently develop a system of ATIII-reactive capillaries. Appearance of these ATIII-reactive capillaries shifts the prognosis for graft survival from bad to good.

Antithrombin III Concentrates

ATIII concentrates have been produced by the blood plasma fractionation industry since the mid-1970s using heparin-affinity chromatography methods.[134] The manufacturing process also includes a heat treatment step to inactivate viral pathogens. ATIII concentrates are considered safe, and the transmission of HIV, hepatitis B, or non-A non-B hepatitis has not been reported in patient follow-up studies (reviewed in Refs.[119,135]). However, the pasteurization step used to inactivate viruses induces formation of aggregated protein and latent ATIII molecules, which are devoid of anticoagulant activity because of uptake of the RCL as a central 6th strand in β-sheet A. Sixteen to forty percent of the ATIII in five extensively analyzed batches of one antithrombin concentrate existed as inactive, latent molecules.[136] Another study of 23 lots of four different ATIII concentrates produced by four different manufacturers demonstrated the presence of denatured antithrombin in all preparations except in those that had received additional solvent/detergent treatment and further purification steps.[137] This study also found levels of TAT and cleaved antithrombin to be substantially elevated in concentrates relative to normal plasma. TAT increased 20- to 1,600-fold, whereas cleaved ATIII increased 50- to 230-fold. Therefore, elevated TAT and cleaved ATIII levels in patients who have received ATIII concentrates should be interpreted with caution.

In the United States, ATIII concentrates are mainly utilized for patients with genetic ATIII deficiencies, as discussed in a previous section. However, in some European countries and in Japan, ATIII concentrates are additionally administered to patients with acquired antithrombin deficiencies (e.g., disseminated intravascular coagulation, sepsis, trauma, hematopoietic malignancy) and to patients undergoing BMT, orthopedic surgery, and cardiopulmonary bypass and to obstetrics patients.

Although ATIII supplementation improved hemostatic parameters and survival in several animal models of sepsis (reviewed in Ref.[138]), the large double-blind, placebo-controlled, multicenter phase III KyberSept trial of human plasma-derived high-dose ATIII for patients with severe sepsis failed to show any benefit with respect to 28-day all-cause mortality.[139] The design of the KyberSept study did not exclude concomitant heparin, which was received by a large subset of the patients, and in retrospect heparin was speculated to have interfered with potential therapeutic benefits of the antithrombin. In a subsequent analysis, it was noted that for patients without concomitant heparin, 28-day mortality was lower for the antithrombin group compared to the placebo group, and that this trend continued through 90 days, whereas it was not observed with groups receiving heparin.[140]

A recombinant form of human antithrombin (rhAT) has been produced in the milk of transgenic goats.[141] Glycosylation of rhAT differs from that of the human plasma-derived protein,

leading to fourfold higher heparin affinity[141] and more rapid clearance and a shorter *in vivo* half-life than plasma-derived ATIII.[142,143] rhAT was approved in Europe for the prevention of VTE in patients with hereditary antithrombin deficiency in 2006, and the U.S. FDA similarly approved it for the prevention of perioperative and peripartum thromboembolic events in patients with hereditary antithrombin deficiency in 2009.[144]

Anti-Inflammatory Properties of Antithrombin III

ATIII exhibits anti-inflammatory properties that are both independent of, and related to, its ability to inhibit thrombin. Work in endotoxemia and ischemia/reperfusion models attributed ATIII anti-inflammatory effects on leukocyte accumulation, vascular permeability, and TNF-α and iNOS induction to endothelial prostacyclin release.[145–148] Prostacyclin administration mimicked the ATIII-mediated anti-inflammatory effects, whereas indomethacin pretreatment antagonized these responses to ATIII. Anti-inflammatory effects were lost in experiments using chemically modified and recombinant ATIIIs with reduced affinity for heparin, suggesting that anti-inflammatory effects require ATIII interaction with cell-surface HSPGs. ATIII has also been demonstrated to block thrombin-mediated, neutrophil-dependent ischemia-reperfusion damage. Postreperfusion treatment with ATIII attenuated the P selectin–mediated leukocyte recruitment response, and similar results were also observed in an *in vitro* model using human blood neutrophils and cultured endothelial cells.[149]

Direct ATIII effects on inflammatory responses of peripheral blood cells have also been demonstrated. ATIII inhibits chemokine-induced migration of neutrophils, lymphocytes, and monocytes, and, in the absence of chemokines, stimulates their migration.[150–152] In monocytes and endothelial cells, ATIII potently blocks activation of nuclear factor kappa beta (NFκB), a transcription factor involved in immediate early gene activation during inflammation.[153] ATIII-mediated NFκB downregulation also involves binding of ATIII to cell-surface HSPGs, and this interaction has been proposed to interfere with signal transduction leading to NFκB activation.

Therefore, on the basis of the recently described anti-inflammatory and the well-established anticoagulant properties of ATIII, ATIII supplementation has been proposed for clinical reversal of microvascular dysfunction in acute inflammatory disorders such as sepsis. High-dose ATIII failed to improve overall 28-day survival in severe sepsis patients of the KyberSept trial; however, decreased mortality was noted for patients who did *not* receive heparin.[139] It has been suggested that clinical benefit was observed in this subgroup because concomitant systemic heparin did not block the anti-inflammatory effects of ATIII.[151]

Antithrombin III—Antiproliferative and Antiangiogenic Activities

Independent of its anticoagulant and anti-inflammatory properties, ATIII also has antiproliferative and antiangiogenic activities. ATIII inhibits thrombin-induced proliferation of human arterial smooth muscle cells and human mesangial cells *in vitro*[154,155] and is also reported to inhibit rat spleen T-cell proliferation in a thrombin-independent manner.[156]

Several conformations of ATIII lack anticoagulant activity but exhibit antiangiogenic and antitumor growth properties that are of potential physiologic and therapeutic importance. These activities are not present in the native, inhibitory conformation of antithrombin, but are acquired after the protein undergoes structural conversion to cleaved and latent forms, which feature incorporation of the reactive loop polypeptide as strand four of the central A β-sheet.[157,158] A "prelatent" conformation of ATIII, which has undergone only limited conformational change and is still able to bind heparin and inhibit thrombin, also exhibits potent antiangiogenic and tumor inhibition activities.[159,160] Antiangiogenic forms of ATIII block FGF-2 and VEGF ligand-receptor signaling on endothelial cells by competing for binding to the heparan sulfate coreceptor, which then alters endothelial gene expression profiles in a manner that produces overall downregulation of proangiogenic functions and upregulation of antiangiogenic functions.[161,162]

HEPARIN COFACTOR II

Overview

HcII is a plasma serpin with 30% homology to ATIII. Compared with ATIII, there are similarities as well as differences in HcII interactions with coagulation enzymes and GAGs. Like ATIII, HcII inactivates thrombin; but unlike ATIII, it is not a broad-spectrum inhibitor of coagulation enzymes and does not inhibit FXa or other clotting factors. The circulating form of HcII, like the circulating form of ATIII, is an inefficient inhibitor of thrombin. Both are activated more than three orders of magnitude by heparin; however, the structural mechanisms for heparin activation of HcII and ATIII are distinct. Dermatan sulfate, which only minimally activates ATIII, is an efficient activator of HcII. Proposed physiologic roles of HcII include extravascular thrombin inhibition, inflammatory signaling, and thrombin regulation during pregnancy. In contrast to ATIII, HcII deficiency is not strongly associated with venous thrombosis but contributes to the prevention of arterial thrombosis.

Biochemistry and Molecular Genetics

HcII (also known as leuserpin 2 and SERPIN D1) is a 66-kDa glycoprotein with a pI of 4.95 to 5.15.[163] HcII contains approximately 10% carbohydrate, two sulfated tyrosine residues,[164] and three free sulfhydryls. The plasma concentration and plasma half-life of HcII are, respectively, approximately 1.2 μM (?80 μg/mL) and approximately 2.5 days.[165,166] The complete amino acid sequence of the 480-residue mature protein is known from complementary deoxyribonucleic acid analysis.[167,168] The serpin domain is approximately 30% homologous to ATIII and other serpins, uses leucine as its P1 residue, and is preceded by a unique *N*-terminal polypeptide sequence that contains two highly acidic hirudin-like repeats. In contrast to ATIII, which is a broad-spectrum inhibitor of thrombin, factor Xa, and other intrinsic and extrinsic pathway coagulation enzymes, HcII inhibition of clotting pathway enzymes is limited to thrombin (FIGURE 18.1). However, HcII also inhibits cathepsin G[169] and chymotrypsin[170] in accordance with its P1 leucine residue. HcII inhibition of thrombin, but not of chymotrypsin, is potentiated by heparin and dermatan sulfate.[170] HcII–thrombin inhibitory complexes are rapidly cleared by LRP,[99] which also clears ATIII inhibitory

complexes. The HcII gene spans 14 kb of DNA in the q11 region of chromosome 22 and has five exons and four introns.[171]

Target Enzyme Specificity and Mechanism of Glycosaminoglycan Activation

The leucine P1 residue of HcII confers primary specificity for inhibition of cathepsin G and chymotrypsin, which prefer medium-sized nonpolar residues, but not for thrombin, FXa, or related coagulation factors, which cleave arginine-containing substrate sequences. Despite having a nonarginine P1 residue, HcII inhibits thrombin, and this specificity is conferred by a GAG cofactor binding–dependent interaction between two negatively charged hirudin-like sequences in the amino-terminal domain of HcII and thrombin's anion-binding exosite I.

FIGURE 18.6A shows the 2.35-Å structure of native HcII.[172] As in native ATIII (FIGURE 18.3A), the RCL is partially inserted into central β-sheet A. Binding sites for heparin and dermatan sulfate occupy overlapping areas in the helix-D region, and most of the positively charged HcII residues that interact with these GAGs are located in homologous positions to basic residues of the ATIII heparin-binding site (FIGURE 18.3A,B). As

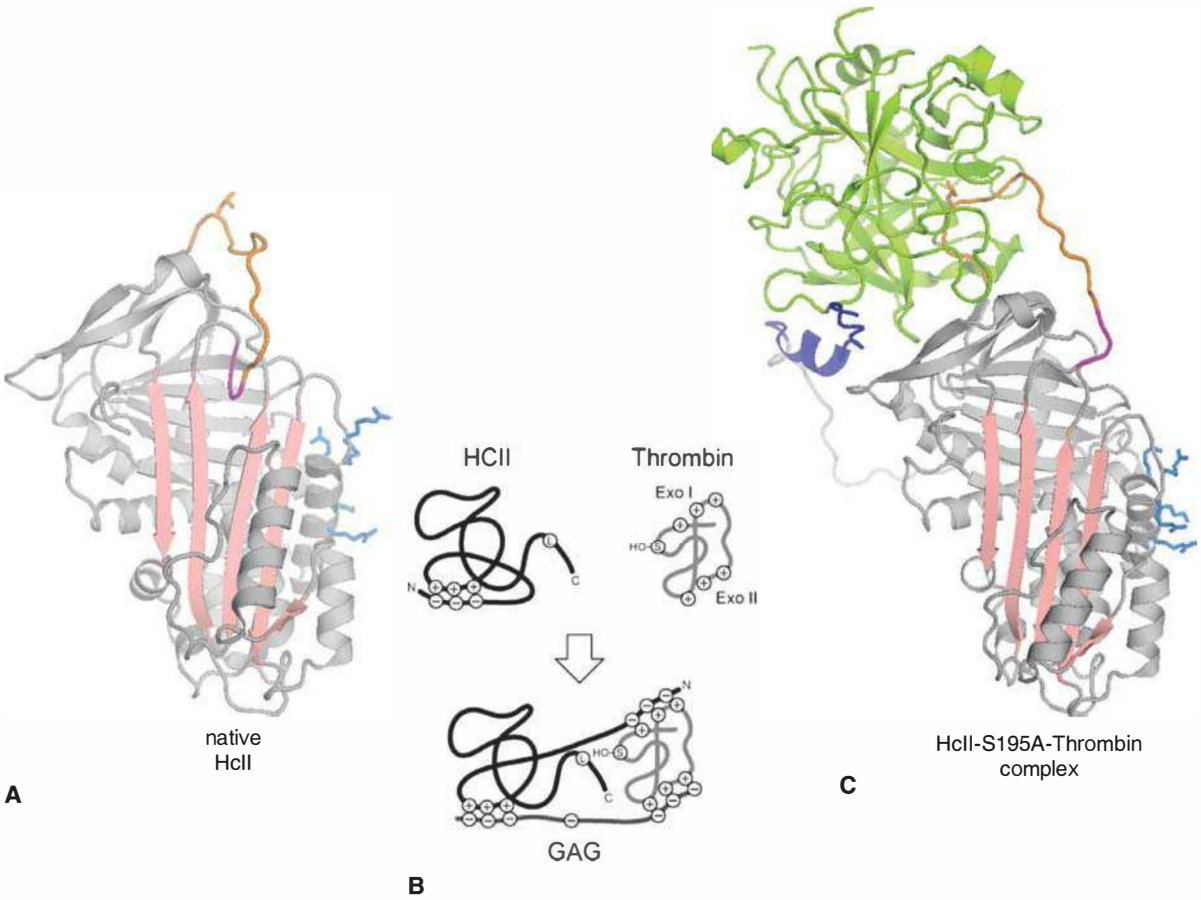

FIGURE 18.6 HcII inhibition of thrombin. **A**: *Native HcII*. The RCL (*orange* and *purple*) of native HcII is constrained due to incorporation of its *C*-terminal end (*purple*) into β-sheet A (*pink*). The leucine P1 residue of the RCL is drawn in *orange* sticks, and side chains of positively charged residues in the heparin- and dermatan sulfate–binding sites on helix D are drawn in *marine blue*. The *N*-terminal hirudin-like polypeptide of HcII was not visible in this structure because perturbations caused by intermolecular contacts in the dimeric native HcII crystal structure largely prevented assignment of its location. **B**: Cartoon model of native HcII shows intramolecular binding of its *N*-terminal, negatively charged hirudin-like and positively charged GAG-binding domains, as inferred from mutagenesis studies. Activation occurs when a heparin or dermatan sulfate GAG molecule binds HcII and competitively displace its *N*-terminal domain from its GAG-binding site. This uncoupling leads to (a) allosteric HcII conformational changes and (b) HcII *N*-terminal domain binding to the thrombin anion-binding exosite I, which together increase the specificity, rate, and strength of the inhibitory complex formation. **C**: *S195A-thrombin—HcII Michaelis complex*. In this structure, the *N*-terminal anionic binding site (*dark blue*) interacts with exosite I on thrombin (*green*), and the allosteric change leading to expulsion of the RCL (*purple* and *orange*) from the A β-sheet has occurred. (**Panels A** and **C** drawn from 1JMJ.pdb and 1JMO.pdb, respectively. Modified from Tollefsen, DM, *Arterioscler Thromb Vasc Biol* 2007;27:456, with permission.)

a result of perturbations caused by intermolecular contacts in the dimeric native HcII crystal structure, the location of the anionic hirudin-like *N*-terminal domain of HcII could not be assigned for the most part. However, from functional studies with HcII *N*-terminal anionic and helix-D GAG-binding region mutants, it is inferred that for the native molecule in the absence of GAG cofactors, there is intramolecular binding of the hirudin-like repeats with the positively charged GAG-binding region, as is schematically depicted in FIGURE 18.6B cartoon.[173–175] Upon exposure to dermatan sulfate or heparin, cofactor binding and occupancy of the GAG-binding site makes dual contributions to activating HcII for more efficient thrombin inhibition. First, GAG binding competitively displaces the hirudin-like amino-terminal polypeptide from the helix-D GAG-binding site, freeing it to interact with exosite I on thrombin,[176] as illustrated in the FIGURE 18.6B cartoon and in FIGURE 18.6C 2.2-Å crystal structure of the HcII–S195A–thrombin complex.[172] Second, by analogy to the mechanism for heparin activation of ATIII (FIGURE 18.3), the release of the binding interaction between the *N*-terminal hirudin domain and the helix-D GAG-binding domain through mutation,[173,176] or cofactor binding, is inferred to induce a long-range protein conformational change, which contributes to expulsion of the RCL from sheet A and improved reactivity with thrombin.[172] The binding sites for heparin and dermatan sulfate on HcII overlap, but are not identical (reviewed in Ref.[177]), and distinct protein conformational changes are induced by the GAGs.[178] In contrast to heparin cofactor inhibition of thrombin by ATIII, both heparin and dermatan sulfate primarily utilize an allosteric mechanism, rather than a template mechanism, to accelerate thrombin inactivation.[179] In the presence of heparin, large decreases in rates of HcII and ATIII inhibition of fibrin clot-bound thrombin occur as a result of the formation of thrombin–fibrin–heparin ternary complexes, which reduce the access of heparin–serpin complexes to the enzyme.[87] However, in the presence of dermatan sulfate, HcII inhibition of clot-bound thrombin remains almost as efficient as its inhibition of free thrombin because of the lack of dermatan sulfate–fibrin cross-linking in the DS–thrombin–fibrin complex that is formed.[180]

Biologic Role of Heparin Cofactor II

HcII participates in thrombin inhibition in the body as indicated by the presence of low levels of thrombin–HcII inhibitory complexes in normal plasma samples[181] and human organ extracts.[103] However, in contrast to ATIII deficiency, HcII deficiency in humans is not strongly associated with venous thrombosis and contributes to thrombotic risk only in combination with other deficiencies (reviewed in Ref.[182]). Furthermore, approximately 1% of apparently healthy individuals (no history of thrombosis) have HcII levels of <60%,[183] and the frequency of HcII deficiency is similar in the general population and venous thrombosis patients.

Recent clinical studies suggest that HcII contributes to the prevention of arterial, rather than venous, thrombosis. Elderly subjects with low plasma HcII activity have a higher incidence of in-stent restenosis after percutaneous coronary intervention,[184] increased carotid artery plaque thickness,[185] and increased prevalence of peripheral arterial disease.[186] These clinical observations imply that HcII protects against the pathologic remodeling response that is initiated by vascular injury and are consistent with results from mouse models of HcII deficiency.

The biologic consequences of HcII deficiency have been investigated in two independently generated mouse models.[187,188] Although there were notable differences in the phenotypes of the St. Louis and Tokushima mice, work with both supports the view that HcII attenuates the vascular injury–induced intimal hyperplasia and atherogenesis.[187,189] St. Louis HcII mice were fully viable and did not exhibit spontaneous thrombosis or other apparent abnormalities.[188] However, complete HcII deficiency in these (−/−) mice was associated with more rapid occlusion after carotid artery injury, and prevented by prior infusion of purified HcII.[188] Moreover, on an apolipoprotein E (apoE)–null genetic background and atherogenic diet, St. Louis HcII null mice exhibited accelerated atherogenesis and neointima formation compared to HcII wildtype mice with the same genetic background and diet.[190] In contrast to the normal viability and growth of the St. Louis HcII-null mice, Tokushima HcII-null mice were embryonically lethal.[187] However, in arterial injury models, heterozygous HcII Tokushima animals exhibited increased intimal hyperplasia and cellular proliferation compared to wildtype. On an apoE-knockout background, the heterozygous HcII deficiency also caused acceleration of aortic plaque formation with increased PAR-1 expression and oxidative stress. Vascular injury–induced intimal hyperplasia in Tokushima HcII$^{+/-}$ mice was abrogated by human HcII supplementation.

In conjunction with the aforementioned clinical observations, the results from the St. Louis and Tokushima mouse models of HcII deficiency imply that it plays a protective role against the pathologic consequences of vascular remodeling, including atherosclerosis. Vascular injury allows HcII molecules from circulating blood to enter the vessel wall, and there bind subendothelial dermatan sulfate, which activates HcII inhibition of thrombin. In this context, HcII serves to block the noncoagulant proliferative and chemotactic functions of thrombin, and thereby protect against intimal hyperplasia, plaque formation, and atherogenesis. This view is consistent with the original proposal of HcII as an extravascular thrombin inhibitor that is activated by fibroblast and smooth muscle cell dermatan sulfate proteoglycans.[191]

HcII has also been suggested to have a biologic role in thrombosis prevention during pregnancy. Dermatan sulfate proteoglycans (presumably of placental origin) increase in the maternal and fetal circulations during pregnancy,[192] and thrombin–HcII complexes are prominent in placental extracts.[103] Elevated HcII has been reported in pregnant women, with levels increasing to three times normal at term.[193,194] HcII levels have also been observed to decrease in preeclampsia patients.[195] However, the previously discussed mouse models of HcII deficiency are confusing with respect to the role of HcII during pregnancy and early development. St. Louis HcII (−/−) mice are born at the expected Mendelian frequency and are reproductively fit,[196] in contrast to Tokushima HcII (−/−) mice, which are embryonic lethal.[187] Thus, the role of HcII in pregnancy is not fully understood.

Finally, a role for HcII in inflammation, wound healing, and tissue repair has also been proposed, on the basis of the ability of proteolytically derived *N*-terminal peptides of HcII to act as leukocyte chemoattractants.[197]

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CHAPTER

19

Protein C, Protein S, Thrombomodulin, and the Endothelial Protein C Receptor Pathways

LAURENT O. MOSNIER AND JOHN H. GRIFFIN

THE PROTEIN C PATHWAYS—OVERVIEW

Control of coagulation reactions is essential for normal hemostasis. As part of the tangled web of host defense systems that respond to vascular injury, the blood coagulation factors act in concert with the endothelium and blood cells, especially platelets, to generate a protective fibrin-platelet clot, forming a hemostatic plug. Pathologic thrombosis occurs *inter alia* when the protective clot extends beyond its beneficial size, when a clot occurs inappropriately at sites of vascular disease, or when a clot embolizes to other sites in the circulatory bed. For normal hemostasis, both procoagulant and anticoagulant factors must interact with the vascular components and cell surfaces, including the vessel wall and platelets. Moreover, the actions of the fibrinolytic system must be integrated with coagulation reactions for timely formation and dissolution of blood clots. The protein C pathways provide diverse important functions to maintain a regulated balance between hemostasis and host defense systems in response to vascular injury. On one hand, the anticoagulant protein C pathway is designed to regulate coagulation, maintain the fluidity of blood within the vasculature, and prevent thrombosis. On the other hand, the cytoprotective protein C pathway provides anti-inflammatory and cytoprotective activities and it acts to prevent vascular leakage. This chapter summarizes current knowledge of the protein C pathway components and their activities, with particular emphasis on the recently elucidated, novel direct cellular effects due to activated protein C (APC) and its cofactors, protein S, thrombomodulin (TM), and endothelial protein C receptor (EPCR). Several reviews are available on the biochemistry and clinical impact of the anticoagulant and cytoprotective protein C pathway.[1,2,3–5,6,7,8,9,10–12,13,14]

Thrombin is central to the protein C pathways. Thrombin that is generated by the blood coagulation pathways (see Chapters 8, 9, and 14) binds with high affinity to the cell-surface receptor, TM, and converts the protein C zymogen that is bound to EPCR to APC. For its anticoagulant activity, APC acts in concert with cofactors, including protein S, to inactivate factors Va and VIIIa, the major cofactors for thrombin generation. Cytoprotective APC activities that include antiapoptotic activity, anti-inflammatory activity, regulation of gene expression, and stabilization of endothelial barrier protection can be initiated when APC targets two key receptors, protease-activated receptor-1 (PAR-1) and EPCR.[6,15–17,18–20] Target cells for APC's cytoprotective activities are not limited to the endothelium but also include epithelial cells, various lymphocytes, dendritic cells, and neurons, and additional receptors may also play key roles.

Many questions arise related to APC's effects on cells, such as (a) what are the signaling pathways and/or molecular mechanisms underlying APC's cytoprotective effects on each type of cells; (b) what are the target(s) for APC's effects on cells that allow for APC's many pleiotropic properties; (c) to what extent are molecular mechanisms for APC's antiapoptotic activity, anti-inflammatory activity, regulation of gene expression, and endothelial or epithelial barrier protection shared, overlapping, or distinct; and, perhaps most critically for mechanistic insights, (d) what are the relative contributions of APC anticoagulant activity and APC's cytoprotective activities for the observed beneficial effects, including mortality reduction, in various *in vivo* injury models? Partial answers to these questions are provided in this chapter. Because of their critical roles in the body's host defense system, APC and the protein C pathway components are ideal targets for translational medicine research, and they provide opportunities for therapeutic treatment of complex and challenging medical disorders, including thrombosis, severe sepsis, and stroke among others.

IDENTIFICATION OF THE PROTEIN C PATHWAY COMPONENTS

Although protein C was originally identified as "autoprothrombin-IIa" more than 50 years ago, it was subsequently rediscovered by Stenflo and named "protein C" because of its elution in the third peak ("peak C") on an ion-exchange chromatogram.[21,22] The identification of TM, an endothelial cell cofactor for thrombin-catalyzed activation of protein C, provided a rationale for how a kinetically inefficient enzyme, such as thrombin, could be a physiologically significant activator of protein C.[23,24] The clinical importance of the protein C pathway was based on discovery of deficiencies in protein C or in protein S linked to thrombosis and, for severe or total deficiency, to inflammation.[25,26,27–29] Efforts to elucidate the molecular basis of the anti-inflammatory effects of APC were sparked by the identification of the EPCR.[30] The finding of the PROWESS trial in which recombinant APC (Xigris) reduced 28-day all-cause mortality in adult severe sepsis patients was a watershed event.[31] However, 10 years after that trial, in late 2011 APC's mortality reduction efficacy was not reproduced in the PROWESS-SHOCK trial, raising multiple questions about each trial and their comparisons. Nonetheless, many studies between 2001 and 2011 were driven by the PROWESS trial's success, including major breakthroughs in understanding APC cellular effects involving the direct action of APC on cells that induce signaling through activation

of PAR-1 when bound to EPCR and possibly other receptors.[16] Additional dimensions to studies on the protein C pathway include preclinical studies showing beneficial effects of second-generation recombinant APC mutants in sepsis and ischemic stroke, the generation of protein C– or protein S–deficient mice, and the findings that protein S can exert direct receptor-mediated effects on cells.[16,17,18–22,23,25,27–29,30,31,32–38,39,40,41,42,43–47]

Protein C

Protein C was discovered in search for vitamin K–dependent coagulation factors that are not detected by clotting assays.[21] The protein C gene (PROC) is homologous to the genes for factors VII, IX, and X and is comprised of nine exons and eight introns, located on chromosome 2q13-14 (Table 19.1).[48–51] After removal of the prepro leader peptide, mature protein C contains 419 amino acids (Mr ~ 62 kDa) (FIGURE 19.1) and is synthesized mainly in the liver. Protein C in plasma circulates at 70 nM (4 μg/mL) with a half-life of approximately 8 hours.[25] A significant posttranslational modification involves the cleavage of the zymogen between residues 157 and 158 by a furin-like enzyme, followed by the loss of the Lys156–Arg157 dipeptide connecting the light chain (residues 1 to 155; Mr ~ 25 kDa) to the heavy chain (residues 158 to 419; Mr ~ 41 kDa). As a result, 85% to 90% of human protein C is a two-chain form, linked by a single disulfide bond between Cys141 and Cys277. Other posttranslational modifications involve β-hydroxylation at Asp71, *N*-linked glycosylation at residues 97, 248, 313, and 329, and γ-carboxylation at the first nine Glu residues in the γ-carboxyglutamic acid (Gla) domain. The three subforms of the heavy chain, designated alpha (Mr ~ 41 kDa), beta (Mr ~ 37 kDa), and gamma (Mr ~ 32 kDa), represent tri-, di-, and monoglycosylated heavy chain due to partial glycosylation of Asn residues 329 and 248.[52] Ca^{2+}-binding sites that are located in the Gla domain are required for binding to phospholipids and EPCR, and such sites are also present in the first epidermal growth factor (EGF) domain and the protease domain.[53–55] The characteristic structural elements of protein C include an amino-terminal Gla domain (residues 1 to 37), an aromatic stack (residues 38 to 45), two EGF-like regions (EGF-1, residues 46 to 92 and EGF-2, residues 93 to 136), an *N*-terminal activation peptide (residues 158 to 169) on the heavy chain, and the serine protease domain (residues 170 to 419) (FIGURE 19.1).

Table 19.1

Characteristics of the protein C pathway components

Protein	Gene	Locus	cDNA	Residues	Theoretical Mass (kDa)	Observed Mass (kDa)	Plasma Concentration (nM)	$\varepsilon_{1\%;\ 1\ cm}$
Coagulation Factors								
Protein C	PROC	2p13-14	NM_000312	419	47	62	70	14.5
Protein S	PROS1	3q11.2	NM_000313	635	71	77	320[a]	9.5
FV	F5	1q23	NM_000130	2,196	249	330	25	8.9
FVIII	F8	Xq28	NM_000132	2,196	265	330	0.7	11.9
C4b-binding protein[b]	C4BPA	1q32	NM_000715	549	70	500–570[b]	260	14.1
	C4BPB	1q32	NM_000716[c]	235	45			
Cell Receptors								
TM	THBD	20p12-cen	NM_000361	557	60	75 (105)[d]	0.05[e]	9.2[f]
EPCR	PROCR	20q11.2	NM_006404	221	25	46	2.5[g]	11.3[f]
PAR-1	F2R	5q13	NM_001992[h]	400	47	65	n/a	ND
ApoER2	LRP8	1p34	NM_004631[i]	899	102	180	ND	24[f]

[a]The total protein S concentration (320 nM) includes free protein S (130 nM) and protein S in complex with C4BP (190 nM).
[b]C4BP contains two polypeptide chains in varying proportions and circulates in different isoforms, alpha-7-beta-1 being the most common. About 17% (45 nM) of C4BP contains only alpha-chains and lacks the beta-chain that binds protein S. The α_6, $\alpha_6\beta_1$, and α_7 isoforms have molecular weights of approximately 500, 530, and 570 kDa.
[c]C4BP beta-chain variants 1 (NM_000716), 3 (NM_001017365), and 5 (NM_001017367) encode for the longer isoform 1 but vary in the 5′-untranslated region, whereas variants 2 (NM_001017364) and 4 (NM_001017366) encode for isoform 2 with a shorter *N*-terminus due to an in-frame alternate splice site.
[d]The TM apparent molecular weight is dependent on the presence of the GAG attachment.
[e]Soluble TM is believed to be the product of proteolysis of TM on the cell surface and comprises different molecular forms (Mr 35–65 kDa) that lack the transmembrane sequence. The concentration of soluble TM in normal individuals is approximately 2 to 5 ng/mL but can increase many-fold under pathologic conditions. Increased soluble TM levels are regarded to be a sign of endothelial cell activation.
[f]Values are given for the soluble extracellular domain of the receptor. Soluble ApoER2 is truncated after EGF-like domain B but includes all seven ligand-binding domains.
[g]Soluble EPCR is derived from proteolysis of EPCR on the cell surface and lacks the transmembrane domain (Mr ~ 43 kDa).
[h]The reference sequence was adjusted in 2008 between revision 2 (NM_001992.2) and 3 (NM_001992.3), resulting in two amino acid changes compared to the sequence originally published for PAR-1.[284]
[i]Several cell-type specific ApoER2 isoforms are reported. Transcript variant 1 (NM_004631) contains all 19 exons, transcript variant 2 (NM_033300) lacks exons 5 and 6 encoding the ligand-binding domain (LBD) 4–6 and 7. Transcript variant 3 (NM_017522; e.g., found in endothelial cells [HUVEC]) lacks exon 5 (LBD4-6), exon 15 (*O*-linked sugar), and exon 18 (part of the intracellular domain). Transcript variant 4 (NM_001018054) is similar to full-length transcript variant 1 except for the lack of exon 18. In addition, U937 cells were found to express an additional transcript that lacks exons 5 and 18 but that contains exon 15.[43]

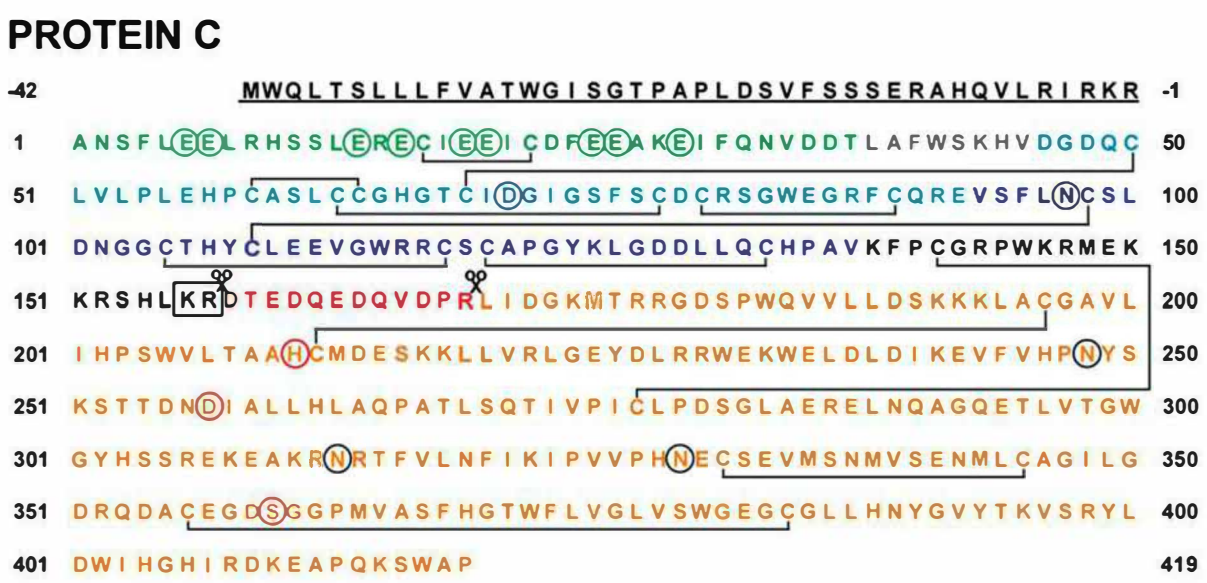
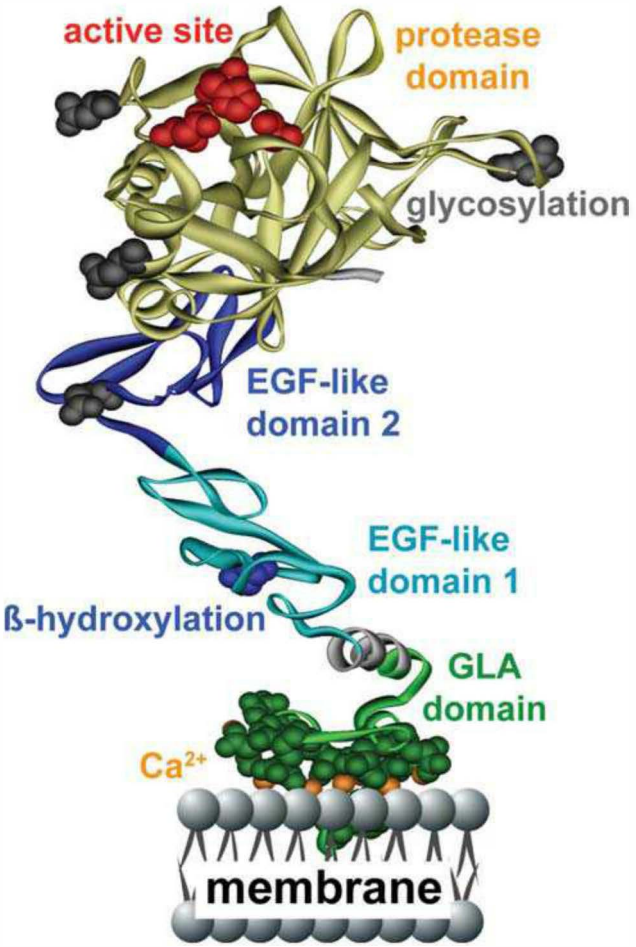


FIGURE 19.1 Amino acid sequence of protein C and ribbon polypeptide scheme of APC. Amino acids are numbered from the amino-terminus of the mature protein with the signal peptide sequence *underlined*. Specific domains are color coded as indicated in the *ribbon cartoon*. *Green circles* depict γ-carboxylation, *blue circles* depict β-hydroxylation, and *black circles* represent sites of *N*-linked glycosylation. Sites of proteolytic cleavage during posttranslational processing and protein C activation are indicated by the scissors. The dipeptide that is proteolytically removed during the posttranslational processing of most protein C molecules in the liver is identified by the box. The serine, aspartic acid, and histidine residues that constitute the active site triad are identified by *red circles*.



Thrombin cleavage of the zymogen at Arg169 removes the activation peptide and generates APC, a trypsin-like serine protease with a typical serine protease active site triad (His211, Asp257, and Ser360). Endogenous circulating levels of plasma APC are low (<40 pM), and inactivation of APC in plasma is driven by serine protease inhibitors (SERPINs) that contribute to a remarkably long circulation half-life of APC in man of approximately 20 to 25 minutes. Most important inhibitors of APC in plasma are protein C inhibitor (PCI) and α_1-antitrypsin and, to a lesser extent, α_2-macroglobulin and α_2-antiplasmin.

Protein S

Protein S, named after its birth city, Seattle, is a vitamin K–dependent plasma glycoprotein and is best known for its function as a nonenzymatic cofactor to APC in the inactivation of factors Va and VIIIa. The relatively large gene for protein S (PROS1) is located on chromosome 3 (3q11.2) and contains 15 exons (Table 19.1). Chromosome 3 also contains an inactive pseudogene for protein S (PROSP at location 3p21-cen). After removal of the prepro leader peptide, mature protein S contains 635 amino acids (Mr ~ 77 kDa) (FIGURE 19.2) and is predominantly synthesized in

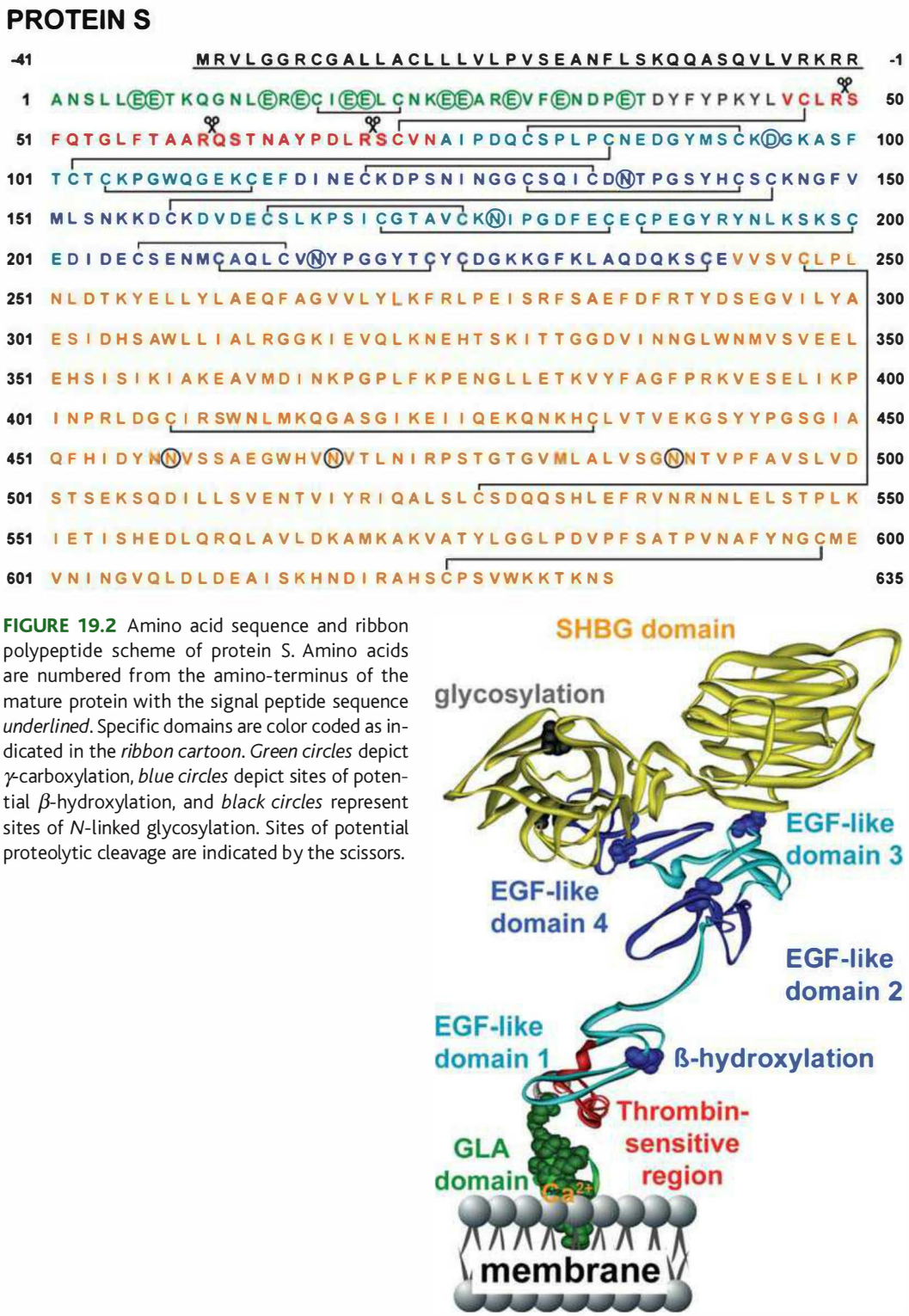


FIGURE 19.2 Amino acid sequence and ribbon polypeptide scheme of protein S. Amino acids are numbered from the amino-terminus of the mature protein with the signal peptide sequence *underlined*. Specific domains are color coded as indicated in the *ribbon cartoon*. *Green circles* depict γ-carboxylation, *blue circles* depict sites of potential β-hydroxylation, and *black circles* represent sites of *N*-linked glycosylation. Sites of potential proteolytic cleavage are indicated by the scissors.

the liver but is also made by endothelium and other cells. Protein S circulates in plasma at a total concentration of 320 nM, and approximately 60% of the protein S is noncovalently complexed with C4b binding protein (C4BP) such that free protein S is 130 nM in plasma.[56] C4BP is an octopus-like shaped glycoprotein of approximately 500 to 570 kDa and consists of six or seven disulfide-linked, identical alpha-chains and one smaller beta-chain (see Table 19.1).[57] C4BP regulates protein S functions by binding via the beta-chain.[58] Protein S binds to the C4BP beta-chain in a 1:1 stoichiometric complex with high affinity ($K_d \sim 0.1$ to 0.6 nM).[59] Although C4BP is an acute-phase response protein, only alpha-chains are increased causing the levels of free protein S levels to remain stable during an acute-phase response.[60]

Mature protein S has a defined structural organization consisting of five distinct domains, including an *N*-terminal Gla domain (residues 1 to 37) and aromatic stack (residues 38 to 45), an unique 29–amino acid sequence known as the thrombin-sensitive region (TSR; residues 46 to 74), a string of four EGF-like domains (EGF-1 [residues 75 to 115], EGF-2 [residues 116 to 159], EGF-3 [residues 160 to 201], and EGF-4 [residues 202 to 242]), and finally a large 393–amino acid domain on the *C*-terminus referred to as the sex hormone–binding globulin-like domain (residues 243 to 635) that is composed of two laminin G-type domains (FIGURE 19.2).

Thrombomodulin

The endothelial cell receptor TM (CD141) was discovered and named for its ability to modulate the protease specificity of thrombin (FIGURE 19.3).[24,61] The gene for TM (THBD) is on chromosome 20 (20p12-cen) and is intron-less (Table 19.1).[62,63] Based on its cDNA sequence, TM consists of 575 amino acids in

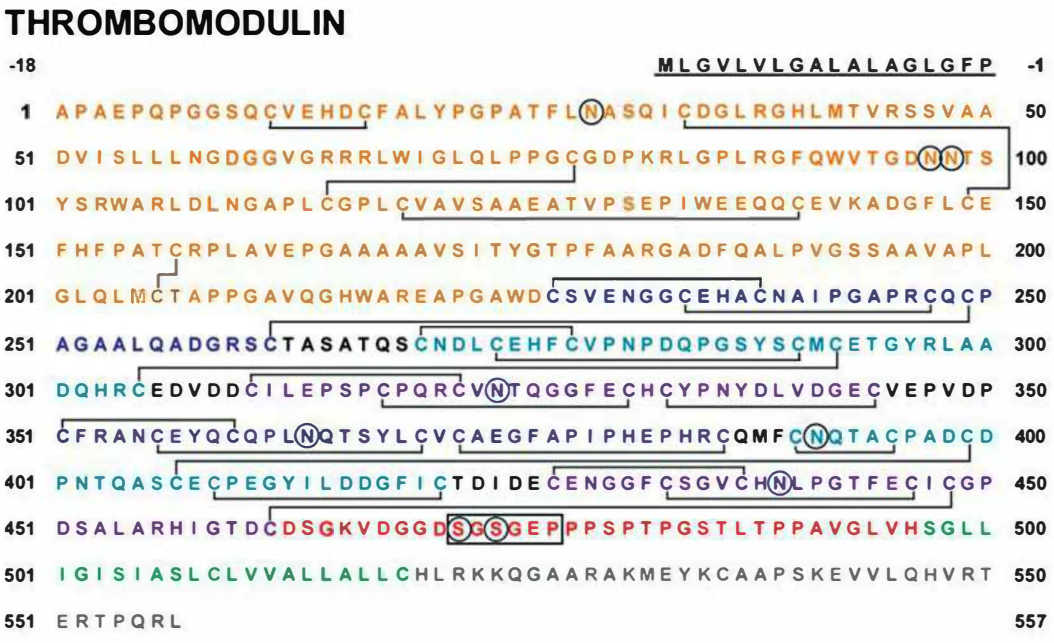
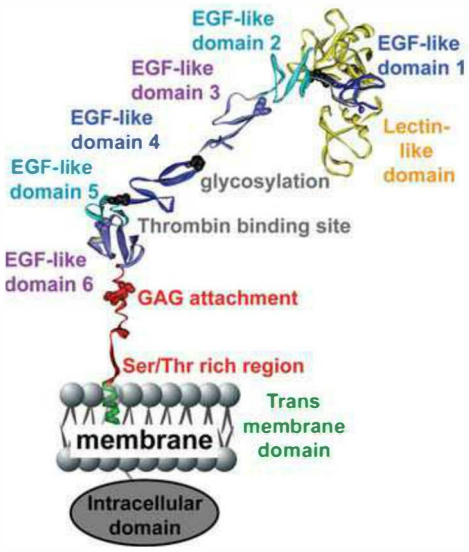


FIGURE 19.3 Amino acid sequence and ribbon polypeptide scheme of TM. Amino acids are numbered from the amino-terminus of the mature protein with the signal peptide sequence *underlined*. Specific domains are color coded as indicated in the *ribbon cartoon*. *Blue circles* depict sites of potential *β*-hydroxylation, *black circles* represent sites of *N*-linked glycosylation. The consensus sequence for the GAG attachment is *boxed*.



the form of a 60.3-kDa single-chain transmembrane protein.[63] Posttranslational *O*-linked and *N*-linked glycosylation accounts for approximately 20% of the apparent molecular weight of TM, and other modifications include covalently bound chondroitin sulfate moieties.[64]

Functionally, five regions of the mature TM can be identified (FIGURE 19.3)[63]: An *N*-terminal lectin domain (residues 1 to 226), a repeat of six EGF-like domains (EGF-1 [residues 227 to 262], EGF-2 [residues 270 to 305], EGF-3 [residues 311 to 344], EGF-4 [residues 351 to 386], EGF-5 [residues 390 to 421], and EGF-6 [residues 427 to 462]), a serine- and threonine-rich region (residues 463 to 496), a transmembrane region (residues 497 to 519), and finally a *C*-terminal intracellular tail (residues 520 to 557). The EGF-like modules 5 and 6 mediate thrombin binding, whereas EGF-like module 4 is required for interactions with protein C. The major site for the glycosaminoglycan (GAG) attachment is within the chondroitin consensus sequence of Ser472-Gly-Ser474-Gly-Glu-Pro at Ser472 or Ser474. GAG-containing TM has somewhat different properties than TM devoid of a GAG attachment as the presence of GAG increases the affinity for thrombin approximately 10-fold, reduces thrombin's ability to clot fibrin and to activate platelets, and modulates the Ca^{2+}-dependent profile of protein C activation. Furthermore, the GAG on TM can bind a second thrombin molecule and facilitate inactivation of thrombin by the SERPINs, PCI, and antithrombin (AT).[65] Besides promoting protein C activation by thrombin, TM also supports activation of a plasma carboxypeptidase, known as thrombin-activatable fibrinolysis inhibitor (TAFI). Activated TAFI is not only a potent attenuator of fibrinolysis but also an effective inactivator of a variety of proinflammatory peptides such as the activated complement components C3a and C5a, bradykinin, thrombin-cleaved osteopontin, and other peptides.[66] For a more detailed discussion of TAFI, see Chapters 20 and 21.

Low levels of soluble TM, generated by shedding from the endothelium due to proteolysis, circulate in plasma and are generally considered to be a marker of endothelial cell damage.[61] The functional significance of circulating TM is unknown, although variations in its plasma level arise in different clinical conditions.

Endothelial Protein C Receptor

The EPCR (CD201[67]) was discovered in a search for the endothelial cell receptor responsible for binding of APC.[30] The gene for EPCR (PROCR) is on chromosome 20 (20q11.2) (Table 19.1).[68] EPCR is homologous to the CD1/MHC superfamily. MHC class I molecules are type 1 integral membrane proteins and consist of three extracellular domains, alpha-1, alpha-2, and alpha-3. The latter domain typically associates noncovalently with β_2-microglobulin. EPCR lacks the alpha-3 domain and thus does not associate with β_2-microglobulin; rather, in EPCR, the transmembrane domain is connected directly to the alpha-2 domain (FIGURE 19.4).[68] The alpha-1 and alpha-2 domains form a binding groove by each providing an alpha-helix that

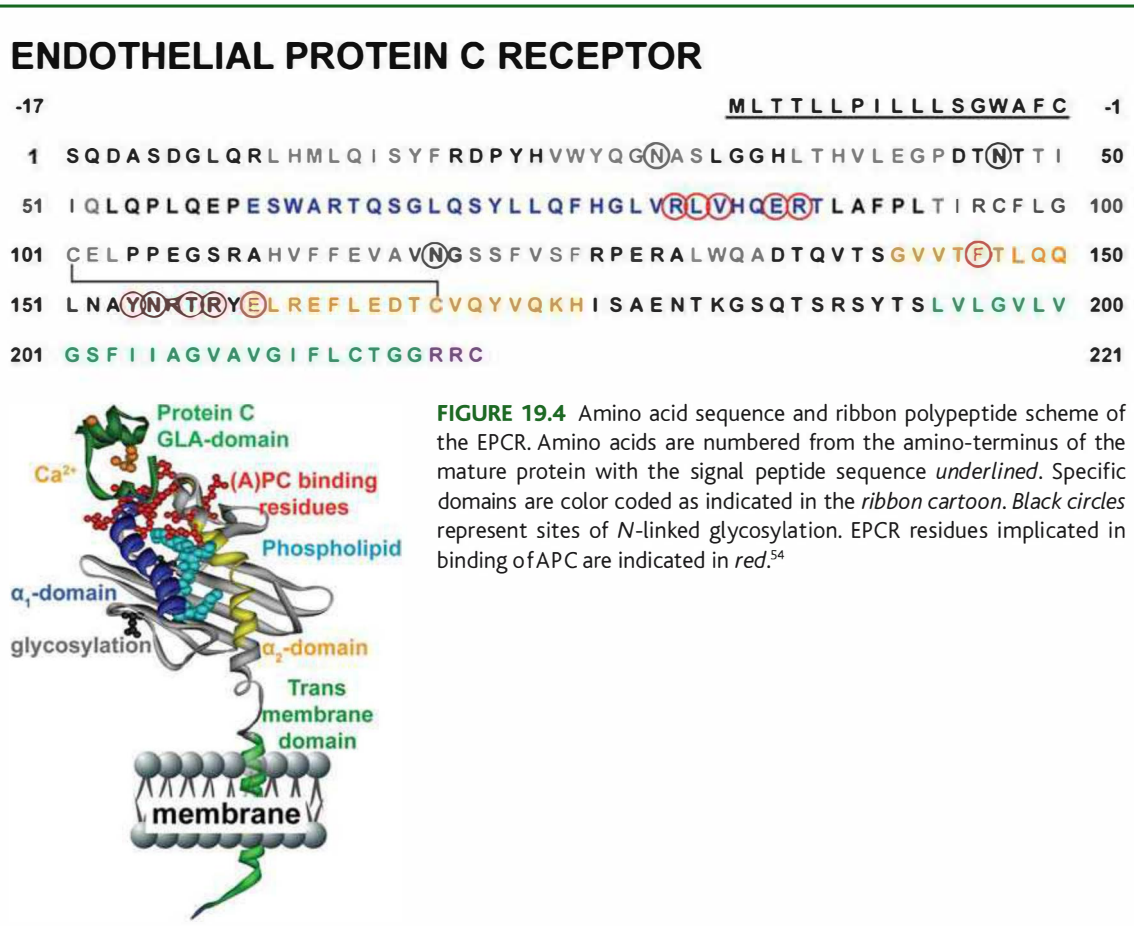


FIGURE 19.4 Amino acid sequence and ribbon polypeptide scheme of the EPCR. Amino acids are numbered from the amino-terminus of the mature protein with the signal peptide sequence *underlined*. Specific domains are color coded as indicated in the *ribbon cartoon*. *Black circles* represent sites of *N*-linked glycosylation. EPCR residues implicated in binding of APC are indicated in *red*.[54]

is located along opposing edges of a planar platform generated by an eight-stranded antiparallel beta-sheet. The crystal structure of soluble EPCR revealed that a phospholipid, most likely phosphatidylcholine, was bound in this groove between the two alpha-helices.[69,70] EPCR's short cytoplasmic tail (Arg–Arg–Cys–CO_2H) suggests that induction of direct cell signaling is unlikely and that palmitoylation of the *C*-terminal Cys residue may help localize EPCR to certain lipid rafts or caveolae and facilitate modulation of cell-signaling pathways that depend on EPCR.[71,72]

In addition to binding protein C and APC with similar affinity (K_D ~ 60 nM), EPCR also binds factor VII and factor VIIa to the same end of an EPCR alpha-helix that binds to the ligand's Gla domain (FIGURE 19.4). Although EPCR is named after its expression on endothelial cells, EPCR is also present on epithelial cells, monocytes, macrophages, neutrophils, eosinophils, natural killer cells, and in mice on specific bone marrow–derived dendritic cells.[30,73–79]

EPCR is susceptible to shedding of the EPCR ectodomain due to proteolysis in the region of residues 193 to 200, just above the transmembrane domain, by tumor necrosis factor-alpha (TNF-α)–converting enzyme/ADAM17 (TACE). Several proinflammatory cytokines, for example, TNF-α and interleukin-1β, induce soluble EPCR formation and increase soluble EPCR levels in mice exposed to endotoxemia; soluble EPCR is increased in plasmas of patients with a prothrombotic and proinflammatory tendency.[80–84]

THE ANTICOAGULANT PROTEIN C PATHWAY

The protein C system is a dual function cofactor-dependent system that exerts direct cytoprotective activities on cells as well as its well-studied anticoagulant activity anticoagulant. By targeting factors Va and VIIIa, major procoagulant cofactors for thrombin generation, APC regulates coagulation and prevents thrombosis. The physiologic importance of thrombosis prevention by the anticoagulant protein C system is evident from clinical observations of thrombotic complications in individuals with deficiencies of protein C and protein S.

Anticoagulant Protein C Pathway Defects and Thrombosis

Homozygous and compound heterozygous severe protein C deficiency typically presents itself as neonatal purpura fulminans, a rapidly progressing hemorrhagic necrosis of the skin due to microvascular thrombosis, inflammation, and disseminated intravascular coagulation (DIC).[85] Infants with severe protein C deficiency suffer massive thrombotic complications, and if they survive due to maintenance replacement therapy, they tend to present with mental retardation and/or visual impairment.[85–87] Replacement therapy with fresh-frozen plasma or with purified plasma protein C concentrate (CEPROTIN) is beneficial.[88–90] The incidence of clinically significant protein C deficiency carries a significantly increased risk for venous thrombosis (see Chapters 80 to 82).[91–93] Hundreds of mutations have been identified (see protein C mutation databases[92,94,95]), and the basis for hereditary protein C defects can most often be rationalized based on the structure of protein C.[96–98] Type I mutations, characterized by reductions of both antigen and activity, typically involve amino acid mutations that affect protein folding, secretion, or stability. In contrast, most type II mutations, characterized by reduction of anticoagulant activity but not antigen, involve residues that cause dysfunctional molecules either by affecting thermodynamic stability or by protein–protein interactions important for expression of anticoagulant activity. Acquired protein C deficiency occurs upon initiation of vitamin K antagonists (coumarin/warfarin) therapy due to the relatively short half-life of protein C ($T_{1/2}$ ~ 8 hours) compared to most procoagulant vitamin K–dependent factors ($T_{1/2}$ ~ 20 to 48 hours); this potential problem is addressed by using heparin during initiation of therapy, or even protein C concentrate for special circumstances.[99,100]

Severe (homozygous or compound heterozygous) protein S deficiency, although extremely rare, manifests with purpura fulminans in the neonatal period similar to severe protein C deficiency.[101–103] Hereditary protein S heterozygous deficiency conveys a significantly increased risk of thrombosis.[29,93,104–106] Due to the binding of protein S to C4b-binding protein in plasma, testing to establish protein S deficiency is challenging.[105] Deficiencies are classified as follows: type I, reduction of both free and total protein S antigen; type II or qualitative deficiency, functional protein S defect with a low functional activity but normal antigen level; and type III, with a low level of free protein S level but a normal level of total protein S antigen.[29,102,104–109] Mutations are found in the majority of patients and families with hereditary protein S deficiency (see Chapters 80 to 82 and protein S mutation databases).[105,110] Two polymorphisms causing deficiencies are known as protein S Heerlen (Ser460Pro), a type III defect among subjects of European ancestry, and protein S Tokushima (K196E), a type II defect.[111–116] Protein S Tokushima is a common risk factor for venous thrombosis among Japanese and is found in approximately 10% of venous thrombosis patients.[105,111,117–119] It is likely that the protein S Tokushima mutation of K196E (residue 155 in mature protein S) arose from a founder effect in Japan and is a balanced polymorphism, like factor V Leiden or prothrombin 20210A that carries a beneficial effect (e.g., prevention of excess fatal bleeding) and a mild risk factor for venous thrombosis. In contrast, the Heerlen polymorphism that alters glycosylation is not as obvious a risk factor. Acquired protein S deficiency is common during pregnancy and also may arise during the use of certain oral contraceptives, or after initiation of oral anticoagulant treatment.[120–124]

APC resistance conveys an increased risk for venous thrombosis and is defined as an abnormally reduced anticoagulant response of a plasma sample to APC. It can be caused by many potential abnormalities in the protein C anticoagulant pathway but among whites a mutation in factor V, which arose in a European ancestor involving an APC-cleavage site (Arg506Gln, factor V Leiden), is the most common cause for hereditary thrombosis.[38,39,125] Other known APC cleavage site mutations in factor V that contribute to an increased thrombotic risk are Arg306Thr (factor V Cambridge) and Arg306Gly (factor V Hong Kong).[126–131] APC resistance with no identifiable genetic or acquired abnormalities is well described in patients with venous and arterial thrombosis, and further studies are needed to identify the causes of APC resistance in such patients.[132–135]

TM genetic mutations may be associated with increased risk of arterial thrombosis and myocardial infarction, whereas there is less supportive data for association with risk for venous thrombosis.[61,136–142] Mutations in TM have also been linked to atypical hemolytic-uremic syndrome patients; this syndrome

is strongly linked to excessive complement activation.[143] The lectin-like domain of TM (FIGURE 19.3) can contribute to inhibition of complement activation and provide direct anti-inflammatory activity, although the molecular basis for this remains somewhat unclear.[144–147]

The potential association of EPCR defects with venous and arterial thrombosis remains a bit controversial and seems difficult to prove (see Chapters 81 and 82).[148–155] To date, four EPCR gene haplotypes have been characterized containing certain polymorphisms.[68] Interestingly, the H1 haplotype was associated with a reduced risk of venous thromboembolism and diminished risk of thrombosis in carriers of the factor V Leiden mutation.[156,157] In contrast, the H3 haplotype of EPCR that is characterized by a Ser219Gly mutation in EPCR causes enhanced EPCR shedding from the membrane, resulting in increased levels of soluble EPCR in the circulation, which might be associated with an increased risk of venous thrombosis.[81,156,158–160]

Protein C Activation

Activation of zymogen protein C by thrombin is due to cleavage at the Arg169–Leu170 peptide bond (FIGURE 19.1) in a reaction that is accelerated by TM and EPCR on the surface of the endothelial cells (FIGURE 19.5).[1,6,7,23,161,162] Binding of thrombin to TM shields thrombin's procoagulant exosite I and unveils its anticoagulant properties.[7,163–165] Subsequent activation of protein C by the thrombin–TM complex is facilitated by localization of protein C on the endothelial cell surface by binding to EPCR.[166,167] Available data suggest that EPCR and TM must be in close proximity on cell surfaces, though this has yet to be experimentally demonstrated. In blood vessels, TM is generally considered more abundant in the microcirculation while EPR is relatively more abundant in large vessels than in small vessels.[1,168,169]

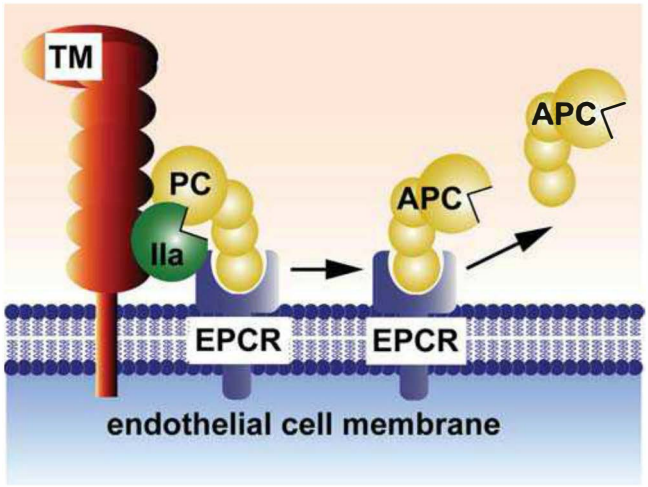


FIGURE 19.5 Protein C activation on endothelium. Physiologic activation of protein C (PC) by the thrombin (IIa)–thrombomodulin (TM) complex occurs on the surface of endothelial cell membranes. The most efficient activation of protein C occurs when protein C is bound to the endothelial receptor (EPCR). Since protein C and APC have a similar affinity for EPCR, after activation APC can either dissociate from EPCR to exert anticoagulant activity or remain bound to EPCR where it might express multiple direct cellular activities. (This scheme was originally published in *Blood*. Mosnier et al. The cytoprotective protein C pathway. *Blood* 2007;109(8):3161–3172. © the American Society of Hematology.)

APC is constantly generated in the resting state because APC is present at approximately 40 pM in all healthy human subjects' plasma. The concentration of zymogen protein C in plasma is an important determinant for circulating APC levels, reflecting the kinetics of the protein C activation on cultured cells *in vitro*.[166,167,170–177] An inverse correlation between circulating fibrinopeptide A and APC levels suggests that APC is a significant *in vivo* regulator of basal thrombin activity.[178–180] Impaired activation of protein C occurs in patients with inflammation and sepsis.[181] Protein C levels in patients with severe sepsis often are markedly decreased, due to a combination of protein C consumption, dysfunctional protein C synthesis in the liver, and/or reduced levels of TM or EPCR on the endothelium.

APC Anticoagulant Activity

Anticoagulant activity of APC involves proteolytic inactivation of factors Va and VIIIa (FIGURE 19.6).[182,183] Multiple cofactors, including protein S, negatively charged phospholipids, neutral glycosphingolipids, and high-density lipoprotein (HDL), are known to enhance APC's inactivation of factor Va and/or factor VIIIa.[184–188]

Factor Va is composed of a heavy chain (A1–A2, residues 1 to 709) and a light chain (A3–C1–C2, residues 1,546 to 2,196) that form a noncovalent heterodimeric complex.[189–195] In the prothrombinase complex, factor Va binds both the substrate prothrombin and the enzyme factor Xa on the surface of negatively charged phospholipids, thereby increasing the rate of thrombin generation by approximately 10,000-fold.[182,183,196] Inactivation of factor Va by APC effectively shuts down thrombin formation via a complex process involving three cleavages at Arg306, Arg506, and Arg679. Cleavage at Arg506 is rapid and much faster than cleavage at Arg306, but it only partially inactivates factor Va, whereas cleavage at Arg306 results in the complete inactivation of factor Va.[182,183,197] Cleavage of the connection between the A2 domain and the A1 domain at Arg306 results in the dissociation of the A2 domain fragment, whereas the A1 domain remains associated with the light chain.[198] Protein S might enhance the APC-mediated cleavage of factor Va by lowering the active site of APC closer to the membrane.[199] In addition, protein S is able to diminish the ability of factor Xa to protect factor Va from inactivation by APC when protein S, factor Xa, and factor Va are simultaneously present. Mutagenesis studies show that positively charged residues in exosites on the APC protease domain surface are required for rapid inactivation of factor Va.[200–205]

Although factors Va and VIIIa are generally homologous proteins with similar domain structure and procoagulant function, factor VIIIa, in contrast to factor Va, is quite unstable with a half-life of only 2 minutes due to spontaneous dissociation of the A2 domain (see Chapter 12). This is because factor VIIIa is a heterotrimer, whereas factor Va is a heterodimer due to different cleavage patterns that cause their respective activations.[206] Factor VIIIa, expressing full cofactor activity, enhances factor Xa formation by the tenase complex approximately 200,000-fold.[207]

The physiologic relevance of APC-mediated factor VIIIa inactivation was questioned due to the spontaneous inactivation of factor VIIIa; however, several arguments, including stabilization of factor VIIIa by factor IXa, support a role for APC in the inactivation of factor VIIIa.[208–210] Proteolytic inactivation

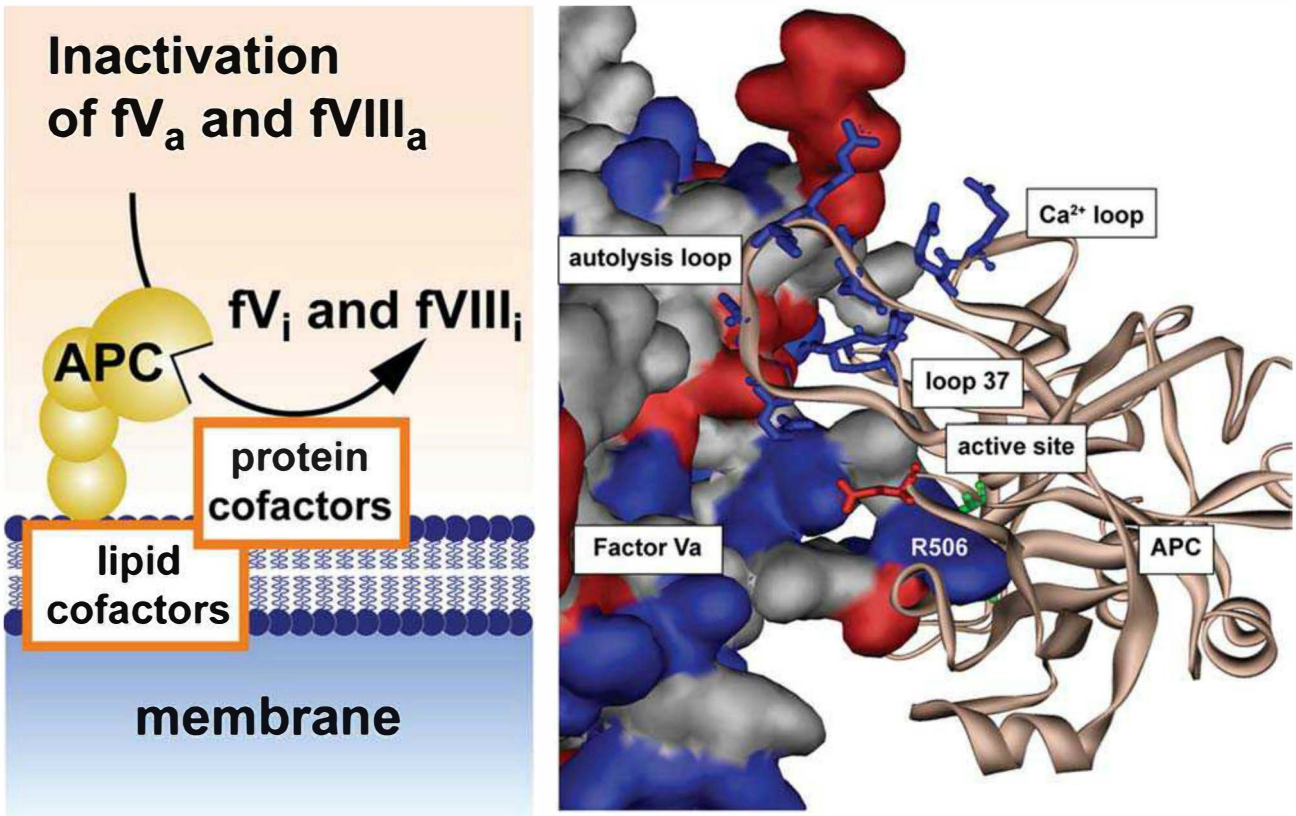


FIGURE 19.6 APC anticoagulant activity. Schematic model of mechanisms for APC anticoagulant activity **(left)**. APC anticoagulant activity involves proteolytic cleavages of the activated cofactors Va (FVa) and VIIIa (FVIIIa) to yield the inactivated cofactors, FVi and FVIIIi, when APC is bound to phospholipid surfaces. Different protein and lipid cofactors, for example protein S, factor V, and various lipids (e.g., phosphatidylserine, cardiolipin, glucosylceramide, etc.), enhance inactivation of factors Va and VIIIa by APC. A close-up scheme **(right)** of the exosite interaction between APC and factor Va. In this APC:FVa complex model (from Pellequer et al.[195]), exosite specificity is based on the juxtaposition of positively charged residues (*blue*) of APC in the autolysis loop, 37-loop, and calcium-binding loop near negatively charged residues (*red*) of FVa. These exosite interactions between APC and factor Va are required for positioning of Arg506 of factor Va in the active site pocket of APC and thus for cleavage of factor Va at Arg506 and for normal anticoagulant activity **(left)**. (This scheme was originally published in *Blood*. Mosnier et al. The cytoprotective protein C pathway. *Blood* 2007;109(8):3161–3172. © the American Society of Hematology.)

of factor VIIIa by APC occurs upon cleavage at Arg336 and Arg562, homologous to Arg306 and Arg506 in factor Va. Protein S only moderately enhances cleavage at Arg336 (~twofold), whereas it stimulates the cleavage at Arg562 only slightly more (~ fivefold).[210–212] Factor V can further enhance the APC cofactor effect of protein S.[213–215] Thus, factor V and protein S act as synergistic cofactors for factor VIIIa inactivation by APC.[213] It also appears that factor V promotes APC's inactivation of factor Va.[216] Because factor V Leiden lacks this APC cofactor activity, it was hypothesized that risk for thrombosis in factor V Leiden carriers is caused by both defective APC cofactor activity and resistance to APC inactivation, with each defective mechanism assumed to contribute equally to increased risk.[217–219]

Protein S Anticoagulant Cofactor Activity

The APC cofactor function of protein S involves a complex set of interactions of protein S with APC and with components of the prothrombinase complex, factor Xa, and factor Va.[220–227] Molecular mechanisms for enhanced APC-mediated cleavage of factor Va at Arg306 by protein S have been partially elucidated and involve a protein S-induced change in the geometry of APC relative to the membrane. Protein S binding lowers APC's active site closer to the membrane, presumably placing it in a better position to cleave Arg306.[199,228] In addition, protein S reduces the ability of factor Xa to protect factor Va from APC, based on the presumption that factor Xa blocks access of APC to factor Va and that protein S competes with factor Xa for binding to factor V.[223–226,229]

Protein S molecular regions (FIGURE 19.2) that bind APC are located in the Gla domain, the TSR, and in both EGF-1 and EGF-2 domains. The sequence in EGF-1 from Lys97 to Pro106 is thought to support direct interactions as well as a supporting structural role for the Gla domain, the TSR, and EGF-2.[222,230–232] The protein S–binding regions on the surface of APC (FIGURE 19.1) are thought to involve hydrophobic helix at the end of the Gla domain, several EGF-1 residues, and the *C*-terminus of the light chain, but such

interactions are speculative in the absence of better structural information.[233,234,235,236]

The protein S anticoagulant APC cofactor function is regulated by at least two mechanisms. First, noncovalent and reversible binding of protein S to C4BP results in the loss of protein S APC cofactor function, which enhances Arg506 cleavage in FVa. Protein S molecules bound to C4BP can still bind to phospholipid membranes, interact with APC, and enhance cleavage at Arg306; however, this cleavage is not well reflected in typical coagulation assays for APC anticoagulant activity.[220,237–241] Second, proteolysis of protein S in the TSR at Arg49, Arg60, and/or Arg70 reduces the affinity of protein S for calcium and phospholipids and causes loss of APC cofactor activity.[242–245] Patients with DIC showed markedly increased levels of cleaved protein S, indicating that cleavage of protein S in the TSR can occur under certain pathological conditions, although this has been labeled nonphysiologic due to inhibition of thrombin-mediated cleavage by calcium ions *in vitro*.[246]

APC-Independent Protein S Anticoagulant Activity

Protein S can directly exert anticoagulant activity independent of its role as an APC cofactor. Initial reports demonstrated that direct binding of protein S to factor Xa, factor Va, and factor VIIIa was responsible for inhibiting functional assembly of the prothrombinase and tenase complex. This activity of protein S was subsequently attributed as due to either an artifact involving protein S multimers or simply a competition of protein S for limited amounts of procoagulant phospholipid surfaces.[220,224,227,247–249] It has been debated whether protein S multimers are artifactual and created during protein S purification, or whether protein S multimers are physiologic and exist in plasma.[248–251] More recent research has provided some evidence for mechanisms by which protein S might directly inhibit thrombin generation, including protein S with tissue factor pathway inhibitor (TFPI), with Zn^{2+} ions, or with tissue factor; each of these interactions, in addition to APC enhancement, may contribute to the antithrombotic activities of protein S.[14,252–255] Few data are currently available to decipher the relative *in vivo* importance of these mechanisms.

Lipids and Lipoproteins

Negatively charged phospholipids, especially phosphatidylserine, markedly enhance both procoagulant and anticoagulant reactions in the test tube. However, certain phospholipids (e.g., phosphatidylethanolamine and cardiolipin) and sphingolipids (e.g., glycosphingolipids and sphingosine) enhance the anticoagulant APC pathway much more than the procoagulant pathways.[184–188,256–260] In particular, glucosylceramide (GlcCer) improves APC anticoagulant activity by enhancing the interaction of APC with phospholipid membranes, and remarkably, GlcCer deficiency is a biomarker and may be a potential risk factor for venous thrombosis.[186,187,259,261]

Most lipids in blood are in lipoproteins. HDL enhances the anticoagulant activity of APC, at least in part, by stimulation of APC's cleavage at Arg306 in factor Va that is protein S dependent.[188] HDL inhibits DIC induced by endotoxin infusion in baboons and inhibits ferric chloride–induced arterial thrombosis in rats.[262,263] The components responsible for this APC cofactor activity of large HDL but not small HDL particles have not been identified, in part, because HDL is extensively heterogeneous in both protein and lipid composition.[258] Moreover, the heterogeneous nature of purified lipoprotein particles makes them challenging for functional coagulation studies; notably, it is possible to draw invalid conclusions about HDL's enhancement of APC activity when studying HDL that was not properly handled or was frozen.[264] Although HDL can exert antithrombotic activity via multiple potential mechanisms, observations from clinical venous thrombosis cohorts are consistent with the hypothesis that deficiency of large HDL particles is a risk factor for venous thrombosis based on reports that recurrent venous thrombosis and venous thrombosis in males are associated with a pattern of dyslipoproteinemia and low HDL.[258,265–267]

In addition, hyperlipidemia and vascular disease can affect protein C activation in primates. Thrombin infusion into healthy animals always generates anticoagulant activity due to APC formation (FIGURE 19.5). But thrombin infusion into hyperlipidemic monkeys with atherosclerosis generates significantly less APC compared with infusion into normolipidemic control monkeys; moreover, hyperlipidemia causes a diminished *ex vivo* response of monkey plasma to APC, thereby showing that dyslipidemia affects the protein C systems *in vivo*.[174,268,269]

THE CYTOPROTECTIVE PROTEIN C PATHWAY

Control of coagulation reactions does not occur in the absence of an integrated host defense system which is comprised of multiple overlapping and integrated pathways, including inflammatory processes, blood coagulation reactions, fibrinolysis, and thrombotic processes (see reviews[1,6,7,161,265,270–272]). In addition to its anticoagulant actions, APC acts directly on cells to cause multiple cytoprotective effects including (a) antiapoptotic activity; (b) anti-inflammatory activities; (c) protection of endothelial barrier function; and (d) alteration of gene expression profiles (FIGURE 19.7). For simplicity of discussion below, these multiple, distinct actions are sometimes, collectively, referred to as APC's cytoprotective effects although the mechanism for each type of activity must involve distinct types of cell-signaling actions. Several components of the anticoagulant pathway, in addition to protein C itself, contribute not only to anticoagulant activity but also to cytoprotective functions and continue to stimulate new directions for the protein C cytoprotective pathway.

Mechanisms and Major Components

The currently best established paradigm for the direct cytoprotective actions of APC is that EPCR-bound APC activates PAR-1 to initiate signaling, and this is widely supported by both *in vitro* and *in vivo* evidence (see reviews[2,5,6,9,13,273,274]) (FIGURE 19.7). However, other receptors may mediate, directly or indirectly, APC-initiated signaling in various cells (see below). Such other receptors include sphingosine-1-phosphate receptor 1 (S1P1), apolipoprotein E receptor 2 (ApoER2), glycoprotein Ib, several integrins, PAR-3, Tie2, and epidermal growth factor receptor.[19,20,43,47,275–282]

APC proteolytic actions can also indirectly protect cells from the detrimental effects of histones or of neutrophil-derived histone–DNA complexes because APC can cleave extracellular histones, and this activity appears to protect against sepsis in mice.[283]

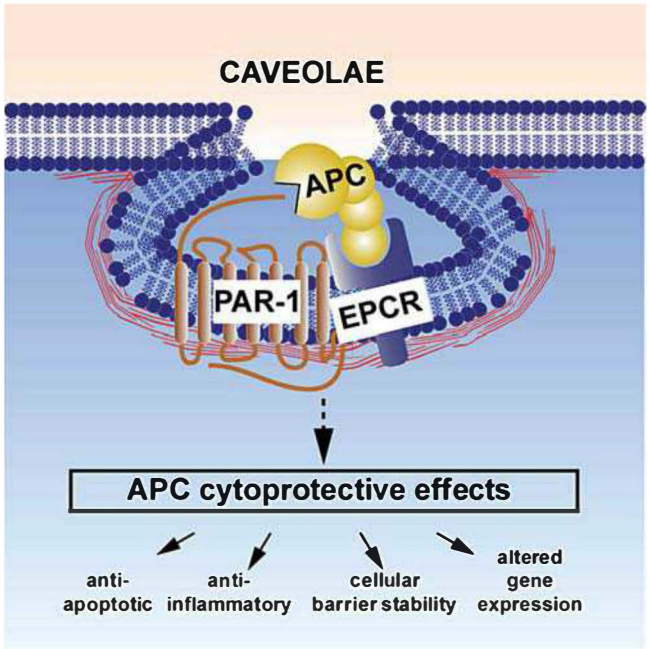


FIGURE 19.7 Beneficial direct effects of APC on cells. The cellular receptors EPCR and PAR-1 are required for APC's beneficial effects on endothelial cells. These activities include APC-mediated antiapoptotic activities, anti-inflammatory activities, protection of endothelial barrier functions, and alteration of gene expression profiles. This paradigm in which EPCR-bound APC activates PAR-1 to initiate signaling is consistent with many *in vitro* and *in vivo* data. Localization of APC signaling in the caveolin-1–rich microdomains (caveolae) may help differentiate mechanisms for cytoprotective APC signaling versus proinflammatory thrombin signaling.[72,311] Additional mechanisms for APC effects on cells may also involve other receptors. These effects include APC anti-inflammatory effects on leukocytes or cytoprotective effects on dendritic cells and neurons, and other receptors may include S1P1, apoER2, PAR-3, and specific integrins (e.g., CD11b). Transactivation of the S1P1 receptor by the PAR-1/EPCR/APC complex is required for APC's ability to protect endothelial barrier functions and EPCR coimmunoprecipitates with S1P1 in the presence of APC.

Protease-Activated Receptor-1

PAR-1 is a thrombin receptor that is the prototype of a four-member subfamily of protease-activated G-protein–coupled receptors (GPCRs).[284–290] Each PAR contains seven transmembrane helical domains and an extracellular *N*-terminal tail that upon cleavage by various proteases generates a new amino-terminus acting as a tethered ligand to trigger cell signaling, most often but not always via coupled G proteins.[285,291,292] The gene for PAR-1 (F2R) is on chromosome 5q13 and contains only two exons (Table 19.1), and its expression varies substantially among different cells and tissues.[284–290,293] Although thrombin activates human platelets via PAR-1 and PAR-4, curiously, murine platelet activation involves PAR-3 and PAR-4, but not PAR-1 which is lacking on murine platelets.[286,294,295]

Mechanisms for PAR-1–initiated signaling are complex and likely involve a spectrum of conformational states and a diverse family of agonists, partial agonists, coreceptors, and allosteric effectors that typify the complexity of a GPCR.[296–300] While EPCR acts as an essential cofactor for PAR-1 activation by APC, EPCR can also modulate PAR-1 activation *in vitro* by factor VIIa and factor Xa.[16,17,18,41,301–306]

Mechanisms on Endothelial Cells

A useful but incomplete paradigm for APC-induced signaling on endothelial cells is one in which EPCR-bound APC cleaves and activates PAR-1 (FIGURE 19.7), and extensive *in vitro* and *in vivo* data support this model (see reviews[6,15–17,42,71,274,276,307–309,310,311]). Yet, the puzzle remains of how PAR-1 can be activated by both APC and thrombin with contrasting outcomes.[2,71,72,311,312] Some suggest that PAR-1's signaling properties are altered in relationship to the localization of PAR-1 and EPCR in membrane rafts, specifically in caveolae (FIGURE 19.7).[71,311,313,314] Accordingly, localization of PAR-1 and EPCR in membrane rafts, possibly induced by occupancy of EPCR, is required for cytoprotective signaling properties via PAR-1 as APC's PAR-1 signaling but not thrombin's PAR-1 signaling is caveolin-1 dependent.[72] Furthermore, thrombin-cleaved PAR-1 is rapidly internalized, whereas APC-activated PAR-1 tends to remain on the cell surface, which could prolong signaling, by APC compared to thrombin.[72,315]

APC's cytoprotective effects are multiple and vary widely in nature (FIGURE 19.7). Certainly, the initiation and integration of signaling pathways that mediate each type of cytoprotective activity of APC are, at most, only partially overlapping. Much needs to be elucidated before even a partial picture emerges for intracellular mechanisms that mediate APC's various cytoprotective actions. It is unclear whether each of these activities is indeed almost completely separate or whether these activities are intimately intertwined and dependent on each other. For example, does APC mediate antiapoptotic activity because APC downregulates certain proapoptotic gene expression, or because it upregulates certain antiapoptotic gene expression, or because APC somehow intervenes more specifically at certain steps of the apoptotic transformation of the cell, or because of all of these?[15–17,276,316–318] Moreover, the mechanisms likely depend on the cell type and the context of cells, tissues, and organs.

Some insights into mechanisms for APC's ability to stabilize endothelial barriers and to minimize vascular permeability are available. Whereas thrombin-mediated PAR-1 activation results in activation of barrier disruptive RhoA, Rho kinase-mediated inactivation of myosin light-chain phosphatase, and actin–myosin contractility due to increased myosin light-chain phosphorylation, APC prevents such morphologic transformations and maintains endothelial barrier functions both *in vitro* and *in vivo*.[19,20,42,319–323] In reactions that require EPCR, PAR-1, S1P1, and caveolin-1, APC activates barrier-protective Rac1 via PAR-1, β-arrestin, and the dishevelled-2 scaffold.[72,311,312,324] APC's barrier protective effects via Rac1 activation are similar to endothelial barrier stabilization by S1P, an endogenous bioactive lipid, in that APC causes EPCR-dependent clustering and transactivation of the S1P1 in membrane lipid rafts and with potential sphingosine kinase 1 (SphK1) "inside-out" signaling by S1P.[19,20,323,325]

Mechanisms on Lymphocytes

Lymphocytes are an important target for APC's anti-inflammatory effects where APC can downregulate inflammatory mediator release and expression of procoagulant activity. Interestingly, the APC–PAR-1–EPCR signaling complex seems to play a less

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prominent role on lymphocytes. Instead, additional receptors and receptor complexes, including ApoER2, EPCR, integrins, or combinations thereof, have been implicated to play a role in mediating APC's anti-inflammatory activities. APC indirectly influences leukocytes by preventing expression of adhesion molecule expression (such as ICAM-1, VCAM-1, and E-selectin) on endothelial cells, resulting in downregulation of lymphocyte adhesion and tissue infiltration.

Integrins are important modulators of APC signaling and anti-inflammatory effects on lymphocytes. On macrophages, APC binds integrin CD11b/CD18 ($\alpha_M\beta_2$, Mac-1, CR3) within specialized membrane microdomains/lipid rafts where it presumably replaces the role of EPCR in initiating PAR-1–dependent signaling by APC. Enhanced production of barrier-protective S1P and suppression of the proinflammatory response of activated macrophages is dependent on CD11b/CD18 but not on EPCR.[282] Soluble EPCR and proteinase-3, a serine protease with elastase-like properties stored in granules of neutrophils, bind CD11b/CD18 on activated neutrophils. When soluble EPCR is bound to proteinase-3, soluble EPCR retains the ability to bind APC. Thus, although speculative at present, soluble EPCR may contribute to CD11b/CD18-mediated APC cellular signals and/or activation of protein C on leukocytes.[326] CD11b/CD18 is a ligand for ICAM-1 and plays a pivotal role in the adhesion and migration of leukocytes.[327,328] Accordingly, binding of soluble EPCR to CD11b/CD18 might interfere with leukocyte adhesion as an antibody that blocks β_2-integrin–mediated adhesion also inhibited soluble EPCR binding to CD11b/CD18.[326] An RGD (Arg–Gly–Asp) sequence is present in human protein C and APC has been implicated in binding β_1- and β_3-integrins and inhibiting neutrophil migration.[278] Furthermore, glycoprotein Ib and ApoER2 are also able to bind APC to cell surfaces.[275] With the arsenal of APC receptors on vascular cells expanding, it seems less and less likely that there is one unifying mechanism for each of APC's cytoprotective effects on various cell types. Instead, APC cellular activities and its underlying mechanisms seem to be dictated by cell-type specific expression of APC receptor complexes. Dendritic cells have been identified as mediating APC's EPCR-dependent ability to reduce mortality in endotoxemia.[79]

Mechanisms on Other Cells

The beneficial effects of APC on neurons, epithelial cells, keratinocytes, and tenocytes have been described, often requiring EPCR. Only little is known about receptors or mechanistic steps for APC's effects on these cells, but EPCR is almost always required.[47,276,279,281,329–331] The remarkable effects of APC include the ability to promote wound healing, likely based on its effects on keratinocytes. Generally, it appears that APC is a homeostatic protease that stabilizes stressed or challenged cells of various types.

Apolipoprotein E Receptor 2

The observation that ApoER2 (gene symbol LRP8) (low-density lipoprotein receptor–related protein 8) binds APC with high affinity and that APC can mediate activation of signaling pathways via ApoER2 expanded the receptor repertoire for APC's cytoprotective effects on cells.[43,275] ApoER2 is found in platelets and endothelial cells, but it is best known for its central role in the "Reelin signaling pathway," which plays a crucial role in the correct positioning of neurons during embryonic brain development.[332–357] Reelin, a very large protein (>3,400 residues), initiates signaling via Dab1 by binding simultaneously to very low-density lipoprotein receptor and ApoER2.[358] Dab1-dependent activation of the Src family kinases ultimately results in the activation of PI3K-Akt signaling cassette, a well-known survival pathway, and a major mechanism for promoting cell survival. APC can activate a "Reelin-like signaling pathway" on monocytes via rapid ApoER2-dependent Dab1 phosphorylation at Tyr220 and phosphorylation of Akt at Ser473.[43] The *in vivo* significance of apoER2 as a physiologic or pharmacologic receptor for APC has not been assessed.

ENDOGENOUS CYTOPROTECTIVE PROTEIN C ACTIVITIES

While much evidence indicates that the cell-signaling activities of APC are primary for pharmacologic applications of APC's ability to reduce mortality, recent studies have established an important physiologic role for cell signaling by the endogenous protein C system in mice. Mice that are genetically engineered to be null for EPCR exhibit embryonic lethality, whereas mice with high levels of EPCR expression show resistance to lipopolysaccharide-induced death, showing there is a physiologically essential role for EPCR.[359–361] The cytoprotective activities of endogenous APC are required for protection in murine sepsis models and also appear to be necessary for survival of the initial shock injury in a different model of trauma/hemorrhagic shock.[362,363]

PHARMACOLOGIC PROTEIN C THERAPIES

Preclinical and clinical studies of APC and protein S have established these molecules as antithrombotic agents with anti-inflammatory activity. The additional *in vivo* effects of APC therapies include antiapoptotic activity and inhibition of vascular leakage. Studies that provided mechanistic insights are noted elsewhere in this chapter. Clinical studies and animal model systems in which APC therapy provided promising results are reviewed elsewhere.[6,9,13,274,364,365]

APC provides remarkable neuroprotective effects *in vivo* and increases survival in murine ischemic stroke studies.[276,366–368] Beneficial effects of daily dosing of APC have proven efficacious in animal models of amyotrophic lateral sclerosis and appear promising in animal models of multiple sclerosis and Alzheimer disease.[13,280,369] Thus, APC conveys disease-modifying effects in both acute neurologic injury (e.g., stroke and nerve crush injury) and chronic neurodegenerative disorders. These neuroprotective actions appear to be based on APC's cytoprotective properties.[13] In murine models of brain injury, APC's neuroprotection required PAR-1, EPCR, and PAR-3.[17,47,276,277,309,370] Whereas tissue plasminogen activator (tPA) is capable of directly causing damage to neurons, APC effectively counteracts tPA's neurotoxic effects.[368,370–373] Moreover, APC prevents tPA-induced hemorrhagic transformation during ischemic stroke therapy.[309] Thus, preclinical studies suggest that APC's cytoprotective activities are promising for providing neuroprotective therapies.

Purified plasma-derived protein C concentrates have been developed and approved for replacement therapy in patients with deficiency of plasma protein C. This protein C product is made using plasma fractionation methods and has been used both for standard replacement therapy and for clinical investigations of the value of protein C therapy.[89,374,375]

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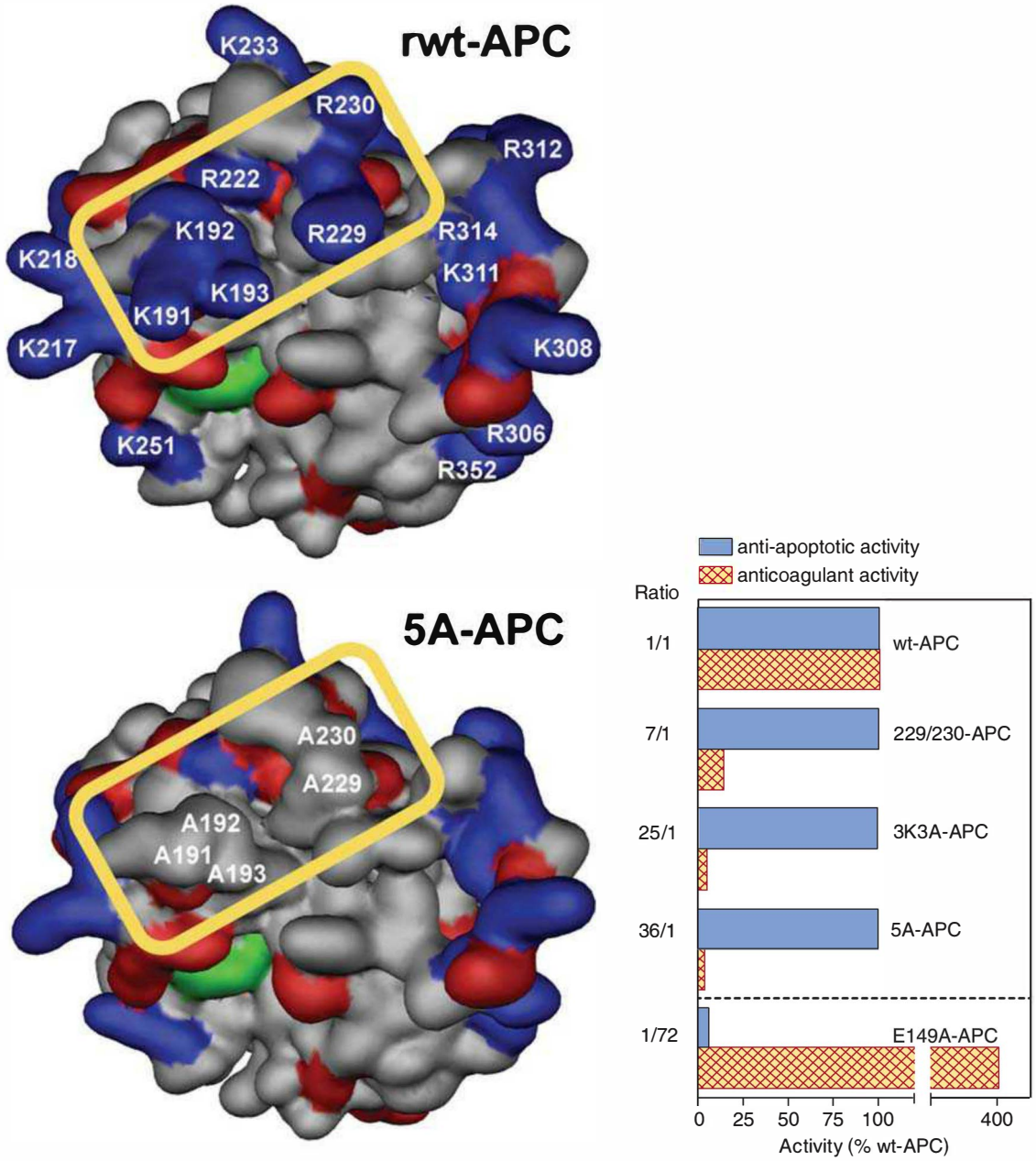

FIGURE 19.8 APC exosite residues on the protease surface that mediate APC anticoagulant activity but not antiapoptotic activity. Interactions of APC with its macromolecular substrates (factor Va, factor VIIIa, and PAR-1) are dictated by exosite interactions of APC protease domain surface loops. Residues in the 37-loop (Lys191, Lys192, and Lys193) and in the calcium-binding loop (Arg229 and Arg230) are required for normal cleavage of factor Va at Arg506 and thus for normal anticoagulant activity while they are not involved in proteolytic activation of PAR-1 that generates antiapoptotic activity. Based on this exosite specificity difference, the 3K3A-APC and 5A-APC variants may provide reduced risk of bleeding due to reduced anticoagulant activity but provide full cytoprotective activity.[381] The surface contours with positive (*blue*) and negative (*red*) side chains are shown for wildtype APC (wt-APC) and for the 5A-APC variant (containing five Ala substitutions at Lys191, Lys192, Lys193, Arg229, and Arg230). The neutral side chain of Ala at 191, 192, 193, 229, and 230 appears *gray*. Comparison of the protease domain for 5A-APC **(bottom left)** to that of wt-APC **(top left)** highlights the loss of five positively charged surface residues (*blue*) from an exosite that is required for normal recognition of FVa. The values for antiapoptotic activity (*gray bars*) and anticoagulant activity (*cross-hatched bar*) for wt-APC and each APC variant are shown where the reference values for wt-APC are defined as 100% **(right)**. The ratio of antiapoptotic to anticoagulant activity is shown, where 1/1 is the reference ratio for wt-APC. The 3K3A-APC and 5A-APC variants have greatly reduced anticoagulant activity relative to cytoprotective activity, while the converse is the E149A-APC variant. (Reprinted with permission from Griffin et al. *J Thromb Hemost* 2007;5:73–80.)

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Protein S has beneficial antithrombotic and neuroprotective effects in several preclinical studies; however, few data are available to clarify the relative importance of antithrombotic actions versus cell-signaling actions induced by protein S.[46,376,377]

ENGINEERED APC MUTANTS

Engineering of APC mutants in the last century was driven by efforts to clarify why human mutations in protein C–deficient patients caused thrombosis and to explore structure–function relationships for APC's anticoagulant activity, and many APC mutants have been studied.[2,6,378,379] However, the success of the PROWESS sepsis trial, combined with the failure of other anticoagulants, AT, and TFPI in similar sepsis trials, helped to stimulate discoveries of APC's cytoprotective actions. It was also disturbing that bleeding was a serious side effect of APC in the PROWESS trial.[31,380] These considerations stimulated the authors to try to prepare APC variants with a reduced risk of bleeding due to reduced anticoagulant activity but with normal direct effects on cells.[381]

Efforts to engineer cytoprotective-selective APC mutants were based on the assumptions that (a) anticoagulant activity is based on APC exosite's recognition of factor Va; (b) antiapoptotic activity is representative of APC's cytoprotective effects *in vivo*; (c) APC's cytoprotective effects require APC-mediated PAR-1 activation; and (d) cleavage at Arg41 in the *N*-terminal tail of PAR-1 has different APC exosite requirements than cleavage at Arg506 in factor Va. This would mean that the factor Va exosites and the PAR-1 exosites on APC are, at least partially, nonoverlapping. Thus, we attempted to alter factor Va exosites in APC without affecting exosites that might recognize PAR-1.[6,381]

The interaction of APC with the Arg506 cleavage site in factor Va that is critical for APC's normal anticoagulant activity involves a positively charged surface of the protease domain that includes loop 37 (protein C residues 190 to 193, equivalent to chymotrypsin [CHT] residues 36 to 39), the Ca^{2+}-binding loop (residues 225 to 235, CHT residues 70 to 80), and the autolysis loop (residues 301 to 316, CHT residues 142 to 153) (see FIGURE 19.8).[40,200,202–205,382] To provide proof of concept, two APC variants were selected with Ala mutations in two exosites defined by two APC surface loops; these mutants involved Ala replacements of Arg229 and 230 and/or of Lys191, 192, and 193. When tested in assays of staurosporine-induced endothelial cell apoptosis, these two APC variants, 3K3A-APC and 5A-APC, had reduced anticoagulant activity but retained normal antiapoptotic activity that requires PAR-1 and EPCR and exhibited a normal ability to cleave a PAR-1 *N*-terminal peptide at Arg41.[381] When the anticoagulant and antiapoptotic activities of these APC variants were normalized to wildtype APC (wt-APC), the two APC variants exhibited 25 times and 33 -times greater antiapoptotic activity relative to anticoagulant activity when compared to wt-APC (FIGURE 19.8). Other APC mutants with altered activity profiles include the cytoselective mutants, L38D-APC, an engineered disulfide APC mutant, and anticoagulant-selective mutants with mutations at residues 330 or 333.[234,383–386]

When murine cytoprotective-selective, 3K3A-APC and 5A-APC mutants lacking most anticoagulant activity were compared *in vivo* to wt-APC for APC's ability to reduce mortality in sepsis or to provide neuroprotection in ischemic stroke models, these mutants were fully active and appeared indistinguishable from wt-APC.[42,47,79,387] In contrast, the murine anticoagulant-selective E149A-APC mutant lacking antiapoptotic activity failed to reduce mortality in mice. Moreover, murine 5A-APC also reduced death in murine pneumonia.[323] These data support the idea that APC's cytoprotective actions are primarily responsible for APC's *in vivo* benefits in sepsis and neuroprotection for stroke and they set the stage for efforts to develop APC mutants as second-generation biologics with greatly reduced risk of bleeding. Cytoprotective-selective APC mutants also should permit trials with altered dosing regimens, consistent with APC's ability to alter cell signaling rather than to provide constant, prolonged anticoagulant therapy with anticoagulant APC.

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Full Reference Lists can be found online

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CHAPTER

20 Plasminogen Activation and Regulation of Fibrinolysis

NICOLA J. MUTCH AND NUALA A. BOOTH

Hemostasis requires mechanisms both to stop bleeding by formation of a hemostatic plug and to dissolve this plug following wound repair, thereby allowing normal blood flow through the vessel. The latter function relies on local activation of plasminogen (PLG) to plasmin, which dissolves fibrin. The system is finely tuned, to allow healing of a vascular lesion without compromising the early stability of the hemostatic plug and to limit fibrinolytic activity to the injured area. There is a dynamic equilibrium between coagulant and fibrinolytic activities and, within these systems, a further balance between proteolytic and inhibitory proteins. Excessive local or systemic fibrinolytic activity can result in bleeding, often occurring after a delay, as the weakened plug is dissolved. Conversely, an inadequate fibrinolytic response may retard lysis of a thrombus and contribute to its extension.

Plasmin is the central enzyme responsible for thrombus degradation. It is a trypsin-like serine protease generated by activation of the zymogen, PLG. The central players governing the system are plasminogen activators (PAs) and inhibitors of both the activation stage and plasmin activity. Additional players include fibrin and cell surfaces and further proteins that interact with the PLG activation system. This chapter first introduces the key components and then considers how they interact to regulate the activation of PLG and the degradation of fibrin.

PROTEASES AND SUBSTRATES

Fibrinolytic proteases include plasmin, the enzyme that degrades fibrin, and two major PAs that occur in the circulating blood, tissue-type plasminogen activator (tPA) and urinary-type plasminogen activator (uPA), also called urokinase (UK). Like the serine proteases of blood coagulation, they generally exist as a zymogen, a single-chain form of the molecule that is activated by cleavage of a single peptide bond. All the active serine proteases cleave arginine and/or lysine bonds and originated from a simple trypsin-like ancestor protease.[1,2] In the case of the fibrinolytic proteases, the products are all in a two-chain form held together by one or more disulfide bonds. An exception to this rule is tPA, which is an active protease in its single-chain form; tPA can be considered to be at one end of the scale of zymogenicity, with PLG as a totally inactive example at the other.[3]

The typical structure of these proteases (FIGURE 20.1) is that they have an *N*-terminal A chain containing several independently folded modules or domains, which have particular binding properties, and a *C*-terminal B chain, which contains the protease domain. The kringle domain—named after a Danish pastry—is approximately 80 residues long and is held together by three internal disulfide bonds. The finger domains are homologous to type I and type II fibronectin regions. Type I finger domains are approximately 50 residues long, contain two internal disulfide bonds, and may play a role in fibrin binding. The epidermal growth factor (EGF) domains, which are homologous with a module in the EGF precursor, contain 53 residues and three internal disulfide bonds. In several proteins, these domains bind to specific cell-surface receptors or binding proteins. The protease domain (about 250 residues, including four disulfide bonds) contains the catalytic triad, Ser195, His57, and Asp102 (chymotrypsin numbering). The protease domain is well conserved, with particularly high-sequence identity in the vicinity of the three active site residues and at the *N*-terminus.

The individual components of the fibrinolytic system are now discussed in more detail, beginning with the central enzyme, plasmin, and its action on fibrin.

PLASMIN DEGRADATION OF FIBRIN(OGEN)

Fibrin is the substrate most relevant to this chapter but many studies of cleavage sites used fibrinogen, a soluble protein that is more amenable to study (reviewed by[4]). Plasmin cuts primarily at lysyl and arginyl bonds. Plasmin initially cleaves the α chain of fibrinogen to release the αC fragments, thereby producing fragment X (~260 kDa; FIGURE 20.2). Further cleavage of fragment X in the α, β, and γ chains along one-coiled coil, connecting the central E and terminal D domains of fragment X, splits the molecule asymmetrically generating fragment Y (~160 kDa) and fragment D (~100 kDa).[5] Plasmin then cleaves fragment Y into two additional products, a second fragment D and fragment E (~60 kDa), which contains the amino-terminal portion of all six polypeptide chains. Fragment X can be subsequently clotted by thrombin while fragments Y and D have antipolymerizing effects.

Plasmin digestion of non–cross-linked fibrin is identical to that of fibrinogen, supporting the hypothesis that fibrinogen does not undergo major structural reorganization during polymerization.[6] Cross-linked fibrin is degraded more slowly by plasmin and produces different fibrin degradation products (FDPs). Initially a fragment, termed D-dimer, consisting of two fragment D moieties from adjacent fibrin monomers covalently bound between their γ-chain remnants is generated (FIGURE 20.2). Fragment E exists free and in a noncovalent complex with D-dimer (DD/E complex). A variety of large degradation products are released from cross-linked fibrin. These complexes represent a noncovalently bound two-stranded, half-staggered fibrin protofibril. The DD/E fragment is the smallest complex, with larger forms representing longitudinal strings of DD/E held together by uncleaved coiled coils.[5] These larger FDPs are able to associate, in a size-dependent manner, both with themselves and with the clot.[6]

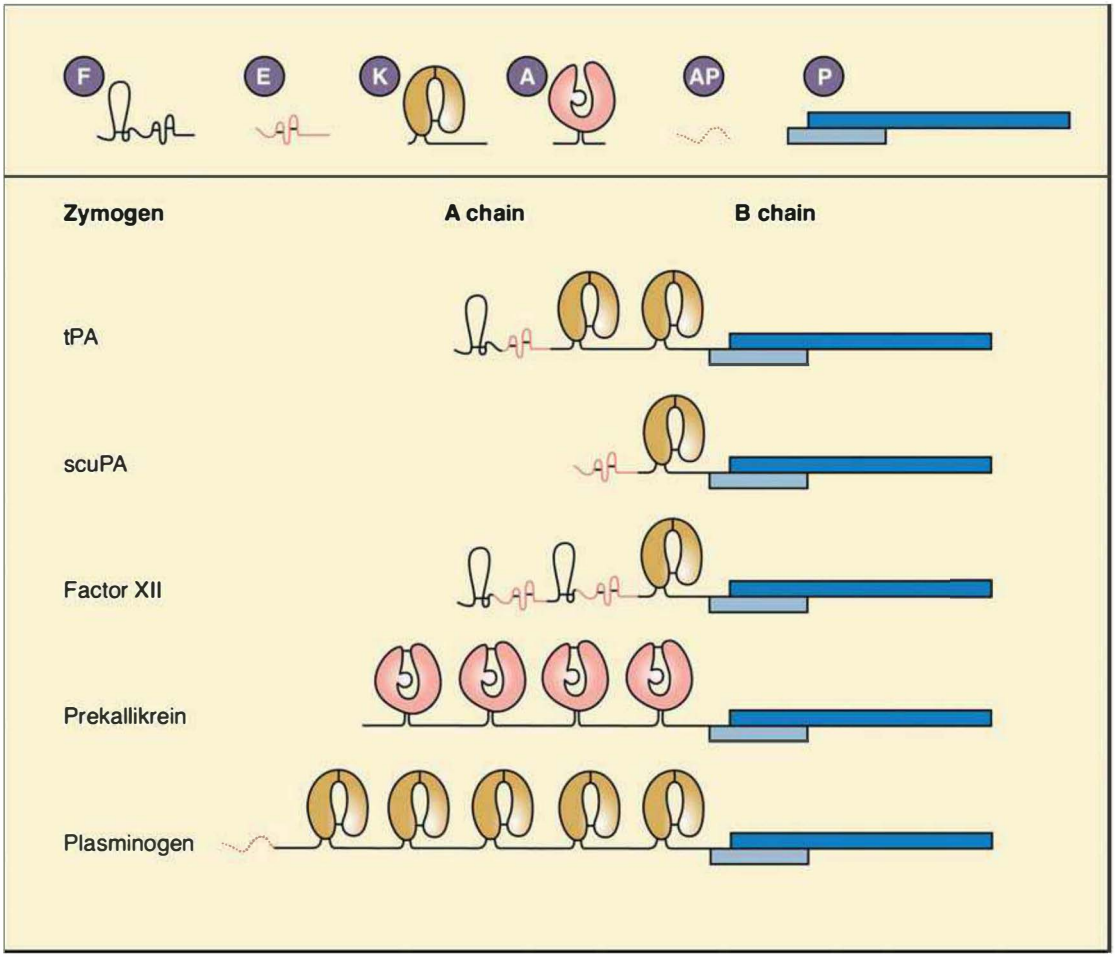

FIGURE 20.1 Structural elements of several fibrinolytic serine proteases. *A*, apple domain (~80 residues); *AP*, activation peptide; *E*, epidermal growth factor domain (~32 residues); *F*, fibronectin finger domain (~40 residues); *K*, kringle (~90 residues); *P*, protease domain (B chain; ~250 residues); scuPA, single-chain urokinase; tPA, tissue-type plasminogen activator.

PLASMIN(OGEN)

Human PLG is the zymogen form of plasmin (EC 3.4.21.7). PLG is a single-chain glycoprotein of 92 kDa, consisting of 791 amino acid residues and approximately 2% carbohydrate (Table 20.1). In the human adult, the plasma concentration of PLG is approximately 200 mg/L, or 2 μM (Table 20.1) and is generally rather stable, but there is an increase in the acute-phase response.[7] The circulating half-life ($t_{½}$) of native PLG, which has a glutamic acid residue at its *N*-terminus (Glu–PLG), is approximately 2.2 days; the partially cleaved Lys–PLG (see subsequent section, "Activation of Plasminogen") has a much shorter $t_{½}$ of 0.8 days.[8] The principal production site for PLG is the liver, but PLG is present in the extravascular space of most tissues. Some tissues, such as eosinophils, kidney, cornea, brain, and adrenal medulla, may be capable of synthesizing it.[9–12]

Expression and Structure

The PLG gene is 52.5 kb and is located on chromosome 6q26–q27,[13] close to two genes for apolipoprotein (a) and for the PLG-related genes A and B.[14,15] Two sequence elements of CTGGGA common to acute-phase reactant genes are at position 76 to 81 and −553 to −558.[16] Three interleukin-6–responsive elements are at positions −830, −518, and +117 in the 5′-flanking region.[17] Two main regions conferring liver specificity of expression to the PLG gene were found in the *cis*-acting element, AAAAATA, at −2,194 to −2,188 and +48 to +61.[18] Several other putative regulatory transcription elements are present in the 5′-flanking region (reviewed in Ref. 17). On the 3′-flanking region, the location of the consensus polyadenylation sequence AATAAA and of potential CAYTG sequence indicates the presence of three polyadenylation sites.[16]

PLG consists of an *N*-terminal activation peptide, five kringles, and the protease domain with the catalytic triad His603, Asp646, and Ser741[16] (FIGURE 20.3). The kringles are well conserved between species[19] and consist of some 80 residues, the structure being shaped by a characteristic 1–6, 2–4, 3–5 disulfide bond pattern using the conserved Cys residues in positions 1, 22, 51, 63, 75, and 80 (kringle 5 numbering). K2 and K3 are covalently linked by a further disulfide bond.[20] Functionally, the kringles give PLG the ability to bind to exposed lysyl residues in fibrin,[21,22] $\alpha_2$antiplasmin (α_2AP),[23] tetranectin,[24] histidine-rich glycoprotein (HRG),[25] collagen,[26] high molecular weight (HMW) and low molecular weight (LMW) kininogen,[27] thrombospondin,[28] and cell-surface receptors or binding proteins for PLG (discussed

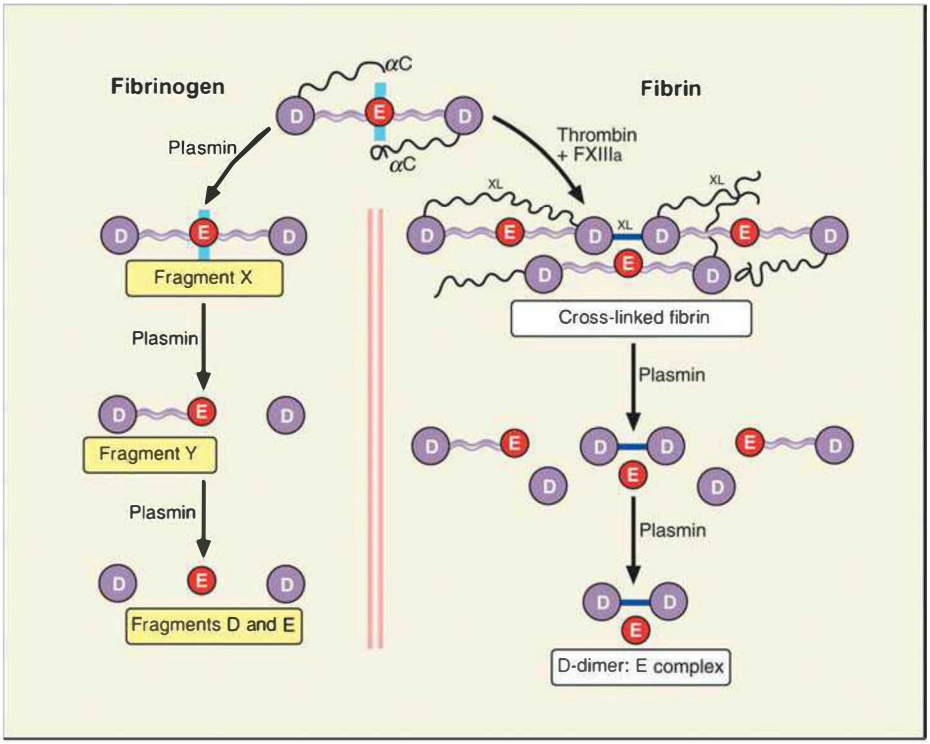

FIGURE 20.2 Degradation of fibrin(ogen) by plasmin. The principal structure of fibrinogen **(top)** is a three domain globular protein with extending αC domains. Through the action of plasmin the degradation fragments D, E, α-helical coiled coils that connect them, and the Aα chains that extend from the carboxy-terminus of the D-domains. Fibrinogen is degraded asymmetrically **(left panel)**. Intermediate degradation product, fragment X, consists of all three domains connected by coiled coils, but lacks the Aα chains and the Bβ1-42 sequence. Fragment Y is composed of the central E-domain connected by a coiled coil to the D-domain. Cross-linked fibrin **(right panel)**, occurs through the enzymatic actions of thrombin and activated factor XIII (FXIIIa). Thrombin cleaves fibrinopeptides A and B from the E-domain, and FXIIIa cross-links (XL) fibrin longitudinally between D-domains and within the α-chain extensions (see Chapter 16). Plasmic cleavage of the two-stranded protofibrils initially removes the cross-linked α chains, then the coiled coils to liberate a series of FDPs, the smallest of which is DD/E. Larger complexes, such as DY/YD, are also liberated from cross-linked fibrin, but these are subsequently degraded to the DD/E moiety.

in more detail below). The affinity of the kringles for lysine is exploited in affinity chromatography.[29] The kringles also bind to ω-aminocarboxylic acids, such as 6-aminohexanoic acid (εACA, 6-AHA), *p*-aminomethyl benzoic acid (*p*-AMBA), and *trans*-4-amino-methyl-cyclohexane-carboxylic acid (*trans*-AMCA, also known as tranexamic acid). These are used experimentally and clinically to inhibit the functional activity of plasmin by competing with the binding of plasmin(ogen) to fibrin and cell surfaces.

Human PLG kringles have different affinities and specificities for ω-amino acids, such as εACA. Kringle 1 exhibits the tightest binding (K_d 9 μM),[30,31] followed by kringle 4[30,32,33] and kringle 5,[32,33] with kringle 2 interacting only weakly and kringle 3 not at all.[34] Kringle structures have been solved both in solution and in crystals and show the general feature of a hydrophobic groove with acidic and basic residues that are ideally spaced for docking of zwitterions such as lysine or εACA, the opposite charges of which are approximately 7 Å apart. The lack of binding by kringle 3 is explained by the positive charge at position 57, which in the other kringles is negatively charged.[35]

Experimentally, kringle 4 can be isolated from PLG by digestion with elastase, typically yielding three major components: a fragment containing kringles 1 to 3, kringle 4, and kringle 5 attached to the protease domain.[20] The latter fragment, also called *mini-PLG*, can be activated to plasmin by PAs,[36–38] as can microplasmin, which has only the last 31 amino acid residues of the A chain and a complete B chain.[38] Kringle 1 can be obtained from kringles 1 to 3 by further digestion with other enzymes, such as *Staphylococcus aureus* V8 protease.[39] Other fragments of PLG have effects on tumor growth and metastasis; these fragments are known as angiostatins. Their activity depends crucially on their disulfide pattern.[40]

Activation of Plasminogen

All known PAs cleave the Arg561–Val562 bond in PLG (FIGURE 20.4), generating a two-chain plasmin molecule in which the A and B chains remain attached by two disulfide bonds. PLG exists in different conformations, affected by its *N*-terminus and also by the presence of lysine analogues. A particularly important consideration is whether PLG is free in solution or bound to a surface, either fibrin or cells, as discussed more fully in the section on "Balance of Plasminogen

Table 20.1

Properties of proteins of the fibrinolytic system

Factor	M_r (kDa)	Residues	Concentration (mg/L)	Molar Concentration	$t_{½}$	Function
PLG	92	791	200	2 μM	2.2 d	Zymogen
tPA	68	530	0.005	70 pM	4 min	Protease
uPA	54	411	0.002	40 pM	7 min	Zymogen
PAI-1	52	379	0.02	400 pM	8 min	Inhibitor
PAI-2	46/70	393	<0.005	<70 pM	—	Inhibitor
Factor XII	80	596	30	0.375 μM	2–3 d	Zymogen
PK	88	619	40	0.45 μM	7–10 d	Zymogen
HK	110	626	70	0.60 μM	9 h	Cofactor
Vitronectin	78	459	350	4.5 μM	—	Cofactor
C1-inhibitor	105	478	180	1.7 μM	70 h	Inhibitor
α_2-Antiplasmin	70	452	70	1 μM	3 d	Inhibitor
α_2-Macroglobulin	725	1,451	2,500	3 μM	—	Inhibitor
HRG	75	507	100	1.5 μM	3 d	Inhibitor
Tetranectin, monomer[a,b]	22.5	181	10	0.5 mM	3 d	Cofactor
Apolipoprotein(a)[c]	300–800	—	<7	—	—	Modulator
TAFI (pro-CpU)	60	401	5	75 nM	10 min[d]	Zymogen, Inhibitor
uPAR	55	313	—	—	—	Receptor
Annexin II[a,e]	38	338	—	—	—	Receptor
LRP (α_2-MR)	600	4,525	—	—	—	Receptor, inhibitor
α-Enolase	54	433	—	—	—	Receptor
Mannose receptor	175	1,437	—	—	—	Receptor

[a] M_r of nonglycosylated form as listed in Swiss protein base ExPASy.
[b] Heavy chain of homotrimer.
[c] Many isoforms due to 13 up to 37 type 4 kringle motifs.
[d] Activated form.
[e] Heavy chain of heterotetramer (formed by two heavy and two 10-kDa light chains).
tPA, tissue-type plasminogen activator; uPA, urinary-type plasminogen activator; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; HMW, high molecular weight; C1-inhibitor, complement component 1 esterase inhibitor; TAFI, thrombin-activatable fibrinolysis inhibitor; uPAR, urinary-type plasminogen activator receptor; LRP, low-density lipoprotein receptor–related protein; MR, macroglobulin receptor.

Activation and Regulation of Fibrinolysis" At this stage of our discussion, it can be assumed that the binding is primarily dependent on exposed lysine residues, and that the binding to fibrin and to cell receptors for PLG are generally equivalent.

Cleavage of Glu–PLG generates trace amounts of plasmin cleaved at the *N*-terminus at one or several of the following bonds: Arg68–Met69, Lys77–Lys78, or Lys78–Val79.[41,42] The final product obtained is termed Lys–plasmin (FIGURE 20.4). These two conformations differ in several features, including efficiency of activation and binding to fibrin. Small-angle neutron scattering revealed that Glu–PLG has a more closed form than Lys–PLG,[43] making it less readily activated. In the closed form, the five kringle domains and the catalytic domain interact to produce an ellipsoid shape. The open form of Lys–PLG, in contrast, has increased flexibility, which facilitates the binding of PAs some 10-fold.[44–46] Binding of εACA to Glu–PLG elicits a similar, but probably not identical change in conformation.[43] Lys 50 of Glu–PLG binds to kringle lysine binding site (LBS),[47,48] making them less available for binding to *C*-terminal lysine in fibrin, PLG receptors, and other proteins, so that Lys–PLG binds with higher affinity to fibrin than does Glu–PLG. The susceptibility of Glu–PLG to activation is dependent on additional factors, such as the presence of anions, which in the human plasma are mainly chloride ions, and of the divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+}. These ions counteract the effect of εACA and stabilize PLG in the Glu-form.[46,49]

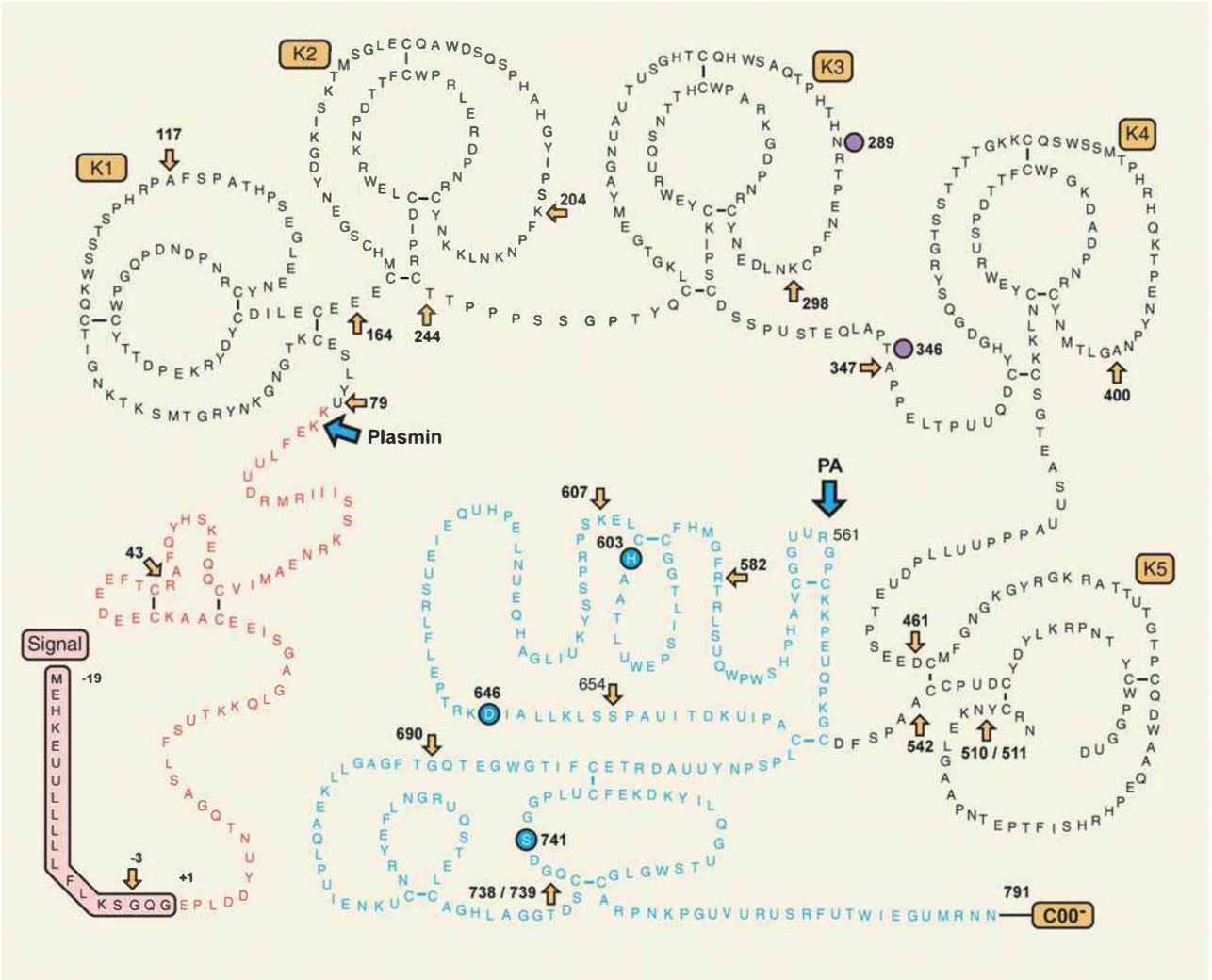

FIGURE 20.3 Sequence of the human PLG molecule. Positions of the 18 introns are indicated by *yellow arrows* at (for type 1 and 2 introns) and between (for type 0 introns) residues. The 19-residue signal peptide (shown in the *pink box* with negative numbers) is cleaved by the signal peptidase at the Gly–Glu peptide bond. Conversion of PLG to plasmin by PAs occurs after the peptide bond Arg561–Val562 (shown by a *blue arrow*). Plasmin cleaves primarily at Lys77–Lys78 bond (shown by a *blue arrow*) to release the activation peptide (*red lettering*) generating Lys–plasmin(ogen). K1–K5 kringles are located in the A chain (*black lettering*). The B chain remains attached to the A chain (*blue lettering*) after cleavage at 561–562 by PAs via the disulphide bonds Cys548–Cys666 and Cys558–Cys566. Carbohydrate attachment sites (Asn289 and Thr346) are shown as *purple circles*. The residues that comprise the classical serine protease Ser–His–Asp catalytic triad are *circled in blue*. (Redrawn based on Petersen TE, Martzen MR, Ichinose A, et al. Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system. *J Biol Chem* 1990;265:6104.)

Variants of Plasminogen

The PLG molecule is subject to considerable variation in the normal population. In addition to the limited proteolysis and activation processes already discussed, the two important sources of variation are glycosylation and genetic polymorphism.

Glycosylation

Human Glu–PLG occurs in two variants that differ in glycosylation. PLG variant 1 is diglycosylated, with *N*-linked carbohydrate moiety of the mannose type with 10 to 11 monosaccharide units on Asn289, and an *O*-linked moiety of three to four residues on Thr346 (FIGURE 20.3).[50] PLG 2, which accounts for a little more than half the circulating PLG, contains only the carbohydrate group at Thr346.[51] Both of these forms exhibit additional heterogeneity with respect to their sialic acid content, generating six additional isoforms with pIs between 6.1 and 6.6.[41,42,52,53] All 12 molecular forms are present in plasma from single donors.[8]

Activation of PLG 1 in solution is faster than PLG 2, but this difference is not evident on fibrin. PLG 1 switches more readily to the open conformation in the presence of fibrin or tranexamic acid than PLG 2.[54] PLG 2 is more rapidly cleared than PLG 1 and bound five times faster to a de-endothelialized

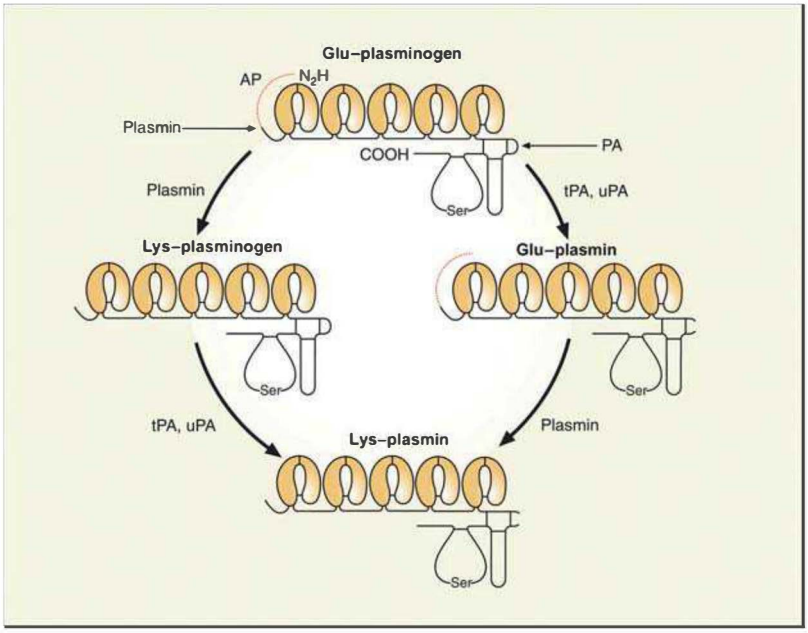

FIGURE 20.4 Activation of native Glu–PLG. PA cleaves at Arg561–Val562, separating the B (light, protease, or catalytic) and the A (heavy, kringle) chains. Glu–PLG and Glu–plasmin forms both contain the amino-terminal activation peptide from Gln1 to Lys76 (shown in *red*). Plasmin has the potential to cleave this activation peptide (*left side*), producing Lys–PLG, an intermediate form that interacts with fibrin more efficiently and is readily cleaved by the PAs (tPA and uPA). Plasmin can also cleave the activation peptide from Glu–plasmin, generating Lys–plasmin (*right side*). The five kringle structures of the A chain are involved in binding of PLG to both fibrin and cell receptors. The catalytic center contains the typical Ser–His–Asp residues and is the major site of interaction with $\alpha_2$antiplasmin.

vessel surface.[55–57] Additional glycosylation sites at Ser249[58] and Ser339[59] have been described; a further serine residue, Ser578, can be phosphorylated.[60]

Polymorphism and Deficiency

Isoelectric focusing of neuraminidase-treated plasma (which removes sialic acid) revealed several bands of activatable PLG, representing the genetic polymorphism of PLG alleles. The two most common variants found in all races are called *PLG A* (A for acidic pI) and *PLG B* (B for basic pI)[61] with Asp and Asn at position 453, respectively.[62] Variants with intermediate pI are designated as PLG M. Variants with more acidic pI than PLG A are assigned a numerical suffix that increases with decreasing pI. Similarly, variants with more basic pI than PLG B receive a numerical suffix that increases with increasing pI. Alleles are designated with an asterisk (e.g., PLG*A3), and silent (null) PLG alleles are designated PLG*Q0 (Q0 standing for quantitatively zero or nonexpressed). The frequency of the allele PLG*A in the Japanese population is considerably higher (95%) than in the Western population (70.1%).[63]

Recurrent thrombosis is associated with a deficiency of PLG antigen and activity (type I), or a normal level of antigen but reduced activity (type II, dysplasminogenemia). In most instances, a thromboembolic event or ligneous conjunctivitis prompt family studies. Cases in which the molecular defect has been identified are listed in Table 20.2[62–76]; updates can be found on the OMIM (Online Mendelian Inheritance in Man) Web site. The data do not prove conclusively that the heterozygous presence of one mutant PLG gene results in an increased incidence of thromboembolism.[77,78] Homozygous deficiencies, however, are clearly associated with ligneous conjunctivitis, caused primarily by Arg216 to His or Trp597 to stop mutations,[67] and replacement therapy with PLG is effective.[69] It is not clear why some mutations cause ligneous conjunctivitis and why very low functional PLG can be tolerated in some individuals, without thrombosis occurring.[67]

PLG-deficient mice also develop ligneous conjunctivitis.[79] These mice are predisposed to severe thrombosis and deposit intravascular and extravascular fibrin in many tissues.[80,81] PLG-deficient mice die prematurely and exhibit retarded growth and frequent rectal prolapse, but ovulation, embryonic development, and reproduction are normal.[82] Wound healing and tissue remodeling are impaired,[83,84] and response to an inflammatory stimulus is diminished.[85] After electric injury to the femoral artery, wound healing, removal of necrotic debris, leukocyte infiltration, smooth muscle cell immigration, and arterial neointima formation were greatly delayed in $Plg^{-/-}$ mice,[86] suggesting that PLG deficiency might reduce development of atherosclerosis. However, $Plg^{-/-}$ mice crossed with apolipoprotein E–deficient mice develop early atherosclerotic lesions.[87] $Plg^{-/-}$ mice crossed with $fibrinogen^{-/-}$ mice were restored to normality in many of these cases, demonstrating the causative role of intravascular and extravascular fibrin deposition.[88,89]

PLASMINOGEN ACTIVATORS

There are two physiologic PAs, tPA (tissue-type) and uPA (urinary-type, UK), named after the original sources of purified proteins. A further activation system, which is dependent on factor

Table 20.2

Congenital PLG mutations

Residue	Mutation	Type	Other Names	Clinical Manifestations	References
9	Thr to Asn	I	—	—	69
19	Lys to Glu	I	—	Ligneous conjunctivitis	69
128	Lys to Pro	I	—	Ligneous conjunctivitis	64,69
133	Cys to stop	I	—	—	67
134	Arg to Lys	I	—	—	64
212	Lys deletion	I	—	Ligneous conjunctivitis	69
216	Arg to His	I	—	Ligneous conjunctivitis	68
355	Val to Phe	II	Nagoya	Thrombophilia	70
374	Val to Phe	—	Nagoya-I	Thrombophilia	70
453	Asp to Asn	—	—	—	64
460	Glu to stop	I	—	—	71
513	Arg to His	I	—	Ligneous conjunctivitis	69
572	Ser to Pro	I	—	Thrombophilia (secretion defect)	71,72
597	Trp to Cys	I	—	Ligneous conjunctivitis	68,69
597	Trp to stop	I	—	Ligneous conjunctivitis	67,68
601	Ala to Thr	II	Tochigi, Osaska II	Thrombophilia	70,75
620	Ala to Thr	I	Nagoya-II, Tochigi, Kagoshima	Thrombophilia	70
675	Ala to Thr	I		Thrombophilia?	72,74
676	Asp to Asn	II	Osaka-II, III	None	75,76
693	Gly to Arg	II	—	—	64
732	Gly to Arg	II	Kanagawa-I	—	77
8,771	Ala to Gly	I	—	Ligneous conjunctivitis	78

XII (FXII), prekallikrein (PK), and high molecular weight kininogen (HK), is known as the contact PA pathway. There are also PAs from bacteria and the vampire bat, which are briefly included here. The two human proteases, tPA and uPA, are also implicated respectively in neurobiology (for review see Ref. 90) and tumor biology (for review see Refs. 91 and 92). These aspects are not considered further in this chapter, which focuses on PAs in hemostasis.

TISSUE-TYPE PLASMINOGEN ACTIVATOR

tPA, a serine protease of 68 kDa (EC 3.4.21.68), previously known as vascular PA or extrinsic activator, is a glycoprotein of 527 residues (FIGURE 20.1). It exerts its effect primarily in the vascular system, because it is produced and secreted by endothelial cells[93]; many other cells in culture also synthesize tPA. In normal plasma, the antigen concentration of tPA is approximately 5 μg/L, which corresponds to approximately 70 pM (Table 20.1),[94] but most is in complex with its primary inhibitor, plasminogen activator inhibitor 1 (PAI-1).[95,96]

Expression and Structure

The gene for tPA is located on bands p12–p11 on chromosome 8[97]; it contains four exons[98] and comprises 32.7 kb. The mature protein exists in two forms, the *N*-terminus being Gly or Ser in Bowes melanoma cell tPA,[99] but predominantly Ser in recombinant tPA,[100] and the numbering in this chapter is based on Ser at position one. tPA expression in cultured cells can be stimulated through multiple intracellular signaling pathways. Vasoactive substances, such as thrombin and histamine, increase tPA synthesis in human umbilical vein endothelial cell (HUVEC), acting through their G-coupled receptors and the protein kinase C pathway.[101–103] Steroid hormones[104,105] and retinoids can increase synthesis of tPA.[106,107] The expression of tPA has been reviewed[108] and will not be covered in detail here. It is important to note that not all endothelial cells synthesize tPA *in vivo* and that expression is restricted to small vessels.[109,110]

The structure of tPA (FIGURE 20.1) shows a finger and EGF domain, and two kringle domains in the A chain, whereas the protease domain resides in the B chain. The finger domain extends from residues 6 to 43. It contains a binding site for fibrin, as mutants lacking kringle domains are still capable of binding fibrin.[111] Consistent with this, a degraded form of tPA that had lost the *N*-terminal 12 kDa bound less well to fibrin than wild-type tPA.[112] The binding of the finger domain is independent of LBS, in that it cannot be blocked by εACA.[113] Structurally, the finger domain is very similar to the seventh type 1 repeat of human fibronectin.[114]

The EGF domain (residues 44 to 92) is structurally similar to other EGF structures, as shown by nuclear magnetic resonance (NMR).[115] There is some evidence from deletion mutagenesis that the EGF domain binds to the mannose receptor and is involved in tPA clearance.[116] Kringle 2 has affinity for lysine, ω-amino acids, and fibrin, whereas no function has yet been ascribed to kringle 1.[111] The affinity for the binding of εACA (a model for *C*-terminal lysine residues) and of *N*-acetyllysine methyl ester (a model for intrachain lysine residues) is approximately equal, suggesting that unlike PLG, tPA does not have a preference for *C*-terminal lysine residues binding.[113] Intact tPA and a variant consisting only of kringle 2 and the protease domains were found to bind to the cyanogen bromide (CNBr) fibrinogen fragment FCB-2, which also binds PLG and acts as a stimulator of tPA-catalyzed PLG activation.[111] In both cases, binding was completely inhibited by εACA, pointing to the involvement of LBS in this interaction.[111]

The structure–function relations among kringle 2 and ω-amino acids and lysine have been studied in detail, using NMR,[117] microcalorimetry,[118] crystallography,[119] and site-directed mutagenesis.[118,120,121] The crystal structure of kringle 2 resembles that of PLG kringle 4; there are, however, differences in the lysine-binding pocket. The core of kringle 2 is formed by a hydrophobic cluster of three tryptophan residues in positions 25, 63, and 74, surrounded by aromatic and hydrophobic side chains that form, at the surface of the kringle, a hydrophobic grove. Ligand binding appears to rely mostly on the integrity of Trp63 and Trp74, and an aromatic residue at position 76, which is normally Tyr.[122] Mutation of the critical amino acids Lys33, Asp55, Asp57, or Trp72 markedly diminished binding to lysine–Sepharose, εACA, or both.[123]

The protease domain of tPA has the typical catalytic triad His322, Asp371, and Ser478. The 2.3-Å crystal structure of the protease domain revealed strong structural similarity with other trypsin-like serine proteases, thrombin in particular.[124] The active site cleft is shaped and narrowed by four surface loops. The loop around Arg299 exhibits five additional residues, Arg298–Arg–Ser–Pro–Gly302, compared with chymotrypsin. It projects out of the molecular surface as a β-hairpin and is of fundamental importance for the interaction with PAI-1.[3] The 60-loop around Arg327 is similar to but shorter than the corresponding loop in thrombin. Further loops are found around Ser381 and Gly465. The fully solvent-exposed hydrophobic region, comprising amino acids 420 to 423 of tPA, which forms a surface loop near one edge of the active site of tPA, is an important secondary site for the interaction of tPA with PLG in the absence of fibrin.

tPA is secreted as a single-chain molecule (sctPA) that is converted to the two-chain form (tctPA) by plasmin by cleavage at Arg275–Ile276. Unlike most serine proteases the single-chain form is not a zymogen but a protease that, in the presence of fibrin, is nearly as active as the two-chain form.[125] Mutagenesis studies have revealed that tPA lacks a specific zymogen triad Asp194, His40, and Ser32 (chymotrypsin numbering).[3,126] The constructed double mutant Phe305His and Ala292Ser was more zymogenic.[126]

Release

The major source of tPA in the circulation under basal conditions is thought to be the endothelial cell, from which it is constitutively released.[127] A secondary pool of tPA is present in specialized storage vesicles and is released in response to specific extracellular stimuli.[128–131] High concentrations of tPA are contained within these storage vesicles that occur in various cells, such as endothelial, neuroendocrine, and adrenal chromaffin cells.[129,131] In rats, the tissue stores of tPA were calculated to be sufficient to maintain a steady-state plasma level of tPA for 2 days in the absence of protein synthesis.[132] Compounds that triggered release within a few minutes include bradykinin, histamine, eledoisin, acetylcholine, β-adrenergic agents, platelet-activating factor, endothelin, calcium ionophore A-23187, and acidosis.[133,134] These compounds induce calcium influx into the endothelial cell and activate G-protein–coupled receptors.[135] Some interventions that cause elevated tPA act through decreased clearance, including exercise and α-adrenergic agents.[136] In all situations in which epinephrine levels are increased, such as stress, anxiety, and exercise, tPA antigen levels increase.[96,137]

Acute release of tPA is established from early studies on venous occlusion.[138–140] Young patients with a history of recurrent venous thromboembolism exhibited an abnormal response to venous occlusion and this could be attributed either to subnormal tPA release or, more often, to elevated PAI-1.[95,139,140] In isolated cases with severe forms of von Willebrand disease, a complete lack of response to venous stasis and the infusion of 1-deamino-8-D-arginine vasopressin (DDAVP) was found.[141] These patients may have a functional or structural defect in their endothelial cells. It should be noted, however, that release of von Willebrand factor (vWF) and tPA occur by different mechanisms.[142] Lower PA activity is found in leg veins than in proximal veins and may be one etiologic factor for development of deep venous thrombosis. DDAVP was widely used in the 1980s to study the release potential for tPA in patients with idiopathic or recurrent thrombosis.[143–145] Comparison of the effects of DDAVP and sodium nitroprusside showed that nitroprusside produced a greater increase of forearm blood flow but no increase of tPA; however DDAVP stimulated tPA release from the vascular bed.[146] The increase of circulating tPA levels after intra-arterial infusion of acetylcholine and methacholine was shown to be mediated by muscarinic receptors.[146–148] Bradykinin and substance P both induced tPA release after being infused into the forearm of volunteers,[149,150] and the releasable pool of tPA, rather than the availability of PAI-1 to inhibit it, was the key factor.[151]

Enzymatic Properties of Tissue-Type Plasminogen Activator

tPA is unusually specific among the serine proteases. Its major substrate is PLG, in which it cleaves the Arg561–Val562 bond but is a relatively inefficient activator of PLG in the absence of its cofactor, fibrin.[152] Binding of sctPA and tPA to fibrin is roughly comparable.[153] The K_d for binding of tPA to fibrin clots in the absence of PLG ranges from 140 to 400 nM.[125,153,154] In the presence of PLG, the affinity of tPA to fibrin increases approximately 20-fold (K_d 20 nM).[155] These observations are explained by formation of a ternary complex between tPA, PLG, and fibrin or binding of tPA to PLG, which subsequently, upon binding to fibrin, takes on an open conformation. The isolated A chain of tPA has been shown to bind to Glu- and mini-PLG with a K_d of 100 nM.[156]

There is a wide range of kinetic parameters reported for the activation of PLG by tPA due to many potential experimental variables, such as different protein preparations, substrate

concentrations, and nature of the fibrin stimulator. In the absence of fibrin, K_M values for the activation of Glu–PLG by tPA range from 9 μM to slightly more than 100 μM.[125,157,158] In general, K_M is three to four times lower with tPA than with sctPA for the activation of Glu–PLG in the absence of fibrin. In the presence of fibrin, this difference is less apparent with K_M values typically two orders of magnitude smaller, with only moderate increases of k_{cat}. Values for K_M in the presence of fibrin range from 0.16 to 1.1 μM PLG, and k_{cat} values from 0.1 to 1.1 per second.[125,158] Several authors found nonlinear enzyme kinetics,[156,157,159] and there appear to be two phases in the activation of Glu–PLG by tPA in the presence of fibrin, with an initial K_M of 1.05 μM and k_{cat} of 0.15 per second. Later in the process, as new high-affinity binding sites for PLG and tPA are exposed in partially digested fibrin,[160–164] K_M decreases to 0.07 μM, whereas the k_{cat} is unchanged.[159] The key message is that PLG is not readily activated by tPA except in the presence of fibrin, because it is only in this situation that the K_M for the reaction is consistent with the circulating PLG concentration of 2 μM.

Distinct sites in the fibrin molecule have been identified as accelerating PLG activation; notably, they are not available for binding in fibrinogen but become exposed in fibrin.[165] The D-region of fibrin binds both PLG and tPA in a region that encompasses the residues Aα148–160. Both these associations are of low affinity and in the circulation the Aα148–160 site would bind only PLG, which is present in much higher concentrations than tPA. Another binding in the D-region encompasses γ311–336 and γ337–379, which are linked by a disulfide bond.[166] Antibodies have revealed that there is a tPA binding site that includes γ312–324.[167] Higher affinity sites for binding tPA and PLG are in the *C*-terminus of the α chain, within the region Aα392–610.[168] The conformational changes that occur on fibrinogen cleavage and fibrin assembly[169] reveal new sites for binding of tPA and PLG, and for enhancement of PLG activation.

Mutations have been engineered in tPA to change its characteristics, with the aim of making it an even more effective therapeutic agent. The most striking of these is the series of mutations to make tPA resistant to PAI-1. Madison et al.[170] identified the importance of interactions between the positively charged tPA sequence 298 to 302 and the negatively charged PAI-1 sequence 350 to 355. Mutagenesis of Arg298, 299, and 304 resulted in a mutant that was inhibited 120,000 times less rapidly by PAI-1 than wild-type tPA.[171] These observations led, in part, to the development of the new tPA mutant tenecteplase (TNK–tPA), in which the sequence 296 to 299 (Lys–His–Arg–Arg) is replaced by four alanines.[172] This variant also exhibits slower clearance, by virtue of its changed glycosylation, and its enhancement of PLG activation is more selective for fibrin over fibrinogen and the fibrin degradation product DD/E.[173]

URINARY-TYPE PLASMINOGEN ACTIVATOR

The urinary-type PA (EC 3.4.2.73; urokinase, uPA) is found in urine at 40 to 80 μg/L,[174] and is synthesized by several cell types, particularly cells with a fibroblast-like morphology, but also by epithelial cells[175] and monocytes and macrophages.[176,177] uPA activates PLG by the cleavage of Arg561–Val562, as does tPA, but importantly does not require fibrin as a cofactor. This characteristic has generated the view that tPA functions in fibrinolysis in the vasculature, whereas uPA's primary role is in processes such as degradation of extracellular matrix and cell migration, with consequences for wound healing, inflammation, embryogenesis, and invasion of tumor cells and metastasis.[178,179] These clear roles of the uPA system outside of fibrinolysis do not argue against its importance in fibrin degradation, as discussed in subsequent text.

Expression and Structure

The human uPA gene is located on chromosome 10q24 and is 6.4 kb in length.[180] It contains 11 exons, and the intron–exon organization of the uPA gene closely resembles the tPA gene. The regulation of its expression has been reviewed in detail elsewhere[108] and is not included here. uPA is produced as a 54-kDa, single-chain glycoprotein, 411 residues long (Table 20.1) and containing three domains: an EGF domain, a kringle, and a protease domain (FIGURE 20.1). The EGF domain interacts with urokinase plasminogen activator receptor (uPAR) found on many cells, as discussed in subsequent text. The kringle domain has no affinity for fibrin but has been reported to stabilize the interaction of uPA with uPAR.[181] The protease domain contains the catalytic triad, His204, Asp255, and Ser356, and has approximately 40% sequence identity with tPA.[98] uPA is normally glycosylated at Asn302, whereas a covalently attached single monosaccharide, fucose, to Thr18,[182] appears to affect the mitogenicity of uPA.[183] There are two phosphorylation sites on Ser138 and Ser303. The phosphorylated form diminishes uPA's interaction with cells and PAI-1.[184]

Activation of single-chain urinary plasminogen activator (scuPA, also called *proUK*) to the active enzyme, uPA (UK), is by cleavage of Lys158–Ile159.[185] Many enzymes can cleave this bond but the most important to hemostasis are plasmin,[186,187] factor XIIa, and kallikrein.[188] In PLG-deficient mice activation of scuPA is ascribed to kallikrein.[189] Cleavage close to the activation site (Lys158–Ile159) generates an inactive form of uPA. Thrombin cleaves Arg156–Phe157 in scuPA[188,190,191] and the inactive product can be subsequently activated through release of the *N*-terminal dipeptide of its B chain by cathepsin C or, albeit slowly, by plasmin.[185,192] Granulocyte elastase and cathepsin G cleave the Ile159–Ile160 peptide bond to yield an inactive product.[193] uPA has two active forms: HMW-UK and LMW-UK.[194] These are, respectively, full-length active uPA and a processed form, cleaved at Lys135–Lys136 to yield the amino-terminal fragment (ATF), which interacts with uPAR, leaving 21 residues of the A chain and an intact B chain.

Enzymatic Properties of Urinary-Type Plasminogen Activator

The question of whether scuPA is a true zymogen was debated for several years, a controversy that was fuelled by the problem of trace plasmin or uPA contamination, with reciprocal activation of scuPA and PLG.[195] The issue has been resolved by studies on mutant forms of the proteins and by examining scuPA in the absence of PLG. For instance, the Lys158Glu mutant of scuPA, which cannot be cleaved by plasmin into uPA, still converted PLG to plasmin.[196] The consensus is that scuPA has approximately 0.5% of the activity of uPA.[197,198,199,200] Therefore, scuPA lies on the spectrum between PLG, a true zymogen, and tPA, which is almost fully active in its single-chain form. Lys300 and Asp355 are key structural elements of scuPA's enzymatic

activity. Lys300, situated in the flexible loop region 297 to 313, forms a weak interaction with Asp355 and pulls the adjacent active site Ser356 into the position found in the fully active protease. Site-directed mutagenesis of Lys300 to Ala[201] or of Asp355 to Asn[202] resulted in a 40-fold and 270-fold reduction in activity, respectively, compared to wild-type scuPA. Altering the flexibility of the 297–313 loop changes the intrinsic catalytic activity of scuPA.[203]

Urinary-Type Plasminogen Activator Receptor

A cellular receptor for uPA (uPAR; CD87) was first isolated on human monocytes[204] but is expressed on several cell types. The gene for uPAR (gene code PLAUR) is on chromosome 19 and consists of seven exons and six introns extending over 23 kb.[205,206] Regulation of its expression has been reviewed[108] and is not included here. The 1.4-kb mRNA encodes a signal peptide of 22 and a protein of 313 residues, with a glycosylphosphatidyl inositol (GPI) anchor (FIGURE 20.5). The *N*-terminus of the receptor consists of three homologous domains; region 1 interacts with uPA and regions 2 and 3 enhance binding.[207]

scuPA and uPA bind to uPAR receptor via the ATF.[208] The binding is of high affinity (K_d 10^{-9} to 10^{-11}M) depending on the cell type.[209] uPAR does not play a catalytic role but serves to localize scuPA and PLG on cell surfaces,[210] thereby enhancing plasmin generation approximately 20-fold.[211] Binding of uPA to uPAR elicits a number of intracellular signaling responses, acting through the extracellular, regulated kinase/mitogen-activated protein kinase pathway.[212] uPAR is not a transmembrane protein and therefore other components are enlisted to transmit a signal to the intracellular compartment. Several proteins are known to bind uPAR, including vitronectin and integrins in complex with caveolin.[213] On some cells, the complex uPA/uPAR is associated with Endo 180, also known as uPAR-associated protein,[214] and is involved in collagen IV internalization.[215] Receptor-bound uPA is susceptible to inactivation by PAI-1, but inhibition is less efficient than in solution.[211] The uPA/PAI-1 enzyme/inhibitor complexes are internalized, and the uPAR is recycled to the surface. This process requires the cooperation of low-density lipoprotein receptor–related protein (LRP).[216,217] The structure of uPAR has been solved at high resolution, providing insights on the interaction with its ligand and informing drug design.[218–220]

Urinary-Type Plasminogen Activator in the Circulation

scuPA is found in plasma in relatively stable concentrations of 2 to 4 ng/mL.[221,222] In contrast with tPA and PAI-1, there is little circadian fluctuation in plasma uPA.[223] Venous occlusion did not increase uPA antigen once values were corrected for

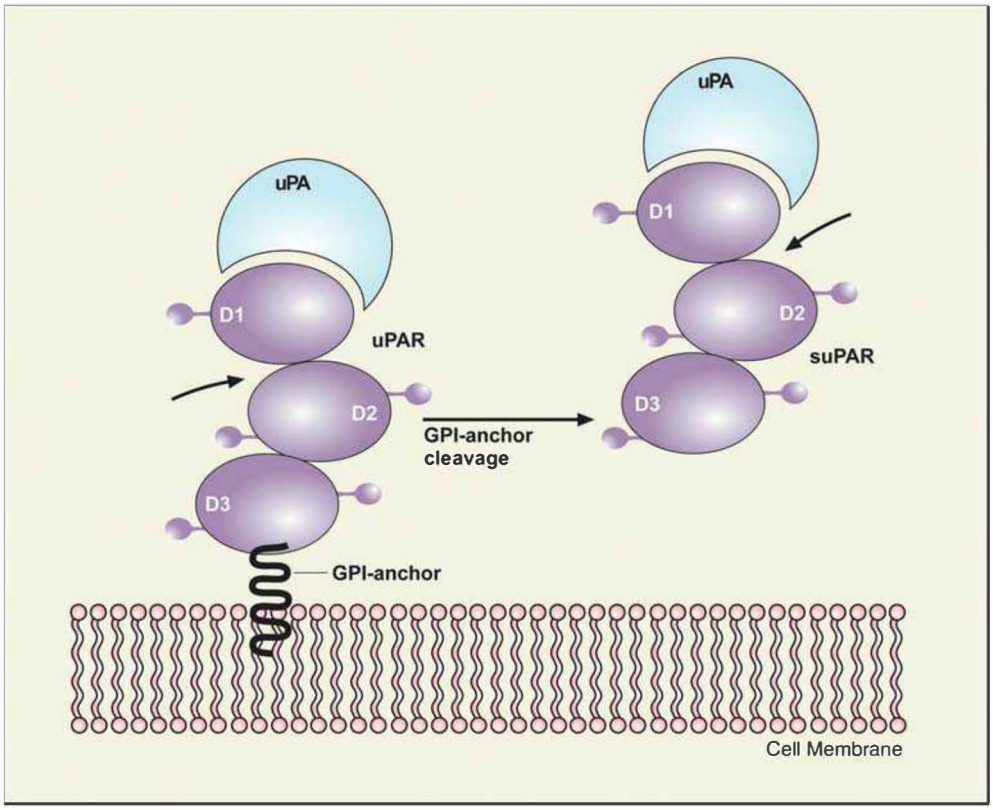

FIGURE 20.5 Domain structure of the uPAR. The *N*-terminal portion of uPAR is composed of three homologous domains with several potential glycosylation sites. uPA binds to domain 1 of uPAR but domains 2 and 3 enhance binding. The receptor is linked via a GPI anchor to the cell membrane. Shedding of uPAR from the cell surface forms the soluble form (suPAR). The region linking D1 and D2D3 can also be cleaved by proteases such as neutrophil elastase, plasmin, and uPA itself (*black arrow*).

the increase of hematocrit,[94] but there are reports of increases of uPA antigen after venous stasis,[222] DDAVP infusion,[224] and strenuous physical exercise.[225] Under normal circumstances, uPA activity is not detected in plasma, but both leukocyte-associated and free scuPA are elevated in leukemia[226] and other disorders, including liver disease.[227] If generated, uPA is cleared quickly from plasma, with a $t_{½}$ of approximately 7 minutes, in a manner that depends on hepatic blood flow.[228] The LRP system binds and internalizes scuPA and uPA–PAI-1 complexes.[216,229,230] The asialoglycoprotein receptor, located exclusively on parenchymal liver cells is also reported to remove uPA from the circulation.[228]

The activity of scuPA is markedly increased when it is bound to uPAR on the surface of monocytes.[231,232] This local activity is relevant to thrombus stability, into which monocytes migrate[233] and express fibrinolytic activity.[176]Indeed, monocyte-bound uPA has been shown to reduce thrombus size in a model of venous thrombosis.[234] In freshly formed model thrombi, there is considerable spontaneous generation of fibrinolytic activity, most of which can be inhibited by antibodies to uPA.[235] Polymorphonuclear cells, primarily neutrophils, generate this local uPA activity, apparently on uPAR[236]; plasma α_1-antitrypsin is crucial in protecting the activity from neutrophil elastase.[237] The integrin $\alpha_M\beta_2$ is important in the generation of such local activity.[238] When scuPA is present on uPAR, for instance on monocytes, it can associate with PAI-1 and other serpins, not in a classic covalent complex but in a reversible manner.[239] This has given rise to the idea that receptor-bound scuPA initiates proteolytic activity and that conversion to uPA is primarily a way of achieving inhibition by PAI-1 and other inhibitors, thereby regulating activity.[240]

scuPA does not bind to fibrin directly, but has some fibrin specificity, in that it degrades fibrin with less systemic degradation of fibrinogen and other plasma proteins than does uPA. It therefore is a more potent thrombolytic agent than uPA.[199,241] The explanations for this include the different but complementary mechanisms by which tPA and scuPA induce fibrinolysis.[242] tPA activates Glu–PLG, which is bound to an internal lysine, probably AαLys157, which is exposed in nondegraded fibrin. As soon as some fibrin is digested by plasmin, new *C*-terminal lysyl residues are generated, increasing the binding of Glu–PLG[160,162] to fibrin. scuPA binds with high affinity to PLG (K_d approximately 65 nM) and appears to selectively activate Glu–PLG, which is bound to *C*-terminal lysines on partially degraded fibrin.[243,244] The generation of small amounts of fibrin-bound plasmin also converts scuPA to uPA, explaining the typical lag phase observed for fibrinolysis by scuPA. A similar phenomenon has been observed with platelet-bound plasmin, where the localized PLG has a potent effect on scuPA activation.[187] Efficient scuPA-mediated lysis occurs after some fibrin has been degraded by tPA. Therefore, sequential administration of tPA followed by scuPA resulted in increased thrombolysis in a rabbit jugular vein thrombosis model; the reverse order was less effective.[245]

CONTACT SYSTEM

The contact pathway is composed of four proteins, FXII, PK, factor XI (FXI), and HK. Reciprocal proteolytic activation of FXII and PK to their active forms, FXIIa and kallikrein respectively, is stimulated by negatively charged surfaces. FXIIa subsequently cleaves downstream FXI to its activated form, FXIa, which feeds into the coagulation pathway. FXI and PK circulate in complex with the nonenzymatic cofactor HK, which assembles these proteases onto the activating surface. Zinc ions induce a conformational change in FXII[246–250] and HK,[251–253] enhancing surface interactions. Artificial anionic surfaces, such as kaolin, ellagic acid, sulphatides, and dextran sulphate, are known to stimulate FXII autoactivation and cleavage by kallikrein.[254–259] Recent work has identified natural surfaces, such as polyP,[260,261] RNA,[262] misfolded proteins,[263] and collagen,[264] that are also capable of triggering this pathway. PK activation can also take place independently of FXII when bound to HK on endothelial cells.[265]

FXII has traditionally been considered a coagulation factor but is structurally related to tPA, uPA, and PLG[266,267] (FIGURE 20.1), and is functionally implicated in fibrinolysis by different mechanisms. Firstly, FXIIa directly activates PLG (FIGURE 20.6) albeit relatively poorly compared to tPA and uPA.[268–270] The reaction can, however, be dramatically enhanced by the presence of dextran sulphate and zinc.[44] The relative plasma concentrations of FXII compared to tPA and uPA (that of FXII is four orders of magnitude higher) suggest it could be as effective as uPA as a PLG activator and may be potentially relevant *in vivo*.[271]

In a second mechanism, generation of kallikrein by FXII dependent[188,272] and independent[265] pathways triggers kinetically favorable activation of scuPA (FIGURE 20.6). Finally, indirect stimulation of fibrinolytic activity arises from kallikrein cleavage of HK. This generates the vasoactive peptide bradykinin, a potent stimulator of tPA release from endothelial cells.[273,274]

These three distinct mechanisms indicate the potential contribution of the contact pathway in regulating fibrinolysis, but further studies are required to define the relative contribution in response to various stimuli and in different local environments.

OTHER PLASMINOGEN ACTIVATORS

Streptokinase

Streptokinase (SK), an extracellular protein produced by streptococci, activates the human fibrinolytic system.[275] SK and other bacterial activators have evolved for the purpose of invasion of the mammalian host[276] and are a key pathogenicity factor in group A streptococcal infection.[277] SK rapidly forms a 1:1 complex with PLG, by inserting its *N*-terminal Ile[1] residue into the PLG amino-terminal binding cleft, activating the catalytic site nonproteolytically.[278–280] The activated SK–PLG′ complex binds a second molecule of PLG and cleaves it at Arg541–Val562 to form plasmin.[279–282] SK–PLG′ retains the ability to bind fibrin and is thereby relatively protected from inhibition by α_2AP.[283]

NMR and crystallographic studies show that SK (47 kDa) consists of three β-grasp tightly folded domains, α, β, γ joined by flexible segments.[284,285] The *C*-terminus of SK regulates the PLG binding and activation.[286] SK residues Arg253, Lys256, and Lys257 mediate plasmin substrate recognition via LBS-dependent interactions with kringle 5.[287] SK is highly antigenic. Most individuals have anti-SK antibodies as a result of previous infections with β-hemolytic streptococci, and anti-SK titers may rise several 100-fold after administration to humans.[288,289] After intravenous injection of SK into humans, dogs, or mice, SK is cleared from the circulation with a $t_{½}$ of 15 to 30 minutes.[290] Clearance mostly takes place in the liver, primarily by LRP.[229,290]

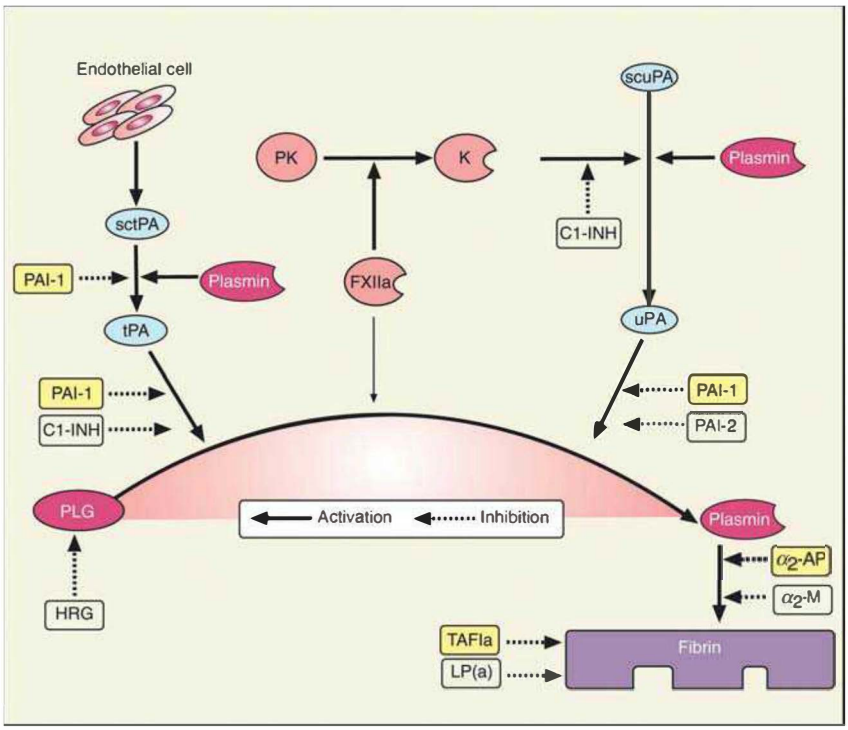

FIGURE 20.6 Activators and inhibitors of the fibrinolytic system. scuPA, single-chain urokinase plasminogen activator; sctPA, single-chain tissue plasminogen activator; α_2AP, $\alpha_2$antiplasmin; α_2M, $\alpha_2$macroglobulin; C1-INH, C1 inhibitor; HRG, histidine-rich glycoprotein; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; Lp(a), lipoprotein a; TAFI, thrombin activatable fibrinolysis inhibitor; PLG, plasminogen; PK, prekallikrein; K, kallikrein.

Staphylokinase

Staphylokinase (SAK), a protein with a M_r of 15,000, produced by *S. aureus*, has been known for more than 40 years to have thrombolytic properties.[291,292] Like SK, SAK is not an enzyme but forms a stable stoichiometric complex with PLG.[293] In the absence of fibrin, it does not activate PLG. Trace amounts of plasmin, such as may be found on the surface of a thrombus, cleave 10 residues from the amino terminus of SAK.[294,295] Removal of this ATF allows SAK to bind PLG, although it still has a relatively low affinity for circulating PLG and preferentially interacts with fibrin-bound PLG. Free plasmin then converts the SAK–PLG complex to SAK–plasmin complex that is a highly fibrin-specific PA. Unlike the SK–plasmin complex the SAK–plasmin complex is not protected from inactivation by α_2AP.[296–298] SAK is antigenic, and extensive mutagenesis has been undertaken to decrease this property.[299] Coupling of SAK mutants to polyethylene glycol of M_r 20,000 was shown to increase the $t_{½}$ of the complex several-fold, which would permit single bolus thrombolytic therapy in humans.[300] Several clinical studies with SAK have been reported. In peripheral arterial occlusions and in patients with myocardial infarction, SAK compared favorably with tPA.[301,302]

Vampire Bat Plasminogen Activator

Many hematophagous animals, such as leeches, hookworms, mosquitoes, and the vampire bat, produce substances that interfere with the clotting or fibrinolysis mechanisms. In the 1960s, a potent PA was found in the saliva of the common vampire *Desmodus rotundus*.[303] The bat lives exclusively on blood and uses the enzyme in its saliva to keep blood in a fluid state. The *D. rotundus* salivary plasminogen activator (DSPA) was cloned independently by two research groups and shows more than 70% identity with human tPA.[304,305] Three major forms were identified: DSPA-α, which contains a finger, EGF, kringle, and protease domain; DSPA-β, which lacks the finger domain; and DSPA-γ, which has just a kringle and a protease domain.

DSPA-α comprises 441 residues and, like SAK, has higher fibrin specificity than tPA.[306] In the absence of fibrin, hardly any PLG activation occurs.[307,308] PLG activation by DSPA is barely stimulated by D-dimer or the DDE complex, whereas these fibrin fragments strongly enhance tPA activity.[306] The crystal structure of the catalytic domain of DSPA-α_1 has been solved at 2.9 Å resolution[309] and shows strong similarity to sctPA. Experiments in animals revealed that the thrombolytic potential of DSPA was at least equal to, and the clearance rate approximately 10 times slower than, that of tPA.[310,311] The hope that high fibrin specificity would result in lesser bleeding at comparable thrombolytic potential was not fully realized,[310] but this agent has several promising features, long clearance time, and fibrin specificity, which permits bolus administration and may be useful in stroke as well as acute myocardial infarction.[312]

INHIBITORS OF THE FIBRINOLYTIC SYSTEM

Several inhibitors control and modulate the expression of fibrinolytic activity. These act at the PLG activation step or on plasmin (see FIGURE 20.6), and they prevent excessive systemic fibrinolytic

activity. The principal inhibitor of tPA and uPA is PAI-1, whereas α_2AP inhibits plasmin. Both are members of the serpin family, which also includes antithrombin and heparin cofactor (see Chapter 18). Serpins function by presenting the target protease a reactive center loop, the P1–P1′ of which mimics a substrate. A stable inactive 1:1 complex is formed between protease and serpin, lowering the effective protease concentration. Dissociation of the covalent complex, which occurs at different rates for different protease–serpin pairs, results in a cleaved form of the serpin, which lacks the short peptide from P1′ to the original *C*-terminus. The serpins have marked specificity for particular proteases, but the specificity is not absolute, so that other inhibitors present at high concentration provide a backup inhibitory system.

PLASMINOGEN ACTIVATOR INHIBITOR 1

The emphasis of this section is the role of PAI-1 in regulation of the PA system in the vasculature. In addition to its inhibition of tPA and uPA, PAI-1 has a role in regulating cell adhesion processes relevant to tissue remodeling, mediated by its binding to the adhesive glycoprotein vitronectin, rather than by proteinase inhibition.[313]

The 52-kDa glycoprotein consists of 379 residues. It is unusual among the serpins in its tendency to lose activity through spontaneous insertion of the reactive center loop into the body of the molecule, forming an extra strand of β-sheet A. This form was termed *latent* because, after denaturation and refolding, activity was regained[314]; there are no physiologic conditions under which this can occur. The noncovalent binding of vitronectin stabilizes the active form of PAI-1,[315] which is present in plasma at low concentrations, approximately 20 ng/mL.[316–318] It has a $t_{½}$ of <10 minutes and consequently its rate of synthesis is high. The variation between normal individuals is large, ranging from 1 to 40 ng/mL, corresponding to 1 to 40 U/mL, the unit being the activity that neutralizes 1 U of tPA in 10 minutes.

PAI-1 is produced by most cultured cells, and early publications identified it as a platelet protein,[319] a product of megakaryocytes,[320] endothelial cells,[321] hepatocytes,[322] and adipocytes.[323–325] The source of resting plasma PAI-1 is not certain. Studies showing that normal endothelium or hepatocytes do not express PAI-1[324] point to adipocytes as the normal source, whereas elevations in the acute phase are likely to result from hepatocytes.[326] Platelets constitute the major pool of circulating PAI-1. Although it is less active than plasma PAI-1, platelet PAI-1 accounts for about half the circulating PAI-1 activity, by virtue of the abundance of the protein in platelets.[317] Large amounts of platelet PAI-1 accumulate in thrombi,[327,328] and this directly correlates with their lysability.[328]

PAI-1 inhibits tPA (tc and sc) and uPA. It does not inhibit scuPA, which is largely inactive, but it does associate with scuPA noncovalently, as discussed in the section on uPA.[239] PAI-1 in plasma is in excess over tPA; therefore, most of the tPA is present in a complex with PAI-1. The second-order rate constants for the interaction with tPA and uPA are $>10^7$/M/s, a little lower for sctPA.

Hereditary deficiency of PAI-1 in humans is quite rare but leads to a lifelong bleeding disorder, presumably caused by premature lysis of hemostatic plugs at sites of vascular trauma, and consistent with this, bleeding occurs after a delay.[329–332] The oral administration of fibrinolytic inhibitors such as AMCA has been effective in normalizing hemostatic function.[331,332]

Mice with a disrupted *PAI-1* gene demonstrate normal development, fertility, and survival. The lack of inhibitor activity induces a mild hyperfibrinolytic state and confers a greater resistance to the prothrombotic effect of endotoxin injected into the footpad.[333] After arterial injury, wound healing in PAI-$1^{-/-}$ mice was accelerated with rapid migration of smooth muscle cells from the uninjured tissue.[334] In an arterial thrombosis model, time to development of an occlusive thrombus was approximately twice as long in PAI-$1^{-/-}$ than in PAI-$1^{+/+}$ mice.[335] In a model of carotid artery injury, partially or completely occlusive thrombi were found in 34% of PAI-$1^{-/-}$ mice and 65% of PAI-$1^{+/+}$ mice.[336]

In contrast to the lack of PAI-1, mice overexpressing human PAI-1 in the endothelium are subject to age-dependent development of arterial thrombosis.[337] In humans, high circulating PAI-1 is clearly associated with a variety of pathologies, including cardiovascular disease[338,339] and cancer.[340] Many studies have been conducted to investigate causal significance, and it appears that high PAI-1 does not independently predict disease, once other factors like obesity, diabetes, and elevated triglycerides are taken into account.[341] A guanine insertion/deletion polymorphism at position 675 in the PAI-1 promoter[342] is associated with differences in circulating PAI-l.[343] Despite promising early findings,[344] the current view is that the predictive power of studying this polymorphism is low.[341,345] Gram-negative septicemic patients have dramatically raised plasma PAI-1 concentrations, as much as 50-fold over normal, which is associated with high mortality.[346]

α_2 Antiplasmin

α_2Antiplasmin (α_2AP), also called *plasmin inhibitor*, is the principal fast-acting inhibitor of plasmin.[347] The gene (symbol PLI) is located on chromosome 17p13.[348] It gives rise to a single-chain protein of M_r of 70,000, comprising 452 residues, the additional mass accounted for by approximately 13% carbohydrates on four potential asparagine glycosylation sites (Table 20.1). The plasma concentration of α_2AP is 70 mg/L (i.e., 1 μM), approximately half the molar concentration of PLG. It is synthesized in the liver and consequently decreased in patients with advanced impairment of hepatic function. The $t_{½}$ of the native inhibitor is approximately 3 days, whereas the plasmin/α_2AP (PAP) complex is cleared with a $t_{½}$ of approximately 0.5 days.[349] The inhibitory activity of α_2AP resides in its reactive center loop, in which the P1–P1′ is Arg–Met; as with other serpins, mutation of Arg to Ala destroys inhibitory function.[350] The rate of interaction of wild-type α_2AP with plasmin is very fast, with a rate constant of 4×10^7/M/s.[351]

α_2AP exists in plasma in several forms, depending on limited proteolysis at *N*- and *C*-termini. This processing appears not to affect the inhibitory capacity of α_2AP, which resides in the core of the protein. The *N*-terminus of α_2AP was originally identified as Asn,[352] but it was later recognized that there is also a proform with an additional 12 residues at the *N*-terminus.[353] Plasma α_2AP was shown to have about equal amounts of the two forms,[354] whereas HepG2 cells produced the Met-form, which, after addition to plasma, was cleaved to the Asn form.[355] An enzyme that removes the 12 residue *N*-terminal fragment has been purified and characterized.[356] The importance of the *N*-terminal processing lies in the accessibility of Gln (at position two in the processed form); the proform terminus blocks access to the cross-linking site.[353] Gln2 in α_2AP is cross-linked by factor XIIIa to the Aα chain of fibrinogen or fibrin, at Lys303.[357,358] Fibrin with cross-linked α_2AP is markedly more resistant to

lysis, an observation that allowed the discovery of the first deficiency of α_2AP.[357] The cross-linking site can be saturated, as elegantly shown by studies with wild-type α_2AP and an active site Arg to Ala mutant.[350] Antibodies specific for cross-linked α_2AP greatly stimulated lysis of fibrin.[359]

The *C*-terminus of α_2AP is extended by approximately 50 residues, relative to other serpins.[352] Two forms are detectable in normal human plasma.[360] The native, full-length form binds PLG, whereas a processed form retains inhibitory capacity but cannot bind PLG.[361] The two forms were distinguished by two-dimensional immunoelectrophoresis, with PLG incorporated in the gel in the first dimension.[362] The ratio of the PLG binding to nonbinding form remains relatively constant in plasma samples, at approximately 65:35, even in patients with advanced liver cirrhosis.[227] The enzyme responsible for processing the *C*-terminal variant of α_2AP has yet to be identified. In addition to binding PLG the *C*-terminus contains an Arg–Gly–Asp sequence that mediates binding of α_2AP to endothelial cells.[363]

The ability of α_2AP to bind PLG interferes with the fibrin–PLG interaction, because both involve the same LBS (FIGURE 20.7). It is also crucial to the fate of plasmin formed on the fibrin surface, to which both tPA and PLG bind by virtue of their LBS. Plasmin formed on fibrin is therefore relatively protected from the action of α_2AP[364] (FIGURE 20.7). This concept arose from experiments using lysine analogues, where α_2AP was shown to be approximately 100 times less effective toward plasmin in the presence of lysine analogues.[351] The exact Lys residues responsible for binding the *C*-terminal region of α_2AP to LBS in PLG are not yet defined. Binding of recombinant *C*-terminal peptide (55 residues) to isolated kringles was most affected by mutation of Lys452, but other internal Lys residues appear to "tether" the kringles.[365] A different study, in which each Lys from position 429 to the *C*-terminal Lys452 was separately mutated, indicated that Lys436 had the greatest effect; their modeling suggested that Lys452 is too close to Phe448 for it to be able to interact with the LBS.[366]

The importance of α_2AP in stabilizing fibrin has been revealed in several *in vitro* systems, including clot lysis.[367,368] A mutant of tPA that is relatively resistant to α_2AP was very efficient in lysing both clots and model thrombi.[369] Deficiency of α_2AP results in delayed bleeding, but initial hemostasis is normal, and this is characteristic of fibrinolytic defects.[370] Inheritance was autosomal recessive in a well-characterized Japanese family.[371,372] An interesting defect in α_2AP Enschede was explained by the abnormal protein behaving as a substrate for plasmin rather than as an inhibitor,[373] as a result of the extended (by one Ala residue) reactive center loop.[374] Mice deficient in α_2AP were essentially normal in many respects, but they did show enhanced endogenous fibrinolytic activity without overt bleeding.[375] In experimental

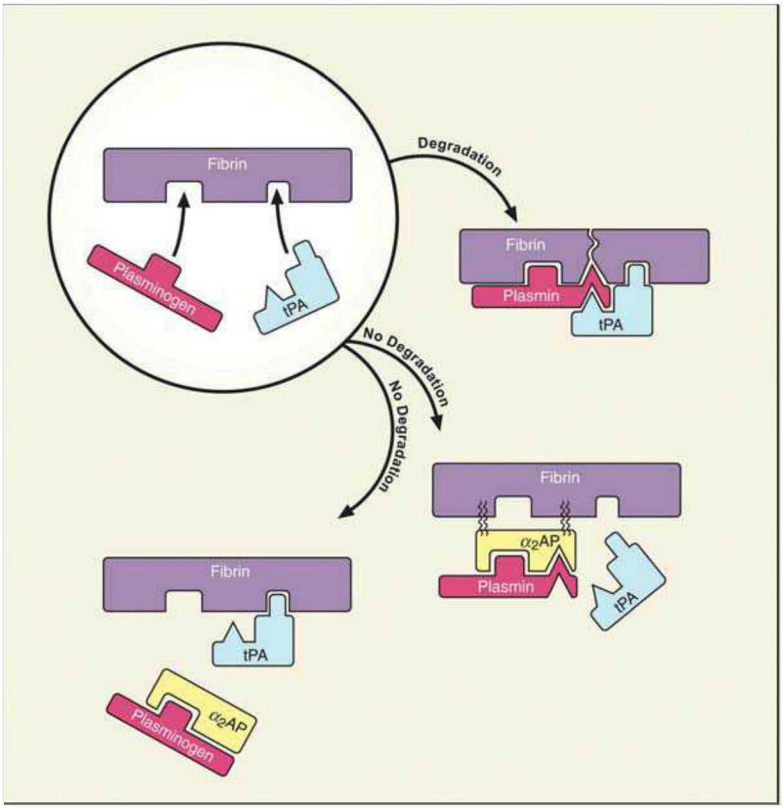

FIGURE 20.7 Interaction of plasmin(ogen), tPA, and fibrin; interference by $\alpha_2$antiplasmin (α_2AP). Plasmin(ogen) and tPA interact directly with fibrin, which acts as a cofactor in PLG activation stimulating fibrin degradation. In solution α_2AP can bind plasmin(ogen) thereby occluding binding to fibrin. α_2AP can also cross-link to fibrin that both prevents plasmin from binding to fibrin and neutralizes the activity of the enzyme. Both these events prevent PLG activation or plasmin activity and thereby inhibit fibrin degradation.

models, the lack of α_2AP had effects on liver regeneration,[376] platelet aggregation,[377] and pulmonary heart failure.[378]

THROMBIN-ACTIVATABLE FIBRINOLYSIS INHIBITOR

The generation of *C*-terminal lysyl residues is an important feedback mechanism for the binding of PLG to fibrin and the enhancement of fibrinolysis (discussed further in the section on "Balance of Plasminogen Activation and Regulation of Fibrinolysis"). These residues can be removed by activated thrombin-activatable fibrinolysis inhibitor (TAFIa) (EC 3.4.17.20; synonyms: carboxypeptidase B, U, or R), further regulating fibrinolysis.[379] The zymogen TAFI is activated by the thrombin/thrombomodulin complex[380] and by plasmin,[381] in a reaction that is enhanced by glycosaminoglycans. It is considered a molecular link between the fibrinolysis and coagulation pathways.[382]

TAFI is primarily produced by the liver and circulates at approximately 75 nM, but the normal variation is wide[379,383] and only a small proportion needs to be activated for full function.[380] There is no known endogenous inhibitor of TAFIa, but it displays a very short $t_{½}$ of approximately 10 minutes at 37°C.[384] The function of TAFIa was shown originally in clot lysis assays; potato tuber carboxypeptidase inhibitor relieves the inhibition.[382,385] The effect of TAFIa has also been shown in model thrombi made from whole blood,[386,387] and in animal studies, where TAFIa activity increased the time taken to restore blood flow in a canine coronary artery thrombosis model.[388] Inhibitors of TAFIa enhanced endogenous fibrinolysis and also thrombolysis by tPA.[389,390] Homozygous-deficient mice show enhanced plasma clot lysis but are comparable to wild-type mice in many thrombus models; mostly those in which time to occlusion rather than thrombus resolution is assessed.[391]

Elevated TAFI has been cited as a mild risk factor for thrombosis, and levels are known to increase in inflammation, correlating with other acute-phase markers.[392] A recent report has indicated that the venous thrombosis risk associated with plasma hypofibrinolysis is related to elevated levels of TAFI and PAI-1.[393] Conversely, it has been shown that deficiency of TAFI in mice increases the risk of intracerebral thrombosis and ischemic stroke.[394] Several polymorphisms in the TAFI gene have been reported. These seem to explain the wide normal range of concentrations but do not correlate strongly with disease.[395] Some methods show bias toward particular polymorphic variants of TAFI[396,397]; associations with disease therefore need to be interpreted with caution.

Increased fibrinolytic activity in hemophilia patients is explained by defective TAFI activation. Most thrombin is formed after clot formation, mainly via the intrinsic pathway by activation of FXI by thrombin. This leads to a positive feedback mechanism and optimal activation of pro-TAFI.[398] Clots composed of factor IX–deficient plasma lysed prematurely, but the defect could be reversed by the addition of factor IX or thrombomodulin to the plasma.[399] Similarly, addition of TAFI, thrombomodulin, or factor VIII to hemophilia A plasma restored normal fibrinolysis.[400] Consistent with this, incorporation of anti–factor XI antibodies or inhibition of TAFI in a rabbit model resulted in an almost two-fold increase in endogenous thrombolytic activity.[401]

OTHER INHIBITORS

Plasminogen Activator Inhibitor 2

PAI-2 was initially identified as a UK inhibitor in human placenta[402] and was purified from human placenta and the monocytoid cell line U-937.[403,404] PAI-2 is the predominant PA inhibitor of squamous epithelia in epidermis, esophagus, cornea, oral mucosa, tongue, and vagina.[405] It is incorporated into the cornified envelope during terminal differentiation of the keratinocyte via transglutaminase-catalyzed crosslinks.[406] Monocytes are an important source of PAI-2[407] and may increase fibrin stability on migration into thrombi, especially because the PAI-2 is cross-linked to fibrin.[408]

PAI-2 is an efficient inhibitor of two-chain uPA (tcuPA), with a second-order rate constant of 10^6/M/s; it reacts approximately five times more slowly with tctPA and quite poorly with sctPA.[404] In normal plasma, there are no measurable levels of PAI-2, but high levels are found in pregnancy, with a steady rise until around 33 weeks, to reach approximately 250 ng/mL.[409] Smaller increases in PAI-2 were observed during intrauterine growth retardation[410–412] suggesting it may be a marker for decreased placental function.[411,412]

PAI-2 is also found in the plasma of patients with acute myeloblastic leukemia of the M_4 and the M_5 type,[413] and in patients with sepsis, in whom PAI-1 is also increased.[414] Speculation remains on the real function of PAI-2. Mice with a disrupted PAI-2 gene do not present any phenotypic abnormalities.[415] The intracellular location of this serpin suggests roles other than inhibition of PA. Protection of cells from the cytopathic effects of alphavirus infection[416] and a role as a retinoblastoma (Rb)-binding protein[417] have been reported. The local activity of PAI-2 appears to be relevant to a number of cancers and this may provide clues to its physiologic function.[418,419]

α_2-Macroglobulin

α_2-Macroglobulin exhibits wide specificity and serves as the backup inhibitor of several proteases, including plasmin. It comes into play when the fibrinolytic system is fully activated and when the capacity of α_2AP is exhausted; it also forms complexes with tPA and uPA.[226] α_2-Macroglobulin is a glycoprotein of M_r 725,000 that consists of four identical chains of M_r 160,000 and contains approximately 8% carbohydrate (Table 20.1). It circulates at a relatively high concentration of 2.5 g/L (3 μM). The tetramer is arranged as a pair of dimers, each consisting of two monomers linked together via a disulfide bond. The complete sequence of this 1,451-residue molecule has been established and the gene locus (gene symbol A2M) attributed to chromosome 12p13.3–p12.3.[420,421] The inhibitor contains two reactive sites. The sequence Arg–Val–Gly–Phe–Tyr–Glu (681 to 686) acts as a bait region that contains sites for proteases of different specificities.[422] Cleavage results in a conformational change and activates the thioester site at positions 949 to 952. Electron microscopic studies showed that the catalytic domain of the rod-shaped plasmin molecule is entrapped inside the α_2-macroglobulin cavity, whereas its *N*-terminal kringle domains protrude outside one end between the two arm-like features of the transformed α_2-macroglobulin structure.[423] It is noteworthy that proteases inhibited by α_2-macroglobulin can still act on small peptide substrates, but their position in the inhibitor cavity makes them inaccessible to larger targets.

C1-Inhibitor

C1-inhibitor is a highly glycosylated 105-kDa serpin, consisting of 478 residues (Table 20.1). The 16 kb gene is located on chromosome 11q12–q13.1.[424–426] It inhibits the activated subcomponents, C1r and C1s, of complement C1, the contact proteases, FXIIa, FXIa, and plasma kallikrein, and the PA proteins plasmin, tPA, and uPA. It circulates in plasma at a relatively high concentration (1.7 μM). In situations in which tPA is present in excess over PAI-1, complex formation with C1-inhibitor is observed.[96,227,427] It has been shown to prevent nonspecific PLG activation induced by a uPA mutant while preserving the fibrin-specific fibrinolysis.[428,429] The role of C1-inhibitor in regulation of fibrinolysis is not extensively described in the literature but by virtue of its inhibitory effect on components of the contact system, it is likely to play a role in controlling contact phase–dependent fibrinolysis and the conversion of scuPA to uPA (FIGURE 20.6).

Lipoprotein (a)

Apolipoprotein (a), a component of lipoprotein (a) (Lp(a)), is linked by a disulfide bond to apolipoprotein B-100. Apo(a) has a wide size distribution, which depends on the number of kringle repeats; these are similar to kringle 4 of PLG.[430] The total mass of Lp(a) therefore varies between 800 and 1,300 kDa. There are also a wide range of plasma Lp(a) concentrations, from 10 to 1,000 mg/L, and a skewed distribution, median value 50 mg/L. The apo(a) gene is located in a cluster on chromosome 6q26–27 with PLG and two apo(a)-related genes.[15,431,432]

Lp(a) binds to fibrin, extracellular matrix, platelets, and cells.[433–436] Interaction with fibrin(ogen) has been shown to involve both Lys-dependent and independent mechanisms.[437] A proportion of the binding is via its kringles to *C*-terminal Lys residues and is inhibited by lysine and εACA. Lp(a) therefore competes with PLG and tPA for binding sites on fibrin exerting an antifibrinolytic effect.[433–435,437] PLG binding and activation on both platelet[436] and fibrin[438–441] surfaces is attenuated by Lp(a). Lp(a) has also been reported to interfere with the self-propagating phase of PLG activation by preventing the plasmin mediated conversion of Glu–PLG to Lys–PLG (FIGURE 20.4).[442] Transgenic mice expressing the human apo(a) gene resolved experimental thrombi poorly when treated with tPA,[443] consistent with the association of high Lp(a) with coronary artery disease.[444–446] Lp(a) size is a key factor with only the smaller forms binding fibrin with high affinity.[447–449] Individuals with the combination of high Lp(a) levels and predominance of the small isoform demonstrate reduced fibrinolytic activity.[449]

Histidine-Rich Glycoprotein

HRG is named for the region between residues 330 and 389, which are similar to the histidine-rich regions in human and bovine kininogen.[450] The 75-kDa protein has 507 residues and approximately 14% carbohydrate (Table 20.1). HRG is synthesized in the liver but can be taken up by platelets and megakaryocytes and released upon thrombin activation.[451] HRG competitively inhibits PLG associations by binding LBS-1 of PLG (K_d 1 μM). It is suggested, on the basis of their relative concentrations, that PLG (2 μM) and HRG (1.5 μM) would circulate predominantly in a 1:1 reversible complex,[25] which would decrease the PLG available for binding to fibrin. However, α_2AP had a greater effect than HRG on binding of PLG to fibrin.[452] HRG has been proposed to tether PLG to the cell surface, which would enhance the cells' migratory potential.[453] Only one family with congenital heterozygous HRG deficiency has been described. The proband, but none of the other five family members with low HRG levels, displayed thrombotic complications.[454] Mice deficient in HRG exhibit shorter plasma prothrombin times and bleeding times as well as faster clot lysis.[455] These results indicate that HRG has both anticoagulant and antifibrinolytic activity.

BALANCE OF PLASMINOGEN ACTIVATION AND REGULATION OF FIBRINOLYSIS

The primary function of the fibrinolytic system is to remove fibrin from the circulation in a controlled manner, thereby preventing excessive accumulation. It is a highly modulated enzyme system, and has evolved to be limited in its action in time and space but with appropriate feedback loops that enhance the fibrinolytic system on the local level. The system is represented schematically in FIGURE 20.8. Circulating PLG is not normally converted to plasmin, which would cause generalized fibrinogenolysis, proteolysis, and degradation of clotting factors, particularly factor V, factor VIII, vWF, and platelet glycoproteins. Instead, fibrin and cell surfaces act as a focus for the generation of plasmin activity, which is then shielded from inactivation by inhibitors. Activation of PLG on fibrin and cell surfaces by the PAs tPA, uPA, and scuPA will be discussed below but it is important to stress that most features of PLG activation on surfaces are unaffected by the activator used.

PLASMIN GENERATION ON FIBRIN

Many modulators increase or decrease the rate of PLG activation; the most important of these is fibrin, which has a dual function, acting both as a cofactor in PLG activation and as the target substrate for active plasmin. PLG binds to fibrin, directing the process of clot lysis to its target, binding that is competed by ω-amino carboxylic acids.[456] Identification of the binding sites was achieved by examining the products of elastase digestion of PLG. Kringles 1 to 3 and miniplasminogen bound to fibrin, but kringle 4 did not.[21,22,457,458] Partial degradation of fibrin by plasmin creates a positive feedback mechanism, whereby native Glu–PLG is bound to newly created *C*-terminal lysines on the fibrin molecules, particularly on the *C*-terminal portion of the α chain.[160–162,459,460] A few studies support the concept that PLG might bridge adjoining fibrin molecules and that probably both kringle 5 and kringle 1 bind to fibrin, albeit not at the same binding site.[16,458,461] There is little consensus on the values of the dissociation constants and number of binding sites for the binding of PLG, particularly of Glu–PLG to fibrin. Taking all reported figures together, it appears that an average K_d for the binding of Glu–PLG to native fibrin is approximately 5 μM and that of Lys–PLG, roughly one order of magnitude lower, representing 10-fold stronger binding. In the presence of partially digested fibrin, the K_d for the binding of Glu–PLG approaches that of Lys–PLG for native fibrin (0.5 μM).[162,460]

Two phases have been identified in tPA-mediated lysis of fibrin (reviewed by Thorsen [462]). During the initial slow phase, sctPA accumulates on fibrin protofibrils and activates the small amount of PLG bound to the fibrin surface. Newly generated plasmin partially degrades the fibrin surface generating *C*-terminal lysine residues that bind additional PLG and possibly tPA. This stimulates the second faster phase of tPA-mediated

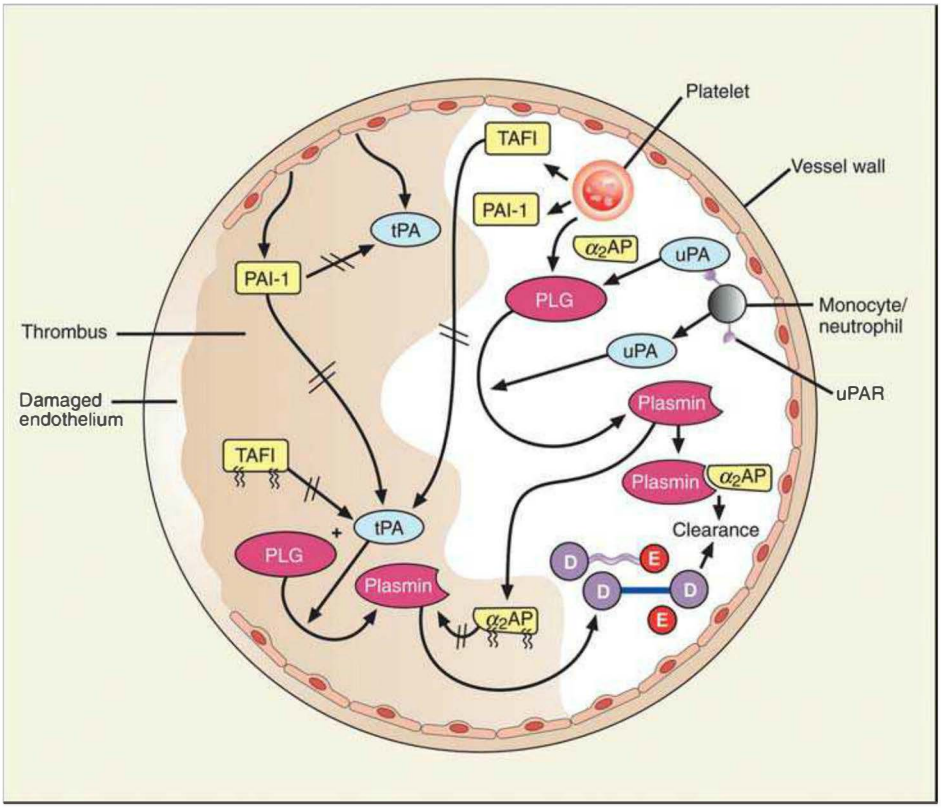

FIGURE 20.8 The balance of fibrinolysis. Schematic representation of the different paths of plasmin formation, inhibition, and clearance within a damaged vessel wall with a partially occluding thrombus. Solution, cell-surface, and fibrin phases of plasmin formation are represented. PLG is present at a relatively high plasma concentration (2 μM) and is readily incorporated into a thrombus as a result of its capacity to bind fibrin. In contrast tPA, which is mainly derived from the endothelium, circulates at low concentrations. tPA is a poor activator of PLG in solution, but it's activity is enhanced several fold when bound to fibrin. This localizes the majority of tPA-mediated PLG activation to the fibrin surface. uPA is found in the circulation and can be released from cells such as monocytes and neutrophils. uPA does not exhibit fibrin specificity and can activate PLG both in solution and while bound to its cell surface receptor, uPAR. Binding to uPAR colocalizes uPA and PLG, providing a focal point for plasmin generation. Plasmin degrades fibrin into FDPs, represented here as D-dimer and DD/E complexes. The system is under the control of several inhibitors; only the principal ones are shown here for simplicity. Complexes of active enzyme and inhibitor are rapidly cleared from the circulation. PAI-1, which is present at low concentrations in plasma, is released from damaged endothelium, but the major circulating active pool is contained within platelets. Once released, PAI-1 can inhibit tPA and uPA in solution. It can also inhibit PA activity in the presence of fibrin or cell surfaces, but this process is generally less efficient. $\alpha_2$antiplasmin (α_2AP) is in abundance in plasma and can be released from platelets. It can act on plasmin generated in solution but that formed on cell or fibrin surfaces is relatively protected from inhibition. α_2AP can be cross-linked onto fibrin localizing it at the site of plasmin formation. The carboxypeptidase inhibitor TAFI is found in plasma and platelets and can be cross-linked to fibrin. TAFI is activated by thrombin/thrombomodulin complex to generate TAFIa that attenuates PLG activation by removing the C-terminal lysines residues from fibrin that are important for binding of PLG and to a lesser extent tPA. This complex series of interactions and events serve to regulate fibrin accumulation in the body in a controlled manner. The many different feedback loops and surfaces involved localize the reactions and prevent excessive generation of plasmin activity.

fibrinolysis. The molecular interactions and specific binding sites involved have been extensively reviewed.[165] Low affinity binding sites (K_d 1 μM) for tPA (γ312–324) and PLG (Aα148–160) are located in the D-region of fibrinogen, only about 45 Å apart. The close proximity of these sites allows a cyclic ternary complex to form, facilitating activation of PLG by tPA. The αC domain of fibrin(ogen) is also reported to play a role in tPA-mediated PLG activation. A fragment corresponding to the αC domain (Aα221-612) binds tPA and PLG with high affinity (K_d 16 to 33 nM) and enhances plasmin generation. It is noteworthy that the binding sites for tPA and PLG in the D region and αC domain are cryptic in circulating fibrinogen and are exposed only during fibrin formation. Binding sites localized within the αC domains are probably uncovered during the switch from intramolecular interaction in fibrinogen to intermolecular interaction in fibrin. During fibrin assembly, conformational

changes in the D region, involving insertion of the B knob in the E region into the αC domain of the D region, are likely to expose tPA and PLG binding sites.

Exposure of *C*-terminal lysine residues in partially cleaved fibrin during the second phase of clot lysis dramatically accelerates the lysis rate.[463] Recent observations, using tPA variants, indicate that the finger domain of tPA plays a more dominant role in the interaction with fibrin than the kringle 2 interaction with *C*-terminal lysine residues.[464] This in turn indicates that the binding of PLG to partially degraded fibrin is the critical step in accelerating the second phase of fibrinolysis.

The structural characteristics of the fibrin network also influence the ability of fibrin to act as a cofactor in PLG activation.[465] Thin fibers are reported to lyse at a more rapid rate than coarse fibers, but yet fibrin networks composed of thin fibers lyze more slowly. These contrasting observations are most likely explained by the increased fiber density in clots composed of thin fibers, which regulates fibrinolytic speed and impairs movement of tPA and PLG into the dissolving clot.[466]

Unlike the single-chain form of tPA, scuPA has very low intrinsic catalytic activity (~0.5% of tcuPA). In the presence of fibrin scuPA, but not tcuPA, induces fibrin specific lysis.[199] The ability of scuPA to activate PLG bound to internal lysine residues is relatively low but partial digestion of fibrin by plasmin and exposure of *C*-terminal lysine residues dramatically increases the ability of scuPA to stimulate PLG activation. In the presence of a fibrin surface, activation of PLG by scuPA does not require conversion to the two-chain form of the activator, suggesting a conformational change in either PLG or scuPA that accelerates the interaction.[467] A further aspect of fibrin-specific lysis by scuPA is that the plasmin formed on the surface is relatively protected from inhibition, as discussed below.

PLASMIN GENERATION ON CELL SURFACES

The roles of fibrin and of cell surfaces in stimulating PLG activation can be viewed as broadly equivalent. PLG binding sites are broadly distributed on eukaryotic cells with most interactions being regulated by LBS within the kringle domains of PLG. Pretreatment of cells with carboxypeptidase B, an enzyme that removes *C*-terminal lysine residues, dramatically impairs the ability of cells to stimulate PLG activation.[468] Gangliosides and a class of glycolytic enzymes with *C*-terminal lysine residues, such as α-enolase, mediate binding of PLG to cell surfaces. Other PLG receptors include histone H2B,[469,470] TIP49a,[471] and p11[469] on monocytoid cells and integrin $\alpha_M\beta_2$ on neutrophils.[238] Annexin A2 on HUVECs binds both tPA (K_d 30 nM) and PLG (K_d 114 nM). Consistent with a role for this receptor in PLG activation, mice deficient in annexin A2 exhibit fibrin deposition in the microvasculature and incomplete clearance of injury-induced arterial thrombi.[472] Injection of recombinant annexin A2 increased the patency of vessels in a rat carotid artery model and enhanced activation of PLG in solution approximately 60-fold.[473]

A novel integral membrane receptor that is synthesized with a *C*-terminal lysine, Plg–R_{KT}, has recently been identified.[474] Plg–R_{KT} is broadly expressed in normal human tissues and is upregulated during differentiation. PLG binds with high affinity to the *C*-terminus of Plg–R_{KT} and regulates cell-surface activation of PLG. The *C*-terminus of Plg–R_{KT} also binds tPA in an overlapping binding site. However, the relative concentrations of PLG and tPA in the circulation should allow simultaneous binding of both ligands to the cell surface, with each tPA molecule bound within the vicinity of multiple PLG molecules. Interestingly, the receptor colocalizes with uPAR, suggesting that it can function to localize PLG with scuPA/uPA to promote cell-surface PLG activation.

Cell bound PLG is activated approximately 10-fold faster than PLG in solution. There are two alternative explanations for this, activation of Glu–PLG to Glu–plasmin or plasmin-mediated conversion to Lys–PLG, which is then activated to Lys–plasmin (FIGURE 20.4). On cell surfaces, and in contrast to solution phase, local plasmin is generated and converts Glu–PLG to Lys–PLG.[475,476] This echoes what happens on fibrin, where small amounts of plasmin are formed, sufficient to convert Glu–PLG to Lys–PLG.[477]

Binding of scuPA to its cellular receptor uPAR accelerates plasmin generation, as a result of the effect on PLG activation and on feedback activation of scuPA to uPA by formed plasmin. Both these steps depend on the association of PLG with the cell surface. Cell-bound plasmin is protected from inhibition by α_2AP, although the (sc)uPA is still susceptible to inhibition by PAI-1 and PAI-2. Direct anchoring of a GPI-linked uPA variant to the cell surface stimulates PLG activation to a similar degree as uPAR.[478] This suggests that the principal function of uPAR is to localize uPA to the cell surface and not to enhance activity of the PA.

In addition to uPAR, other receptors for uPA may exist on different cell types. An example of this is $\alpha_M\beta_2$ on neutrophils that binds both PLG and uPA to stimulate plasmin generation.[238] Platelets also exhibit the ability to bind scuPA[479,480] and indeed tPA.[481,482] None of these receptors have been identified conclusively. Plasmin activity associated with platelet membranes is capable of activating added scuPA, the local plasmin being much more active than in solution.[187]

uPA on monocytes or endothelial derived microparticles specifically activates PLG bound to platelets, fibrin, or extra cellular matrix proteins (e.g., fibronectin, laminin).[483] This recent evidence suggests that components of the fibrinolytic system need not be assembled on the same surface to induce PLG activation. This cross-talk mechanism of plasmin generation fulfills the requirement for PLG to be bound to a biologic surface for efficient activation (i.e., fibrin or cell surface)[438] but differs in that uPA is expressed on an adjacent cell or microparticle. Interestingly, cells bearing tPA were not able to elicit a cross-talk response, indicating that this is a specific feature of uPA-mediated PLG activation.

Balance of Proteases and Inhibitors

The PA and their inhibitors are subject to large variation, suggesting that regulation of fibrinolytic activity occurs mainly via subtle changes in the balance of PA and their inhibitors. This view arises both from the relative concentrations of PA, many orders of magnitude lower than the PLG concentration, and the very short half-lives of PA compared to PLG, minutes rather than days (Table 20.1). In normal individuals, steady-state levels of PLG in plasma are rather stable. Plasma levels reflect synthesis, release, and also clearance, primarily by the LRP family of receptors.[229] Liver endothelial cells express a mannose receptor that specifically interacts with the mannose-type carbohydrate on kringle 1 of tPA. A calcium-dependent receptor on the surface of

liver parenchymal cells recognizes the finger and EGF domains of tPA[116] and most likely represents LRP, which is also important for clearing tPA–PAI-1 complexes from the circulation.[229]

The fibrinolytic system is normally quiescent as a result of two factors: the relative abundance of inhibitors over proteases and the requirement for fibrin or cell surfaces for initiation of activity. In healthy individuals, there is a molar excess of active PAI-1 in plasma over tPA (Table 20.1). Consequently, most of the tPA in plasma is in the form of an inactive complex with PAI-1.[484] Small amounts of free tPA occur in the circulation as a result of the high synthesis rate of both proteins and the finite time it takes for complex formation to occur. Despite the stabilization of plasma PAI-1 in an active conformation by vitronectin[485,486] it still loses activity. This increases the possibility that plasma tPA can, in some circumstances, overcome inhibition by its primary inhibitor. Normally, tPA activity is detectable at approximately 0.1 to 0.5 U/mL of plasma, even when blood is collected in an acidified anticoagulant to prevent complexing of any free tPA by PAI-1.[487] The maximum activity normally seen is equivalent to 1 μg/L; tPA has a specific activity of 500,000 to 700,000 U/mg.[488] This low tPA activity is generally close to the borderline for detection, which is why procedures like euglobulin preparation, which removes most plasma inhibitors, but notably only about half the PAI-1,[317] are used. The levels of free tPA increase several-fold, after strenuous exercise, venous occlusion, or intravenous injection of DDAVP to healthy volunteers, but despite this no circulating plasmin is generated because PLG activation does not occur within this physiologic range of tPA concentrations. This does not apply during therapy with recombinant tPA, during which circulating plasma concentrations of tPA are achieved that are up to 1,000-fold higher than those observed in normal plasma.

Changes in the balance of tPA and PAI-1 are a clear example of how fibrinolytic activity can be initiated. Indeed, these components of the system fluctuate remarkably during a 24-hour period.[489] tPA activity is lowest at night and in the early morning and then increases up to three-fold during the day. PAI-1 antigen and activity are highest in the early morning hours and then gradually decline during the day, reaching their lowest level in the early afternoon. Various factors may interfere with the normal circadian rhythm.[223] There is a large body of literature on the predictive value of tPA antigen and PAI-1 as risk factors for acute myocardial infarction, stroke, and peripheral vascular disease.[339,344,345,490] High tPA antigen is found to be associated with disease in a number of studies, and this probably reflects tPA/PAI-1 complex.[484,491]

It is generally assumed that PLG, present in plasma at approximately 2 μM, is not rate-limiting for fibrinolysis. Even though its concentration is so much higher than the activators (Table 20.1), it is indeed possible for it to become a limiting factor. The need to consider local PLG concentration, especially when the activators are used therapeutically, has been clearly shown.[463] Even *in vitro* in platelet-rich plasma, platelet-associated PLG was shown to have a marked effect on scuPA activation, despite the presence of plasma PLG, pointing to the importance of local pools.[187] The outcome of variations in available PLG is highlighted in studies on TAFIa.[492] The effect of TAFIa on tPA-initiated lysis is usually stressed, in line with tPA's known fibrin dependence, but TAFIa also modulates uPA and scuPA-induced lysis, pointing to its primary effect being on PLG binding.[386]

Fibrinolytic activity is modulated by serpins. tPA and uPA are efficiently inhibited by PAI-1. More than 90% of blood PAI-1 is located in platelets and is promptly released upon activation. Although platelet PAI-1 is less active than the plasma pool, there is still enough active PAI-1 in platelets to stabilize platelet-rich thrombi, as occur in the arterial circulation where blood pressure is high and premature lysis of a hemostatic plug is undesirable. Plasmin bound to fibrin is partly protected from inactivation by α_2AP. This assures that fibrinolysis proceeds on the surface of a clot. However, free plasmin that spills over into the general circulation is rapidly inactivated by α_2AP. After massive activation of the fibrinolytic system has taken place, such as in thrombolytic therapy, the amount of α_2AP does not suffice to inhibit all circulating plasmin, and α_2-macroglobulin acts as a scavenger protease inhibitor.

The activity of the fibrinolytic system can be viewed as being regulated by a number of different factors: the local generation of plasmin on fibrin or cell surfaces rather than free in the circulation, the relative abundance of protease zymogens and inhibitors, which in turn is dictated by synthesis, release, and clearance. There are several means to activation of the system, whether by tPA or (sc)uPA, or the direct use of plasmin. Similarly inhibition is multifaceted, with TAFIa modulating plasmin generation on fibrin or cell surfaces, PAI-1 directly inhibiting tPA and uPA, and α_2AP directly inhibiting plasmin. The relative potencies of the proteases and the inhibitors can appear different depending on the experimental system or the clinical situation, reflecting the subtle interplay of interactions. Thus, while the system is superficially much simpler than coagulation and boasts only a couple of steps, it should not be interpreted simplistically and its subtleties require further work to reveal all their secrets.

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CHAPTER

21 Inhibitors of Fibrinolysis

BJÖRN WIMAN AND ANNA ÅGREN

The fibrinolytic system has many different functions *in vivo*. Within the blood vessels, its main function is to remove fibrin deposits and keep the blood vessels open after clots have been formed. But the fibrinolytic system also seems to play roles in other processes such as reproduction, tissue repair and remodeling, invasive growth, and metastasis.[1] When activated, the fibrinolytic system results in the formation of active plasmin, a very powerful proteolytic enzyme, with an action very similar to that of trypsin. Plasmin eventually has the capacity to degrade almost all of the plasma proteins *in vivo*. It is therefore of utmost importance that both its action and its formation are highly regulated.[2] This is achieved in two distinct ways. Firstly, activation of plasminogen to plasmin physiologically occurs preferentially at the surface of fibrin. Secondly, free plasmin becomes inactivated rapidly. In that way, the action of the fibrinolytic system in the blood vessels is localized to eventual deposits of fibrin. Fibrinolytic inhibitors play important regulatory roles in different stages of this process. The inhibitors are mainly antiplasmin and *PAI-1* (plasminogen activator inhibitor 1), two inhibitors belonging to the serine proteinase inhibitors or the serpin superfamily of proteins. In addition, other substances influence the process both in a positive and a negative way. They may also be of great physiologic significance. A schematic picture of the fibrinolytic process and its regulation is demonstrated in FIGURE 21.1. In this chapter, we mainly discuss the inhibitors involved in this regulation, such as those inhibiting plasmin, which are antiplasmin and α_2-macroglobulin, those inhibiting plasminogen activators, which are *PAI-1* and *PAI-2* (plasminogen activator inhibitor 2), and finally, other compounds influencing the process, such as fibrin and TAFI (thrombin-activatable fibrinolysis inhibitor).

From the pathophysiologic point of view, an increased function of the fibrinolytic system leads to an increased bleeding tendency. This is, for example, seen in patients with antiplasmin deficiency and may result in bleedings as severe as in patients with hemophilia. On the other hand, a decreased fibrinolytic function, as is found in patients with increased plasma levels of *PAI-1*, gives a thrombotic tendency both in the arteries and in the veins.

ANTIPLASMIN

Discovery and Basic Information

Antiplasmin is a serpin with a molecular weight of about 70,000. It was discovered in 1976 by three different groups.[3–5] It is produced in the liver,[6] and therefore its plasma concentration is decreased in severe liver diseases where protein biosynthesis is diminished.[7] The plasma concentration of antiplasmin is about 70 mg/L (1 μmol/L), which is approximately half the concentration of plasminogen in plasma on a molar basis. Therefore, under normal circumstances, when only little plasminogen is activated, the antiplasmin concentration is in far excess of the formed plasmin. However, during thrombolytic treatment, especially with non–fibrin-specific plasminogen activators, such as streptokinase or urokinase, large amounts of the plasminogen will become activated rapidly with the risk of all the antiplasmin becoming depleted. Under those circumstances, α_2-macroglobulin might become an important inhibitor of plasmin. The half-life in the circulation of native antiplasmin is approximately 3 days.[8]

Antiplasmin has several different functional properties. Its main physiologic activity is inhibition of plasmin by forming an irreversible enzymatically inactive complex with plasmin.[9,10] It inhibits plasmin extremely rapidly.[9] In addition, it inhibits the binding of plasminogen to fibrin, which is a very important regulatory phenomenon in the fibrinolytic process.[2,11] It may also become cross-linked to the α-chains of fibrin during clotting by the action of coagulation factor XIIIa.[12,13] Antiplasmin bound to fibrin in this way is believed to protect the formed fibrin from becoming prematurely degraded, keeping the fibrinolytic activity somewhat delayed until the healing processes get started.

Structure

The gene of antiplasmin has been localized to chromosome 17p13.[14] This inhibitor is a glycoprotein consisting of a single polypeptide, containing approximately 13% carbohydrate.[15] Holmes et al.[16] determined the primary structure of mature human α_2-antiplasmin by DNA sequencing of overlapping cDNA fragments prepared from human liver mRNA. It was found to contain 452 amino acids as deduced from the cDNA sequence, with Asn as NH_2-terminal amino acid. Later, it was found that native antiplasmin has an additional 12 amino acids at the NH_2-terminal end and is rather composed of 464 amino acids with an NH_2-terminal Met.[17,18] In plasma, Met-antiplasmin is converted to Asn-antiplasmin.[19] There are four potential asparagine-linked glycosylation sites in the molecule. Antiplasmin has not yet been possible to crystallize, so much of its structure had to be anticipated by homologies with other proteins. It belongs to the serpin superfamily of proteins. It seems to contain all the important structural elements of a serpin. Of these, the reactive center loop (RCL), which contains the scissile peptide bond (red string in FIGURE 21.2) and the A β-sheet (yellow part in FIGURE 21.2), is of special functional importance. RCL is the part catching the protease and thus the most important portion of a serpin. It might occasionally be inserted into the A β-sheet as a new central strand and then form an inactive,

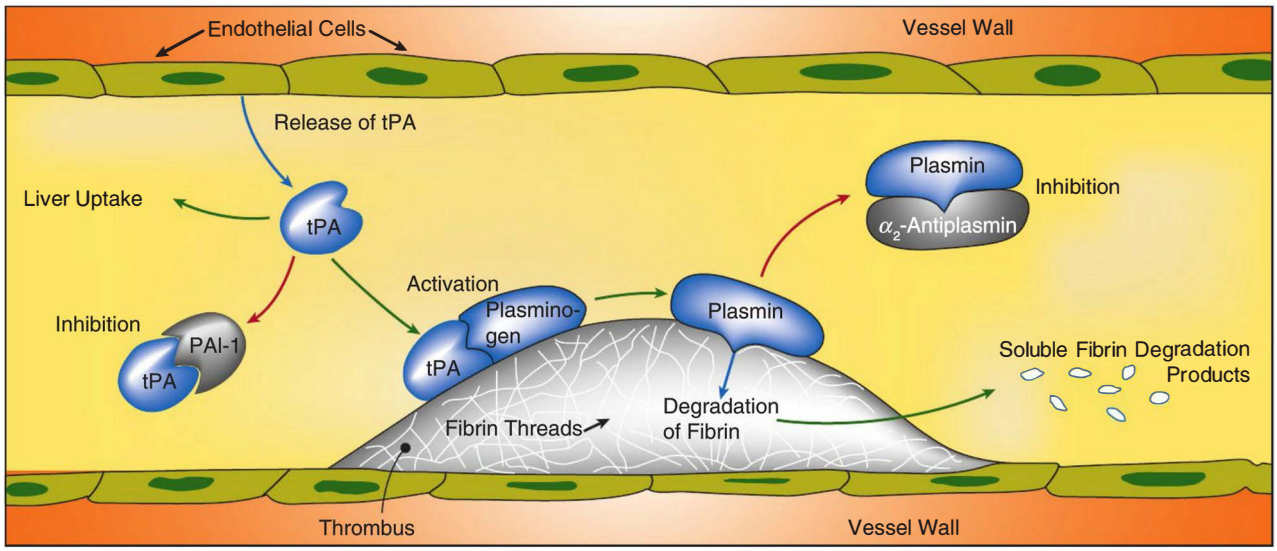

FIGURE 21.1 A schematic picture of the fibrinolytic system and its activation is demonstrated. Activation of plasminogen occurs preferably at the surface of fibrin, while plasmin becomes inhibited only after it has left the fibrin surface. (Adapted from Wiman, MFR informerar, 1987.)

latent form that may be reactivated, if treated with denaturing substances, followed by dialysis. Alternatively, the RCL might be inserted into the A β-sheet of another molecule resulting in a tendency of polymerization.

Reaction with Plasmin

Antiplasmin is capable of reacting with and forming stable enzymatically inactive complexes with plasmin. The reaction is very rapid, with a rate constant of about 2×10^7 $M^{-1}s^{-1}$, and occurs in several steps involving several different interacting sites.[9,10,11,20,21] The first step, which seems to be the rate limiting, takes place between one of the lysine-binding sites in the plasmin molecule and a complementary site in the very COOH-terminal (CT) portion of the antiplasmin molecule. The second is a noncovalent interaction between the substrate-binding pocket in the plasmin active site and the scissile peptide bond in the RCL of antiplasmin. Subsequently, peptide bond cleavage occurs.[22] After formation of an ester bond between plasmin and antiplasmin, major conformational changes occur both in the plasmin molecule and in the antiplasmin molecule.

The lysine-binding-site-mediated interaction has an important role in regulating the fibrinolytic system. The same site in the plasmin molecules are also involved in the interaction with fibrin. Therefore, fibrin-bound plasmin reacts much slower with antiplasmin than free plasmin, thereby keeping the fibrinolytic process localized.[2,9,11] It was initially believed that the CT lysine (Lys^{452}) in antiplasmin is responsible for the lysine-binding-site-mediated interactions with different proteins.[11,23–26,27,28–31] Current evidence indicates that K436, but not K452, is important in this reaction.[32] Using single-site mutants, the variant K436E reacted much more slowly than wild-type antiplasmin (wt-antiplasmin) or the other variants including K452E or K452T. In addition, in the presence of 6-aminohexanoic acids, the reaction rate decreased about 10-fold or more for wt-antiplasmin and most variants. The variant K436E was less affected by 6-aminohexanoic acids, again suggesting that this residue is involved in the lysine-binding-site-mediated interaction between plasmin and antiplasmin. The data were further supported by studies using surface plasmon resonance to examine the interaction of antiplasmin mutants to immobilized plasminogen or an elastase fragment from plasminogen constituting "kringles" 1–3. The only antiplasmin variant with decreased affinity was the K436E, again demonstrating that the lysine residue 436 is important in this interaction.[33] Thus, available data clearly demonstrate that Lys^{436} of antiplasmin is involved in the lysine-binding-site-mediated interaction with plasminogen and fibrin.

Stability

Many of the serpins seem to be metastable proteins, with quite interesting mechanisms regulating their stability. In contrast to some of the other serpins, human antiplasmin is quite stable at physiologic conditions, but that activity is rapidly lost at acidic conditions[34] or at neutral pH at elevated temperatures.[35] At temperatures above 45°C, the rapid inactivation was found to be a polymerization process. However, the inactivation occurring at low pH occurred mostly without any visible polymerization tendency. However, such material could gradually recover activity at neutral pH, and if treated with guanidinium chloride, followed by dialysis, antiplasmin activity was again recovered,[35] in a similar fashion as for *PAI-1* (see below).

Using site-directed mutagenesis, four single-site mutants, His197Thr, His341Thr, Glu353Ala, and Glu382His, were found to display significantly increased stability.[36] The mutant His341Thr was found to have the longest half-life at pH 4.9, about sevenfold increased stability as compared to wt-antiplasmin. One mutant, Glu346Thr, had a shorter half-life

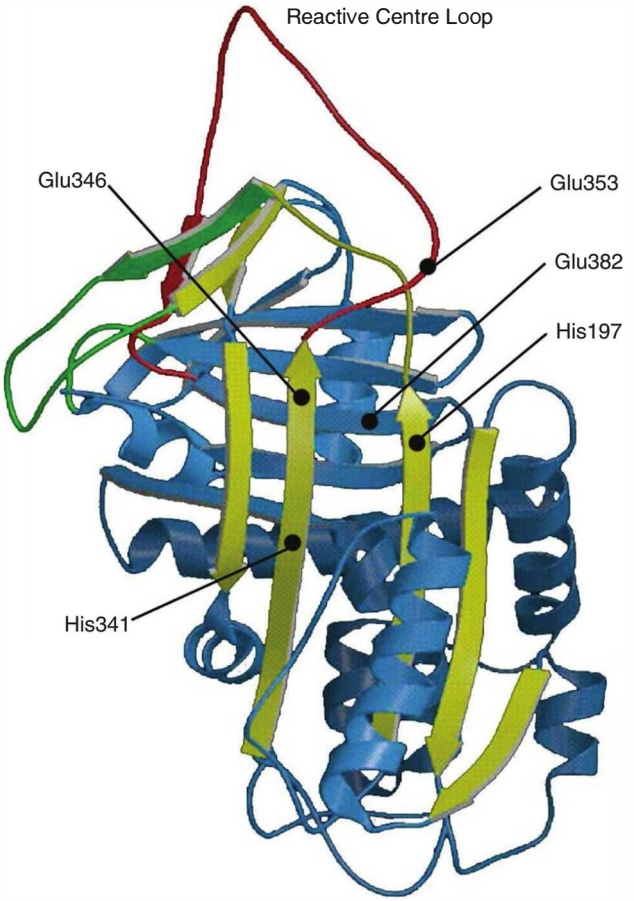

FIGURE 21.2 A schematic picture of active *PAI-1* (Adapted from Aertgeerts et al. 1994). The expected sites for some mutations if this was in antiplasmin are indicated. Antiplasmin has not yet been crystallized. Therefore, we show a model of *PAI-1* instead, since most serpins are expected to have a very similar basic structure. The RCL is labeled red and the A β-sheet yellow.

than wt-antiplasmin. All these residues are believed to be situated in the vicinity of the point where the RCL is inserted into the A β-sheet to get latent molecules (FIGURE 21.2). Unfortunately, so far it has not been possible to crystallize antiplasmin. However, the serpin superfamily of proteins all seem to be very similar in their overall structure. Therefore, in FIGURE 21.2, a schematic picture of *PAI-1* is shown as adapted from Aertgeerts et al.[37] 1994. The expected places for where the important mutations in antiplasmin, as discussed above, would be situated are indicated in this figure.

Incorporation into Fibrin

Antiplasmin is incorporated into the α-chains of fibrin during the clotting process through the action of activated coagulation factor XIII. This interaction involves the NH_2-terminal portion of antiplasmin, more specifically the Gln residue number 2. Only a fraction of the fibrin monomers will have an antiplasmin incorporated, but these molecules are believed to be very important from the physiologic point of view. They catch and inactivate the initial plasmin molecules formed upon activation of the fibrinolytic system. In that way, fibrin is initially protected and the fibrinolytic process becomes somewhat delayed.[38] This permits the repair processes to get started before the fibrinolytic process really gets accelerated.

Clinical Significance

A few individuals have been identified with congenital antiplasmin deficiency.[39] They suffer from a severe bleeding tendency, similar to individuals with classical hemophilia. However, when correctly diagnosed, these patients are very easily treated with antifibrinolytic drugs. Tranexamic acid or earlier 6-aminohexanoic acid is the most frequently used drug to achieve this. It interferes with the interaction between plasminogen and fibrin and prevents formation of the ternary complex between fibrin, plasminogen, and tPA (tissue plasminogen activator). This causes a dramatic decrease in the activation rate of plasminogen and thus, an antifibrinolytic effect is obtained. Aprotinin, a low molecular weight and very efficient inhibitor of trypsin and plasmin, has also been used. Decreased plasma concentrations of antiplasmin are seen in connection with severe liver disease.

PAI-1

Discovery and Basic Information

PAI-1 is a serpin with a molecular weight of about 50,000. It was initially discovered in endothelial cells [40] in human plasma [41,42,43] and in hepatocytes.[44] It is now known that *PAI-1* is produced by several types of cells, including vascular endothelial cells, adipocytes, platelets (or rather megakaryocytes), and placental cells.[1] *PAI-1* is also produced by smooth muscle cells in atherosclerotic arteries.[1]

The concentration of active *PAI-1* in human plasma is 0.6 nmol/L (about 30 μg/L), much lower than for the other serpins. Some individuals have 5 to 10-fold higher plasma concentrations. This is true for individuals with increased body weight, especially those with an increased waist/hip ratio, or those with an increased inflammation, but it is also true for pregnant women.

PAI-1 efficiently inhibits tPA and uPA (urinary plasminogen activator) by forming a 1:1 very stable complex that resists strong denaturing substances such as SDS.[45] The reaction between *PAI-1* and tPA/uPA is also extremely fast with second-order rate constants above 10^7 M^{-1} s^{-1}.[46,47] During complex formation, the scissile peptide bond Met^{346}-Arg^{347} in *PAI-1* becomes cleaved.[45,48]

The *PAI-1* Gene

The human *PAI-1* gene is located on chromosome 7 and is approximately 12.2 kbp long. The gene contains nine exons separated by eight introns. Several groups have sequenced and analyzed this gene.[49,50,51,52–55] Two different *PAI-1* mRNA species seem to exist in humans with lengths of 2.4 and 3.2 kb.[50] They both contain the entire coding region and just differ in the length in their 3′-untranslated region.[51,56] Both these messengers seem to function similarly, but they differ in stability, with the 2.4-kb form being more stable.[57] The different transcripts are the result of the presence of multiple polyadenylation splice sites.[58]

Several polymorphisms within the *PAI-1* gene have been described. The most frequently studied one is the 4G/5G polymorphism located 675 bp upstream of the transcriptional start site.[59,60] It is a polymorphism of functional importance since it correlates with the plasma *PAI-1* concentration (see below). Other reported polymorphisms are a *Hin*dIII polymorphism in the 3′-region,[56] a $(CA)_n$ repeat in intron 3,[60] a G/A substitution at −844 in the promoter [61,62] and at +1334[194], a T/G substitution at +11053,[62] a 9-nucleotide insertion/deletion between +11320 and 11345,[62] and a G/A substitution at position +9785 in intron 7.[62] None of these have yet been shown to be of any practical importance.

Structure

Human *PAI-1* is a single-chain glycoprotein with a molecular weight of about 50,000.[42] Recombinant *PAI-1* expressed in *Escherichia coli* has a molecular weight of approximately 43,000 due to the lack of glycosylation in the bacteria. After removal of a signal peptide of 23 amino acids, the mature protein consists of 379 amino acids. *PAI-1* lacks cysteine residues and contains three potential glycosylation sites.[50] It has the typical serpin structure (FIGURE 21.2).

The *PAI-1* molecule is fully active upon synthesis,[63] but under physiologic conditions it seems to be spontaneously transformed into an inactive form. This form can, however, again become active if treated with some denaturing (preferably hydrogen bond–cleaving) substances and allowed to refold.[40,63,64] This form has come to be called "latent" *PAI-1*. The crystal structure of latent *PAI-1* was solved in 1992[65] and revealed that in the latent conformation the RCL (red string in FIGURE 21.2) is fully inserted forming a new strand in the middle of the A β-sheet[37] (yellow portion in FIGURE 21.2). In the active conformation, the RCL is exposed on the surface of the molecule (see FIGURE 21.2).

The Reaction between *PAI-1* and Plasminogen Activators

As mentioned above, the reactions between *PAI-1* and the different plasminogen activators, tPA or uPA, are very rapid. This seems to depend on the presence of second-site interactions [46] in analogy with the reaction between plasmin and antiplasmin.[9,20] Besides the interaction between the active site in the activators and the scissile peptide bond Met[346]-Arg[347] in the RCL in *PAI-1*, an interaction between Glu350 in *PAI-1* with Arg[304] in tPA also seems to be of importance.[66,67]

The formation of the stable complex between a serpin and a serine proteinase occurs by the binding of the RCL to the active site of the proteinase, followed by cleavage of the reactive center peptide bond and formation of an ester bond between the carboxyl group of the P1 residue of the serpin and the hydroxyl group of the active-site serine of the proteinase (P1P1′).[22,48,68,69] The amino acid residues at positions P1–P16 are then inserted into the A β-sheet, and the serine proteinase is transported toward the opposite pole of the RCL in the serpin.[48,69–71]

The inhibition of plasminogen activators by *PAI-1* involves an interaction between the RCL of the inhibitor and the catalytic groove of the proteinase in question. The mobility of the RCL is required for the inhibitory function of *PAI-1* but not for the initial recognition of the plasminogen activators.[72] The basic residue at the P1 position within the RCL plays a crucial role for the uPA inhibitory activity.[73–75] However, the presence of a neutral or hydrophobic residue at this position does not affect the inhibitory activity toward tPA.[76]

Different Molecular Forms of *PAI-1* and Stability

The existence of latent *PAI-1* was described above. The transformation of active to latent inhibitor is a spontaneous process at physiologic conditions. The importance of some residues on β-strand 5A for the activity and latency transition of *PAI-1* has been investigated.[77] It was shown that the side chain of Gln[303] is important since substitution of this residue toward a His increased the latency transition fourfold. Binding of a murine monoclonal antibody produced against the human tPA-*PAI-1* complex induced a 4,000-fold decrease in the half-life of active *PAI-1*.[78] Binding of this antibody seems to cause a partial insertion of the RCL into the *PAI-1* A β-sheet. Different parts of helix F in the *PAI-1* molecule seem also to be involved in the conversion from active to the latent form. When substituting Asp[125] by Lys and Arg[133] by Asp, these variants were rapidly transformed into latent forms.[79] The residue Lys[325], situated at β-strand s5A and normally forming a hydrogen bond with Asn[150] (located on the s3A/hF loop), also seems to be important in regulating the latency transition.[80]

Several studies have been performed to increase the understanding and mechanisms regarding *PAI-1* stability, by generating and investigating the properties of mutants of the molecule. The importance of the positively charged residues (Arg[186], Arg[187], His[190], or Lys[191]) on the turn connecting s4C with s3C for the functional stability of *PAI-1* has been nicely demonstrated by generating double-site mutants.[81] It was earlier suggested that the mobility of loop 1 (His[185]-Pro[200], the turn connecting strand s4C with s3C) is required for transition to the latent form.[82] It has also been demonstrated that mutation of His[185] and His[190] significantly decreased the stability of *PAI-1* at pH 7.5 as well as the pH dependence of stability.[83] Using random mutagenesis,[84] a very stable mutant with a half-life of 145 hours was constructed. This variant had four residues mutated, and it was suggested that large increases in stability require contributions from several amino acid substitutions.

PAI-1 has a significantly increased stability at slightly acidic pH.[85] A decrease in pH is accompanied by protonation of imidazole groups, and it is therefore likely that histidine residues are involved in stabilizing the active form of *PAI-1*. It was speculated that His[143] in helix F, situated in the vicinity of the A β-sheet in the three-dimensional structure of *PAI-1*, could be involved.[86] However, the introduction of mutations at this position did not favor that hypothesis.[87] Instead, it was found that His[364] close to the CT end of the molecule was important for the pH-dependent stability.[83] Other structures may of course also be involved.

By molecular modeling of *PAI-1*, the normal inhibitory function of this molecule has been transformed into a substrate form as well.[88,89] After complex formation, the ester bond is in this case no longer stable, but becomes rapidly hydrolyzed. Substrate behavior has also been induced by insertion of synthetic peptides corresponding to the RCL[90] or by binding of certain monoclonal antibodies directed against the human tPA-*PAI-1* complex.[91]

Interaction with Vitronectin

Already at the discovery of *PAI-1* in plasma, it was found that its gel filtration behavior deviated from expected[42] and suggesting that it was bound in plasma to a specific binding protein.[92] It was subsequently demonstrated that this binding protein was vitronectin.[93,94] Vitronectin is a 75,000 Mr glycoprotein[93] present in plasma at a concentration of about 200 mg/L. When *PAI-1* is purified from human plasma, it copurifies with vitronectin.[94] Although the interaction between vitronectin and latent, or cleaved, forms of *PAI-1* or *PAI-1* in complex with plasminogen activators is weak, vitronectin binds active *PAI-1* with very high affinity.[95,96] The vitronectin-binding sites in *PAI-1* have been localized to the region from Gln^{55} to Lys^{145}.[97–99] In addition, the deletion of residues 127 to 158 decreased the binding of *PAI-1* to vitronectin by a factor of 3,000.[100] This suggests that the most important part for this interaction most probably resides in the portion residues 127 to 145. It has been proposed that, although vitronectin does not bind to the RCL, interactions with vitronectin induce conformational changes in the reactive center of *PAI-1*.[101] The corresponding site in vitronectin seems to be localized to the NH_2-terminal region[102,103] as studied by the binding of fragments from vitronectin to active *PAI-1*.

In plasma, active *PAI-1* is virtually completely bound to vitronectin. This stabilizes the active form of *PAI-1* and prolongs the half-life from 2 to 4 hours regarding the transition of active to the latent molecule.[93,94] Increasing the concentration of NaCl from 50 mM to 1 M dramatically increases the stability of *PAI-1*.[104] It was demonstrated that an anion-binding site is located between strand 5 of the central β-sheet and a small domain constituting the residues 128 to 161 in helix F and the loop connecting to s3A.[105] During the active to latent transformation, the domain containing these residues is directed away from the main body of the protein while RCL is inserted. Thus, a chloride ion in the anion-binding site stabilizes the active conformation.

Interaction with Heparin

Heparin is a cofactor for several serpins and stimulates the inhibition of uPA by *PAI-1*.[106] When *PAI-1* is bound to heparin, the inhibition of thrombin by *PAI-1* is greatly enhanced.[107–110] Based on site-directed mutagenesis studies, the positively charged residues Lys^{65}, Lys^{69}, Arg^{76}, Lys^{80}, Lys^{88}, [108] and Arg^{115} and Arg^{118} [79] in *PAI-1* were suggested to be involved in the *PAI-1* binding to heparin. In addition, it was reported that four residues His^{3}, His^{77}, His^{112}, and His^{229} could also be partly involved in this interaction.[83] The affinity between heparin and *PAI-1* has been used practically to purify *PAI-1* in a gentle and efficient way.[111] However, it is not known if this interaction really is of physiologic significance.

Regulation of *PAI-1*

There is a large diurnal variation of the *PAI-1* concentration in plasma.[112–114] The *PAI-1* concentration is highest in the early morning and then decreases quite rapidly, causing the tPA activity to be lowest in the early morning and instead increasing during the day. The reason for this is that the *PAI-1* gene transcription is regulated by clock genes.[115] *PAI-1* also acts as an acute-phase reactant, which increases during an inflammatory response. Lipopolysaccharide or certain cytokines increase the *PAI-1* expression in endothelial cells in culture.[116–118] The synthesis and secretion of *PAI-1* is, however, affected by many other factors. Insulin induces an increase in *PAI-1* secretion from hepatocytes in culture.[119,120] It was suggested that insulin is a major regulator of the plasma *PAI-1* concentration.[121] In fact, a very strong correlation has been found between plasma insulin antigen and *PAI-1* levels in a study of about 1,500 patients with angina pectoris.[122] On reducing plasma insulin levels and insulin resistance by exercise, by weight loss, or by medication with metformin, the plasma *PAI-1* activity becomes decreased.[123–125] The decreased fibrinolytic capacity frequently seen in type 2 diabetes is most likely due to increased *PAI-1* levels. In a rat model, it was found that hyperglycemia in diabetes mellitus is associated with increased *PAI-1* content in several different types of tissue.[126]

Plasma *PAI-1* correlates positively with the concentration of serum triglycerides.[127,128,129] *In vitro*, it has also been shown that very low density lipoprotein (VLDL) and oxidized low density lipoprotein (LDL) stimulate the production of *PAI-1* in cultured endothelial cells.[130–133]

The 4G/5G polymorphism located 675 bp upstream of the transcriptional start site [59,60] correlates to the plasma *PAI-1* concentration. Individuals who are homozygous for the 4G allele have higher concentrations of *PAI-1* in plasma. It has been proposed that both the 4G and the 5G alleles bind a common factor which may work as a transcriptional activator.[134] It was also proposed that a repressing nuclear protein binds to the 5G allele, which decreases the binding of the activator protein, thus reducing the transcription of *PAI-1*.[134] VLDL seems to induce transcription of *PAI-1* in endothelial cells through binding of a VLDL-inducible transcription factor to residues −672 to 657 in the *PAI-1* promoter.[135)]

Endothelial cell growth factor combined with heparin and agents increasing intracellular cAMP (e.g., forskolin) are among the few substances that down-regulate the *PAI-1* gene expression in endothelial cells in culture.[136,137] The most important factors influencing the plasma *PAI-1* concentrations are summarized in FIGURE 21.3.

Clinical Significance

Increased plasma levels of *PAI-1* have been reported to correlate with the development of thrombotic disease, both venous and arterial.[127,138,139–143] In contrast, a total *PAI-1* deficiency[144–149] has been found to be associated with increased bleeding problems. The genetic background has not been examined in most of the reports concerning individuals with increased bleeding and low or nonmeasurable plasma *PAI-1* concentration. However, a child with complete deficiency of *PAI-1* due to a frameshift mutation was reported.[148] This mutation was a dinucleotide insertion in exon 4 leading to a new stop codon in the *PAI-1* gene. In a later study, this mutation was found among 26 other individuals, 7 of which were homozygous and with a complete *PAI-1* deficiency. These individuals experienced increased bleeding problems after trauma and surgery.[149] The heterozygous individuals, however, did not have any clinical signs of increased bleeding tendency. In addition, individuals with very low or immeasurable *PAI-1* concentrations in plasma, but with a normal gene did not display any significantly increased bleeding tendency.[150,151]

As already mentioned, increased plasma concentrations of *PAI-1* are associated with the development of thrombotic events, such as deep vein thrombosis,[140–143,152] myocardial

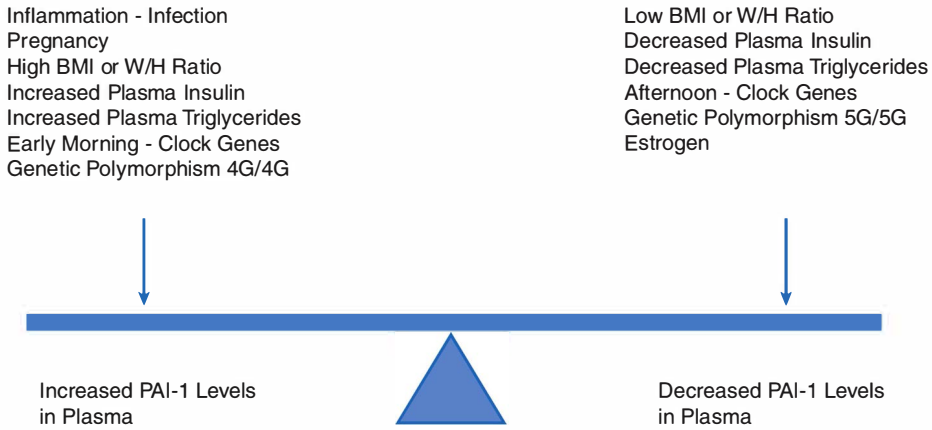

FIGURE 21.3 Factors that influence the plasma *PAI-1* concentration. BMI is body mass index; W/H ratio is waist-to-hip ratio.

infarction,[127,138,139,153] and stroke. *PAI-1* concentrations in plasma are not normally distributed in humans, but it correlates quite well with tPA/*PAI-1* complex that is normally distributed. This complex is also more stable in plasma. For this reason, it might be an advantage to measure this complex in plasma rather than *PAI-1* and use the complex as a risk indicator for myocardial infarction.[153] Further support that *PAI-1* is etiologically involved in the development of thrombosis comes from studies on transgenic mice overexpressing *PAI-1*.[154] The data regarding increased plasma *PAI-1* concentration in connection with myocardial infarction is getting impressive. Prospective data from the SHEEP study of reinfarction in patients with a previous infarction demonstrated that individuals with increased *PAI-1* levels or tPA/*PAI-1* complex in combination with smoking or type II diabetes had five- to eightfold increased risk of reinfarction.[155]

The *PAI-1* in plasma may have different origins. Thus, high plasma concentration in individuals with high waist-to-hip ratio mostly has its origin in the visceral fat, while that seen during an inflammatory process mostly have its origin in the liver. It has been demonstrated that *PAI-1* of different origin has different glycosylation patterns.[156] This can of course be used to determine the origin of plasma *PAI-1*. It is not known if *PAI-1* in plasma of different origin has deviating clinical significance.

PAI-1 is also present in platelets in quite high concentrations.[157,158] However, most of it seems to be latent, even if some individuals can have a large portion of active inhibitor.[159] The reason for this is not known with certainty, but it has been demonstrated that biosynthesis of *PAI-1* may occur within the platelets.[160] If the platelets contain much active *PAI-1*, it might play a role in stabilizing clots and slows down fibrinolysis *in vivo*.[161] The high concentration of mostly inactive *PAI-1* in platelets might cause problems in blood sampling for measurement of *PAI-1* in clinical studies. Under conditions where *PAI-1* leaks from the platelets, such as if the sodium citrate concentration is too low, the *PAI-1* antigen concentrations in plasma might be artifactually increased. The *PAI-1* antigen concentrations in serum are typically about 10 times higher than in plasma.

PAI-2

PAI-2 was first discovered in human placental extracts.[162] Later, it was found in macrophages and in several other different cell lines in culture.[163] In plasma from healthy nonpregnant individuals, it is typically not found in measurable concentrations. However, during pregnancy, *PAI-2* is produced by trophoblasts in the placenta and released into plasma. As a result, high concentrations are found also in plasma from pregnant women.[164,165] *PAI-2* has been purified from placenta and from plasma from pregnant women.[166] The plasma concentration of *PAI-2* increases steadily during pregnancy and reaches concentrations above 35 μg/L, but decreases sharply after delivery.[165,167] This suggests that *PAI-2* may have a hemostatic role during pregnancy and delivery. Also in patients with acute myeloblastic leukemia, plasma *PAI-2* could frequently be found in high concentrations.[168] In fact, *PAI-2* is mostly found as an intracellular protein, and as such, many other functions have been suggested for this serpin.[169]

PAI-2 is a single-chain protein, consisting of 425 amino acids.[170,171] The gene spans 16.5 kb and is located on chromosome 18q22.1.[170,172] *PAI-2* exists in two forms: a secreted glycosylated form with a molecular weight of about 70,000 and an intracellular nonglycosylated form with a molecular weight of about 46,000. *PAI-2* is an efficient inhibitor of uPA.[165] It also inhibits two-chain tPA, but it is a very poor inhibitor of single-chain tPA.[165] *PAI-2* may spontaneously and reversibly polymerize by insertion of the RCL from one *PAI-2* molecule into the A β-sheet in another *PAI-2* molecule.[173,174] The exact physiologic role of *PAI-2* is not known, but a decreased fibrinolytic activity could be important in late-state pregnancy and even more so during delivery. Pregnant women with placental insufficiency typically have low concentration of *PAI-2*.[175] Preeclampsia patients also seem to have lower concentrations of *PAI-2* and higher concentrations of *PAI-1* than women during a normal pregnancy.[176] For a recent review of *PAI-2*, see Croucher et al.[177]

α_2-MACROGLOBULIN

Human α_2-macroglobulin is a large plasma protein, with a molecular weight of about 720,000. It is composed of four identical subunits, each with a molecular weight of 180,000, held together by disulfide bridges and noncovalent interactions.[178] In the central portions of these subunits, a bait region is present, which is easily cleaved by a number of proteases of completely different classes. Cleavage results in a dramatic conformational change, resulting in entrapment of the responsible protease, frequently

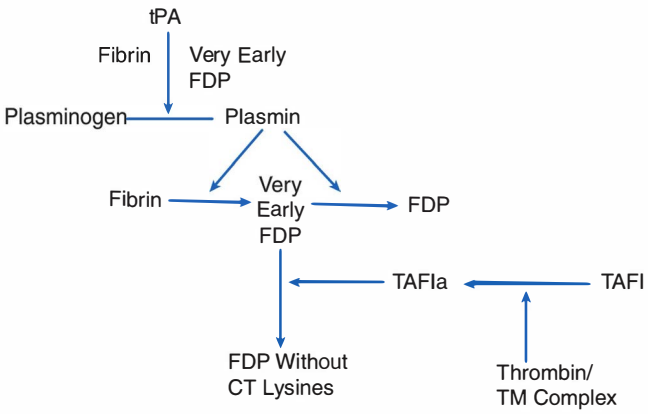

FIGURE 21.4 A schematic picture of the function of TAFI. Fibrin and early fibrin degradation products (FDPs) stimulate plasminogen activation by tPA. If the formed CT lysines are removed by activated TAFI, the FDP no longer stimulates plasminogen activation. TAFI is activated mainly by thrombin in complex with TM.

preserving the active site of this protease, especially toward low molecular weight substrates. α_2-Macroglobulin reacts with plasmin, forming a 1:1 complex. It can typically bind two molecules of low molecular weight proteases/molecule of α_2-macroglobulin, but only one mol/mol of a large protease such as plasmin. Its concentration in plasma is typically between 1.5 and 4 g/L, and its half-life in the circulation is about 6 days. However, α_2-macroglobulin in complex with proteases is rapidly cleared from the circulation by uptake to the reticuloendothelial system by binding to LDL receptor–related protein followed by internalization.[179] Under normal circumstances, α_2-macroglobulin is not a very good inhibitor of plasmin. The reaction rate is too slow to be of physiologic significance. However, during conditions where antiplasmin is severely depleted as is frequently seen in connection with thrombolytic treatment, especially with non–fibrin-specific plasminogen activators (streptokinase or urokinase), α_2-macroglobulin might be very important as a fibrinolytic inhibitor. In fact, under such conditions, when antiplasmin is depleted to a large extent, fibrinogenolysis is often seen and the risk of bleeding complications increases drastically. It is possible that a depletion of α_2-macroglobulin as well would rapidly lead to a deteriorated situation.

TAFI

TAFI is a glycoprotein with Mr 60,000, consisting of 417 amino acids, which is synthesized in the liver. Even if TAFI has the capacity to inhibit or slow down the fibrinolytic process, it is not an inhibitor by itself, but activated, it is a carboxypeptidase type B. As such it has the capacity to remove CT lysine residues, preferably from partially degraded fibrin.[180] Partially degraded fibrin is an important stimulator regarding plasminogen activation by tPA, because the presence of CT lysines increases the number of binding structures for plasminogen (via lysine-binding sites). This causes a rapid and localized activation of plasminogen. Removal of those CT lysine residues in fibrin will slow down the fibrinolytic process (FIGURE 21.4). TAFI therefore may act as an inhibitor of fibrinolytic activity. It is also known as procarboxypeptidase U or plasma procarboxypeptidase B, names that in fact are more accurate. It can be activated by thrombin, trypsin, kallikrein, or plasmin into the active enzyme TAFIa. The most efficient activator seems, however, to be the complex between thrombin and thrombomodulin (TM).[181,182] Activation occurs by releasing a 92-residue activation peptide, generating the active 35-kd enzyme. The physiologic or clinical importance of TAFI still remains to be settled. TAFI-deficient mice did not display any major clinical problems.[183] However, such mice had an easily measurable enhanced endogenous fibrinolytic activity, suggesting that it might play a pathophysiologic role.[184]

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CHAPTER 22

Overview of Megakaryocyte and Platelet Biology

JOEL S. BENNETT

Platelets are circulating anucleate fragments of bone marrow megakaryocytes that provide primary hemostasis by forming hemostatic plugs at sites of vascular injury; likewise, platelet thrombi are predominantly responsible for the morbidity and mortality of arterial vascular disease. The discovery of platelets as formed elements of the blood, the identification of their central role in hemostasis and thrombosis, and the research that has led to our current understanding of the mechanism and regulation of platelet production and function have been outlined in excellent reviews by Coller[1] and Coller and Shattil.[2] The chapters in this section of the textbook provide detailed analyses of the current understanding of platelet production and function.

PLATELET PRODUCTION FROM MEGAKARYOCYTES

Megakaryocyte development and the subsequent production and release of platelets are unique phenomena that are discussed in Chapters 23 to 25. Megakaryocytes are relatively rare cells in the bone marrow, constituting <1% of marrow cells, but they are also the largest marrow cells with diameters of up to 40 to 50 μm. They are located in the extravascular compartment of the bone marrow along the albuminal face of the sinusoidal endothelium, frequently in clusters (FIGURE 22.1). Despite their apparent scarcity, sufficient numbers of megakaryocytes are present in the marrow to produce the 10^{11} platelets per day required to maintain the normal steady-state level of circulating platelets.[3] In order to meet this need, megakaryocytes have evolved a unique maturation process in which the diploid DNA of megakaryocyte precursors undergoes repeated cycles of duplication (endomitosis), reaching a modal DNA level of 16N or greater, but without cell division.[4] This results in a cell with a single polylobulated nucleus and with an expansive cytoplasm whose fragmentation is the source of circulating platelets. Although the mechanism responsible for endomitosis in megakaryocytes is not known, it is thought to be related to failure to develop and sustain a cleavage furrow during the late stage of mitosis with subsequent absence of cytokinesis. Recent evidence indicates that this is associated with the RUNX1-induced downregulation of the nonmuscle myosin IIB heavy chain,[5] a component of the contractile ring that generates the forces necessary for cytokinesis, and with downregulation of the RhoA-specific guanine-nucleotide exchange factors (GEFs) GEF-H1 and ECT2,[6] thereby preventing the RhoA signaling required for the formation of the contractile ring.

As discussed in Chapter 24, the conversion of megakaryocyte cytoplasm into platelets has been studied predominantly *in vitro* using cultured murine megakaryocytes. This process begins with the generation of large pseudopod-like structures from one pole of the megakaryocyte that elongate, in a microtubule-driven process, into long thin branching tubular projections termed proplatelets, which are themselves comprised of tandem arrays of platelet-sized swellings connected by thin cytoplasmic bridges.[7] When this process was observed *in vivo* in mouse skull bone marrow using multiphoton intravital microscopy, megakaryocytes were observed to extend cellular processes, either pseudopodia or immature proplatelets, into bone marrow sinusoids.[8] This implies that intravascular shear forces may help to separate proplatelets from the megakaryocytes and that proplatelet morphogenesis into platelets occurs in the peripheral blood.

The primary humoral regulator of megakaryocyte proliferation and differentiation is thrombopoietin (TPO), a 331-amino acid homologue of erythropoietin and a member of the four-helix bundle family of cytokines[3] (see Chapter 25). TPO is synthesized predominantly by the liver, but constitutive TPO mRNA expression can be detected in kidney, smooth muscle cells, and other tissues as well. Moreover, TPO mRNA transcription can be induced in bone marrow stromal cells by substantial degrees of thrombocytopenia, and transcription in the liver can be enhanced by the inflammatory response mediator IL-6, perhaps accounting for the reactive thrombocytosis that often accompanies inflammatory states. However, absence of TPO or its receptor (see below) does not preclude platelet production, and platelet counts remain approximately 10% of normal following their genetic elimination in mice. This suggests that other hematopoietic cytokines also support platelet production. Although IL-3, IL-6, and IL-11 stimulate megakaryocyte colony formation *in vitro*, crossing TPO-receptor null mice with mice null for signaling by these cytokines does not eliminate basal thrombopoiesis. On the other hand, the chemokine SDF-1 synergizes with TPO to support megakaryopoiesis, suggesting that it may support at least a portion of basal platelet production.

The receptor for TPO on megakaryocytes and platelets is c-Mpl, the protooncogene of the murine myeloproliferative leukemia virus and a member of the type 1 hematopoietic cytokine receptor family.[3,4] c-Mpl is also present on hematopoietic stem cells that require TPO stimulation for maintenance and expansion. The *c-Mpl* gene encodes a 610-amino acid protein that resides on cell surfaces as a constitutive homodimer. TPO binding to the homodimer extracellular domain is thought to induce a conformational change in the homodimer that enables autophosphorylation and activation of JAK family kinases (JAK2 and TYK2) associated with its cytoplasmic domains. In addition to phosphorylating the c-Mpl cytoplasmic domains

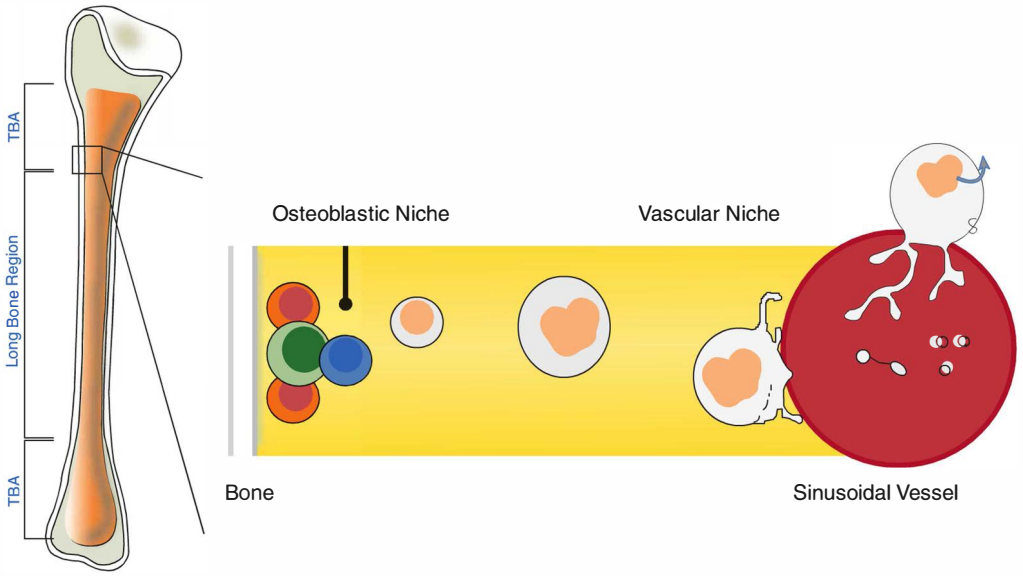

FIGURE 22.1 Diagrammatic representation of megakaryocyte production and platelet production. Hematopoietic stem cells located in the osteoblastic niche next to the endosteal bone surface migrate to the center of the marrow cavity where they undergo successive cycles of endomitosis to produce polyploidy megakaryocytes. Mature megakaryocytes then extend proplatelet elongations into marrow sinusoidal blood vessels where the proplatelets are released and ultimately release individual platelets. (Adapted from Thon JN, Italiano JE. Platelet formation. *Semin Hematol* 2010;47: 220–226, with permission.)

themselves, thereby providing binding sites for signaling molecules, activated JAK phosphorylates a number of signaling molecules that promote cell survival and proliferation, including members of the signal transducer and activator of transcription (STAT 1, 3, and 5), MAPK, and phosphotidylinositol-3 kinase pathways, as well as proteins that limit cell signaling including the phosphatases SHP1 and SHIP1 and members of the suppressors of cytokine signaling (SOCS) family. There are also a small of number of copies of c-Mpl expressed on the platelet surface (25 to 100 per platelet). Although TPO binding to platelet c-Mpl does not by itself result in platelet activation, it does prime platelets for activation by the classical platelet agonist, perhaps enhancing platelet reactivity in the presence of thrombocytopenia. On the other hand, TPO binding to c-Mpl on platelets and megakaryocytes is thought to play an important role in regulating the concentration of TPO in plasma. TPO adsorbed to the platelet surface is internalized and metabolized. Thus, in the presence of thrombocytopenia, the TPO concentration in plasma increases, stimulating megakaryocyte proliferation; conversely, the TPO concentrations decrease in the presence of thrombocytosis because of an increase in platelet-mediated clearance.[9] The low TPO concentrations observed in autoimmune thrombocytopenia has been attributed to increased TPO clearance by the hyperplastic megakaryocytes seen in this disorder.[10]

ELEMENTS OF PLATELET FUNCTION

Platelet Adhesion

Platelets circulate for 7 to 10 days in a nonreactive state in humans until they are needed for hemostasis. The nonreactive state of circulating platelets is maintained, at least in part, by the inhibitory effect of nitric oxide and prostacyclin secreted by endothelial cells, which increase the platelet content of cyclic AMP (cAMP) and cyclic GMP (cGMP), respectively,[11] by the ability of the endothelial cell ecto-ADPase CD39 to metabolize ambient plasma adenosine diphosphate (ADP),[12] through the inhibitory effect of platelet proteins such as PECAM-1,[13,14] and because the negative surface charge of circulating platelets inhibits their interaction with each other and with erythrocytes, leukocytes, and the vascular endothelium. Further, the intact endothelium presents a barrier separating platelets from adhesive substrates in the subendothelial connective tissue matrix. Disruption of the endothelial barrier by trauma, or by a disease such as atherosclerosis, allows platelets to come in contact with and adhere to the subendothelial matrix.

At the low shear rates present in the venous circulation, the integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ can mediate the binding of unactivated platelets to the subendothelial matrix components collagen, fibronectin, and laminin, respectively, at least *in vitro*.[15] However, as discussed in Chapters 28 and 29, at the higher shear rates present in arteries, arterioles, and the microcirculation, efficient platelet adhesion requires the additional presence of subendothelial von Willebrand factor (vWF).[16,17] vWF is an elongated multimeric glycoprotein synthesized by endothelial cells and megakaryocytes that binds to the GPIb-IX-V complex on unactivated platelets and to the integrin $\alpha_{IIb}\beta_3$ on activated platelets.[18] Although vWF is present in plasma, it is normally not present on the platelet surface. However, exposing vWF *in vitro* to the antibiotic ristocetin or to the snake venom protein botrocetin induces vWF binding to GPIb-IX-V.[19,20] As discussed in Chapter 26A, the factors that enable platelets to

adhere to vWF *in vivo* are likely shear stress-induced changes in the conformations of vWF, GPIb-IX-V, or both and/or changes in the conformation of vWF that occur when it is incorporated into the subendothelium.[21,22] Regardless, the bonds between vWF and GPIb-IX-V are characterized by rapid on and off rates so that platelets tend to translocate along, rather than firmly adhere to, vWF-coated surfaces.[23,24] This reduces platelet velocity, facilitating subsequent adhesion and activation events that would otherwise not occur under high-flow velocity conditions.

Collagen is not only a substrate for platelet adhesion, but it is a binding site for vWF in the subendothelium and an agonist for platelet aggregation and secretion, as discussed in Chapters 28 and 30. Although a number of platelet proteins have been found to interact with collagen, only two, the immunoglobulin superfamily member glycoprotein VI (GPVI)[25] and the integrin $\alpha_2\beta_1$ (also known as GPIa-IIa),[26] are physiologic platelet receptors for collagen. As currently understood, the relatively low affinity interaction of GPVI with collagen, mediated by the more distal of its two extracellular Ig-C2–like domains[27] and glycine-proline-hydroxyproline triplets in collagen,[28] initiates a signal transduction activation pathway via the Fc receptor γ chain noncovalently associated with GPVI.[29] This eventually results in the activation of phospholipase Cγ2 and the hydrolysis of the relatively scarce membrane phospholipid phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3) to activate protein kinase C (PKC) and increase the intracellular concentration of Ca^{2+}, respectively.[30] In turn, the latter increase the affinity of α2β1 for glycine-x-hydroxyproline-glycine-glutamic acid-arginine (GxOGER) motifs, enabling activated platelets to adhere firmly to collagen.[31,32]

Platelet Aggregation

Following adhesion, platelets aggregate to form hemostatic plugs or platelet-rich thrombi. In contrast to platelet adhesion, platelet aggregation is an active metabolic process in which agonist binding to specific membrane receptors initiates signaling pathways that enable the integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) to bind soluble macromolecular ligands such as fibrinogen or vWF.[33] The fibrinogen and/or vWF bound to $\alpha_{IIb}\beta_3$ then crosslinks platelets into a hemostatic plug or thrombus. The events responsible for platelet aggregation are summarized in Chapters 26B and 31.

$\alpha_{IIb}\beta_3$, the platelet structure directly responsible for platelet aggregation, is a calcium-dependent, noncovalent heterodimer composed of two type I transmembrane glycoproteins, α_{IIb} and β_3.[33] Each glycoprotein contains a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain that can interact with a variety of cytoskeletal and signaling proteins.[34] There are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ per platelet, making it the most densely expressed platelet membrane protein.[35] Based on the estimated platelet surface area, $\alpha_{IIb}\beta_3$ molecules are spaced <200 Å apart. Rare congenital deficiency or dysfunction of $\alpha_{IIb}\beta_3$ produces the autosomal recessive bleeding disorder Glanzmann thrombasthenia (GT).[36] Patients with GT usually present in infancy with mucocutaneous bleeding that can be severe.

Crystal structures for the extracellular domains of $\alpha_{IIb}\beta_3$ and the related integrin $\alpha_v\beta_3$ indicated that these domains are bent over on themselves when the integrins are inactive; accompanying electron microscope images suggested that the molecules are extended when the integrins are active.[37–39] Ligand binding to $\alpha_{IIb}\beta_3$ requires that its extracellular domain shifts from a closed low-affinity to an open high-affinity conformation, the end result of a concerted "switchblade-like" rearrangement of the entire heterodimer that begins with separation of the cytoplasmic and transmembrane domains.[38] The resting conformation of $\alpha_{IIb}\beta_3$ appears to be maintained by an extended interface involving portions of its cytoplasmic, transmembrane, and extracellular stalk domains.[40] $\alpha_{IIb}\beta_3$ activation occurs when this interface is disrupted by binding of the cytoskeletal proteins talin and kindlin-3 to the β_3 cytoplasmic domain.[41] In turn, this causes transmembrane and stalk domain separation and the exposure of ligand-binding sites on the $\alpha_{IIb}\beta_3$ extracellular domain (FIGURE 22.2).

The major $\alpha_{IIb}\beta_3$ ligand is fibrinogen.[33] Fibrinogen is composed of pairs of Aα, Bβ, and γ chains folded into three nodular domains. Although peptides corresponding to the carboxyl terminal 10 to 15 amino acids of the γ chain or containing the arginine-glycine-aspartic acid (RGD) motif present at two places in the α chain can inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$, fibrinogen binding is mediated by the γ chain sequences.[42,43] Nevertheless, clinical studies confirm that RGD-containing peptides inhibit $\alpha_{IIb}\beta_3$ function.[44] Based on the three-dimensional structure of fibrinogen, the ability of RGD-based peptides and peptidomimetics to inhibit $\alpha_{IIb}\beta_3$ function would seem paradoxical, but crystal structures of RGD and γ chain peptides bound to $\alpha_{IIb}\beta_3$ revealed that the RGD and γchain–binding sites on $\alpha_{IIb}\beta_3$ overlap.[45]

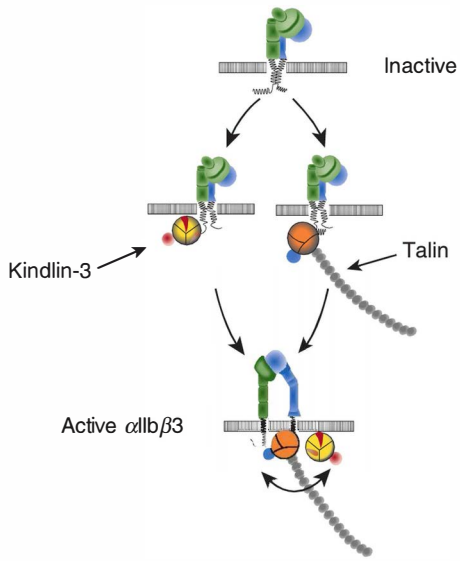

FIGURE 22.2 Activation of $\alpha_{IIb}\beta_3$ by talin and kindlin. Cartoon illustrating that both talin and kindlin-3 must bind to the β_3 cytosolic domain to cause $\alpha_{IIb}\beta_3$ activation by inside-out signaling. Whether there is a preferred sequence of protein binding to β_3 remains to be determined. (Adapted from Moser M, Legate KR, Zent R, et al. The tail of integrins, talin, and kindlins. *Science* 2009;324:895–899, with permission.)

Platelet Agonists

Agonist-stimulated platelet activation is required to enable binding of soluble ligands to $\alpha_{IIb}\beta_3$. As discussed in Chapter 33, the receptors responsible for platelet activation by the classical platelet agonists have all been identified. The serine protease thrombin is the most potent platelet agonist. Human platelets contain two signaling receptors for thrombin, PAR1, and PAR4.[46,47] Although PAR1 and PAR4 are members of the large family of GTP-binding protein (G-protein)–coupled seven transmembrane domain receptors, they are unique in that they are activated when thrombin cleaves their amino-termini to expose new termini that act as tethered intramolecular ligands.[48,49] Tethered ligand binding, in turn, activates the G-proteins G_q, $G_{12/13}$, and G_i to stimulate phospholipase Cβ2 activity, increase cytosolic calcium, induce platelet shape change, and inhibit platelet adenylyl cyclase. Since higher thrombin concentrations are required to cleave PAR4 than PAR1, it is likely that PAR1 is the predominant receptor for thrombin at the low thrombin concentrations needed to activate platelets, but once activated, PAR4 signaling is more prolonged than PAR1.[50,51]

ADP, the first platelet agonist to be identified, is secreted by stimulated platelets from storage sites in platelet-dense granules and may be released from damaged tissue and red cells. There are at least three platelet receptors for ADP.[52] The first, $P2X_1$, is an ATP-gated nonselective Ca^{2+} channel whose physiologic function in platelets is unclear, although $P2X_1$ stimulation by ATP *in vitro* can amplify the platelet response to low concentrations of other agonists.[53] The other two receptors are G-protein–coupled seven transmembrane domain proteins. ADP binding to $P2Y_1$ activates phospholipase Cβ via the G-protein G_q and induces platelet shape change, perhaps via the G-protein $G_{12/13}$.[52,54] ADP binding to $P2Y_{12}$ inhibits platelet adenylyl cyclase via the G-protein G_i.[52] $P2Y_{12}$ is the pharmacologic target for clopidogrel and other thienopyridines.[52] Simultaneous stimulation of both $P2Y_1$ and $P2Y_{12}$ is required to induce platelet aggregation.[54,55] Moreover, because antagonism of either the G_q- or G_i-coupled ADP receptor can be overcome by stimulating another platelet receptor coupled to G_q or G_i, respectively, it appears that signals emanating each G-protein are needed to activate $\alpha_{IIb}\beta_3$.[56]

Agonist-stimulated platelets synthesize prostaglandins from arachidonic acid released from membrane phospholipids by the enzyme phospholipase A_2[57] (see Chapter 34). The released arachidonic acid is then converted by the enzyme prostaglandin endoperoxide H synthase-1 (cyclooxygenase-1) to the prostaglandin endoperoxide intermediates PGG_2 and PGH_2 and subsequently to TXA_2 by the enzyme thromboxane synthase.[58] The endoperoxide intermediates and TXA_2 exit from the platelet and function as platelet agonists and vasoconstrictors by binding to the G_q-coupled thromboxane receptor TP.[59] Two TP isoforms (α and β) result from alternative splicing of the TP mRNA transcript, but only TPα protein has been detected in human platelets.[60] Inhibition of TXA_2-stimulated platelet aggregation by a $P2Y_{12}$ antagonist indicates that like thrombin and ADP, concurrent stimulation of a G_i-coupled receptor is required to complement TP-mediated activation of G_q in order to induce platelet aggregation.

Epinephrine stimulates platelets by binding to a_2A-adrenergic receptors that are coupled to the G-protein G_i and acts in synergy with agonists like thrombin and ADP to induce platelet aggregation.[61] Platelets also express G_q-coupled receptors for serotonin ($5\text{-}HT_{2A}$),[62] vasopressin (V_1),[63] and platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF-acether).[64] The latter is a lipid synthesized by a variety of cells, including neutrophils, eosinophils, monocytes, platelets, endothelial cells, and mast cells, and activates platelets at nanomolar concentrations.

In contrast to the platelet stimulatory effect of hydrolyzing platelet membrane phosphatidylinositol 4,5 bisphosphate, increasing platelet concentration of cAMP and cGMP inhibits most aspects of platelet function.[65] cAMP is synthesized by a membrane-associated enzyme complex, adenylyl cyclase, which converts ATP to cAMP. cAMP increases result from stimulation of adenylyl cyclase by prostaglandins such as PGD_2, PGE_1, and PGI_2 (prostacyclin) or by low concentrations of adenosine, as well as by inhibition of the phosphodiesterases responsible for cAMP degradation.[66] cGMP synthesis results from stimulation of platelet guanylyl cyclase by nitric oxide.[67] Whereas stimulating adenylyl or guanylyl cyclase inhibits $\alpha_{IIb}\beta_3$ function, inhibiting these enzymes by stimulating agonist receptors coupled to G_i does not cause $\alpha_{IIb}\beta_3$ activation.[68] Nonetheless, stimulating these receptors does prevent the increased cAMP synthesis stimulated by PGD_2, PGE_1, and PGI_2.

There is limited understanding of the biochemical pathway coupling agonist stimulation to $\alpha_{IIb}\beta_3$ activation. It is known that increases in cytosolic free calcium, PKC activation, and the active form of the diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) activate the small GTPase Rap1.[69–71] By interacting with an effector scaffold, Rap1-GTP-interacting adaptor molecule or RIAM, activated Rap1 directs RIAM-bound talin to the plasma membrane where it can bind to β integrin cytoplasmic tails, causing integrin activation.[72–74] Whether this is the only, or even the predominant, pathway for $\alpha_{IIb}\beta_3$ activation in platelets is not known. Although the Rap1-RIAM-talin complex reconstitutes $\alpha_{IIb}\beta_3$ activation in transfected CHO cells, deletion of Rap1b from murine platelets attenuated, but did not eliminate, agonist-stimulated platelet aggregation and fibrinogen binding to $\alpha_{IIb}\beta_3$,[75] implying that other pathways are present to couple agonist stimulation to $\alpha_{IIb}\beta_3$ activation.

Platelet Secretion

The platelet cytoplasm contains four types of granules: three to eight dense (δ) granules containing ADP, ATP, calcium, serotonin, and polyphosphate; approximately 50 α-granules containing a variety of proteins, some derived from the plasma such as fibrinogen, vWF, factor V, albumin, and IgG and others synthesized by the megakaryocyte such as platelet factor 4, β-thromboglobulin, platelet-derived growth factor (PDGF), and thrombospondin; lysosomes containing acid hydrolases; and microperoxisomes containing a peroxidase activity. δ-Granule ADP is thought to be involved in propagating the primary platelet response, α-granule PDGF in wound repair, and α-granule factor V in thrombin generation. The function of other α and δ-granule substances is unclear, since many are already present at high concentration in plasma. Nevertheless, platelet secretion disorders are a frequent cause of a mild- to-moderate bleeding, implying that platelet secretion plays a role in supporting normal hemostasis.

The contents of these granules are extruded following platelet stimulation, a process known as platelet secretion. The α-granule membrane also contains many of the same proteins as the platelet plasma membrane ($\alpha_{IIb}\beta_3$ and GPIb-IX-V), as well as others specific for α-granules (P-selectin and osteonectin). Each α-granule membrane protein can be translocated to the platelet surface during secretion. Secretion stimulated by weak agonists such as ADP and epinephrine requires platelet aggregation, the synthesis of prostaglandin endoperoxides and thromboxane A_2, and is prevented by inhibiting prostaglandin synthesis.[76] On the other hand, secretion stimulated by strong agonists such as thrombin and collagen can bypass the aggregation and prostaglandin requirements.

Platelets contain components of the secretory machinery similar to those found in neurons where granule secretion is governed in part by matching a vesicle SNARE (v-SNARE) with a target membrane SNARE (t-SNARE).[77,78] As discussed in detail in Chapter 32, platelets contain four v-SNARES (VAMPs 2, 3, 7, and 8), and studies using platelets from knockout mice suggest that VAMP-3, and in particular VAMP-8, are important for platelet secretion. Platelets also contain members of two t-SNARE families (syntaxins 2, 4, 7, 11 and SNAP 23, 25, 29). SNAP-23 is phosphorylated on serine residues after platelet stimulation and may be a link between platelet activation and granule membrane fusion. Similarly, there are data suggesting roles for syntaxins 4 and 2 in α-granule and lysosome secretion and in α-granule, δ-granule, and lysosome secretion, respectively. It is noteworthy that syntaxin 11 is mutated in familial hemophagocytic lymphohistiocytosis type 4, and platelets from FLH4 patients have impaired platelet secretion.[79] Platelets also contain a number of SNARE regulators, including members of the Munc18, Rab, Munc13, synaptotagmin, and tomosyn families, whose apparent roles in platelet secretion are to enhance the efficiency of SNARE-mediated exocytosis, primarily by interacting with syntaxins or syntaxin-containing complexes. Thus, substantial progress has been made in identifying the components of the platelet secretory mechanism, but how these components assemble to couple platelet stimulation to fusion of platelet granule and plasma membranes is not yet understood.

Platelet Procoagulant Activity

The plasma membrane of activated platelets provides a procoagulant surface for the assembly of the "tenase" and "prothrombinase" complexes that activate coagulation factor X and prothrombin, respectively, a result of the calcium-induced exposure of anionic phospholipids on the platelet surface[80] (see Chapter 35). The phospholipid composition of the platelet plasma membrane bilayer is asymmetric with phosphatidylcholine and sphingomyelin localized predominantly in the outer membrane leaflet and phosphatidylserine and phosphatidylethanolamine concentrated in the inner leaflet.[81] This asymmetry is established by two enzymatic activities: a "flippase" directing the inward transport of lipids[82] and a "floppase" that directs the transport of choline- and amino-phospholipids to the outer leaflet.[80] Following an agonist-stimulated rise in intraplatelet Ca^{2+}, membrane asymmetry is lost with exposure of anionic phospholipids on the platelet surface, as well as on the surface of shed platelet membrane vesicles or microparticles[83] (see Chapter 36). The Ca^{2+}-induced movement of phospholipids between membrane leaflets in platelets and other cells appears to be mediated by a membrane-associated phospholipid scramblase activity, an essential component of which is the protein TMEM16F.[84] It is noteworthy that when TMEM16F expression is knocked down in Ba/F3 cells, Ca^{2+} ionophore-stimulated anionic phospholipid exposure is decreased. Moreover, TMEM16F mutations have been identified in two patients with isolated congenital deficiency of platelet procoagulant activity (Scott syndrome), further supporting its role as a component of scramblase.[84,85]

PLATELET FUNCTION UNRELATED TO HEMOSTASIS

Although the most important function of platelets is to provide primary hemostasis, it is also becoming clear that platelets contribute to a number of processes in addition to hemostasis such as inflammation,[86] tissue regeneration,[87] host defense,[88,89] angiogenesis,[90] lymphatic development,[91] and tumor metastasis.[92] A role for platelets outside of hemostasis has been the topic of a number of recent reviews.[86,93,94,95]

Activated platelets express on their surface and secrete a variety of bioactive molecules that participate in the processes listed above[93] (Table 22.1). Platelet α-granule contents contain growth factors that support processes such as wound repair and immunomodulation including platelet-derived growth factors A, B, and C (PDGF A, B, C); vascular endothelial growth factor (VEGF); fibroblast growth factor (FGF); epithelial growth factor (EGF); connective tissue growth factor; hepatocyte growth factor; transforming growth factor β (TGFβ); and insulin-like growth factor-1. For example, PDGF is instrumental in initiating and regulating tissue wound repair and platelet α-granules are the largest source of TGFβ in the blood where it functions as an antiproliferative and immunosuppressive factor. The α-granule membrane also contains CD40 ligand (CD40L, CD154) that is translocated to the platelet surface following platelet activation,[86,96] where it either remains membrane bound or is cleaved from the platelet surface, primarily by MMP-2, as soluble CD40L (sCD40L).[97] In either case, it transmits the effect of platelet activation to CD40 expressed by endothelial cells, leukocytes, macrophages, dendritic cells, smooth muscle cells, fibroblasts, and keratinocytes, affecting fundamental process such as atherogenesis and innate and adaptive immunity.[98]

Besides functioning as a neurotransmitter, serotonin is a mitogen and modulates tissue remodeling.[99] In a mouse model of liver regeneration following partial hepatectomy, thrombocytopenia and impaired platelet function were associated with decreased liver cell proliferation, effects that were reversed by a serotonin receptor agonist.[87] Because the vast majority of serotonin in the blood is present in platelet δ-granules, partial hepatectomy was performed in tryptophan-hydroxylase 1–deficient mice whose platelets lack serotonin, resulting in a decrease in all markers of hepatocyte proliferation. Restoration of platelet δ-granule serotonin by infusing 5-hydroxytrytophan also restored the markers of hepatocyte proliferation, consistent with a role for platelet-derived serotonin in the initiation of liver regeneration.[87]

Platelet activation leads to the release of more than a dozen cytokines and chemokines from platelet granules including platelet factor 4 (PF4, CXCL4), CXCL7, and IL-1.[94] PF4 binds with high affinity to glycosaminoglycans (hence its designation

Table 22.1

Platelet-associated molecules with immune function

Molecule	Location within Platelet and Mechanism of Release	Activation State	Functions	Cellular Targets
Histamine	Synthesized, location unknown; released during platelet activation	Preformed	Promotes allergic type hypersensitivities	Endothelial cells, monocytes, PMNs, NK cells, T cells, B cells, eosinophils
Serotonin (also known as 5-HT)	Dense granules; released during platelet activation	Preformed	Actions on central nervous system; promotes coagulation and T-cell activation	Monocytes, macrophages, platelets
Thromboxane A2	Plasma membrane	Synthesized	Promotes inflammation and coagulation	Platelets, macrophages, T cells
PAF	Plasma membrane	Synthesized	Promotes inflammation	Platelets, PMNs, monocytes, macrophages
PDGF	α-Granules; released during platelet activation	Preformed	Promotes wound healing	Monocytes, macrophages, T cells
TGFβ	α-Granules; released during platelet activation	Preformed	Growth inhibition; immunosuppression	Monocytes, macrophages, T cells, B cells
CXCL7 (also known as NAP2)	Not known	Cleavage product of performed precursor	Chemokine	PMNs
CXCL4 (also known as PF4)	α-Granules; released during platelet activation	Preformed	Chemokine	PMNs, platelets
CXCL1 (also known as GROα)	α-Granules; released during platelet activation	Preformed	Chemokine	PMNs
CXCL5 (also known as ENA78)	α-Granules; released during platelet activation	Preformed	Chemokine	PMNs
CCL5 (also known as RANTES)	α-Granules; released during platelet activation	Preformed	Chemokine	Monocytes, eosinophils, basophils, NK cells, T cells, dendritic cells (DCs), platelets
CCL3 (also known as MIP1α)	α-Granules; released during platelet activation	Preformed	Chemokine	Monocytes, eosinophils, basophils, NK cells, DCs
CCL7 (also known as MCP3)	Not known	Preformed	Chemokine	Monocytes, basophils, NK cells, DCs
IL-1β (and IL-1 precursor protein)	Not known	Synthesized	T-cell activation; multiple effects	Monocytes, macrophages, DCs, T cells
HMGB1	Not known	Preformed	Inflammatory gene regulation	Macrophages, PMNs, endothelial cells
Thrombocidins 1 and 2	α-Granules; released during platelet activation	Preformed	Antibacterial peptides	Bacterial cells
CD40	Plasma membrane	Preformed	Costimulation; endothelial interactions	T cells

Table 22.1

Platelet-associated molecules with immune function *(Continued)*

Molecule	Location within Platelet and Mechanism of Release	Activation State	Functions	Cellular Targets
CD154	α-Granules and plasma membrane; released and cleaved	Preformed	Costimulation; endothelial interactions	B cells, DCs, macrophages, monocytes, endothelial cells
TLR1, TLR2, TLR3, TLR5, TLR6, TLR7	Plasma membrane	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes, platelets
TLR4	Plasma membrane	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes
TLR9	Unknown internal site; upregulated on plasma membrane during platelet activation	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes
TREM1 ligand	Plasma membrane; expression increased during platelet activation	Preformed	Pathogen detection	PMNs, DCs, macrophages, monocytes

Adapted from Semple JW, Italiano J E Jr, Freedman J. Platelets and the immune continuum. ***Nat Rev Immunol*** 2011;11:264–274, with permission.

as platelet antiheparin factor), as well as to a chemokine receptor CXCR3.[100,101] Although the physiologic function of PF4 is not known with certainty since it has both pro- and anti-inflammatory and both pro- and antiproliferation activities, it has been reported to be a negative autocrine regulator of megakaryopoiesis *in vivo*[102] and to impair T-cell proliferation and cytokine release and inhibit the angiogenic activity of endothelial cells in *in vitro* experiments. CXCL7 is derived from the proteolysis of pre-platelet basic protein into platelet basic protein, connective tissue–activating protein III, β-thromboglobulin, and CXCL7 (neutrophil-activating peptide 2).[94] By binding to CXCR1 and CXCR2, CXCL7 induces neutrophil adhesion and migration. IL-1 is noteworthy because platelet stimulation has been reported to induce splicing of IL-1β precursor mRNA present into platelets to IL-1β mRNA, which is then used as a template for IL-1β synthesis.[103] The synthesized IL-1β is then packaged into microparticles. Platelet microparticles have been detected in the joint fluid of patients with rheumatoid and other forms of inflammatory arthritis where the IL-1 they contain was thought to be proinflammatory, eliciting cytokine responses from synovial fibroblasts.[104]

Platelets have been found to express the pattern recognition Toll-like receptors 1 to 9 (TLRs), implying that platelets participate in innate immune responses.[93] TLR4 is a binding site for bacterial lipopolysaccharide (LPS), and LPS-induced thrombocytopenia has been attributed to LPS binding to TLR4.[105] Moreover, platelets contain all of the components of the LPS-signaling complex (TLR4, CD14, MD2, and MYD88), which may explain the ability of LPS to cause platelet activation.[106] In addition, LPS binding to TLR4 induces platelet binding to adherent neutrophils, neutrophil activation, and the release of intravascular webs of histone-containing DNA (neutrophil extracellular TRAPs, NETs) in liver sinusoids and pulmonary capillaries that have the capacity to trap circulating bacteria.[107] TLR2 is a natural ligand for lipoteichoic acid, a major constituent of the cell wall of gram-positive bacteria, but mixed and often contradictory effects of this interaction on platelet function have been reported.[89,108]

Thrombocytopenia has been associated with poorer outcomes in patients infected with malaria.[109] Because platelets bind to erythrocytes infected with malarial parasites via CD36,[110] it has been postulated that platelets can either positively or negatively affect the course of malaria. Platelets have been found to kill intraerythrocytic malarial parasites and have a favorable effect on survival after infection, an effect that appears to be reversed by aspirin treatment.[109, 111] Nonetheless, the effect of platelets is not straightforward since PF4 secreted by platelets has been reported to promote the development of experimental cerebral malaria by enhancing KLF4-dependent cytokine production by monocytes; in this case, the deleterious effects of platelets were reduced by platelet depletion or aspirin treatment.[112,113]

Besides directly supporting the integrity of the vasculature,[114] platelets participate in various aspects of vascular development. For example, platelets contribute to lymphatic vascular development by interacting with lymphatic endothelial cells through the C-type lectin-like receptor 2 (CLEC-2) and a platelet-signaling pathway that includes the Syk pathway–signaling component SLP-76.[91] Platelets have also been found to contribute to the postnatal occlusion of the ductus arteriosus, at least in newborn mice, by promoting its thrombotic occlusion and subsequent luminal remodeling.[115] New vessel development or angiogenesis can be either promoted (VEGF, bFGF, EGF, PDGF, and various matrix metalloproteinases [MMP]) or inhibited (endostatin, PF4, thrombospondin-1, α2-macroglobulin, PAI-1, and angiostatin) by factors present in platelet α-granules. Recent evidence suggests that pro- and antiangiogenic factors are segregated into separate α-granule populations that are also differentially secreted in response to platelet agonists.[116] Thus, proangiogenic

factors are secreted in response to platelet PAR1-selective agonists, whereas antiangiogenic factors are secreted in response to PAR4-selective agonists, suggesting a mechanism by which platelets can either stimulate or inhibit angiogenesis.

In addition to affecting the growth of tumors by either promoting or inhibiting tumor angiogenesis depending on microenvironmental circumstances, a number of mouse models suggest that platelets also play a role in tumor cell metastasis.[92] Thus, heteroaggregates of platelets and tumor cells or platelets, leukocytes, and tumor cells may protect circulating tumor cells from destruction by natural killer (NK) cells, may facilitate either transient or stable adhesion of tumor cells to vascular endothelium, and may modulate the extravasation of tumor cells out of the blood stream into target organs, either directly or by increasing vascular permeability. Despite the elegance of many of these models of tumor metastasis, they are by necessity contrived to greater and lesser extents. Thus, whether platelets play an important role in tumor metastasis in humans and whether intervention with antiplatelet therapy can be clinically beneficial will require continued investigation.

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CHAPTER

23

Platelet Production: Cellular and Molecular Regulation

ELISABETH CRAMER BORDÉ AND WILLIAM VAINCHENKER

Platelets are derived from the fragmentation of the cytoplasm of giant bone marrow cells, the megakaryocytes (MKs). MKs are the largest cells of the marrow, up to 50 μm in diameter, and are characterized by a single polylobulated nucleus. They constitute <0.05% of marrow cells. MKs arise and mature in the bone marrow, along with the precursors of other blood cells. Immature MKs are located near the osteoblastic niche and migrate to the subenthothelium of venous sinusoids during maturation (FIGURE 23.1). Given a normal platelet count (150 to 400 $\times$ 10^9/L) and platelet life time (8 to 10 days in humans), an average of 10^{11} platelets are produced every day to maintain the blood platelet level.

MKs are unique cells that display a single polyploid nucleus and grow by undergoing a unique phenomenon called endomitosis during which the nucleus increases its deoxyribonucleic acid (DNA) content without dividing. Following polyploidization, the cytoplasmic mass of MK increases roughly proportionally to the ploidy status. After achieving full maturation, the MK cytosol is filled with a network of membranes formed by invagination of the plasma membrane, is loaded with secretory granules, and is ready to deliver a myriad of newly formed platelets. Platelets detach from cytoplasmic MK protrusions called proplatelets that extend into the lumen of bone marrow sinusoids and are shed into the peripheral blood from the tips of the extensions. Study of MKs is difficult because of their low numbers in the bone marrow, but scientific advances and techniques such as intravital imaging have aided our understanding of MK maturation and platelet formation. The development of *in vitro* MK culture systems, allowing the preparation of pure MK populations and the identification of specific and early markers permitting the immunologic detection of MK and their precursors, have made the study of MK feasible. Finally, the identification, purification, and cloning of thrombopoietin (TPO), the major regulator of platelet production, and the availability of recombinant TPO for experimental purposes have facilitated the preparation of large enriched MK populations.

This chapter focuses on the cellular and molecular aspects of MK differentiation and its regulation in humans. Descriptions of relevant animal models are presented as well. TPO and its receptor MPL-R are discussed in detail in Chapter 25 and details of platelet maturation are provided in Chapter 24.

MEGAKARYOCYTE DIFFERENTIATION

The Megakaryocytic Cell

Platelet production ultimately results from the differentiation of a multipotent hematopoietic stem cell (HSC) into MK. HSCs are the only long-lived cells of the hematopoietic system and are capable of regenerating all the hematopoietic tissue by virtue of their self-renewal capability. When HSCs commit to MK differentiation, they lose self-renewal capability and multipotency. Committed cells are called hematopoietic progenitors. The loss of multipotency occurs through precise developmental stages. In a first step, the HSC gives rise to a common myeloid progenitor and a common lymphoid progenitor. The common myeloid progenitors subsequently commit toward specific lineages. The erythroid and MK lineages derive from a common progenitor called either MK/erythrocyte bipotent progenitor (MEP) or BFU-E/MK.[1] This bipotent progenitor has been characterized in mice and humans and gives rise to colonies containing mostly erythroblasts and a few MKs. The phenotype of this progenitor has been clearly assessed in the mouse,[2] whereas in human, its purification is more preliminary.

At a subsequent developmental stage, the MEP is only committed toward the MK lineage and its following differentiation stages can be divided into three developmental steps. The MK progenitor cell is capable of proliferation, giving rise *in vitro* to MK colonies. It further differentiates, through a variable number of mitosis, to a transitional cell (promegakaryoblast [PMKB]) that enters an endomitotic process with an average of three DNA cycles of duplication (modal ploidy of 16N). These cells correspond to MK that have begun to synthesize the main platelet proteins and are morphologically identifiable. Cytoplasmic maturation subsequently accelerates to yield a typical MK. Finally, the mature MK sheds platelets in the circulation outside the marrow itself.

Megakaryocytic Progenitors

In the hematopoietic hierarchy, MK progenitors derive from a common MEP, but the MK lineage begins with the appearance of committed MK progenitors. These were first defined by their proliferative capacities in several clonal assays, allowing the identification of three main types of MK progenitors according to colony size and the time required for colonies to develop.[3] More recently, immunologic phenotyping has been used for more precise delineation of MK progenitor compartments and better understanding of megakaryopoiesis, especially at the molecular level.

BFU-MK. These are the most primitive MK-committed progenitors, producing colonies composed of more than 50 cells organized into subcolonies. BFU-MK follow the same pattern as BFU-E–derived colonies. They mature in 12 days in mice[4] and in 21 days in humans.[5]

CFU-MK. These cells differ from the BFU-MK by a lower capacity for proliferation. They give rise to colonies containing 3 to 50 cells in 5 days in mice[6] and in 12 days in humans.[7] Unlike BFU-MK that are in the G0/G1 phase of the cell cycle, CFU-MK are in an active cell cycle.

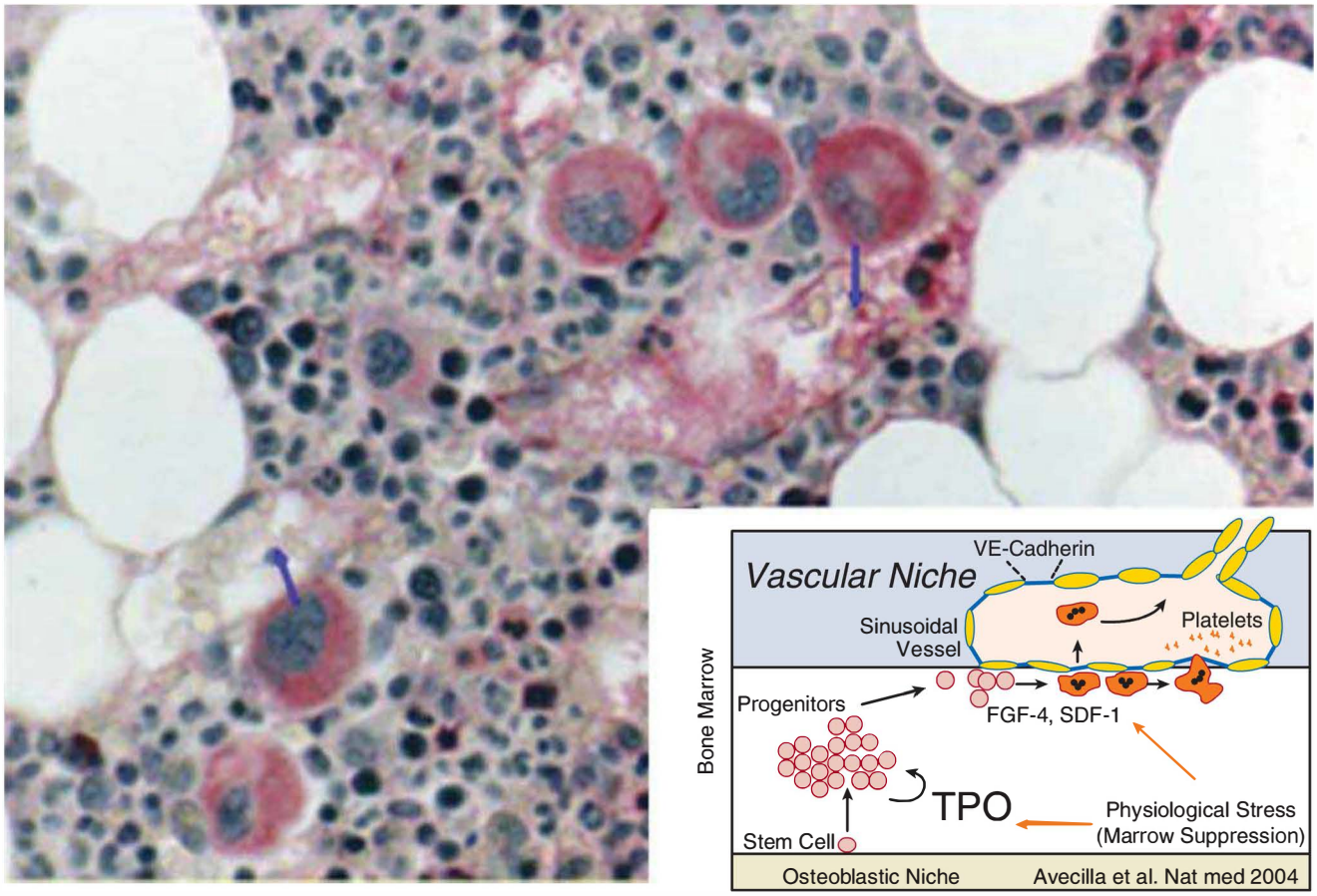

FIGURE 23.1 Normal human bone marrow biopsy immunostained for fibrinogen. Labeling detects blood vessels and MKs. MKs are frequently located along a vascular sinusoid (*V*) depicting the vascular niche. Fibrinogen displays a centrifugal staining pattern typical of an α-granule protein. The staining intensity is weak in the small immature MKs (*arrow*) and it is maximal in large, mature MKs (*arrowheads*). (Adapted from de Larouziere et al. Inverse immunostaining pattern for synthesized versus endocytosed alpha-granule proteins in human bone marrow megakaryocytes. *Br J Haematol* 1998;101:618–625, with permission.) **Inset:** Location of MK in vascular niche. (Adapted from Avecilla et al. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nat Med* 2004;10:64–71, with permission.)

Late progenitors. In the mouse, these are MK progenitors with a density of <1.050 mg/mL. In 2 to 3 days, they give rise to colonies composed of a few MK with high ploidy.[8] The developmental stage of these progenitors is extremely close to that of the transitional cell that has switched toward an endomitotic process.

Megakaryocyte Diploid Precursors

The size of diploid precursors is that of a small lymphocyte and they have no characteristic morphologic features. Thus, their identification depends on the presence of specific immunologic markers.[9] Electron microscopy is also a sensitive tool for the cytochemical detection of platelet peroxidase.[10]

Classification of megakaryocyte maturation stages. MK differentiation is a continuous process, but for the sake of clarity it has been classified into three distinct maturation stages (FIGURE 23.2).[11] Megakaryocytic differentiation becomes morphologically identifiable because cell size increases after nuclear ploidy becomes a multiple of 2N. On panoptic staining, the type I or immature MKs (FIGURE 23.2A) have a basophilic cytoplasm and can be distinguished by a large polyploid nucleus, frequent cytoplasm blebs, and relatively large cell volume (i.e., >14 μm in diameter). At this stage, no further cell division occurs and the cells only undergo endomitosis. The nuclear to cytoplasm ratio (N/C) is high, chromatin thin, and cytoplasm basophilic without azurophilic granules. Type II MKs of intermediate maturation (FIGURE 23.2B) are larger (i.e., 15 to 40 μm), with a polylobulated nucleus and a deep blue cytoplasm. The intense basophilia coincides with high RNA content, a large number of free ribosomes, and a well-developed rough endoplasmic reticulum (ER), indicative of active protein synthesis. In some, an azurophilic area is visible near the cell center where granule formation begins. As the cell matures, the N/C ratio decreases while the amount of cytoplasm dramatically increases and becomes azurophilic, the nuclear lobes become more distinct, and the chromatin condenses. The mature MK type III (FIGURE 23.2C) has a low N/C ratio. The nucleus is dark purple, with a dense chromatin texture and clearly distinct nuclear lobes. The cytoplasm is vast, uniformly granular, and azurophilic. At this stage, the MK is ready to fragment and liberate platelets. It has been estimated that each MK can produce 400 to 8,000 platelets.[12]

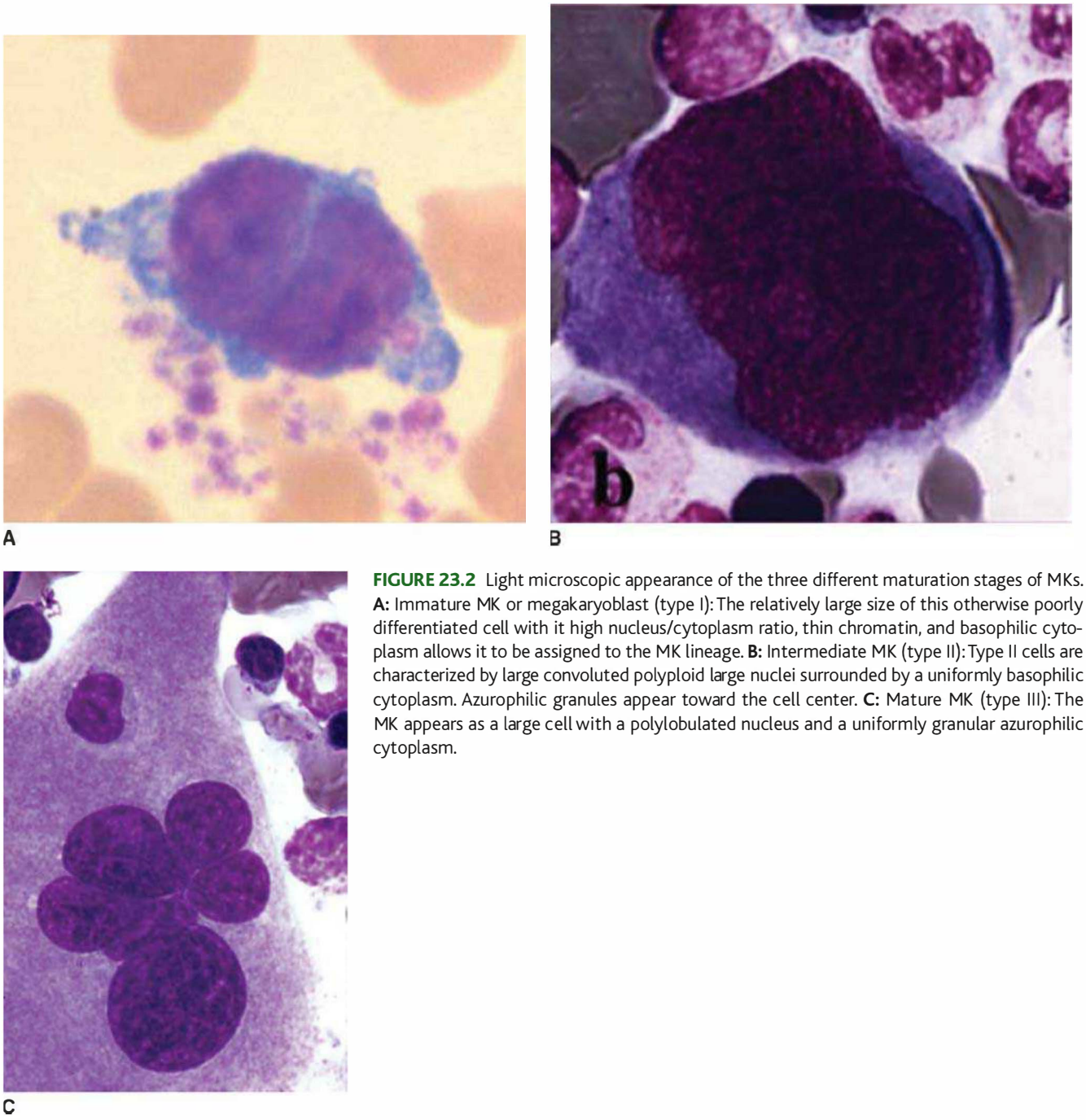

FIGURE 23.2 Light microscopic appearance of the three different maturation stages of MKs. **A:** Immature MK or megakaryoblast (type I): The relatively large size of this otherwise poorly differentiated cell with it high nucleus/cytoplasm ratio, thin chromatin, and basophilic cytoplasm allows it to be assigned to the MK lineage. **B:** Intermediate MK (type II): Type II cells are characterized by large convoluted polyploid large nuclei surrounded by a uniformly basophilic cytoplasm. Azurophilic granules appear toward the cell center. **C:** Mature MK (type III): The MK appears as a large cell with a polylobulated nucleus and a uniformly granular azurophilic cytoplasm.

ULTRASTRUCTURE OF MK DIFFERENTIATION

MK differentiation is characterized by structural changes that are easily observed under the electron microscope. Immunoelectron microscopy, in particular immunogold labeling, is a sensitive tool that has greatly contributed to the description of this process.

Demarcation membrane system. During the maturation process, the demarcation membrane system (DMS) develops as a network of smooth membrane channels located initially near the plasma membrane. These channels derive from multiple invaginations of the MK plasma membrane[13] (FIGURE 23.3). The demarcation membranes are always in contact with the extracellular medium, as demonstrated by the penetration of electron-dense extracellular tracers such as horseradish peroxidase. The DMS grows rapidly and becomes widespread within the whole cytoplasmic volume, expanding by more than 700% within 72 hours. Although some differences between the protein composition of the DMS and plasma membrane have been detected by freeze fracture studies,[14] the main platelet surface (glycoprotein [GP]) receptors are expressed equally on both.[15]

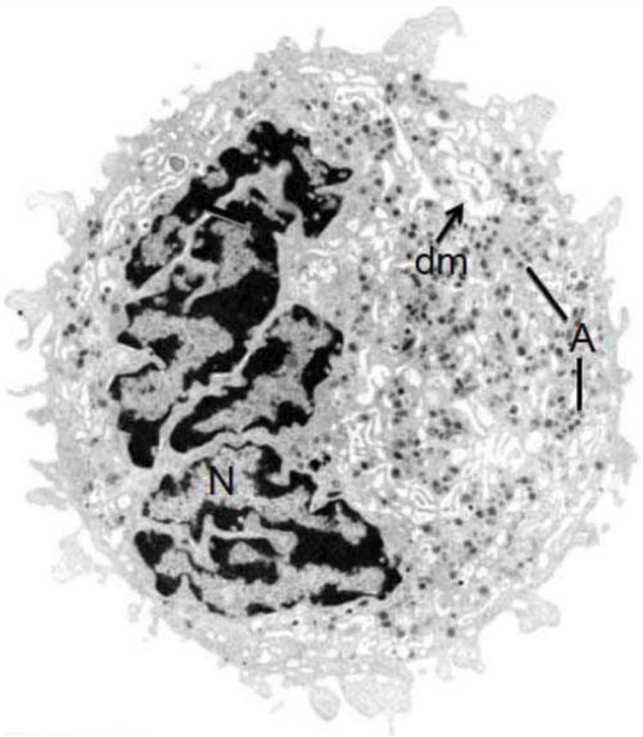

FIGURE 23.3 Electron micrograph of maturing MKs. Mature MKs are characterized by their large size and multilobed nucleus. The cells contain specific organelles (α-granules [*A*]) and a well-developed demarcation membrane system (*dm*) regularly scattered throughout the cytoplasm. (Magnification ×5,940.)

The DMS is the source of proplatelet membrane[16] and is the precursor of the membranes of future platelets (plasma membrane and surface connected canalicular system); the mechanism of platelet shedding is discussed in detail below.

Because mature MKs accumulate near marrow sinusoids and represent a potential physical barrier for other marrow cells migrating into the circulation, migrating cells are able to penetrate the open channels of the DMS and transmigrate across the MK volume. This natural phenomenon, termed "emperipolesis," is occasionally observed in normal bone marrow and is distinct from phagocytosis since the emigrating cell maintains its integrity.[17]

Golgi complexes and centrioles. Several Golgi complexes within a single MK follow the migration of several centrioles. The localization of centrioles within the cell is random, as are the Golgi complexes. The latter are found more frequently near the cell center in the perinuclear region. Their number appears to be roughly proportional to ploidy. Endogenously synthesized platelet granule proteins can be initially detected immunologically in the trans-Golgi network (TGN) where they undergo glycosylation, concentration, and precipitation. Granule proteins endocytosed from the extracellular medium are not detected in the TGN.[18] Membrane GPs also transit through the Golgi complex. It is noteworthy that immunolabeling of the GP Ib/IX/V complex is weak, whereas GPIIb/IIIa and P-selectin, major components of the α-granule membrane, are seen more prominently in the Golgi cisterns and arising vesicles.[19]

Endoplasmic reticulum. Rough ER and free ribosomes are abundant in immature MK. The smooth ER that derives from the rough ER after loss of ribosomes remains abundant until the end of the maturation process and is closely associated with the DMS. It contains a number of enzymes, including NADH–cytochrome *c* reductase, cyclooxygenase, thromboxane synthase, and Ca^{2+} Mg^{2+} adenosine triphosphatase. In humans, a peroxidase activity, the platelet peroxidase, is located in the ER and reflects the presence of cyclooxygenase, an early marker of the cell line. The ER gives rise to the platelet-dense tubular system containing the same cytochemical activity.[10] In rodents, acetylcholinesterase (AchE), a marker absent from mature human MK, is also specifically located in the ER.

Formation and packaging of cytosolic granules. Four different types of granules are formed during MK maturation: α-granules, dense granules, lysosomes, and peroxisomes. Their contents are detailed in Table 23.1; the following sections focus on their mechanism of formation.

α-Granules. α-Granules appear early during MK maturation, concurrent with the development of the DMS.[20] They are the more numerous granule population in the mature MK (FIGURE 23.3). α-Granules arise from the TGN, where a dark nucleoid is rapidly

Table 23.1

α-Granule proteins

Matrix	Membrane
PRESENT IN PLASMA	**α-GRANULE MEMBRANE RESTRICTED**
(Endocytosed)	P selectin (CD62P)
Fibrinogen	GMP33
Albumin	Osteonectin
Fibronectin	
IgG, IgA, IgM *(MK synthesized)*	**α-GRANULE MEMBRANE AND PLASMA MEMBRANE**
vWF	GP IIb/IIIa (α_{IIb}/β_3)
Coagulation factor V	GP IV
Thrombospondin	CD9
ABSENT FROM PLASMA *(Platelet specific)*	PECAM1
Multimerin	GP Ib/IX/V
β-Thromboglobulin, PF4,	rap1b
MIXED	
VEGF/VPF	
PDGF, BFGF EGF, TGF-β	
A_2-macroglobulin, Matrix Metalloproteinases	
Endostatin, angiostatin	
Plasminogen activator inhibitor 1	

Ig, immunoglobulin; MK, megakaryocyte; vWF, von Willebrand factor; PDGF, platelet-derived growth factor; BFGF, basic fibroblast growth factor; EGF, endothelial growth factor; TGF-β, transforming growth factor-β; GP, glycoprotein; PECAM1, platelet/endothelial cell adhesion molecule-1; VEGF/VPF, vascular endothelial growth factor/vascular permeability factor.

visible within budding vesicles. α-Granules are unique organelles in that they acquire their protein contents by two distinct mechanisms: (a) MK biosynthesis[21] and (b) plasma protein endocytosis, either receptor mediated or through a fluid phase.[22,23] Endocytosis may continue in platelets.[24,25] The cytoplasmic markers are initially detected in a diffuse staining pattern when located in the cisterns of synthesis and later in a granular pattern when packaged in granules. Two types of information have been provided by immunolabeling α-granules proteins. First, endocytosed proteins (e.g., fibrinogen and immunoglobulins) appear later in the MK than endogenously synthesized proteins (e.g., von Willebrand factor [vWF]),[18] and their distribution patterns within the maturing MK cytoplasm are distinct. Endocytosed proteins are centrifugal and maximal at the cell periphery, whereas synthesized proteins are centripetal and predominant in the juxtanuclear area.[26] vWF multimers are apparent in the form of 20-nm tubular structures, first appearing in the TGN and then in the α-granules.[27] Stored proteins may not be uniformly distributed among α-granules. For example, angiogenic proteins seem to be selectively located in distinct granule populations according to their function, pro- and antiangiogenic, leading to differential release from platelets.[28] The α-granule membrane contains numerous receptors that appear during MK maturation: P-selectin (CD62P), GPIIb/IIIa, CD36, CD9, and PECAM1.[19,29] Some are detectable in the Golgi apparatus and appear to be transported directly to α-granules, but others appear to transit *via* the plasma membrane and to be internalized. Evidence based on intracellular trafficking suggests that the GPIIb/IIIa present in the α-granule membrane originates directly from an internalized plasma membrane pool.[30] Finally, fully formed platelet α-granules appear as highly elaborated and compartmentalized structures, storing hemostatic and adhesive proteins and numerous growth factors (Table 23.1). Multivesicular bodies, present in MK and only occasionally in platelets, represent a sorting compartment, intermediately between the TGN and α- and dense granules.[31,32] In contrast to secretory cells such as endothelial cells, virtually no constitutive granule secretion occurs in MK; secretion follows the regulated pathway and secreted proteins are packaged within secretory granules.[33]

Dense granules. Dense granules are the final marker of the maturation of MK cytoplasm at the ultrastructural level.[34] Indeed, these granules acquire their dense appearance quite late, but their limiting membrane and intrinsic receptors are probably formed earlier.[35] They arise from TGN and their constituents are sorted from the α-granule components in multivesicular bodies.[32] They are lysosome-related organelles containing high concentrations of several low molecular weight molecules necessary for normal blood hemostasis. These include calcium, serotonin, adenine nucleotides, pyrophosphate, and polyphosphate. Their absence results in the hemorrhagic δ-storage pool deficiency disorders. In the Hermansky-Pudlak syndrome, δ-storage pool deficiency is associated with tyrosinase-positive albinism, revealing a relationship between platelet-dense granules and the melanosomes of melanocytes. There are similarities in the biogenesis and secretory code of both types of granules that are linked to the small G-protein rab27 deficient in the ashen strain of mice.[36] Murine models have also shown that a specific gene (*Slc35d3* gene) is involved in dense granule formation independent of lysosomes and melanosomes.[37]

Lysosomes and peroxisomes. These granules can be detected cytochemically within maturing MK because of their specific enzymatic content–acid hydrolases[38] and catalase, respectively.

The MK nucleus. The nucleus of MK displays several sparse rounded lobes with abundant euchromatin and conspicuous nucleoli (type I) (FIGURE 23.2A). Its maturation is characterized by a constant size increase and segmentation and by chromatin clumping (type II) (FIGURE 23.2B). Through the unique process endomitosis, nuclear lobulation and ploidy increase without division, with the DNA duplicating to levels of 4N to 64N (rarely 128N). A correlation between ploidy and the number of nuclear lobes has been found on squash preparations. Close to the final maturation stage of MK, the nucleus changes texture and aspect (type III) (FIGURE 23.2C). Electron microscopy shows that the nuclear lobes tend to elongate and gather, the chromatin becomes coarser, and long clefts of cytoplasm extend between each lobe. These structural changes herald the start of proplatelet formation and platelet shedding.

Megakaryocyte activation. MKs are able to react to agonists in a manner similar to platelets.[39] Thrombin induces dramatic morphologic changes as early as the megakaryoblastic stage, indicating the early expression of protease-activated receptors. In the presence of agonists, the MK plasma membrane becomes bristled and sends out numerous thin pseudopods, the nucleus becomes eccentric, cytosolic organelles centralize, and secretory granules fuse with DMS after which they discharge their contents. This phenomenon, which leads to inappropriate secretion of granule contents, including growth factors, may be implicated in pathologic states such as fibroblast activation and myelofibrosis.[40]

STAGE-SPECIFIC DIFFERENTIATION MARKERS

Megakaryocyte Progenitors

Monoclonal antibodies against platelet proteins such as GPIIb/IIIa (CD41b), the GPIb complex (GPIbα, GPIbβ, GPIX and GPV, or CD42a, b, c and d), CD9, and CD150 have been used to identify MK progenitors.

In mice, it has been possible to purify MK progenitors close to homogeneity based on the expression of several antigens: Lin^- c-$kit^+$$Sca\text{-}1^-$ $FcgR^{low}IL7R\alpha^-$ $Thy1.1^-$ $CD34^{low}$ $CD9^+CD41^+$ and more recently, Lin^- c-$kit^+$$Sca\text{-}1^-$ $CD41^+CD150^+$.[41,42]

In humans, MK progenitors were initially characterized by the expression of differentiation markers on their surface that are also present on other hematopoietic progenitors, such as CD34, CD31, and CD133. Based on the expression of HLA-DR, it is possible to distinguish between BFU-MK ($HLA\text{-}DR^{low}$) and CFU-MK ($HLA\text{-}DR^{high}$).[5] Late MK progenitors can also be isolated in the $CD34^+CD41^+$ population which is enriched in CFU-MK.[43]

Megakaryocyte Precursors

Markers have been identified for later stages of MK differentiation. In the mouse, AchE staining, easily detected with light microscopy, is a convenient marker for investigating murine megakaryopoiesis *in vitro*.[44] In humans, AchE predominates in the erythroid lineage and cannot be used to identify megakaryocytic cells.

Studies in the mouse have shown the presence in the marrow of small AchE-positive cells with 2N ploidy. These cells are the direct precursors of MK and differentiate into polyploid MK in a few days.[45] Most of the 2N cells have no proliferative capacity

and undergo cycles of endoreplication. Their number is greatly increased or reduced in induced thrombocytopenia or thrombocytosis, respectively.

Megakaryocytes

Recent studies have used monoclonal antibodies directed against platelet proteins, permitting a better understanding of MK differentiation. Most studies have focused on the main platelet GPs, GPIIb/IIIa ($\alpha_{IIb}\beta_3$ or CD41b) and the GPIb complex (GPIbα, GPIbβ, GPIX, and GPV, or CD42a, b, c, and d).

CD41b is composed of CD41a (GPIIb, αIIb) and CD61 (GPIIIa, β3). CD61 is not specific for MK because as the β subunit of the vitronectin receptor $\alpha_v\beta_3$, it can be detected on nonmegakaryocytic cells such as endothelial cells, activated macrophages, and osteoclasts. By contrast, CD41a appears more specific for the MK lineage, especially in adults. In the marrow, a minute cohort of small cells (0.8 to 2.5 × 10^4 marrow cells) expressing platelet proteins can be detected using an anti–GPIIb antibody.[46–48] During CFU-MK culture *in vitro*, a large number of these small cells are present just before the development of recognizable MK colonies (day 6).

CD41 can also be detected on $CD34^+$ cells. Most $CD34^+$ $CD41^+$ cells in the adult correspond to late MK progenitors capable of giving rise to clusters of MK in 5 days[43] and to typical CFU-MK. Other experiments in transgenic or knock-in mice, and using the thymidine kinase gene under control of the GPIIb promoter, have shown that after ganciclovir treatment, most hematopoietic progenitors, as well as some HSCs, were killed.[49,50]

Recently, it has been shown that CD41 is an ontogenic marker of murine hematopoiesis. It is the first marker of hematopoiesis in embryonic stem (ES) cells, as well as in the embryo.[51,52] During later development, CD41 becomes restricted to the megakaryocytic lineage, although it is also expressed during mast cell differentiation.[51,53] In human neonates, CD41 is not restricted to the megakaryocytic lineage, but it is detected on some hematopoietic progenitors, especially on the bipotent erythroid/MK progenitors, lymphoid progenitors, and some HSCs capable of reconstituting hematopoiesis in immunodeficient animals (SCID-RC).[54] It is noteworthy that the expression of Mpl, the TPO receptor, mimics the expression of CD41. It is expressed at a low level on HSCs and all types of hematopoietic progenitors. Its expression markedly increases after commitment toward the MK lineage.[55]

CD41 expression precedes the detection of other major platelet proteins. The CD42 antigen is detected slightly later on a cell that expresses the CD34 and CD41 antigens. These cells are capable of giving rise to a single MK or a cluster of less than four MKs. The proliferative capacity of these cells is quite similar to those of the human CFU-MK defined in the agar medium.[56] $CD41^+CD42^+$ cells have been called *transitional* cells because they can switch from a mitotic stage to an endomitotic stage. In contrast to the CD41, CD42 expression is restricted to the MK lineage, although some late erythroid progenitors may express this marker. To detect the CD42 antigen on the MK surface, the GP Iα (CD42b), GP Iβ (CD42c), and GP IX (CD42a) must be expressed together, whereas GP V (CD42d) is not required and may be synthesized later than the other proteins.[57]

CD42 expression corresponds to a later differentiation stage that is correlated with the appearance of CD36 (GPIV) and the detection of α-granule proteins such as platelet factor 4 (PF4) and vWF, as well as a marked increase in the expression of Mpl, GPVI, and the integrin $\alpha_2\beta_1$. CD34 disappears during the endomitotic process.

Finally, immature MKs express CD4, which may be relevant for the mechanisms of thrombocytopenia in HIV infection.[58–60] CD4 expression tends to decrease during MK maturation and is virtually absent from mature MKs and the platelet surface.

ENDOMITOSIS

At the end of the proliferative phase, *2N* MK precursors become polyploid. MK polyploidization is unique among mammalian cells in that a multiple of 2N chromosomes is enclosed in a single nucleus surrounded by a single nuclear membrane. To achieve polyploidization, MKs undergo several rounds of DNA duplication and the nucleus segments but without nuclear and cytoplasmic division.

MKs are the only cells that become polyploid during their normal differentiation, whereas other cells may become polyploid after a stress. Polyploidy is a way to increase platelet production because the MK cytoplasm volume increases in parallel with ploidy. An increase in MK ploidy is one of the first events to occur after the induction of acute severe thrombocytopenia in mice: within 24 hours, the modal MK ploidy increases from 16N to 32N. This increase is maximal at 48 hours and ploidy returns to basal values within 120 hours.

As a first maturation step, MKs increase their ploidy and stop DNA duplication at any point between 2N and 64N, rarely going up to 128N. In humans, as in most mammals, the modal ploidy is 16N. However, in some mouse species such as C3H mice, the modal MK ploidy is 32N.[61] In the fetus and embryo, MKs have a lower ploidy; most fetal MKs are only 2N or 4N in culture.[62] Nonetheless, low ploidy MKs, either on a physiologic basis or due to malignancy, are able to shed platelets *in vitro*.

MK endomitosis is a rare event that remains difficult to observe. Originally, the term endomitosis was given to the mechanism of polyploidization because it was suggested that the mitotic process was occurring without rupture of the nuclear envelope. However, ultrastructural studies have allowed a description of the endomitotic process. The first steps are identical to those of mitosis. They begin with chromosome condensation and disappearance of the nuclear membrane. Several mitotic spindles are subsequently formed with two centrosomes for a tetraploid nucleus. Each centrosome is active and participates in elongation of the mitotic spindle. At metaphase, chromosomes are aligned at the equator and have sizes similar to those of mitotic cells.

MK culture in the presence of TPO, in conjunction with immunolabeling of various components of the mitotic apparatus, has made it easier to precisely describe the endomitotic process. It was initially thought that endomitosis represented mitosis that had skipped anaphase B and cytokinesis.[63,64] In fact, the initiation of endomitosis is identical to a normal mitosis with duplication of the centrosomes, development of a mitotic spindle, chromosome condensation (prophase), rupture of the nuclear envelope, chromosomal alignment at the equatorial plate (metaphase), and separation of the sister chromatids (anaphase).[65] Initially, the late phases of endomitosis were not observed because of their rarity. However, with the use of time-lapse videomicroscopy, it has been observed that endomitotic

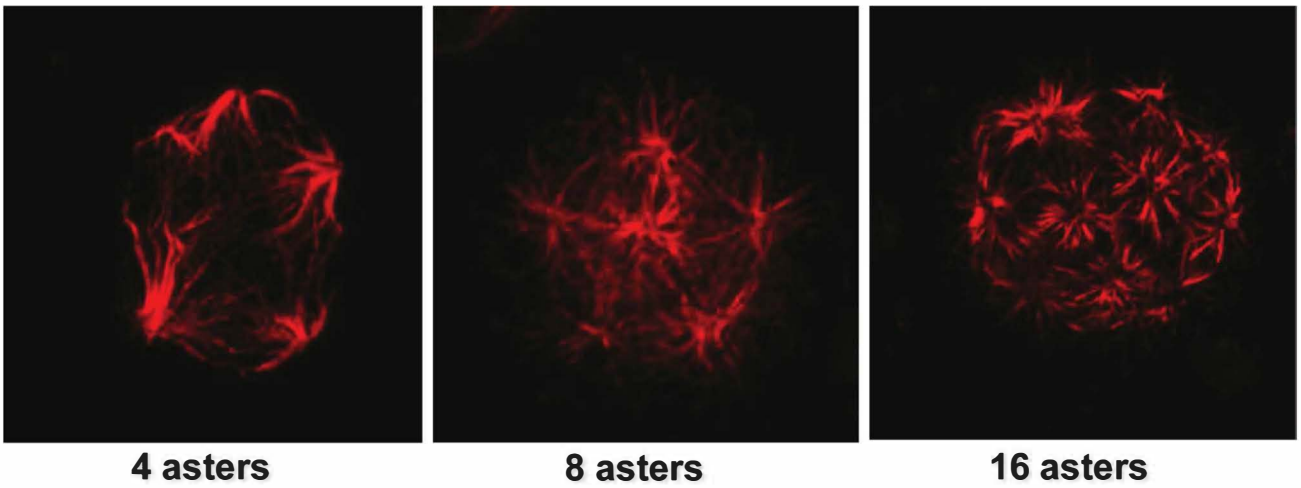

FIGURE 23.4 Immunofluorescent labeling of α and β tubulin in MKs undergoing endomitosis. These images of prometaphases of endomitosis from 4N to 8N, 8N to 16N, and 16N to 32N show the structure and asymmetry of the multipolar mitotic spindles.

MKs go through telophase and begin a cytokinesis that fails.[66,67] At the 2N and 4N transition, the two daughter cells that are nearly fully separated make a backward movement and fuse together. The structure of the central spindle has been a matter of debate, but it appears normal at the 2N and 4N transition. The main abnormality involves the cleavage furrow and its ingression which is incomplete. At higher ploidy, the abnormality is more pronounced with only incomplete cleavage furrow ingression.[66–69] When the MK is polyploid, its spindle is multipolar with the number of poles corresponding to the ploidy level; however, the number of chromosomes at each pole does not correspond to 2N because segregation of chromatids is asymmetrical during endomitosis[65] (FIGURE 23.4). It is surprising that a defect in cytokinesis leads to mononucleated cells because it usually induces multinucleated cells. This suggests that anomalies in chromatid separation may be also present during endomitosis. Preliminary evidence suggests that a large fraction of 4N MKs have two nuclei as a consequence of a pure defect in late cytokinesis. Because the abnormality is more pronounced in MK with higher ploidy, the chromatids are less separated, leading to a defect in karyokinesis and to a single nucleus. However, it has recently been shown that polyploid MKs are able to undergo a mitotic cycle with the generation of 2N cells.[70]

The cell cycle of a MK undergoing polyploidization is very similar to a mitotic process. There is clearly a succession of G1/S/G2/M phases, but the M phase is incomplete. After the M phase, the cell reenters G1 to undergo a subsequent cell cycle of DNA duplication. However, the molecular mechanisms responsible for the switch from a mitotic to an endomitotic cycle, as well as the mechanisms involved in stopping the process, are still incompletely understood.

REGULATION OF ENDOMITOSIS

The usual regulators of mitosis are active during the endomitotic process. Cyclin D3, cyclin E, and cyclin A are normally expressed during G, S, and G2/M phases, respectively.[71] The presence of cyclin B1 during endomitosis has been a subject of debate. There is now a large body of evidence indicating that cyclin B1 is expressed during the endomitotic cycle and is degraded in anaphase.[64,65] The presence of cyclin B1 is absolutely required for an entry in mitosis because the cdc2 (CDK1) kinase activity is indispensable for rupture of the nuclear envelope and chromatid condensation. Cyclin E may also play an important role in ploidization. Cyclin $E^{-/-}$ mice have a marked defect in MK ploidization,[72] but a proliferation defect was not observed in the other mitotic cells. In addition, cyclin E overexpression increases MK polyploidy.[73]

The anaphase-promoting complex is functional in MKs. This proteasome complex regulates the end of mitosis by degrading successive mitotic regulators such as the p55cdc20 subunit and cdh1. During the endomitotic process, cyclin B1, p55cdc20, and Aurora B are normally degraded.[65,74] On the other hand, CDK inhibitors such as p21 and p27 are expressed at high levels in polyploid MKs, but are not directly involved in endomitosis. These inhibitors do not seem to contribute to mitotic arrest as MK ploidy is normal in $p21^{-/-}$, $p27^{-/-}$ and double KO mice.[75] However, $p19^{Ink4d}$ appears to play a role in endomitotic arrest because $p19^{Ink4d}$ KO mice have MK with a 64N modal ploidy.[76] It is noteworthy that $p19^{Ink4d}$ is transcriptionally regulated by RUNX1 as well as several genes coding for platelet function. Thus, RUNX1 may be a link between the regulation of the end of endomitosis and terminal cytoplasmic differentiation.

There are also differences between endomitosis and mitosis. Levels of cyclin D3 and cyclin D1 are extremely high in MK,[71,77] and an increase in cyclin D increases MK ploidy. Cyclin D1 expression is regulated by GATA1 in MK.[77] In addition, it has been shown that STAT1, which is downstream of GATA1, also increases polyploidy and MK maturation.[78] Interferon γ, which induces STAT1 phosphorylation, induces MK polyploidization as well.

Whatever the molecular mechanisms involved, there are two main abnormalities in the cell cycle of the endomitotic MKs. One abnormality occurs at the end of mitosis and is responsible for the failure in cytokinesis. It has been suggested that this is related to a defect in Aurora B and survivin expression in polyploid MK,[79,80] but no major defect in the expression of these proteins and in their

localization in the central spindle has been found at the 2N to 4N transition.[74,81] Such abnormalities may be present in higher ploidy MKs.[80] Perhaps more germaine to the process, there is accumulation of myosin II at the contractile ring.[74] Myosin II normally plays a central role in cytokinesis by promoting the contractile ring forces required for cytokinesis. It is presently unknown if the defect in myosin II accumulation is primary or is secondary to low Rho/Rock pathway activation. The second cell-cycle abnormality occurs at the G1/S transition. A normal cell cannot become polyploid because an as yet unknown "sensor" detects the level of ploidy and blocks subsequent reentry into the S phase. This checkpoint is mainly regulated by p53.[82] However, a role for p53 in polyploidization remains controversial.[83]

Whether polyploidization is advantageous for platelet formation has been unclear. Theoretically, an endomitotic cycle could be shorter than a mitotic cycle because the late phases of the cell cycle are abrogated. However, the time for DNA duplication seems identical in both processes. During terminal differentiation, MK must synthesize large amounts of membrane to produce platelets. The endomitotic process could produce considerable economy of nuclear and plasma membrane synthesis which may aid in the efficiency of platelet production. Polyploidization may also be a way to markedly increase protein synthesis. In favor of this hypothesis, it has been shown that all 2N alleles of a gene remain functional during polyploidization; therefore, polyploidization represents true gene amplification.[84] Whether platelets arising from MK of different ploidy have similar or different sizes and function remains a matter of debate. In addition, polyploidy may induce resistance to mutations leading to haploinsufficiency as multiple gene copies are present. Whether polyploidization modifies protein synthesis and gene expression also remains to be determined. It has been reported that there is a linear correlation between mRNA or protein expression and ploidy. However, at high ploidy, expression may reach a plateau. Gene profiling during polyploidization has shown that the processes of polyploidization and terminal differentiation are partially intermingled because expression of genes involved in platelet function increases with ploidy.[85] Perhaps this is because terminal differentiation and polyploidization are regulated by the same transcription factors, for example, GATA1, FLI-1, or RUNX1.[76,77]

PLATELET FORMATION AND SHEDDING

Platelet formation has been described in vitro[86] and recently proplatelet formation was observed directly in mice in vivo through vital imaging.[87] In vitro, bone marrow MKs produce long extensions of cytoplasm called proplatelets (FIGURE 23.5) giving the MKs, in the process of shedding platelets, the morphologic appearance of an octopus.[88]

TPO-containing cultures have enabled the study of platelet formation and shedding in vitro using human[89] and murine precursors.[90] In addition, electron microscopy has enabled the identification of sequential subcellular events that lead to platelet production.[89,91] First, there is peripheral redistribution, alignment, and dilatation of demarcation membranes (Online FIGURE 23.5A,B). This is followed by extension of a proplatelet containing an axial bundle of microtubules and regular constriction zones (Online FIGURE 23.5C). Finally, there is breakage of a distal constriction rich in microtubules and liberation of a newly formed platelet (Online FIGURE 23.5D,E).

FIGURE 23.5 Photomicrograph of platelet formation by human MKs in culture. After reaching full maturation, the MK cytoplasm is remodeled into long processes termed proplatelets. The proplatelets develop constrictions along their length, giving them a beaded appearance. Finally, there is breakage of distal constrictions rich in microtubules and liberation of newly formed platelets. (See also Online FIGURE 23.5A-E).

Proplatelets bend, branch, and bend again, each elbow and tip being surrounded by a circular microtubule belt.[90] Future platelets detach from these cytoplasmic extensions. Alternatively, proplatelets may detach from the cell core and deliver platelets. The extension of proplatelets is accompanied by trafficking of organelles, mainly granules and mitochondria, in both directions, until they reach the tip of the proplatelet where they are immobilized. Videomicroscopy shows that proplatelets are dynamic and reversible structures and that future platelet organelles move freely along their long axes. It could be the circular microtubule at the proplatelet tip that prevents the organelles from going backward again.

In these models, the platelets that detach from the tip of the proplatelet contain a circular loop of microtubules. To explain the presence of this loop, it has been suggested that some newly formed platelets still bear a longitudinal bundle of microtubules in the circulation, then bend, curl, and form a loop with a central vacuole; both its extremities fuse together, then the vacuole shrinks, and the circumferential belt of microtubule is formed. Finally, blood platelets are still capable of protein synthesis.[92] It has been suggested that platelets can divide *in vivo*. However, it is unclear whether they are capable of duplicating the amount of proteins, such as actin or tubulin, which are fundamental for platelet function.

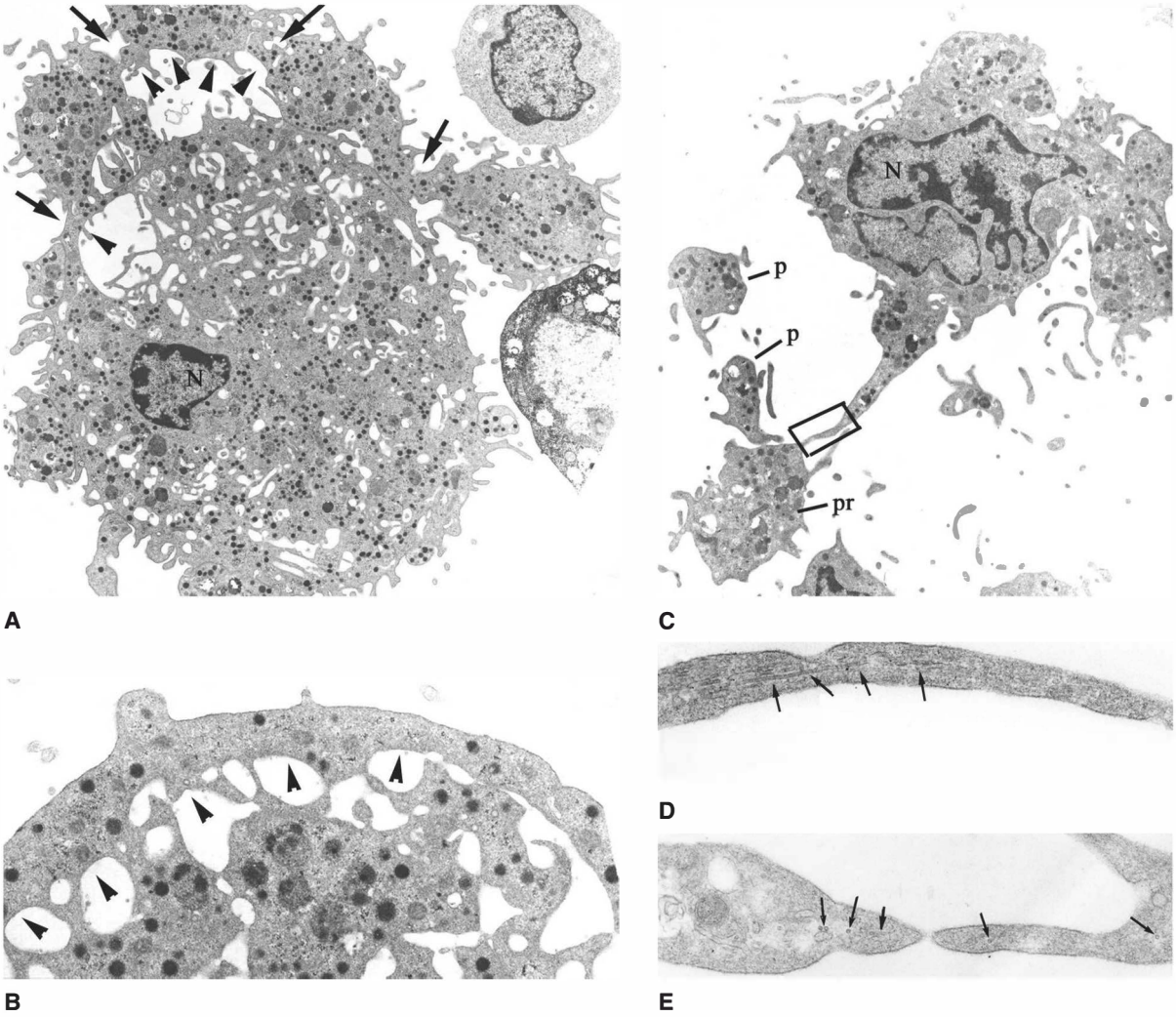

FIGURE 23.5. **A** and **B:** After reaching full maturation, megakaryocytes (MK) display the alignment and dilatation of some peripheral demarcation membranes (*arrowheads*), creating an outer ring of cytoplasm. The peripheral sheet of cytoplasm unfolds from the cell core, forming an elongated proplatelet process. Constriction zones are regularly disposed along this cytoplasmic extension (*arrows*), delimiting future platelet territories (*N*, nucleus) [magnification **(A)**, ×3,520; **(B)**, ×9,560]. **C:** Cytoplasm extends away (*box*) from the core of the mother cell, forming a proplatelet (*pr*). Some platelets (*p*) have detached from its tip (*N*, nucleus). **D:** A high magnification of the constriction zone shows some elongated parallel microtubules stretching along the long axis of the proplatelet (*arrow*) (magnification ×36,650). **E:** At places of constriction along the proplatelets, transverse microtubules appear (*arrows*) that might help increase local fragility, leading to breakage and liberation of a newly formed platelet (magnification ×35,650). Adapted from Cramer EM, Norol F, Guichard J, et al. Ultrastructure of platelet formation by human megakaryocytes cultured with the Mpl ligand. Blood 1997; 89:2336–2346.

The following platelet organelles play a key role in platelet formation:

Demarcation Membranes. Mature MKs evaginate demarcation membrane channels to their surface which elongate to form proplatelet extensions. Thus, demarcation membranes do not delineate platelet territories in MK cytoplasm, even though previous ultrastructural images of murine bone marrow MK had suggested that platelet territories could have been preformed in the maturing cell.[93] Finally, the number and size of platelets released by individual MK are directly dependent on their developmental stage.[86,94]

Microtubules. Pharmacologic inhibitors of microtubule formation prevent extension of proplatelets.[91,95,96] Microtubules are longitudinally oriented along these processes and constrictions occur that delineate the future platelets. It has been suggested that the microtubules in each proplatelet are derived from one centriole, implying a direct relationship between platelet shedding and ploidy.[86] Microtubules in each proplatelet extension appear to be oriented in opposite directions in each bundle and can slide back and forth along each other, facilitating changes in proplatelet length.[97,98] Microtubules play a central role in the redistribution of organelles in proplatelets as well.[99]

Actomyosin. Paradoxically, the process of proplatelet formation is enhanced by disruption of the actin cytoskeleton, indicating that this filamentous network is not a main component in proplatelet extension.[91] On the other hand, it is involved in the bending and branching of these extensions, thereby determining the size and number of shed platelets.[90] Globally, the actin cytoskeleton provides antiprotrusive forces that restrict proplatelet formation, and myosin II activity must decrease to permit proplatelet formation.[100] Actomyosin may also be involved in the scission process required for platelet shedding. Finally, the actin cytoskeleton is a major determinant of the MK migration required for the transit of MK from the osteoblastic to the vascular niche.[101] In support of the importance of the actin cytoskeleton in platelet formation, several inherited platelet disorders characterized by alterations in platelet size are related to mutations in components of actomyosin (MYH9)[102] or its regulators (WASP).[103].

Platelet size. Regulating the size of shed platelets is of importance since large platelets appear to be more functionally active than small platelets. The subcellular structures described above are involved in determining platelet volume. The main regulatory element appears to be the microfilament network. Indeed, the ability of proplatelet bending and kinking seems to be critical for platelet territory formation.[90] Actin filaments are linked to the plasma membrane by actin-binding protein (filamin) bound to the GPIb/V/IX complex, and abnormalities of GIbα, GPIbβ, and GPIX result in the macrothrombocytopenia that is characteristic of the Bernard-Soulier syndrome.[104] Other congenital macrothrombocytopenias, grouped together under the name MYH9-related disorders, are autosomal dominant syndromes that variably affect platelet formation and result from mutations in the human nonmuscle myosin-IIA heavy-chain gene, the only myosin heavy chain present in platelets.[102] It is unclear whether this disease results from haploinsufficiency of MYH9 or from an alteration in myosin heavy-chain function. MYH9 knockout mice develop macrothrombocytopenia that resembles the MYH9 syndrome in humans, but these abnormalities are only observed in MYH9$^{-/-}$ mice.[100] In contrast, knock-in mice for the mutations present in the MYH9 syndrome develop macrothrombocytopenia in the heterozygous state.

In autoimmune thrombocytopenia, platelet volume increases, and this can be compared to the effect of hemolysis on red blood cell volume where accelerated production increases cell size. Accelerated platelet production leads to a quasi-immediate increase in platelet size, and this can be attributed to repartition of the constriction zones, which when closer to the MK core along the proplatelet delimits larger territories.[86,89,97] Finally, pathologic states like myelodysplasia are often characterized by an increased mean platelet volume. Given that acquired mutations of the transcription factor GATA1 that accompany some preleukemic states also lead to macrothrombocytopenia, the increased platelet volume of myelodysplasia likely originates from a complex combination of cellular and molecular abnormalities.

Apoptosis and platelet production. Platelet production and MK death are intimately linked processes. Following platelet release, the nuclei of senescent MK undergo morphologic changes such as chromatin condensation that are characteristic of apoptotic cell death.[105] Recent observations suggest that biochemical processes characteristic of apoptosis, including caspase activation and cytochrome *c* delocalization from mitochondria, appear to precede proplatelet extension during the final step of MK maturation.[106] Evidence also suggests that caspase activation occurs in a localized manner rather than throughout the cytosol, explaining how the intrinsic apoptosis pathway might be activated without causing premature MK death. Furthermore, blocking the intrinsic apoptotic pathway either by caspase inhibition or by overexpression of the antiapoptotic protein Bcl-2 inhibits proplatelet formation. A role for caspases in proplatelet formation has also been shown in studies of the human MK cell line MEG-01 where proplatelet formation is enhanced by activation of the extrinsic apoptotic pathway, by activation of the Fas pathway by receptor ligation, or by overexpression of the downstream effector caspase-8.[107]

A case of familial thrombocytopenia has been reported which is linked to a gain in function mutation affecting cytochrome c.[108] Early platelet formation was observed *in vitro*, together with the presence of mature platelets released in the bone marrow, suggesting a major deregulation of both the timing and the location of proplatelet formation and platelet release.

Mild thrombocytopenia is seen in mice that overexpress Bcl-2 in the hematopoietic compartment[109] or that have lost expression of proapoptotic Bim.[110] However, mice that overexpress Bcl-XL in MK show no alterations in platelet counts, although cultured MKs show reduced proplatelet formation, and platelet recovery *in vivo* is delayed following experimentally induced immune thrombocytopenia.[111]

However, a recent report argues against a role for the classical intrinsic apoptosis pathway in proplatelet formation.[112] Deletion of Bax and Bak in MK, the key proapoptotic Bcl-2 family proteins that trigger loss in mitochondrial membrane integrity and subsequent cytochrome *c* release, had no effect on proplatelet formation, and the observed thrombocytopenia was related to increased platelet clearance. On the other hand, mice lacking MK Bcl-XL had decreased platelet counts and their MKs begin to form proplatelets in culture, but subsequently die. It is unknown whether this is related to species differences in platelet shedding or to the induction of a caspase activation, independent of apoptosis, during proplatelet formation.

Microenvironment and MK localization in the bone marrow. Normal megakaryopoiesis takes place in the bone marrow where HSCs proliferate and differentiate close to the osteoblastic niche. When MKs mature, they are often gathered into small groups, consisting of up to three cells of various size, ploidy, and maturation stage. During the maturation process, MKs tend to migrate from the osteoblastic niche, the favored localization of HSC, to the

vascular niche in proximity to a vascular sinusoid[113] (FIGURE 23.1). During the maturation process, MKs are in close contact with stromal cells, whereas fully mature MKs are closer to endothelial cells.[114] This is important because MKs and endothelial cells share many potential pathways. MKs express mitogenic factors for endothelial cells, like vascular endothelial growth factor (VEGF),[115] whereas endothelial cells express receptors and adhesion molecules that contribute to retaining MK in proximity to the bloodstream where platelets are shed.[116] In mice and rats, but not in humans, MKs are distributed equally in the bone marrow and the spleen, and to a lesser extent the liver.[93] In addition, MKs are occasionally observed in the pulmonary circulation of both human and animals. Mature bone marrow MKs are extremely deformable and not only can adhere to the sinusoidal wall, but can also transmigrate across the barrier to enter the circulation. The first capillary bed that MKs encounter when they exit the bone marrow is the pulmonary microcirculation where they can be easily trapped. Indeed, it has been pointed out that MKs are present in consistent numbers in the small vessels of the lung and are trapped in the pulmonary capillary beds where they release platelets.[117] This hypothesis has been recently supported by injection of mature MKs into the circulation of mice where they are trapped in the pulmonary circulation to produce platelets.[118] These observations suggest the lung as a possible site of platelet production.

MK chemotaxis. MKs express the chemokine receptor CXCR4, a G-protein–coupled seven transmembrane domain receptor. The CXCR4 ligand, stromal cell–derived factor-1 (SDF-1), contributes to retaining HSC in the marrow and to inducing MK chemotaxis *in vitro* in a modified Boyden chamber.[119] Under these conditions, mature MKs were observed to form a unilateral cytoplasmic pseudopod with the nuclear lobes gathered at the opposite pole, enabling MK migration through a transwell of bone marrow endothelial cells. Others also observed that CXCR4 expression increases with MK maturation, but mature MK, as well as platelets, do not respond to SDF-1. This suggests that SDF-1 signaling operates to retain immature MK in the marrow, whereas mature MKs can migrate into the blood.[101,120,121]

SDF-1 is also a chemoattractant for human CD34 hematopoietic progenitors. Thus, it is involved in homing of HSC to the bone marrow[122,123] and plays a major role in the retention of hematopoietic cells in the marrow.[124] Downregulation of CXCR4 by either proteolysis or endocytosis participates in the mobilization of hematopoietic progenitors in blood.[125] Lastly, CXCR4 is a coreceptor for HIV.[126]

MK localization. In normal individuals, platelet shedding does not occur in the marrow but directly into the circulation. In the marrow, MKs are located in the subendothelial region, in close contact with endothelial cells.[127] Long cytoplasm pseudopods appear to pass through the endothelial barrier and enter into the marrow sinusoids.[13,87,113,128] Alternatively, mature MKs can migrate through the endothelial cell barrier and can be detected in the circulating blood.[128] Platelet release occurs by breaking off long proplatelet extension at the level of constrictions, perhaps due to the mechanical force of flowing blood. Initially, proplatelets are released with a longitudinal orientation of the microtubules and their tips elongated (like a teardrop). Subsequently, rearrangement of microtubules occurs, giving rise to a marginal microtubule coil and discoid-shaped platelets. Others have proposed a model in which proplatelet curvature results in platelet formation.[90] However, recently reported intravital microscopy has shown that true proplatelets are released in the circulation, as well as entire MKs.[87] Finally, the demonstration of a role for physical force in platelet shedding from MK provides additional evidence that a substantial proportion of platelet formation occurs in the circulation.

Effect of shear stress on platelet formation and shedding. Because mature bone marrow MKs migrate toward the vascular niche, it is likely that proplatelets or entire MKs enter capillaries where they are exposed to the physical forces of blood flow. Indeed, when mature MKs are placed in a perfusion chamber with shear stresses ranging from 900 to 1,800 s^{-1},[96] they undergo the specific changes that accompany platelet formation. It is noteworthy that these changes occur much faster than under static conditions with the entire process, from a spherical MK to platelet release occurring in <30 minutes; under static conditions in culture, the process takes at least 10 hours.

There are also morphologic differences between platelet production under shear and under static conditions. When platelets are produced under shear, proplatelets extend from the cell core in a bipolar manner and are oriented parallel to the flow. Entire proplatelets detach, followed by the release of platelet subunits; alternatively, platelets may be shed directly from the tip of the proplatelets still attached to the MK core. This process is sustained by microtubules and is completely reversed by the microtubule inhibitor nocodazole. It is also GPIb dependent and is completely blocked by antibodies to GPIb.

Embryonic stem cells and platelet production. By differentiating murine ES cells in coculture with stromal cells, Fujimoto et al.[129] succeeded in producing functional platelets that were able to spread, aggregate, bind fibrinogen, and express P-selectin. There was evidence that these platelets were functional because they recirculated when injected in immunodeficient mice and participate in the formation of clots. This promises to be an extremely useful model because the differentiated ES cells closely mimic cultured MK, including proplatelet and platelet production (FIGURE 23.6). One possible limitation is that the platelets had embryonic/fetal features, and it is not presently known whether they function differently from their adult counterparts. Another approach is based on the induced pluripotent stem (iPS) cell model and the recent observation that fibroblasts expressing Oct4 can proliferate and generate multipotent progenitors in unlimited quantities which could then differentiate

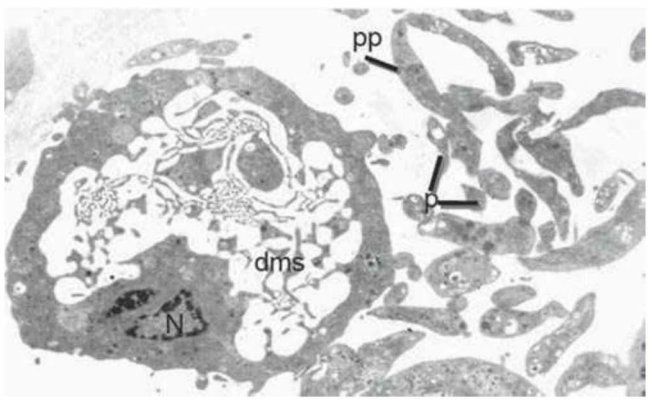

FIGURE 23.6 MK derived from an ES cell. The ultrastructure of this cell is very close to an MK in primary culture, exhibiting a lobated nucleus (*N*), prominent demarcation membrane system (*dms*), adjacent proplatelets (*pp*), and newly formed platelets (*p*).

into MK.[130] The advantage of the reprogramming technique is that it gives rise to adult MKs in contrast to ES cells. In addition, iPS cells could be used to recapitulate congenital platelet disorders.[131]

MOLECULAR REGULATION OF MEGAKARYOPOIESIS

There has been substantial progress in understanding normal MK differentiation, its relationship to erythroid differentiation and platelet shedding, and the basis for congenital and acquired MK abnormalities (FIGURE 23.7).

The GATA transcription factor family and its partners. Sequencing the promoters of MK-specific genes such as GPIIb, PF4, GPIba, β-thromboglobulin, GPIX, and GPV revealed the presence of consensus-binding sequences (WGATAR) for transcription factors of the GATA family previously observed in erythroid-specific genes.[132] MK-specific gene promoters also possess DNA consensus sequences for the Ets family of transcription family (GGA/T), some being in tandem with GATA motifs. Mutations impairing Ets binding reduce the activity of MK promoters by 60%. Therefore, GATA and Ets-binding sites appear equally important for the activity of MK gene promoters. Ets-binding sites are not found in erythroid-specific genes, whereas CGCC or CACC motifs are associated with GATA-binding sites.

Two GATA proteins are expressed during erythromegakaryocytic differentiation. GATA-1 was first described as a specific regulator of erythroid-specific genes but was later detected in MKs. Knockout of GATA-1 in mice is embryonic lethal due to anemia.[133] When GATA-1 was specifically knocked out in MK, the mice were viable but presented with profound macrothrombocytopenia and the presence of small immature MK in the marrow.[134] The latter exhibited increased proliferation capacity. In addition, synthesis of the major platelet GPs was markedly decreased.

GATA-2 is mainly involved in the proliferation of multipotent progenitors but is also required for normal MK development in GATA-1 mutant cells. GATA-2 prevents induction of a latent myeloid gene expression program. In the absence of GATA-1, GATA-2 contributes to cell-cycle progression and the maintenance of MK identity of GATA-1-deficient cells.[135]

The GATA-1 protein is associated with other transcription factors, the most important for MK differentiation include Friend of GATA-1 (FOG-1), TAL-1, RUNX-1, and FLI-1. The significance of GATA-1/FOG-1 association has been underscored in human X-linked congenital thrombocytopenia which is characterized by large platelets and moderate dyserythropoiesis. Four distinct *GATA-1* mutation types have been detected in X-linked disorders,[136,137] including mutations impairing FOG-1 binding, impairing binding to a GATC motif, affecting the N finger of GATA-1 but not disrupting FOG-1 or DNA binding, as well as mutations generating a truncated form of GATA-1 (GATA-1s).

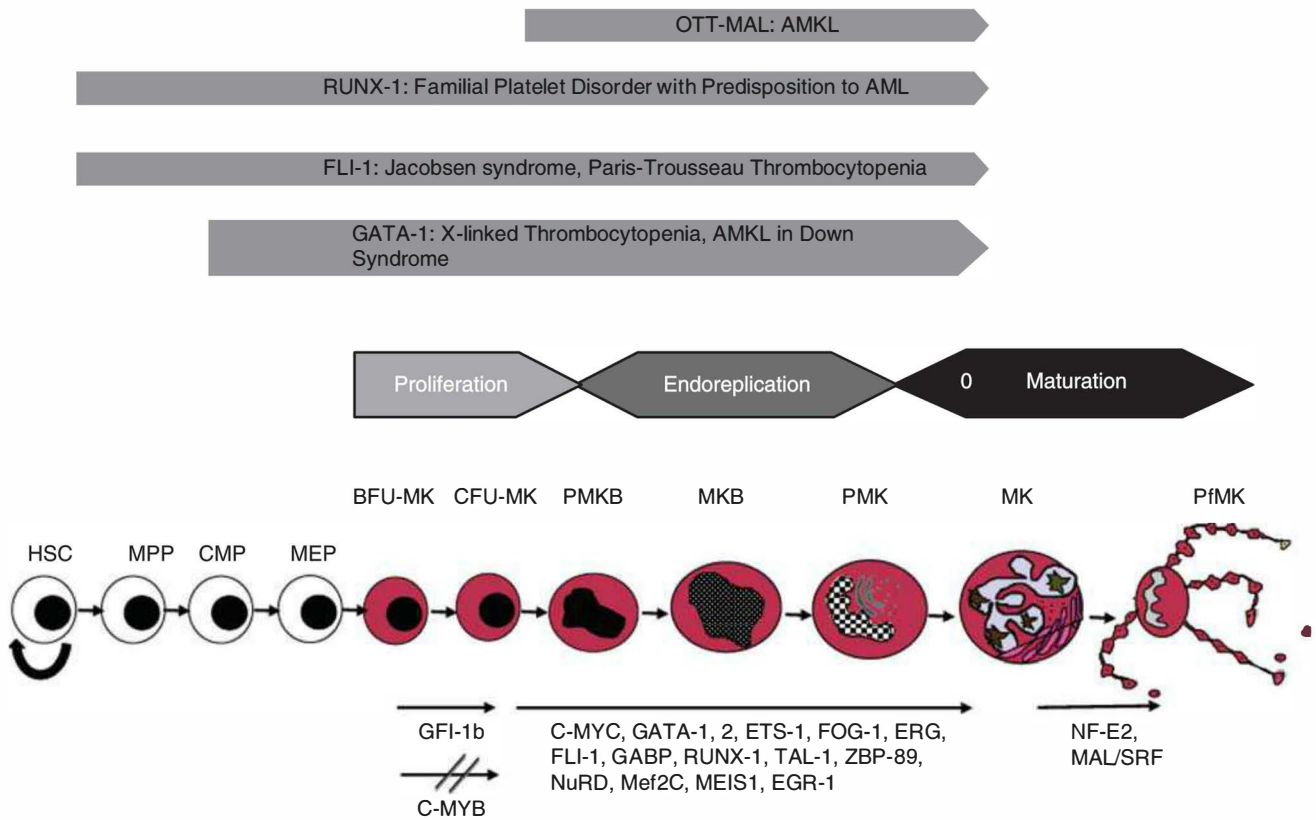

FIGURE 23.7 Molecular regulation of megakaryopoiesis and related disorders. FOG, friend of GATA; BFU, burst-forming unit; CFU, colony-forming unit; GP, glycoprotein; PMKB, promegakaryoblast; PF4, platelet factor 4; vWF, von Willebrand factor.

Acquired mutations in exon 2 of GATA-1 that generate a short GATA-1 variant (GATA-1s) lacking its *N*-terminus are responsible for the transient myeloproliferative disorder associated with Down syndrome which may lead to megakaryoblastic leukemia (AML-M7). It is noteworthy, however, that in a family with germline GATA1s, no myeloproliferative disorder or megakaryoblastic leukemia were described. In some patients with the Bernard-Soulier syndrome, a mutation of the GATA-binding site was detected in the *GPIb* promoter.[138]

FOG-1. FOG-1 was identified by a double hybrid approach. The knockout of FOG-1 gave a phenotype similar to that of GATA-1 in the erythroid lineage but with an additional profound absence of MK progenitors, suggesting that FOG-1 may have another partner in the early stage of the megakaryocytic lineage. GATA-2 appears to be a good candidate because simultaneous inactivation of critical FOG-binding regions of GATA-1 and GATA-2 leads to failure of the early and late stages of MK differentiation. The interaction between FOG-1 and GATA-1 is specifically required for late stages of MK maturation, but not early MK development, suggesting that GATA-1 and -2 are functionally interchangeable only at early stage in megakaryocytic development.[139]

ZBP-89 and NuRD were also identified as being components of multiprotein complexes involving GATA-1 and FOG-1. Studies in FOG-1- and GATA-1-deficient zebrafish and mice revealed a ZBP-89 requirement for megakaryopoiesis and definitive erythropoiesis but not for primitive erythropoiesis.[140] NuRD acts as a repressor or activator of select GATA-1/FOG-1 target genes, and the GATA/FOG1/NuRD axis not only promotes erythroid and MK maturation but is required for maintaining lineage fidelity by constraining lineage-inappropriate gene expression throughout MK-erythroid development.[141]

TAL-1. TAL-1 (also called SCL) is a bHLH transcription factor.[142] TAL-1/GATA-1 are present in a complex that also includes E2A, Ldb1, SSBP2, and LMO2 when regulating erythroid genes and in a complex that includes GFI1B, ZFP143, and the corepressor ETO2 when regulating MK genes.[143] TAL-1 knockout is embryonic lethal because of the absence of HSC.[144] Surprisingly, an inducible *Tal-1* knockout revealed that TAL-1 is not necessary for the stem cell properties of hematopoietic cells but is a master gene in the commitment of a mesenchymatous precursor cell toward hematopoiesis. In addition, TAL-1 is indispensable for the development of the erythroid, megakaryocytic, and mast cells.[52] It is also critical for platelet production during stress thrombopoiesis by directly regulating NF-E2 transcriptional activity.[145] Moreover, deletion of Mef2C, a direct TAL-1 target in MK cells, severely impairs *in vitro* megakaryopoisis and platelet quantity and functions *in vivo*.[146]

RUNX-1. A third transcription factor interacting with GATA-1 is RUNX-1 (AML-1).[147] RUNX-1 is the DNA-binding subunit of the core-binding factor (CBF) transcription complex. RUNX sites consisting of TGT/cGGT motifs have been found in several megakaryocytic promoters. Both RUNX-1 and CBFβ are highly expressed during megakaryocytic, but not erythroid, differentiation. Germ-line heterozygous missense, frame shift, and nonsense mutations, and a nucleotide deletion in a single allele of the *RUNX-1* gene have been detected in cases of familial thrombocytopenia with a predisposition to develop AML (FPD/AML).[148] The majority of these mutations are localized to the RUNT domain and impair DNA binding. By contrast, *C*-terminal domain mutations retain the ability to bind to DNA but lack transactivation properties. Two mechanisms for FPD/AML induction have been proposed based on the consequences of these mutations. One is linked to haploinsufficiency and the second to a negative dominant effect. In the former, mutated RUNX1 is unable to bind to DNA and to interact with CBFβ; in the second, the RUNX-1/CBFβ interaction is conserved, but the complex cannot bind DNA or cannot cause transactivation. The predisposition to develop AML seems to be related to the role of RUNX-1 in HSC homeostasis and may be increased when RUNX-1 mutants display a dominant negative function. Knockout mice indicate that RUNX-1 is essential for establishing definitive hematopoiesis and an inducible knockout of both RUNX-1 and CBFβ causes profound disruption of megakaryopoiesis.[149,150] Recently, several genes, including MYL9,[151] PF4,[152] ALOX12,[153] and MPL,[154] all implicated in megakaryopoiesis, proplatelet formation, and platelet function, were found to be directly regulated by RUNX1.

Ets family transcription factors. The Ets family of transcription factors consists of at least 13 different proteins. The products of two candidate genes, *ETS-1* and *FLI-1*, expressed in MK cell lines and MK, are able to transactivate MK-specific genes. *Ets-1* knockout mice have a marked defect in lymphoid lineages but normal megakaryopoiesis. However, ETS-1 expression increases during MK maturation and its overexpression promotes MK differentiation and enhances the upregulation of GATA-2 and MK-specific genes.[155] In contrast, the Fli-1 knockout is embryonic lethal due to defects in vascular development and megakaryopoiesis.[156] There is marked thrombocytopenia with an excess of small immature MK undergoing apoptosis. These abnormalities are related to those observed in the macrothrombocytopenia present in the Paris-Trousseau syndrome thrombocytopenia, a variant of the Jacobsen syndrome. These syndromes are associated with a deletion in human chromosome 11q23 involving both the *ETS-1* and *FLI*-1 genes. Overexpression of FLI-1 in $CD34^+$ cells partially restores the dysmegakaryopoiesis linked to this syndrome.[157] A direct role for FLI-1 in the late stages of megakaryopoiesis has been demonstrated in a mouse model in which there was an association between the loss of a carboxy-terminal regulatory domain and thrombocytopenia.[158] FLI-1 cooperates with GATA-1, FOG-1, and RUNX-1 to transcriptionally activate late MK genes such as GPIX, GPIbα, and PF4. The ETS transcription factor GABPα also regulates the expression of genes such as GPIIb or c-mpl expressed earlier during MK differentiation.[159] The transcription factor EKLF has been recently shown to act as an antagonist FLI-1 antagonist. While FLI-1 is involved in the commitment of a megakaryocyte–erythrocyte progenitor toward the MK lineage, EKLF plays the same role in the commitment to the erythroid lineage.[160] An inducible knockout of TEL-1, an ETS transcription factor, leads to a dramatic decrease in platelet counts.[161] Finally, the Ets-related gene ERG is also required for normal megakaryopoiesis. ERG is expressed in acute megakaryoblastic leukemia (AMKL) and strongly cooperates with GATA1s present in Down syndrome AMKL to immortalize MK progenitors, suggesting that the additional copy of ERG in trisomy 21 may have a role in Down syndrome AMKL.[162] Simultaneous alterations in FLI-1 and ERG lead to phenotypes more dramatic than single heterozygotes. These include a reduction in the number of committed hematopoietic progenitors, a significant decrease

in MK number, and an MK dysmorphogenesis leading to severe thrombocytopenia. These observations illustrate an indispensable requirement for both FLI-1 and ERG in normal HSC and MK homeostasis and suggest that these transcription factors may coregulate common target genes.[163]

p45$^{NF\text{-}E2}$. A hierarchical relationship between the GATA-1 and p45$^{NF\text{-}E2}$ transcription factors was demonstrated in MKs, showing that p45$^{NF\text{-}E2}$ acts downstream of GATA-1, although only 50% of p45$^{NF\text{-}E2}$ expression depends on GATA-1. p45$^{NF\text{-}E2}$ is a basic leucine zipper transcription factor that forms a heterodimer with proteins of the maf family to form an active NF-E2 transcription factor. p45$^{NF\text{-}E2}$ has also been identified in erythroid cells where it regulates genes involved in heme synthesis. Surprisingly, p45$^{NF\text{-}E2}$ knockout mice die at birth from severe thrombocytopenia with no defect in the erythroid lineage. In these p45$^{NF\text{-}E2-/-}$ mice, there was an increase in MK number with marked maturation defects, especially in the development of demarcation membranes and in the distribution of α-granules. p45$^{NF\text{-}E2-/-}$ MKs were unable to form proplatelets *in vitro*, correlating with acute thrombocytopenia.[164] The partner of p45NF-E2 in MK is predominantly MafG and, to a lesser extent, MafK. Double knockout mice, MafG$^{-/-}$ MafK$^{-/-}$, have thrombocytopenia similar to that of p45NF-E2$^{-/-}$ mice. Recently, it was demonstrated that the MafG C terminus is essential for proplatelet formation and platelet gene activation, but not for p45 binding to the Maf recognition element.[165]

Discovery of the p45$^{NF\text{-}E2}$ target genes has provided an opportunity to characterize genes involved in proplatelet formation. β1-Tubulin is restricted to the megakaryocytic lineage and is expressed at late stages of differentiation.[166] β1-Tubulin knockout mice are thrombocytopenic with abnormal-shaped platelets (i.e., absence of a discoid shape). However, overexpression of β1-Tubulin in p45$^{NF\text{-}E2-/-}$ MK does not restore proplatelet formation. It has also been shown that Rab27b, a small G protein involved in granule trafficking, is a target gene of p45$^{NF\text{-}E2}$ and may play a role in proplatelet formation,[167] whereas thromboxane synthase, also a p45$^{NF\text{-}E2}$ target, has no role in proplatelet formation.[168] The combination of NF-E2, GATA-1, and Ets-1 proteins plays a critical role in regulating expression of αn isoform of the human thromboxane A_2 receptor-α during megakaryocytic differentiation. Another NF-E2 target, Lims1/Pinch1, is implicated in cell migration, spreading, and platelet release.[169] The MK maturation process is closely related to the cellular response to oxidative stress in bone marrow. Thus, NF-E2 regulates not only platelet genes, but also cytoprotective genes that are common targets of p45 and Nrf2. The functional domination by p45 over Nrf2 favors reactive oxygen species (ROS) accumulation by limiting the expression level of cytoprotective genes. ROS accumulation promotes the full activation of platelet genes such as *Txas, Gp6, Selp,* and *Slamf1*.[170]

GFI-1b. GFI-1b, a transcription factor with a SNAG domain, is thought to be a transcriptional repressor. It is mainly expressed during erythroid and megakaryocytic differentiation and regulates the proliferation and differentiation of bipotent MEP.[171] GFI-1b knockout is embryonic lethal due to major abnormalities in erythroid and megakaryocytic differentiation with fatal anemia and thrombocytopenia.[172] Fetal liver progenitors from GFI-1b KO mice give rise to small MK colonies with a decreased level of GPIIb, c-mpl, and NF-E2 compared to wild-type cells. Although GFI-1b target genes responsible for this phenotype as well as its partners are unknown, GFI-1b controls erythropoiesis and megakaryopoiesis at least in part by regulating *TGFBR3* expression in MEP.[171]

Transcription factors linked to AML-M7. Study of AML-M7 has suggested that additional transcription factors are involved in MK development.

Neonatal AML-M7 is usually associated with a t (1;22) translocation leading to a fusion protein between two ubiquitous transcription factors MAL (MKL1) and OTT (RBM15).[173,174] MAL is an important transcription factor for MK development because it is a coactivator of the serum responsive factor (SRF) that is regulated by Rho signaling pathway.[175] MAL is associated with cytosolic G-actin in nonactivated cells. When the Rho pathway is activated, G-actin is sequestered and MAL is translocated to the nucleus. In combination with SRF, it activates target cytoskeletal genes such as *Tpm1, Actb, Flna, MMP9,* and *MYL9* that are required for MK migration and platelet biogenesis.[176] The role of SRF/MAL in polyploidization is controversial.[177]

The transcription factor c-MYB also plays an essential role in MK development. Suboptimal levels of c-MYB favor the differentiation of MKs and macrophages, while high levels are important for erythropoiesis and lymphopoiesis. At the stage of definitive MEP, c-MYB is an essential regulator of erythroid–megakaryocytic lineage bifurcation, while primitive erythropoiesis and megakaryopoiesis are c-MYB independent.[178] The expression level of c-MYB is downregulated in MEP by TPO through induction of miR-150.[179] Loss-of-function mutations are also associated with expansion of megakaryopoiesis and platelet production in mice.[180]

Additional transcription factors. MEIS1, a member of the TALE family of homeobox genes, is essential for MK development since MEIS1-deficient embryos lack MK/platelets and show extensive haemorrhage.[181] The transcription factors EGR-1 and FosB are involved in the megakaryocytic differentiation of the K562 cell line that is induced by phorbol ester.[182,183] EGR1 is also involved in the induction of p21 during MK differentiation leading to a senescent phenotype.[184] Absence of functional Foxp3, a key transcription factor for regulatory T cell development, also leads to defects in MKs and platelets.[185] MLLT3, originally identified as a fusion partner of MLL in acute myeloid leukemia, is a positive regulator of E/Meg differentiation decisions and acts in a regulatory circuit that includes GATA-1.[186] Among the different STATs, STAT1 seems to play a major role in MK differentiation. Finally, c-MYC plays a positive role in MK progenitor proliferation and was reported to be essential for the TPO–c-mpl axis in megakaryopoiesis. In contrast, studies using *c-Myc*–deficient mice showed that absence of the gene led to an increase in the platelet count,[187] while transient activation of *c-MYC* expression is critical for efficient platelet generation from human iPS.[188]

HUMORAL REGULATION OF MEGAKARYOPOIESIS INDEPENDENT OF THROMBOPOIETIN

Study of the cytokine regulation of megakaryopoiesis has largely been dominated by two concepts. First, humoral regulation by a late differentiation factor called TPO,[189–191] analogous to erythropoietin, but for the MK lineage. Second, sequential dual regulation corresponding to different developmental stages: one

regulator acting early in differentiation (*MK-CSF*) and a second acting late (differentiation potentiators or synergistic factors)[192] (FIGURE 23.8). Identification of TPO has verified these hypotheses since TPO acts on both early and late stages of megakaryopoiesis. However, in contrast to erythropoietin, TPO is not the only growth factor that acts on late stages of megakaryopoiesis. In particular, it does not regulate proplatelet formation. Indeed, the thrombocytopenia of c-mpl$^{-/-}$ and TPO$^{-/-}$ mice corresponds to about 10% of the normal platelet number, suggesting that other growth factors also regulate platelet production. Further, negative regulation by feedback may also influence platelet production.

Stimulatory Factors

Stem cell factor (kit ligand or steel factor). Stem cell factor (SCF), one of the most important cytokines, acts as a synergistic factor with most hematopoietic growth factors. It is synthesized by marrow stromal cells. Its predominant effect is in the early stages of hematopoiesis where it is a costimulator with erythropoietin on erythroid lineage. SCF also plays a role in megakaryopoiesis. Steel mice, deficient in SCF, exhibit a decrease in MK number, but also an increase in MK size, resulting in normal platelet count. It is noteworthy that Steel mice have no rebound thrombocytosis after 5-fluorouracil treatment.[193] However, when they are treated with SCF, they are able to undergo rebound thrombocytosis like their wild-type litter mates. *In vitro*, SCF greatly potentiates the effects of other cytokines including TPO on the growth of MK progenitors, increasing both the cloning efficiency and the size of MK colonies.[194–196] *In vivo*, injection of SCF accelerates platelet recovery after myelosuppression. In addition, SCF mobilizes hematopoietic progenitors in addition to MK progenitors. However, its use in clinical situations is limited by its toxicity.

Interleukin-3 (IL-3). IL-3 is a 14 to 22 kDa GP synthesized by T cells, but not by stromal cells, that acts directly on CFU-MK.[197,198] With the exception of TPO, IL-3 is the only cytokine that is able to induce MK colony formation alone. Its effect predominates in the early stages of megakaryopoiesis. It has even been suggested that IL-3 may impair MK terminal differentiation.

Erythropoietin. Epo is synthesized by the kidneys and regulates erythropoiesis. The Epo receptor is present on MK and platelets. There are strong indications that Epo is essentially a maturation cytokine.[199] Therefore, it acts more effectively as a costimulator with other cytokines and is able to favor terminal differentiation. Epo may also potentiate proplatelet formation by purified murine MKs.[200] However, its effect *in vivo* is controversial. It may increase platelet function, but also platelet production. In humans, chronic administration of Epo may slightly increase platelet production.[201,202]

Interleukin-6, Interleukin-11, leukemia inhibitory factor, oncostatin M and ciliary neurotrophic factor. These cytokines have pleiotropic effects on numerous cell types.[203] Their redundancy is explained by the molecular biology of their receptors. All utilize a common signal transducing chain (GP130). They are essentially mediators of the inflammatory response.

IL-6 was the first molecule of this family to be studied on megakaryopoiesis. It is a 21 to 26 kDa GP that is synthesized by a large number of cells including monocyte/macrophages, T cells, stromal cells, and MKs.[204,205] IL-6 has a minimal effect on MK colony formation, especially in humans, but acts in synergy with IL-3 and SCF.[206,207] IL-6$^{-/-}$ mice have no quantitative or qualitative defects of platelets, and IL-6 is not responsible for the basal platelet number in TPO$^{-/-}$ or *c-mpl*$^{-/-}$ mice.[208] IL-6 and IL-11 are probably responsible for the increase in platelet production in inflammatory diseases, although this effect might be indirect and related to increased synthesis of TPO.

In mice, leukemia inhibitory factor (LIF) potentiates the effects of IL-3 on MK colony formation[209]; its injection induces thrombocytosis.[210] However, in humans, its effect on *in vitro* megakaryopoiesis is less pronounced than IL-6.[196,211] LIF knockout mice have normal platelet count.[212] Oncostatin M has similar effects on thrombopoiesis as leukemia inhibitory factor.[213]

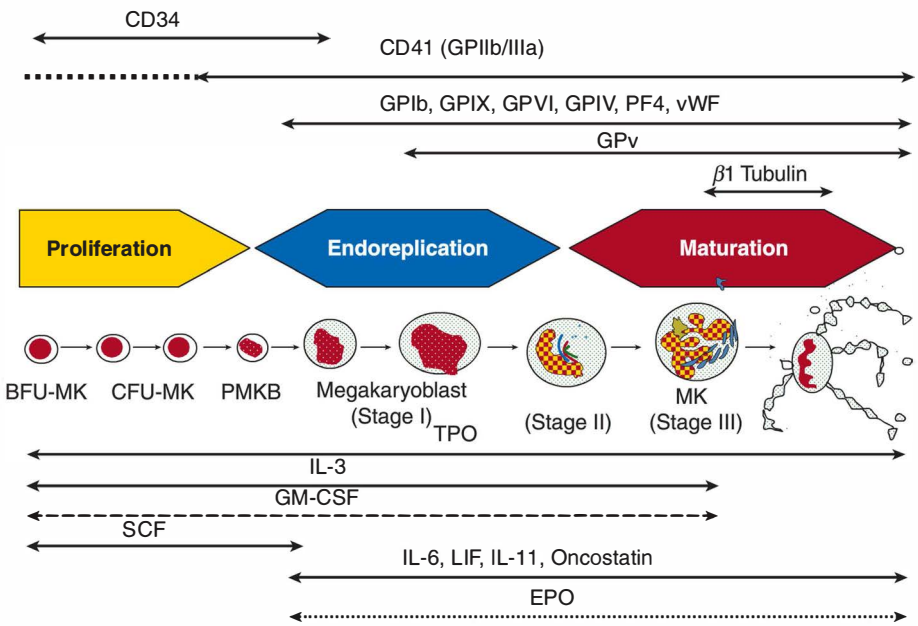

FIGURE 23.8 Regulation of MK development by cytokines. BFU, burst-forming unit; CFU, colony-forming unit; PMKB, promegakaryoblast; MKs, megakaryocytes; TPO, thrombopoietin; SCF, stem cell factor; EPO, erythropoietin.

IL-11 has marked effects on thrombocytopoiesis. *In vitro*, IL-11 has no MK-stimulating activity alone, but acts in synergy with IL-3 to stimulate murine and human colony formation.[214,215] IL-11 acts on the late differentiation by increasing MK size and ploidy in humans.[214] IL-11 has been used a therapeutic agent in human, but clinical trials have been discontinued due to secondary effect.

CXCL12 or stromal cell–derived factor-1 (SDF-1). SDF-1, also called *pre–B*-cell growth stimulating factor, is a CXC chemokine that binds to CXCR4 and is synthesized by stroma, bone marrow, endothelial, and dendritic cells. Disruption of the *CXCR4* and *SDF-1* genes in mice indicates that these molecules play an essential function in different tissues. Mice die at birth from numerous developmental abnormalities including cerebellar and cardiac abnormalities as well as abnormal blood vessel formation. CXCR4 and SDF-1 play major roles in B-cell development and in bone marrow colonization by HSC from fetal liver during ontogenesis. Thus, SDF-$1^{-/-}$ embryos have a marked defect in marrow myelopoiesis, whereas fetal liver myelopoiesis is normal.[216,217]

Besides its chemoattractive effect, SDF-1 is a growth factor for HSC.[218,219] MK progenitors and MK express CXCR4 which increases with maturation.[220] Despite increased expression, mature MKs respond poorly to SDF-1, at least in chemotactic assays.[120,121] SDF-1 has a synergistic effect with growth factors such as TPO on MK colony formation. It can directly affect MK polyploidization and maturation, increasing proplatelet formation.[221] High SDF-1 or fibroblast growth factor-4 expression can restore normal platelet production in $TPO^{-/-}$ and c-$mpl^{-/-}$ mice. This effect is not direct and is related to the chemoattraction of MK precursors toward a hematopoietic vascular niche that is permissive for MK maturation.[114] Therefore, TPO-independent platelet production may not be directly related to a specific cytokine, but rather to the interaction of megakaryocytic cells with the bone marrow environment and endothelial cells.

Bone morphogenic protein 4 (BMP4). BMP4 belongs to the TGF-β family that regulates cellular events such as differentiation, migration, adhesion, and apoptosis. BMP4 is able to regulate human MK differentiation by interfering with TPO signaling through the JAK/STAT and mTOR pathways. In the absence of TPO, BMP4 is able to induce $CD34^+$ progenitor differentiation into MK through all stages. TPO uses the BMP4 pathways to induce megakaryopoiesis. In addition, TPO upregulates the BMP4 autocrine loop by inducing BMP4 production by MK progenitors and upregulates BMP receptor expression.[222]

Inhibitory Factors

It became evident through MK colony assays that the MK lineage was different from the erythroid or granulocytic lineages because MK colony formation was partially inhibited by serum in a dose-dependent manner. However, serum derived from platelet-poor plasma had no effect,[223] implying that platelets store and secrete factors that inhibit megakaryopoiesis. It was subsequently found that these inhibitory proteins are present in platelet α-granules and consists of four different proteins: *TGF-β*, PF4-associated peptides, β-thromboglobulin, and connective tissue activating peptide (CTAP-III).[224,225]

The inhibitory effect of TGF-β on megakaryopoiesis is extremely potent,[224] but is not specific for megakaryopoiesis as all primitive hematopoietic progenitors, including HSCs, are inhibited.[226] Nonetheless, the effects of TGF-β on the MK lineage differ from those observed in other cell lines since TGF-β not only inhibits MK progenitor growth but also the endomitotic process and cytoplasmic maturation.[227,228] The inhibitory effect may be related to inhibition of TPO signaling.[229] Treatment of mice for 7 to 14 days with TGF-β1 induces a 95% reduction in the platelet count, as well as anemia and an increase in granulopoiesis.[230]

PF4, β-thromboglobulin, and CTAP-III belong to the chemokine family and are able to bind heparin. Their inhibitory effects may be related to a common dodecapeptide sequence, Asn–Gly–Arg–Lys–Ile–Cys–Leu–Asp–Leu–Glu–Ala–Pro.[224] Injection of PF4 into mice induces thrombocytopenia. *In vitro*, PF4 inhibits MK progenitor proliferation, MK polyploidization, and proplatelet formation. However, its effect on megakaryopoiesis is nonspecific. Similar to other members of this family, such as MIP-1α, PF4 acts on primitive hematopoietic cells and may also inhibit angiogenesis.[231] PF4 appears to exert its effect through low-density related protein-1, which is present on large, polyploidy MK and nearly absent from MEP and platelets.[232]

Thrombospondin 1 may inhibit MKs *in vitro* by binding to CD36.[233] Other cytokines such as interferons and TNF-α also inhibit megakaryopoiesis *in vitro*.[234,235] Finally, the Notch ligand, Delta4, also inhibits the terminal differentiation of human MKs,[236] whereas it may favor MK commitment at an early stage of megakaryopoiesis.[237]

The physiologic relevance of platelet inhibitory molecules on platelet production is currently unknown. These molecules could play a role in a feedback regulation process and could be involved in the mechanisms that render platelet production dependent on the platelet mass.

CONCLUSION

Megakaryopoiesis, the cellular process responsible for platelet production, is part of the general hematopoietic proliferation and differentiation process that takes place in the bone marrow. It takes an average of 1 week for an undifferentiated bone marrow stem cell to give birth to mature platelets. Megakaryopoiesis has a number of unique features when compared to the other hematopoietic cell lines. These include:

- Proximity to the erythroid lineage during the early steps of differentiation
- A switch during differentiation from DNA duplication followed by cytokinesis to DNA duplication without cytokinesis, leading to a polyploid cell with a single polylobulated nucleus. This process is quasi-obligatory during differentiation, in contrast to many other cell systems where polyploidization is a stress response
- A complex cellular process leading to platelet release into the blood

Platelets are born from the cytoplasmic fragmentation of MK via a regulated and dynamic process with intermediary proplatelet formation. The number of platelets formed is not the direct reflection of MK number but of MK mass. Nonetheless, the precise mechanism and site in the human body where platelets are released from mature MK still remain to be established.

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CHAPTER

24

Megakaryocyte Structure and Platelet Biogenesis

JOSEPH E. ITALIANO, JR. AND JOHN H. HARTWIG

INTRODUCTION

The process of making platelets is both amazing and bewildering. In the humans, 10^8 megakaryocytes are born each day, as the same number of mature megakaryocytes move to the bone marrow sinusoids and undergo terminal differentiation to release 10^{11} platelets. The ability to culture megakaryocytes, primarily from mice, and follow their maturation and platelet production *in vitro*, is beginning to provide information on the sequence of events leading to platelets and the molecules that regulate and power this process. In general, proplatelet and platelet production are driven by microtubule assembly and sliding, while mature platelet-based hemostasis is an actin-controlled process. In this chapter, we summarize the current knowledge of how megakaryocytes subfragment into platelets.

HISTORY/THE PROPLATELET MODEL

The process by which platelets are generated has been studied for over 100 years. In 1869, Italian biologist Giulio Bizzozero described a "cellule gigantic" up to 45 μm in diameter. However, it was not until 1906 that James Homer Wright discovered that platelets derive from these large precursor cells called megakaryocytes.[1] Wright established that platelets did not emerge in embryos until megakaryocytes appeared and demonstrated platelets coming off the cortex of megakaryocytes in the human, dog, rabbit, guinea pig, pig, and opossum. Many different theories have been proposed to explain how megakaryocytes produce platelets. Early microscopists documented that maturing megakaryocytes became filled with membranes, called demarcation membranes, and hypothesized that this membrane system divided the cytoplasm into "platelet territories" in which individual platelets were formed.[2,3] Release was postulated to occur by the complete fragmentation of the megakaryocyte cytoplasm into thousands of platelets. Later, it was observed that megakaryocytes extend long processes called proplatelets whose shafts are decorated with platelet-sized beads.[4,5] The classical "proplatelet theory" of platelet production was introduced by Becker and De Bruyn who first proposed that megakaryocytes extend long, sinus processes that fragment into platelets.[6] Later, Radley and Haller developed the "flow model," postulating that platelets derived from bead-like structures connected by thin cytoplasmic bridges.[7,8] Proplatelets have been observed both *in vitro* and *in vivo* across species and the bulk of experimental evidence now supports the involvement of proplatelets in platelet production.[9,10,11–14,15] Although the basic idea that proplatelets give rise to platelets is correct, the precise mechanism of platelet release has only recently been established.

CYTOPLASMIC MATURATION

As megakaryocytes mature in the bone marrow, they customize their cytoplasm and membrane systems for platelet formation. Before a megakaryocyte has the capability to produce platelets, it expands significantly to diameters of 50 to 100 μm and fills its cytoplasm with high levels of ribosomes producing platelet-specific proteins. Multiple rounds of endomitosis, a process that amplifies the DNA content by as much as 64-fold, facilitate this rapid cytoplasmic expansion.[16–18] In addition to DNA expansion, megakaryocytes undergo considerable maturation as internal membrane systems, organelles, and granules are assembled during their development. In particular, their cytoplasm, except for the most cortical regions, becomes densely filled with an expansive and interconnected membranous network of flattened cisternae and tubules[19] (FIGURE 24.1) This unique internal membrane system was initially called the demarcation membrane system (DMS) and as mentioned above was thought to divide the megakaryocyte cytoplasm into fields where individual, preformed platelets would assemble and release. This theory of cytoplasmic fragmentation as a mechanism for platelet biogenesis has lost support and DMS membranes are now thought to function primarily as a reservoir that continuously provides membrane for the growth of proplatelets.[8,20,21]

OVERVIEW OF PROPLATELET PRODUCTION

The discovery of thrombopoietin (Tpo) in 1994 has permitted major advances in the study of thrombopoiesis.[22–25] Tpo has facilitated the development of methods to culture bone marrow cells committed to the megakaryocyte lineage and that have successfully reconstituted the transition of terminally differentiated megakaryocytes into functional platelets *in vitro*. Megakaryocytes cultured in the presence of Tpo extend numerous proplatelets, consistent with the flow model. These proplatelets are structurally similar to those seen *in vivo* extending into bone marrow sinusoids and within the bloodstream.[9,10,26] Platelets released from megakaryocytes grown *in vitro* are also structurally and functionally similar to those found *in vivo*. Culture-derived platelets are of 2 to 3 μm diameter, disc-shaped fragments containing a microtubule-based marginal band and are responsive to platelet agonists.

Video microscopy has revealed the spatial and temporal changes leading to the generation of proplatelets[26] (FIGURE 24.2A–C). The production of platelets from megakaryocytes involves an elaborate process that converts the megakaryocyte cytoplasm into long, branched proplatelets upon which platelets are assembled. The process begins at a single position

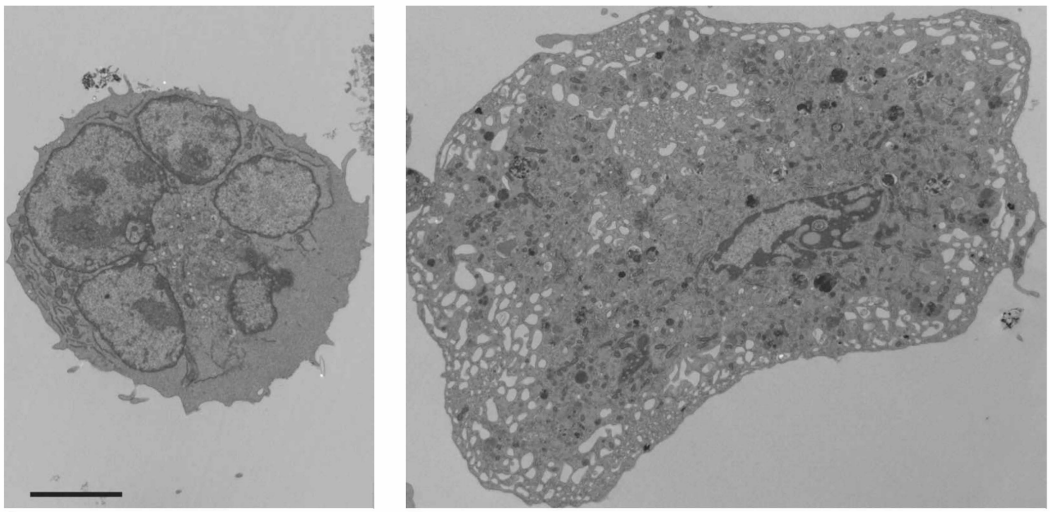

FIGURE 24.1 Maturation of the megakaryocyte DMS. **A:** Thin-section electron micrograph showing an immature mouse megakaryocyte that lacks a well developed internal membrane system. **B:** An extensive and interconnected system of internal membranes and channels that are connected to the plasma membrane characterizes the mature mouse megakaryocyte. The bar is 5 μm.

on the megakaryocyte plasma membrane. From this region, a large pseudopod forms, initially as a thickening of the plasma membrane. Over 4 to 8 hours, the pseudopod continues to extend and narrow into a tubule with an average diameter of 2 to 4 μm. Multiple bulges, termed swellings, each similar in size to a platelet, form along the length of the proplatelet, giving them the appearance of beads connected by thin bridges (FIGURE 24.2D). A teardrop-shaped tip develops at the proplatelet terminus and is believed to be the primary site of platelet maturation. Formation of additional proplatelets continues from the original erosion site and spreads in a wave-like manner throughout the remains of the cell until the megakaryocyte cytoplasm is entirely converted into an extensive and complex network of interconnected proplatelets. The multilobed "naked nucleus" remains in the megakaryocyte cell body as it is transformed into proplatelets. The events involved in platelet release from proplatelets are described below.

THE CYTOSKELETAL MECHANICS OF PLATELET PRODUCTION

The Cytoskeleton of the Resting Platelet

As discussed in Chapter 23, resting blood platelets have a well-defined microtubule cytoskeleton that functions to maintain the discoid shape of the resting platelet, as well as a robust filamentous (F)-actin cytoskeleton that both stabilizes the resting cells and is reorganized to generate the active shapes of spread platelets.[27–29] The microtubule skeleton is composed of an approximately 100 μm long microtubule and multiple dynamic short microtubules that shape the platelet into its discoid form by coiling around the edge of the cell.[30,31] In megakaryocytes, this microtubule cytoskeleton is the "engine" of platelet production (FIGURE 24.3). In the resting platelet, an actin-based membrane skeleton also plays a major role in stabilizing the discoid structure and in organizing the topology of surface receptors.[32,33] Hence, to understand the ultimate steps in platelet release, knowledge of the structure of the actin membrane skeleton in the resting platelet is essential.

Although the actin cytoskeleton of cells is complex, four proteins are critical in defining the architecture of the platelet membrane: spectrin, filamin, the von Willebrand factor receptor (vWfR), and F-actin. Spectrin forms a planar network linked together by the ends of F-actin to support the plasma membrane.[33,34,35] Disruption of spectrin tetramers (the functional unit of spectrin) prevents proplatelet elaboration and destabilizes released forms. Filamin links the GP1bα subunits of the vWfR to underlying F-actin.[32,36,37] The importance of the filamin–vWfR interaction has been established in both humans and mice where loss of expression of either molecule in platelets results in macrothrombocytopenia and bleeding.[38,39]

Two isoforms of filamin are expressed in platelets.[40–43] The most abundant isoform, filamin A (flnA), is expressed at a concentration of 5 μM (12,000 molecules per platelet). FlnA is in 20-fold molar excess over the second isoform filamin B.[44] Expression of FlnA increases dramatically in megakaryocytes in culture on days 2 to 4, particularly on day 4 when proplatelet formation begins. FlnA and FlnB are large elongated homodimers whose monomers are assemblies of an amino-terminus high-affinity actin-binding site followed by 24 Ig-like repeats, each composed of seven runs of β–structure.[45,46] Repeats 15 and 16 and repeats 23 and 24 are interrupted by hinge regions that are sites for calpain cleavage. Repeat 24 self-associates to dimerize the subunits.

In addition to binding and cross-linking F-actin, filamin molecules are scaffolds for the binding of additional proteins, including GP1bα, β–integrin tails, FilGAP, ralA, migfilin, and Syk.[47] The interaction of flnA with GP1bα is now understood at the atomic level. Binding occurs between a short run of

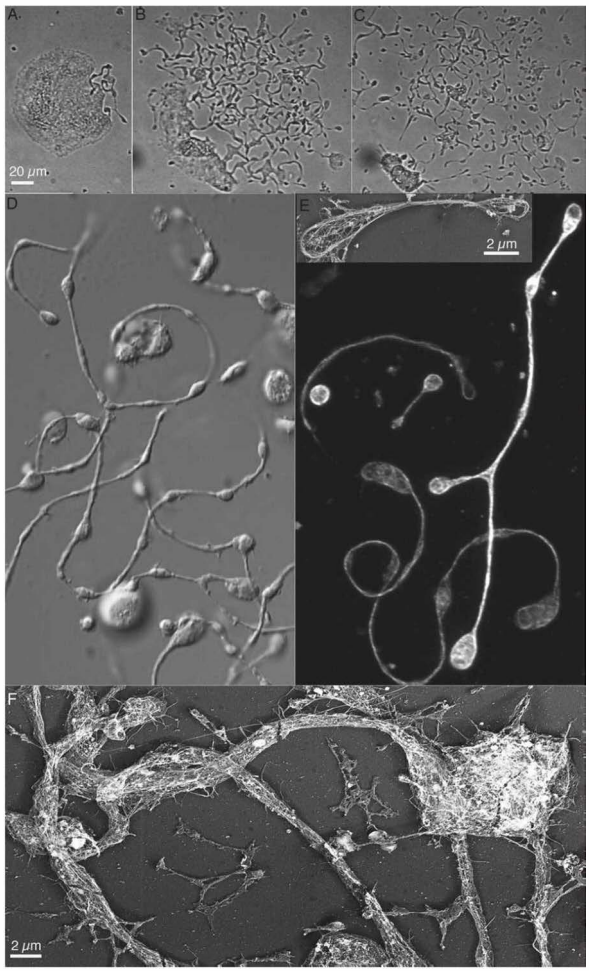

FIGURE 24.2 Visualization of production of platelets by megakaryocytes. **A:** Time-lapse sequence of a maturing megakaryocyte, showing the events that lead to the formation of proplatelet intermediates *in vitro*. Platelet formation initiates when the megakaryocyte begins unraveling at one pole. The bar is 25 μm. **B:** The bulk of the megakaryocyte cytoplasm has been transformed into multiple proplatelets. Proplatelets are highly dynamic and undergo extensive bending and branching. **C:** Proplatelets elongate and thin until the bulk of the megakaryocyte cytoplasm has been consumed. **D:** Differential interference contrast image of proplatelets elaborated by mouse megakaryocytes in culture. **E:** Proplatelets stained with antitubulin antibodies reveal the organization of its microtubules. Microtubules line the length of proplatelets and form coils only at the ends of the proplatelets. **Inset:** Low magnification electron micrograph showing the microtubule-based cytoskeleton of a representative released proplatelet. **F:** Undercoat structure of the proplatelet. The plasma membrane of the proplatelet tube is supported by a fibrous, spectrin-based membrane skeleton that is similar in structure to the membrane skeleton of resting platelets.

β–strand in the GP1bα cytoplasmic tail (amino acids 566 to 577) and two of the β–strands in flnA repeat 17; the GP1bα site binds in an antiparallel fashion between a groove formed between the C and D β–strands of FlnA.[36,37,48] FlnB shares the identical GP1bα binding site. Tight binding between these two proteins requires each GP1bα subunit in a vWfR to be bound to each repeat 17 in a filamin molecule. As there are 25,000 GP1bα chains expressed on the surface of a platelet, the stoichiometry for this interaction is ideal, and biochemical experiments have demonstrated that 90% to 95% of GP1bα is bound constitutively to flnA.[49] The stoichiometry of GP1bα binding to filamin is 1:1. Moreover, biochemical experiments indicate that 90% to 95% of GP1bα is bound constitutively to flnA.[49]

In turn, filamin is linked to underlying F-actin. This interaction organizes the topologic arrangement of the vWfR on the surface of mature platelets, and presumably on the surface of maturing proplatelets and preplatelets, as well. The final result is a linear alignment of vWfR on the platelet surface, a process that stabilizes and adheres the spectrin membrane skeleton to the plasma membrane.[50] The ends of actin filaments are bound on their sides at multiple points by flnA and on their ends by the spectrin tetramers where they cross-link the spectrin strands into a two dimensional lamina under the cytoplasmic surface of the plasma membrane. Curiously, platelets express both erythroid and nonerythroid spectrins.[33,34,35] This maybe due, in part, to the common stem cell origin of platelets and red blood cells or to the necessity of coping with membrane deflections during blood flow in similar fashion to erythrocytes; however, heterodimers of nonerythroid spectrin have lower affinity for one another compared to the nonerythroid molecules. This allows them to undergo dynamic association-dissociations *in situ*.

When and how the GP1bα and F-actin connections are established during platelet formation remains to be established. Failure to express either flnA or GP1bα results in platelets that are approximately three times larger than normal platelets and are extremely fragile, resulting in their rapid clearance from circulating blood.

Extension of Proplatelets is Powered by Microtubules

Proplatelet formation is dependent on microtubules because exposure of megakaryocytes to drugs that disassemble microtubules, such as vincristine and nocodazole, blocks proplatelet production.[8,11,15,51] Microtubules, polymers assembled from tubulin dimers, are the major structural component of the engine that powers proplatelet extension. Each microtubule is 25 nm in diameter and composed of a circular assembly of 13 protofilaments. Examination of the microtubule cytoskeletons of megakaryocytes in the act of producing proplatelets provides clues as to how microtubules power platelet development.[26,52] The microtubule cytoskeleton undergoes a dramatic remodeling during proplatelet formation. In immature megakaryocytes, microtubules emanate from the center of the cell to the cortex. During the initial stage of proplatelet formation, as blunt pseudopodia form, cortical microtubules consolidate into thick bundles positioned just beneath the plasma membrane. The pseudopodia then elongate at an average rate of 0.85 μ/min and microtubules form linear arrays that line the entire length of the proplatelet extensions. The microtubule bundles are thickest in the proplatelet near the cell body of the megakaryocyte, but thin to bundles of 5 to 10 near the proplatelet tips. The distal end of each proplatelet always contains a microtubule bundle that loops just beneath the plasma membrane and reenters the shaft to form a teardrop-shaped structure (FIGURE 24.2D,E).

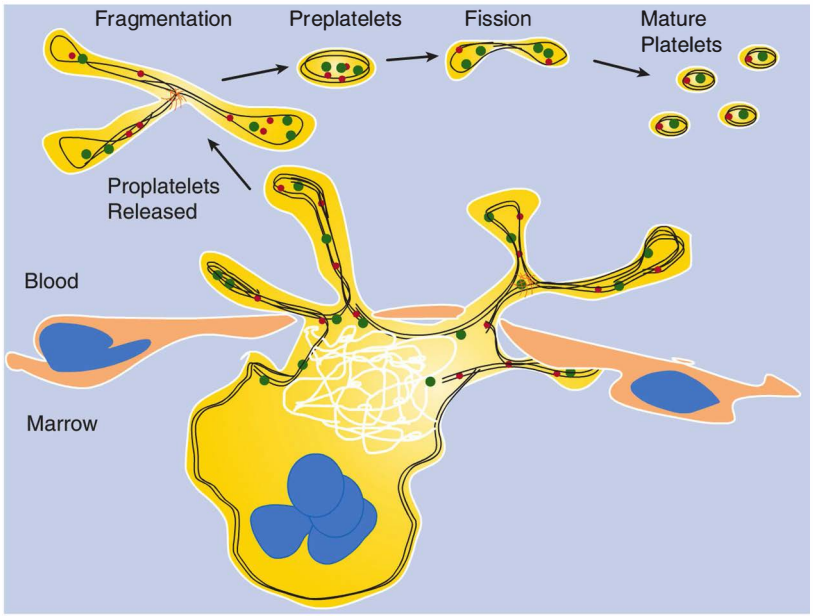

FIGURE 24.3 Proplatelet model of platelet production. Megakaryocytes mature in the bone marrow. In the final stage of development, megakaryocytes mature their cytoplasm, granules, and DMS and move near the marrow sinusoids. Proplatelets are elaborated, many of which, protrude through the sinusoids and release into the flowing blood. Proplatelet production begins with the generation of large pseudopodia that elongate and form thin proplatelet processes with bulbous ends. The proplatelet ends contain a peripheral bundle of microtubules that loops upon itself to form a teardrop-shaped structure. Sliding of overlapping microtubules powers proplatelet elongation as organelles are moved individually over microtubules into proplatelet tips. In the marrow, or in blood, proplatelets mature by fragmentation into smaller sized proplatelets until they reach sizes that can release 2 to 10 platelets, called preplatelets. Microtubule dynamics cause preplatelets to take on barbell shapes that ultimately undergo fission and release the mature platelets from their ends.

Megakaryocytes express different β–tubulin isoforms, but the divergent, hematopoietic b1 isoform is dominant (Table 24.1). β1-tubulin is expressed exclusively in megakaryocytes and platelets during the late stages of megakaryocyte development and several observations reveal it is essential for the generation of normal platelet numbers, as well as the disc shape of platelets.[53] First, β1 tubulin is a downstream effector of the megakaryocyte transcription factor NF-E2.[54] Second, genetic elimination of the β1-tubulin gene in mice causes thrombocytopenia and circulating platelets are spherical, not discoid.[55] Third, megakaryocytes derived from β1-tubulin knockout mice do not form proplatelets in culture and instead extend only a small number of short protrusions.[55]

Direct visualization of microtubule dynamics in living megakaryocytes using green fluorescent protein (GFP) technology has provided insights into how microtubules orient to power proplatelet elongation.[52] End-binding protein 3 (EB3), a microtubule plus end-binding protein associated only with growing microtubules, when fused to GFP and retrovirally expressed in murine megakaryocytes, can be used as a marker to locate microtubule plus ends and follow plus-end dynamics (FIGURE 24.4). Immature megakaryocytes without proplatelets employ a centrosomal-coupled microtubule nucleation/assembly reaction, which appears as a prominent starburst pattern when visualized with EB3-GFP. Microtubules assemble only from the centrosome and grow outward into the cell cortex where they turn and run in parallel with the cell edges. However, just before proplatelet production begins, centrosomal assembly ends and microtubules release and consolidate into the cortex as bundles. Fluorescence time-lapse microscopy of living, proplatelet-producing megakaryocytes expressing EB3-GFP revealed that as proplatelets elongate, microtubules assembled continuously throughout the entire proplatelet. The EB3-GFP studies also revealed that microtubules polymerize in both directions in proplatelets, for example, both toward the tips and cell body, demonstrating that microtubules composing the bundles have a mixed polarity.[52] How is this microtubule polymerization regulated? Ran binding protein (RanBP10) is a β1-tubulin binding factor that concentrates on proplatelet microtubules and may regulate microtubule assembly.[56] RanBP10 contains a guanine nucleotide exchange factor (GEF) activity for related nuclear protein (RAN). To nucleate microtubules, the GEF RCC1 loads Ran with GTP, leading to the activation of Tpx2, a factor that carries positional information for microtubule nucleation (FIGURE 24.4). Accordingly, RanBP10 depletion in megakaryocytes causes disruption of microtubules.[57]

Even though microtubules are continuously assembling at their plus ends in proplatelets, polymerization *per se* does not provide the force for proplatelet elongation. Proplatelets possess an inherent microtubule sliding mechanism. Cytoplasmic dynein, a minus end microtubule molecular motor protein, localizes along the microtubules of the proplatelet and appears to directly contribute to microtubule sliding, since inhibition of dynein through disassembly of the dynactin complex prevents proplatelet formation.[52] Microtubule sliding can also be reactivated in detergent-permeabilized proplatelets. ATP, known to

Table 24.1

Specific molecules involved in thrombopoiesis

Polymer System	Protein	Function	MK KO Phenotype	PP KO Phenotype	Platelet KO Phenotype	Reference
Microtubules	B1-tubulin	Main MT component		Decreased in formation	Decreased platelets, spherical platelets	55
Microtubules	EB-1, EB-3	Promotes + end assembly				52
Microtubules	RanBP10	Ran GEF	MT disruption			57
F-actin	Filamin	Cross-link F-actin, scaffold signaling proteins, link GP1bα to actin	Altered DMS *in vitro*, *in vivo* 5–10× more megakaryocytes	Accelerated formation of large proplatelets	Fragile and large platelets, short circulation times	77
F-actin	GP1bα	vWf binding		Blockage of preplatelet maturation	Fragile and large platelets, short circulation times	39
F-actin	Myosin II	Clot retraction, actin contraction	Abnormal inclusions	Accelerated formation	Decreased formation	59
F-actin	Spectrin	Stabilize membranes	No KO but expression of dimerizing fragment disrupts DMS	Stops proplatelet elaboration		26
F-actin	Cofilin	F-actin turnover	Unknown	Unknown	Macrothrombocytopenia—platelets filled with F-actin	78
F-actin	ADF				Small platelets	78
F-actin	Rac/cdc42 CalDAG-GEF Kindlin Rab27b Dynamin 3	Regulate actin assembly RapGAP Integrin	 Over expression increases ploidy in megakaryocytes	 Dominant negative reduces PP formation *in vitro*	Mild thrombocytopenia Normal numbers, poor integrin inside- out activation Diminished integrin inside-out signaling	79, 80 Bergmeier 81 82 83

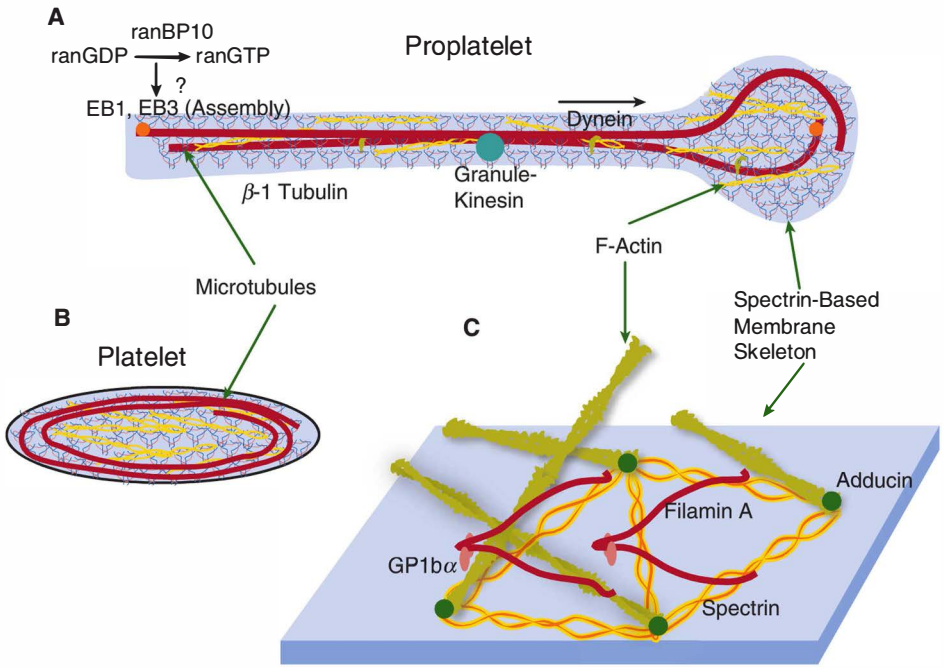

FIGURE 24.4 Cytoskeletal systems that form and stabilize proplatelets and platelets. **A:** Cytoskeleton of the proplatelet. Proplatelets are protruded from the megakaryocyte cell body by microtubule-based forces. Microtubules are assemblies of αβ-tubulin dimers. β1-tubulin is unique to the megakaryocyte lineage and is the major isoform expressed. In proplatelets, microtubules are organized into bundles that run from the cortex of the megakaryocyte into the shafts of proplatelet. These bundles are composed of multiple microtubules having mixed polarity. Individual microtubules are highly dynamic, continuously undergoing assembly-disassembly reactions. Assembly occurs at the plus end of microtubules and is promoted by end-binding proteins such as EB-1 and EB-3. The ends of proplatelets are enlarged and contain loops of microtubules. Elongation of proplatelets is driven primarily by cytoplasmic dynein, which attaches to one microtubule and slides a second past. Small GTPases such as Ran are upstream in these reactions. RanGDP is converted to RanGTP by the ranBP10 GEF. The microtubule bundles are also the tracks used by kinesin-coated granules that move up and down the proplatelet shafts. Proplatelets also have a spectrin-based membrane skeleton **(C)** that is required to stabilize the protruded forms. **B:** The mature platelet. Fission of preplatelets is mediated by the sliding apart of microtubules in the shafts of the preplatelet barbell forms and by their rolling into a coil to define the mature platelet and provide it with a discoid shape. **C:** The membrane skeleton of preplatelets and platelets. A two-dimensional lamina of spectrin tetramers coats the cytoplasmic surface of the plasma membrane. This network is interconnected by the barbed ends of actin filaments that are targeted to spectrin by bound adducin molecules. This network is tightly laminated to the plasma membrane by connections between the sides of actin filaments and the vWfR. These connections are made by fInA molecules that bind to both F-actin and the GP1bα chain of the vWfR.

support the enzymatic activity of microtubule-based molecular motors, activates elongation in permeabilized proplatelets that contain dynein and its regulatory complex, dynactin. Thus, dynein-facilitated microtubule sliding appears to be the key event in driving proplatelet elongation.

Transport and Packaging of Organelles/Granules is Microtubule-Dependent

Nascent platelets, maturing at the proplatelet tip, must ultimately be loaded with their allotment of granules and organelles prior to release. Central to the principle that proplatelets are the precursors of platelets is the requirement that granules/organelles have the ability to move sizable distances from the megakaryocyte cell body. In some cases, these granules/organelles must travel approximately 0.5 mm, a serious challenge to the distribution system. Organelles move individually and bidirectionally along the microtubules of proplatelets by two major mechanisms: (a) translocation of granules over microtubule tracks, and (b) attaching granules to and sliding them with microtubules.[58] However, measurements of the velocity of granule/organelle movement leads to apparent paradoxes because granule/organelle movement is extremely slow relative to the 4 to 8 hours needed to complete proplatelet and platelet formation and because granule/organelle movement changes directions frequently. Thus, although the transport mechanism effectively moves granules/organelles into proplatelets, it does not seem capable of producing rapid unidirectional delivery to proplatelet ends. One solution to this problem employed by the megakaryocyte is to detach the proplatelet and also use the fragmented end for platelet production. A second solution is to sequentially reduce the length of the proplatelet by inward trimming from the ends as platelets release, consequently shortening the distance that granules/organelles must travel. Once granules

arrive in the vicinity of the proplatelet ends, they are captured by the microtubule coil in the nascent platelets and concentrated at the tips.[58] It is also likely that megakaryocytes use some type of counting mechanism to ensure that each platelet receives its correct number of granules and essential organelles.

Amplification of Proplatelet Ends is Driven by Actin

Because platelet maturation is limited to proplatelet tips, efficient platelet production requires generation of large numbers of ends. Analysis of live action video microscopy of proplatelet formation from cultured megakaryocytes has revealed that ends of proplatelets are increased in a dynamic process that repeatedly bends and branches the shaft of the proplatelet.[26] End amplification begins when a proplatelet shaft is bent into a sharp kink, which then folds back on itself, forming a loop in the microtubule bundle. The new loop ultimately elongates, forming a new proplatelet shaft branching from the side of the original proplatelet. Loops lead the proplatelet tip, define the site where nascent platelets will assemble, and where organelles and granules are trafficked. Megakaryocytes treated with the actin inhibitors latrunculin or cytochalasin can only extend long, unbranched proplatelets that are decorated with few swellings along their length.[26] Despite extensive characterization of actin filament dynamics during platelet activation, how actin participates in this reaction and the cytoplasmic signals that regulate bending are not understood. Electron microscopy and phalloidin staining of megakaryocytes undergoing proplatelet formation indicate that actin filaments are distributed throughout the proplatelet and are particularly concentrated within swellings and at proplatelet branch points. One possibility is that proplatelet bending and branching is powered by the actin-based molecular motor myosin. Interestingly, a mutation in the nonmuscle myosin heavy chain-A gene in humans produces the May-Hegglin anomaly, a macrothrombocytopenic disorder.[59,60] Protein kinase Cα also associates with aggregated actin filaments in megakaryocytes undergoing proplatelet formation, and inhibition of protein kinase Cα or integrin signaling pathways prevent actin filament aggregation and proplatelet formation in megakaryocytes.[61] Nonetheless, the function of actin filament dynamics in platelet biogenesis remains to be clarified.

Expression, Assembly, and Importance of the Megakaryocyte Membrane Skeleton

The plasma and demarcation membranes of megakaryocytes are supported by an elaborate skeleton system. The major component of this system is a planar spectrin network. This plasma membrane undercoat structure is made up of spectrin strands crosslinked by actin filaments. Spectrin strands bind to the ends of actin filaments to assemble a continuous mesh-like structure beneath the plasma membrane (FIGURE 24.4C). Pores in this network have a triangular shape and are similar to those observed in the well-characterized erythrocyte membrane skeleton. However, the organization is somewhat different to that found in the red blood cell. The spectrin strands are bound to the ends of long actin filaments instead of short actin oligomers. Electron microscopy reveals that proplatelets, like circulating platelets, have a dense membrane skeleton whose main fibrous component is spectrin (FIGURE 24.2F), although unlike other cells, megakaryocytes and their progeny express both erythroid and nonerythroid spectrins. Assembly of spectrin into tetramers is required for DMS maturation and proplatelet extension, as expression of a spectrin tetramer–disrupting construct in megakaryocytes inhibits both processes. Incorporation of this spectrin-disrupting fragment into a novel octyl glucoside-permeabilized proplatelet system rapidly destabilizes proplatelets, causing blebbing and swelling. Spectrin tetramers also stabilize the "barbell-shapes" of the penultimate stage in platelet production (see section, *The Final Stage Of Platelet Production-Release*), since addition of the tetramer-disrupting construct converts these barbell-shapes to spheres.

THE INFLUENCE OF THE BONE MARROW NICHE ON THROMBOPOIESIS

Megakaryocytes cultured outside of the bone marrow can form highly developed proplatelets in suspension, demonstrating that direct interaction with the bone marrow niche is not a requirement for platelet production. Nonetheless, the efficiency of platelet generation *in vitro* appears to be reduced relative to that observed *in vivo* and the bone marrow environment composed of a complex adherent cell population may play a role in platelet production by direct cell contact or secretion of cytokines. Scanning electron micrographs of bone marrow megakaryocytes extending proplatelets through junctions in the endothelial lining into the sinusoidal lumen have been published, suggesting platelet production occurs in the bone marrow.[4,7,62–64] Bone marrow megakaryocytes are strategically located in the extravascular space on the abluminal side of sinus endothelial cells and appear to send beaded proplatelet projections into the lumen of sinusoids. Electron micrographs show that these cells are anchored to the endothelium by organelle-free projections extended by the megakaryoctes.[65] Several observations suggest that thrombopoiesis is dependent on the direct cellular interaction of megakaryocytes with bone marrow endothelial cell–specific adhesion molecules.[66] It has been shown that the translocation of megakaryocyte progenitors to the vicinity of bone marrow vascular sinusoids is sufficient to induce megakaryocyte maturation.[67] The chemokines stromal cell–derived factor 1 and fibroblast growth factor 4 (FGF-4), known to stimulate expression of adhesion molecules such as very late antigen-4 on megakaryocytes and vascular cell adhesion molecule 1 (VCAM-1) on bone marrow endothelial cells, have been implicated in this process.[68,69] Disruption of bone marrow endothelial cell VE-cadherin–mediated homotypic intercellular adhesion interactions results in a profound inability of the vascular niche to support megakaryocyte differentiation and to act as a means to the bloodstream.

THE FINAL STAGE OF PLATELET PRODUCTION-RELEASE

Although *in vitro* systems have provided insight into platelet generation, the exact mechanism of platelet release *in vivo* is still highly debated. Megakaryocytes are produced in the bone marrow and may undergo fragmentation into platelets at this location. Bone marrow megakaryocytes positioned on the abluminal side of sinus endothelial cells have been visualized extending proplatelets into the vessel lumen, where they have been hypothesized to release into the circulation and undergo

further fragmentation into individual platelets. In 1998, Behnke and Forer proposed a model suggesting that the final stages of platelet maturation occur exclusively in the circulation. In this model of thrombopoiesis, megakaryocyte fragments released into the blood become transformed into platelets while in the circulation. This theory is supported by several observations. First, the presence of beaded megakaryocyte processes in circulating blood has been thoroughly documented.[70,71,72,73,74] Megakaryocyte fragments can make up to 5% to 20% of the platelet mass in plasma. Second, megakaryocyte fragments, when isolated from platelet-rich plasma, have been reported to elongate, undergo curving and bending motions, and eventually fragment to form disk-shape structures resembling chains of platelets.[70] Third, because both cultured human and mouse megakaryocytes can form functional platelets *in vitro*, neither the bone marrow environment nor the pulmonary circulation is essential for platelet production and release.[9,10,26]

Visualization of thrombopoiesis within the bone marrow of living mice also supports a model in which proplatelet morphogenesis continues in the blood. Using intravital fluorescence microscopy to examine the opened cranial marrow cavity of mice, living megakaryocytes have been visualized in the act of elaborating proplatelet processes into the vascular lumen.[75] Multiphoton intravital microscopy of intact bone marrow visualized proplatelet extensions protruding into microvessels, where they appeared to be sheared from their transendothelial stems by blood flow. Most of the shed fragments of megakaryocytes exceeded the dimensions of platelets, suggesting that proplatelet development continues in the blood to produce individual platelets.[75]

The cytoskeletal mechanics of proplatelet maturation and platelet release have recently been established.[76] By isolating released proplatelets and analyzing their conversion into individual platelets *in vivo* and *in vitro*, a new intermediate stage in platelet production, the preplatelet was identified. Preplatelets are anucleate discoid particles 3 to 10 μm across that have the capacity to convert reversibly into elongated proplatelets. The preplatelet to barbell proplatelet transformation is powered by twisting microtubule-based forces. Preplatelets can mature into platelets both *in vivo* and *in vitro*. Platelet release from the ends of proplatelets occurs at an increasing rate during culture as larger proplatelets undergo successive fission.[76] Shear forces accelerate proplatelet fission and individual platelet release in culture.

CONCLUSION

Megakaryocytes are highly specialized precursor cells that function exclusively to generate and release platelets. After completing endomitosis, polyploid megakaryocytes undergo a rapid cytoplasmic expansion phase characterized by the formation of an extensive DMS and the accumulation of cytoplasmic proteins and granules critical for platelet function. During the final stages of development, the megakaryocyte cytoplasm undergoes a massive remodeling in cytoplasmic extensions termed proplatelets. Proplatelets function as the assembly lines of platelet production. Microtubules, actin filaments, and the spectrin membrane skeleton all contribute to the formation of proplatelets and production of individual platelets.

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CHAPTER

25 Thrombopoietin

KENNETH KAUSHANSKY AND IAN HITCHCOCK

HISTORY

The term erythropoietin (EPO) was coined at the turn of the last century to describe the humoral substance responsible for red cell production. At that point in time, blood platelets were barely distinguishable in the best microscopes, prompting their dismissal as "the dust of the blood." However, the pioneering work of Carnot, Wright, and others in the early 20th century defined the critical role of blood platelets in coagulation and their origin from the marrow megakaryocyte, ultimately prompting the Hungarian physician Keleman to coin the term thrombopoietin (TPO) to describe the humoral substance responsible for the recovery of platelet levels following thrombocytopenia.[1]

In the mid-1960s, several groups set out to purify TPO from the plasma of thrombocytopenic animals. At the time, the only reliable assay on which to base a purification strategy was the ^{35}S uptake assay, in which radioactivity incorporated into blood platelets of injected mice or rats served as a measure of thrombopoietic activity. This *in vivo* assay proved very cumbersome; the assay was too insensitive, costly, and time-consuming to be practical, and little progress was made. With the availability of *in vitro* megakaryocyte differentiation assays in the 1980s, additional purifications were attempted; nevertheless, although the availability of what was thought to be partially purified TPO helped to define its expected biologic properties,[2–4] none of the initial purification efforts led to amino acid determination or cloning of the molecule.

Occasionally in science, a finding from one field, although important in itself, can have a profound, catalytic effect on a seemingly unrelated area of research. The discovery and characterization of the murine myeloproliferative leukemia virus had such an influence on the search for TPO. The virus causes an acute myeloproliferative syndrome in infected mice.[5] In 1990, the responsible oncogene was cloned, and the protooncogene obtained 2 years later.[6,7] Based on the predicted presence of four spatially conserved cysteine residues and a juxtamembrane pentapeptide sequence (Trp–Ser–Naa–Trp–Ser), it was immediately evident that the cellular gene was a member of the hematopoietic cytokine receptor family,[8] which includes the receptors for EPO, the granulopoietic factors interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF), and granulocyte macrophage colony-stimulating factor (GM-CSF), and multiple lymphokines. However, in 1992 its ligand was unknown; *c-mpl* encoded an orphan receptor. Based on the origin of the *c-Mpl* cDNA, the bipotent erythroid/MK cell line HEL,[9] and on antisense *c-mpl* knock-out experiments demonstrating a significant reduction in megakaryocytes,[10] many groups postulated that the Mpl ligand might be identical to TPO.

THE CLONING OF THROMBOPOIETIN

While progress in understanding megakaryocyte biology and in purifying TPO was being made using the c-Mpl receptor, others had developed improved bioassays for TPO and continued to make efforts using conventional purification strategies. Using a modified *in vivo* assay in which the polyploidy of marrow megakaryocytes from injected rats was examined, Kuter et al.[11] performed an 11-step purification of ovine TPO, although the publication of this work did not report the cloning of the corresponding cDNA. In contrast, using an *in vitro* megakaryocyte assay, the scientists at Kirin Pharmaceuticals in a 12-step conventional purification scheme obtained sufficient plasma TPO from thrombocytopenic rats to obtain amino acid sequence and cloned cDNA for rat and then multiple species of the protein, including the human hormone.[12]

Using the *c-Mpl* protooncogene product coupled to affinity matrices, scientists at Genetech and at AMGen obtained microgram quantities of porcine and canine TPO, respectively, allowing their amino acid sequencing and cDNA cloning.[13] In contrast to the biochemical purifications utilized by these four groups, an expression cloning strategy was used by Lok et al.[14] to obtain cDNA for murine and then human TPO. In this approach a hematopoietic cell line, BaF3, that requires IL-3 for survival and proliferation, was engineered to express the *c-Mpl* protooncogene. In this way, if the c-Mpl ligand was present in the culture, the cells should remain viable and potentially proliferate after withdrawal of IL-3. The BaF3/mpl cells were chemically mutated and autonomously growing sublines were obtained; although most such sublines were producing their own IL-3, others were producing the c-Mpl ligand. This subline was used as source for cDNA library construction and an additional functional cloning strategy. Remarkably, the cDNA obtained from the above four groups, three of them cloned nearly simultaneously, all encoded the same polypeptide (except for species differences). Initial *in vitro* experiments using the corresponding recombinant proteins demonstrated the effect of each of these substances on megakaryocyte maturation, and injections into normal mice resulted in massive increases in peripheral blood platelet counts and marrow megakaryopoiesis. With these efforts TPO was finally cloned.

STRUCTURE OF THROMBOPOIETIN

The cloned human TPO cDNA encodes a polypeptide of 353 amino acids including the 21–amino acid secretory leader sequence.[15] The mature protein can be conveniently divided into two domains: the amino-terminal 154 residues bear striking sequence homology with EPO and bind to the mpl receptor; the

carboxyl-terminal domain bears no resemblance to any known proteins, but contains multiple sites of both *N*- and *O*-linked carbohydrate. This latter feature accounts for the large discrepancy between the predicted and actual molecular weight of the protein; approximately half of the 70-kDa TPO molecule is carbohydrate.

Based on its amino acid sequence, and like its closest homologue EPO, TPO is predicted to fold into a four-helix bundle protein. This prediction was recently confirmed with solution of the tertiary structure of the receptor-binding domain of TPO in complex with an anti-TPO monoclonal antibody.[16] Comparisons of the two proteins reveal striking similarities, but also some critical differences, likely accounting for the failure of the two hormones to cross react with the receptors for the other hormone.[17]

As noted, TPO can be conveniently divided into two domains based on homology to EPO; expression of a truncated version that represents the domain most homologous to EPO revealed that this amino-terminal half of the molecule is responsible for binding to and stimulation of the Mpl receptor.[18] However, the circulatory half-life of the isolated amino-terminal fragment is substantially shorter than the full length molecule, unless it is modified with polyethylene glycol.[19] In contrast to the stimulatory activity of the amino-terminal domain, the carboxyl terminal domain of TPO acts to enhance secretory efficiency of the protein; the level at which the isolated amino-terminal fragment is expressed from cells that normally produce TPO is reduced about 5- to 10-fold compared to the secretory level of the full length molecule.[20–22] Whether this is due to enhanced folding of the polypeptide, protection from intracellular proteolysis, or other mechanisms is presently under study.

Several investigators have attempted to ascertain the sites on TPO responsible for binding to its receptor. Using site-directed alanine scanning mutagenesis to study the functional activities of 40 solvent-exposed residues of the protein, monoclonal antibody epitope mapping, and a phage display-binding assay system, Pearce et al.[23] determined that the residues vital for hormone binding to c-Mpl are located in the first and fourth predicted α-helices of the molecule and the segment connecting the first and second helices, locations previously shown to be important for functional interactions in several other cytokine/receptor systems.[24] Subsequently, Jagerschmidt produced 20 TPO mutants and compared the mutants' ability to induce the proliferation of a c-Mpl-expressing cell line, M-07e, with that of wild-type TPO. Among the mutations that substantially reduced biologic activity, three were found located in the A helix of the protein (Arg10, Lys14 and Arg17) and four in helix D (His133, Gln132, Lys138, and Phe141). Moreover, they found that Arg10 and Arg17 seem to be specific determinants for TPO/c-Mpl recognition, although Pearce et al. failed to find any reduction in binding affinity of an Arg10 to Ala mutation. As these residues in EPO are Arg and Leu, respectively, the latter residue could help to explain the failure of the two cytokines to cross-react in receptor binding assays.[17]

THE REGULATION OF THROMBOPOIETIN EXPRESSION

As would be expected for the physiologic regulator of platelet production, plasma concentrations of TPO vary inversely with the platelet count in patients with marrow failure.[25] Northern blot analyses of multiple organs reveal that TPO is widely expressed, mRNA being present at highest levels in the liver of normal animals, but the kidney, smooth muscles, and a number of other organs also express transcripts for the hormone.[13,14,18] Based on the capacity of platelets to adsorb the hormone from its surroundings, internalize, and destroy it, Kuter and Rosenberg[26] and other investigators[27] have proposed that the regulation of TPO levels in plasma is dependent entirely on platelet numbers; in the presence of thrombocytosis, the steady-state level of hormone production is overwhelmed by platelet-mediated TPO removal and levels are low, and in states of thrombocytopenia, little of the same amount of TPO production is adsorbed, allowing levels to rise in order to stimulate thrombopoiesis. Initial findings of relatively low TPO levels in patients with immune thrombocytopenia (ITP) were explained by further postulating that the megakaryocytic hyperplasia seen in such states then accounts for the increased TPO adsorption. Ancillary data for this assertion come from the *tpo* hemizygous mouse[28]; loss of one allele of *tpo* leads to a 40% reduction in platelet counts. If active regulation of TPO exists, then increased expression from the second, normal allele should have corrected the reduction in hormone levels and reestablished a normal platelet count, much like what occurs in the EPO system.

These arguments not withstanding, a growing body of evidence suggests that additional mechanisms operate to affect TPO expression. At baseline, it is very difficult to detect specific mRNA in marrow cells. However, transcript levels can be substantially induced in marrow stromal cells in response to thrombocytopenia,[29–32] albeit at platelet levels lower than the 60% found in the *tpo* hemizygous mice. In addition, a number of inflammatory states are associated with TPO levels above that expected for their platelet count[33–35] although this finding has not been universal.[36] It has been shown that IL-6, a well known inflammatory response mediator, increases TPO production both *in vitro* and *in vivo*,[37–39] potentially helping to explain the enhanced levels seen in inflammatory and possibly other pathologic states.

The mechanism by which TPO production is regulated is coming under increased investigation. Using platelet granule proteins, Sungaran et al.[40] determined that the platelet α granule proteins PDGF-BB and FGF2 stimulated, and that platelet factor four, thrombospondin, and transforming growth factor-β inhibited TPO production from cultures of marrow stromal cells; on balance, whole platelet extracts suppressed TPO production. More recently, platelet granule proteins were shown to transcriptionally repress TPO mRNA expression in a marrow stromal cell line as well as in primary bone marrow stromal cell cultures. Like TPO mRNA, secretion of TPO protein was also suppressed by serum. Reporter gene constructs indicate that DNA elements located in an approximately 1.9 kb region between 250 bp upstream of the transcriptional initiation site and the middle of the second intron are able to mediate this transcriptional repression.[41] Thus, accumulating evidence indicates that marrow stromal cells can be induced by exogenous stimuli to produce TPO and help explain the response to inflammatory stimuli.

The *TPO* gene displays an unusual 5' untranslated region structure. Unlike the majority of genes that initiate translation with the first ATG codon present in the mRNA, TPO translation initiates at the eighth ATG codon in the transcript, located

in the third exon of a full-length mRNA (there are two transcription initiation sites that differ because of alternate splicing of the first exon).[42] As the eighth ATG is out of frame but embedded in the short open reading frame (ORF) of the seventh ATG, translation initiation is quite inefficient.[43] Thus, under normal circumstances, little TPO protein is produced for any given amount of mRNA. Although it is not yet certain whether this molecular arrangement has physiologic consequences (i.e., it can be differentially regulated), it is now clear from patients with familial essential thrombocythemia (ET) that mutation of the *TPO* gene in noncoding sequences can lead to enhanced translation efficiency and thrombocytosis. Several cases of autosomal dominant familial thrombocytosis have been linked to mutations in the region surrounding the initiation codon. In two families, a single mutation in different nucleotides of the intron three splice donor sequence results in alternate splicing of the primary transcript, eliminating the seventh and eighth ATG codons and creating a new amino-terminus by fusing of the fifth ORF with the hormone coding sequence. This novel TPO mRNA is efficiently translated resulting in supraphysiologic levels of hormone production and nonclonal expansion of thrombopoiesis.[44,45] In another mutant *Tpo* allele deletion of a single nucleotide within the seventh ORF leads to its fusion with the TPO coding sequence and now enhanced translation of TPO from the seventh ATG codon.[46] Finally, another mutation within the seventh ORF leads to its premature termination, preventing its interference with translation initiation from the usual eighth initiation codon[47] again enhancing protein production. The molecular mechanisms of diseases of mRNA translation have been summarized.[48]

MEGAKARYOPOIETIC ACTIVITIES OF THROMBOPOIETIN *IN VITRO*

As initially defined, TPO was thought to be a potent megakaryocyte differentiation factor but was not thought to influence cellular proliferation. However, as soon as recombinant protein was available it was established that in contrast to the partially purified samples thought to contain the hormone, TPO clearly acts to induce proliferation of megakaryocytic progenitor cells,[49] and in synergy with other cytokines, to support the proliferation of multiple types of immature hematopoietic progenitor cells.[50–53] From the initial studies of its biologic properties, it has been clear that TPO is a potent megakaryocyte colony-stimulating factor. Approximately 75% of a maximal number of megakaryocyte colony-forming cells can be induced to grow using optimal levels of TPO alone.[49,54] A number of other cytokines and hormones, including IL-3, kit ligand, EPO, and IL-11 also act in synergy with lower levels of TPO to promote megakaryocyte colony formation.[55] However, the hormone appears to exert its proliferative effect primarily on progenitors of limited developmental potential. The number of megakaryocytes present in each colony is greater for those that form in the presence of IL-3 or kit ligand, either alone or together with TPO, than in the presence of TPO alone. However, in the absence of the hormone, IL-3- or kit ligand-induced megakaryocyte formation is aborted; the cells display low ploidy values and poor cytoplasmic maturation.[54]

Serum-free suspension culture systems have been most useful for demonstrating the differentiative effects of the hormone on megakaryocytes. Using the recombinant protein several groups have established that TPO increases megakaryocyte cell size, shifts the cells to much higher ploidy classes, and enhances the expression of the lineage specific markers glycoprotein (gp)Ib and gpIIb/IIIa.[49,56–58] When examined under the electron microscope, TPO-induced megakaryocytes display prominent membrane production and numerous platelet specific granules, and the cytoplasm demarcates into regions destined to fragment into mature platelets.[49,54,56] When cultured slightly longer, especially in the presence of plasma, the cells form "proplatelet processes," and fragment into platelets that appear to function normally upon exposure to classic platelet agonists.[59] Moreover, this final process appears to be independent of TPO, instead being more dependent on a substance in normal plasma. One report suggests that the activity in plasma responsible for platelet formation is a complex of thrombin and antithrombin III.[60] At present, this report remains unconfirmed, so that the nature of a (regulatable) stimulus (if there is one) for the final stage of platelet formation is presently unclear.

One relatively consistent property of hematopoietic growth factors has been that the cytokine or cytokines that stimulate the production of a given cell lineage also activate or "prime" the resulting mature blood cells for activation in response to other signals.[61–63] Like that for IL-3, GM-CSF and G-CSF *in vitro* studies indicate that although incubation in the hormone leads to the priming of platelets to respond to an otherwise subthreshold level of classical platelet agonists,[64,65,66] TPO does not lead to frank platelet activation.

THROMBOPOIETIN AND THE HEMATOPOIETIC STEM CELL

Analogous to the lineage specific characteristics of EPO, the biologic properties of TPO were initially thought to be restricted to cells of the megakaryocyte lineage. Nevertheless, early studies of the biologic activities of the hormone included the stimulation of sorted $CD34^+$ cells.[56] As this population of cells includes megakaryocytic progenitors, it remained formally possible that the hormone was lineage restricted until two groups demonstrated that sorted marrow cells highly enriched in hematopoietic stem cells (HSCs) also survived in the presence of TPO alone, and proliferated in synergy with IL-3 or kit ligand.[52,53] Shortly after these *in vitro* findings were reported, genetic elimination of *c-Mpl* was found to result in a significant reduction in the number of hematopoietic progenitors of all lineages[67] and then using competitive repopulation, Solar et al.[68] demonstrated that the number of HSCs were reduced seven to eightfold in *c-Mpl* null mice. As it became appreciated that the congenital form of amegakaryocytic thrombocytopenia are due to a homozygous or mixed heterozygous mutation in the c-Mpl receptor locus, the role of TPO on stem cells has been extended to humans, as nearly all such children develop aplastic anemia due to dwindling numbers of stem cells within the first 2 to 5 years of life.[69,70,71,72] Consistent with this notion, we demonstrated that the self-renewal/expansion of HSCs is also vitally dependent on TPO, as transplantation of normal stem cells into *Tpo* null mice results in a 15- to 20-fold reduced level of stem cell expansion.[73] Thus, it is now clear that TPO joins kit ligand as the only cytokine that plays a nonredundant role in stem cell survival, self-renewal, and proliferation.

IN VIVO ACTIVITIES OF THROMBOPOIETIN

The effects of exogenous TPO on the kinetics of platelet production have been studied extensively. As would be expected from its megakaryopoietic effects *in vitro*, when TPO is administered to animals or people, the platelet count begins to rise after 3 to 5 days, depending on the dose and the recipient species.[13,14,18,74–79] These results indicate that TPO does not cause the immediate release of platelets from megakaryocytes, consistent with its failure to accelerate fragmentation of proplatelet processes, the long exvaginations of megakaryocyte cytoplasm felt to represent the most immediate platelet precursor.[59] Instead, TPO stimulates the development and maturation of new megakaryocytes. Once the size and numbers of megakaryocytes increase, the platelet count begins to rise until a new steady-state level is reached, the height of which depends on a log-linear relationship with the administered dose.[19] Due to a prolonged biologic effect (the result of its slow plasma clearance and action on both primitive and mature hematopoietic cells), enhanced platelet production resulting from parenteral administration of TPO occurs 6 to 16 days after a steady state level of the hormone is achieved.[79,80]

Given the panhematopoietic effects of TPO *in vitro*, careful attention was paid to all of hematopoiesis in animals administered the recombinant hormone; the *in vivo* effects of TPO were found to correlate with its *in vitro* properties. Administration of the hormone to either normal or myelosuppressed animals increased the numbers of all types of hematopoietic progenitor cells,[81] and genetic elimination of *Tpo* or its receptor causes a 65% to 95% reduction in number of transplantable stem cells and hematopoietic precursors of multiple lineages.[67] Thus, TPO should be thought of as a lineage-dominant panhematopoietic cytokine that plays multiple roles in blood cell production.

As noted above, a large body of evidence indicates that cytokines that stimulate hematopoietic cell production also activate the corresponding mature cell type. If this were also true for TPO, platelet activation and thrombosis could result. *In vitro* studies have indicated that the hormone primes platelets to aggregate in the presence of classical agonists, but does not, by itself, lead to platelet activation.[65] Studies performed *in vivo* are consistent with this conclusion; the administration of TPO to nonhuman primates[19] or humans did not result in *de novo* platelet activation,[78] and an excess of thrombotic complications has not been seen in multiple clinical trials of two forms of the hormone (see below). Nevertheless, these clinical trials have all avoided enrolling individuals with any history of pathologic thrombosis, as has the administration of FDA approved small molecule thrombopoietic agonists.

Despite this increased understanding of the vital role of TPO on thrombopoiesis, a remaining major question is what is responsible for the approximately 10% residual platelet formation present in mice and humans in which *Tpo* or its receptor are genetically altered. Based on the megakaryopoietic activities of IL-3, IL-6, IL-11, GM-CSF, and leukemia inhibitory factor, both *in vitro* and *in vivo*, in an attempt to identify the cytokine(s) responsible for residual platelet production in $Mpl^{-/-}$ mice, mice genetically deficient in *IL-3*, *IL6-R*, *IL-11-R*, or the combined signaling subunit of the IL-3, GM-CSF, and IL-5 receptors (β_C) were crossed to *Mpl* null animals; none of these double to quadruple cytokine/cytokine receptor deficient mice displayed more severe thrombocytopenia than found in the $Mpl^{-/-}$ mouse.[82–85] Whether another known (or possibly unknown) cytokine is responsible for the residual platelet production in the absence of TPO function, or as was suggested, this combination of the chemokine SDF-1, which displays a synergistic action on megakaryocyte growth[86] and endothelial cells.[87]

THE Mpl RECEPTOR AND MOLECULAR MECHANISMS OF TPO ACTION

The initial step in hematopoietic growth factor action is binding to its specific, cell-surface receptor. Recent studies of the response of many of these receptors have begun to provide molecular insights into cytokine-induced signaling. It is clear that protein tyrosine phosphorylation of the receptor itself, and several substrates is an integral part of the cellular response to hematopoietic growth factor stimulation, as protein tyrosine kinase inhibitors block the growth induced by many hematopoietic cytokines, and phosphatase inhibitors have the opposite effect.[88,89] However, the TPO receptor does not display any enzymatic activity; rather, cytokine receptors recruit cytoplasmic kinases to activate signaling molecules. The EPO receptor was the first member of the hematopoietic cytokine receptor family shown to recruit and activate a member of the Janus family of cytoplasmic tyrosine kinases (JAKs). Subsequent work has shown that JAK kinases mediate much of the secondary protein tyrosine phosphorylation triggered by the binding of hematopoietic growth factors to their corresponding receptors.[90,91] The role of other cytoplasmic kinases, such as src and the src-related kinases, in growth factor-induced signaling is also under active investigation.[92,93] Although many of the signals involved in thrombopoietic cytokine action are rapidly being identified, major challenges lie ahead in deciphering the molecular basis of signal specificity. For example, the cellular response generated upon TPO binding is distinct from that originating at the IL-3 receptor, but many of the same molecules are activated by both receptors; a thorough analysis of the specific patterns of signaling intermediates activated by each of the cytokines that act directly on megakaryocytes will be required to more fully understand the role played by each during thrombopoiesis.

Progress has been rapid in deciphering the signaling pathways employed by TPO. Because of its homology with EPO, and between c-Mpl and the rest of the cytokine receptor family, initial studies of TPO signal transduction concentrated on the JAK and signal transducers and activators of transcription (STAT) families of proteins. The first reports focused on leukemic cell lines that naturally express the c-Mpl receptor, or ones engineered to do so. These studies identified JAK2 and TYK2 as the immediate kinases that bind to c-Mpl and become activated on exposure to TPO.[94–102] Other proteins reported in various megakaryocytic cell lines to be phosphorylated in response to TPO include STAT3 and STAT5,[94,95,100,103,104] the adapter proteins Grb2,[100,101] Cbl,[101] and son of sevenless (SOS),[101] Shc and its related phosphatase SHIP,[95,100,101,105] phosphoinositol-3-kinase (PI3K) and its downstream kinase Akt,[64,106,107] an inhibitory transcription factor downstream of Akt, FKHRL-1,[108] the Raf-1/MAP kinase,[100,109,110] and hematopoietic cytokine receptor-related phosphatases such as SHPTP-2.[108]

In addition to determining the intracellular signaling pathways activated by TPO, much work has been performed to identify the site of the c-Mpl receptor from which each signal originates (summarized in FIGURE 25.1). For example, Tyr625

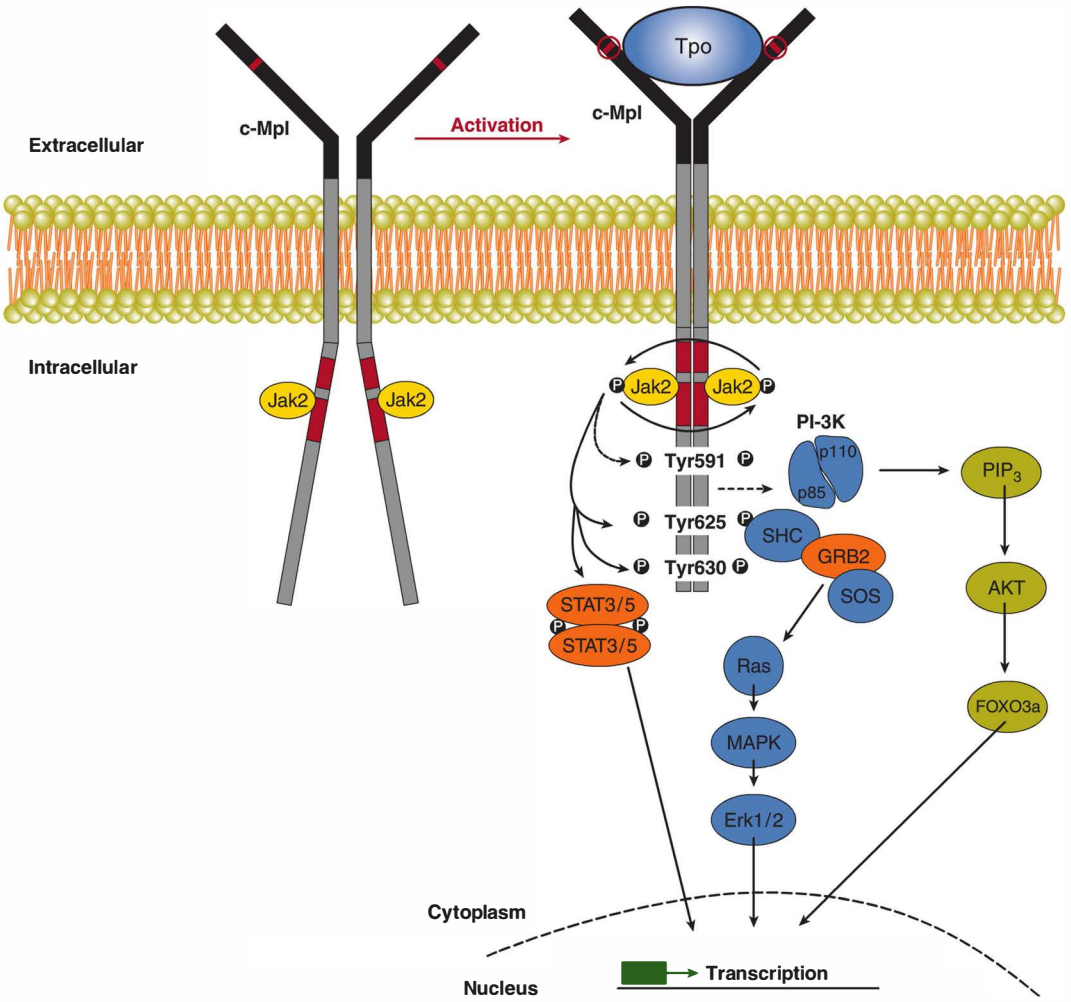

FIGURE 25.1 Schematic representation of c-Mpl signaling following TPO stimulation. TPO binding to its cognate receptor c-Mpl causes a conformation of the receptor homodimer, bringing associated Janus kinase (JAK) 2 proteins in closer juxtaposition, enabling their autophosphorylation. Activated JAK2 leads to phosphorylation and activation of signal transducers and activators of transcription (STAT) proteins and phosphorylation of c-homoogy domain-containing transforming protein (SHC)-1, which associates with growth factor receptor-bound protein (GRB) 2, and son of sevenless (SOS) to stimulate the mitogen-activated protein kinase (MAPK) signating pathway via the protooncogene Ras. In addition, TPO stimulation of c-Mpl activates AKT via phosphinositide-3-kinase (P1-3K) and phosphatidylinositol (3,4,5) triphosphate, upregulating the forkhead box 01 (FOX01) transcription factor.

in murine c-Mpl is phosphorylated in response to the hormone binding and serves to initiate both Shc and STAT3 activation.[96,111] Truncation of the c-Mpl receptor 69 residues from the transmembrane domain eliminated both Shc and STAT3 activation; however, it only reduced activation of STAT5, PI3K, and ERK1/2, leaving a receptor that was still capable of supporting cell growth, conclusions that were verified *in vivo* when a similarly truncated *c-mpl* cDNA was knocked in to the *c-mpl* locus in mice.[112] The resting platelet count of these mice was normal; however, platelet recovery following cytotoxic therapy-induced thrombocytopenia was significantly delayed, indicating that the proximal part of the Mpl receptor includes all signals required for baseline MK proliferation and maturation, but that either the additional signal intensity of STAT, ERK, and PI3K, and/or the distinct signals initiated from the distal part of the receptor are important for high level thrombopoiesis. Similar conclusions that STAT responses serve an amplification role in cytokine signaling are derived from studies of STAT5a/STAT5b double knockout mice.[95]

Given the number and variety of intracellular signaling pathways activated by TPO, stringent mechanisms are required for the negative regulation of c-Mpl to ensure the maintenance of normal cellular responses. Suppressors of cytokine signaling (SOCS) proteins have been shown to bind either directly to the transmembrane receptor or to JAK/STAT proteins, downregulating their activity (reviewed in[113]). Expression of SOCS-3 is significantly increased in response to TPO in c-Mpl-expressing cell lines, while IFN-α negatively regulates TPO-activated signaling pathways via upregulation of SOCS-1.[114] TPO-mediated activation of Lyn, a member of the Src-family kinases, also appears to negatively regulate TPO signaling. Suppressing Lyn activity using chemical inhibitors increased Erk1/2 phosphorylation

and also enhanced the proliferation of Mpl-bearing cell lines and promoted megakaryocyte differentiation of bone marrow cells.[115] Additionally, *Lyn*-deficient mice exhibit increased megakaryopoiesis and enhanced TPO-mediated signaling.[116] Lnk, an adaptor protein (AP) containing SH2-binding and pleckstrin homology domains, also negatively regulates TPO-mediated signaling[117] and has significant effects on HSC maintenance and quiescence.[118] Recent evidence shows that Lnk directly interacts with phosphorylated Y_{613} and Y_{813} on JAK2 following TPO stimulation. Furthermore, HSCs isolated from $Lnk^{-/-}$ mice exhibit a significantly increased JAK2 phosphorylation in response to TPO.[118]

In addition to increasing expression and activity of negative regulators, c-Mpl is rapidly internalized and degraded following TPO stimulation.[119] The intracellular motif Y_{591}RRL is critical for c-Mpl internalization, probably via interactions with AP-2, which drives clathrin-mediated endocytosis. Following its internalization, c-Mpl has three possible fates. It is either polyubiquitinated on both intracellular lysine residues (K_{553} and K_{573}) prior to proteasomal or lysosomal degradation,[120] or it is recycled back to the plasma membrane. However, the exact mechanisms behind c-Mpl endocytosis, degradation, and recycling remain unclear.

The nature of TPO signaling in mature platelets has also been explored[121–124] and reveals that the hormone stimulates the phosphorylation and activation of JAK2, STAT3, and STAT5. As the hormone has been shown to prime platelets for an enhanced response to classical platelet agonists, additional studies now focus on determining how these signaling intermediates might participate in platelet activation.

THE THERAPEUTIC POTENTIAL OF THROMBOPOIETIN

The toxicity and therapeutic efficacy of TPO have been tested in many animal species from mice to man. In all studies, administration of any form of the hormone was safe; there were no signs of hepatic, renal, or pulmonary toxicity, capillary leak, coagulopathy or hematopoietic toxicity, and no evidence of an acute phase response that is characteristic of other cytokines known to affect megakaryocyte development. When tested in animals treated with nonablative doses of either chemotherapy, radiation, or both, the administration of exogenous TPO resulted in higher nadir platelet counts and accelerated platelet recovery.[74,81,125–129] TPO administration also promoted improved recovery of the erythrocyte and granulocyte lineages in these animals, consistent with its *in vitro* stimulatory effects on cells of multiple hematopoietic lineages.[52,53,81] In one study using rhesus monkeys administration of TPO after sublethal irradiation resulted in functional iron deficiency, confirming its effect on erythropoiesis, and arguing for study of prophylactic administration of iron in patients receiving the recombinant protein.[129] Together, these results suggest that the therapeutic efficacy of the hormone might be greater than initially anticipated.

In contrast to the near universal success of the hormone in ameliorating the thrombocytopenia and in many studies the pancytopenia associated with myelosuppressive therapy, its effectiveness in accelerating platelet recovery after myeloablative therapy and stem cell transplantation has been less impressive. The administration of TPO to lethally irradiated mice receiving bone marrow cells accelerated platelet recovery by 2 to 4 days in two studies,[77,130] but did not augment hematopoietic recovery when fewer bone marrow cells were given.[131] In mice receiving peripheral blood stem cell transplants TPO accelerated platelet recovery by only 1 or 2 days as compared with mice receiving transplants but no exogenous hormone.[130] TPO also did not facilitate hematopoietic reconstitution in nonhuman primates undergoing autologous bone marrow transplant or peripheral blood stem cell transplantation.[132] For the most part, the preclinical studies of TPO have accurately predicted the response of patients administered the hormone for reasons of myelosuppression. The results of many clinical trials of TPO therapy given for up to 10 days have now been published.[80,78,133,134,135–139] In all studies, the administration of either recombinant human (rh) TPO or pegylated recombinant human megakaryocyte growth and development factor (PEG-rHuMGDF) to cancer patients was safe. Specifically, there were no significant changes in vital signs or weight, and the frequency of symptoms or signs of bone pain, organ dysfunction, superficial or deep venous thrombosis, or platelet activation was similar in the treatment and placebo groups. It must be noted, however, that patients with a history of cardiac, pulmonary, vascular, or thrombotic disease were excluded from all of the studies. Only a single patient in any of these clinical trials developed antibodies to TPO.[79] However, very few patients have been given more than a single course of treatment in the initial clinical trials, making any conclusions on the immunogenicity of the two products premature.

Due to the dramatic increase in our understanding of the structure and function of TPO in the 1990s, the first generation of thrombopoietic reagents for human use consisted of recombinant proteins aimed at mimicking the role of endogenous TPO. Two such compounds were extensively tested, rhTPO and PEG-rHuMGDF (reviewed in [140,141,142,143]). RhTPO is a glycosylated, full-length peptide identical to endogenous TPO and produced by CHO cells. When rhTPO was administered intravenously to cancer patients, a marked increase in platelet counts was observed after 5 days, which peaked 10 to 14 days later.[79,135] PEG-rHuMGDF, a pegylated product containing the amino-terminal 163 amino acids of human TPO, was produced in *Escherichia coli*. On subcutaneous administration to healthy volunteers, PEG-rHuMGDF caused a rise in platelet numbers in a time course similar to rhTPO.[144,145]

The efficacy of TPO in clinical trials of patients with thrombocytopenia has been mixed. Results from three studies using PEG-rHuMGDF or rhTPO in a total of 101 patients with cancer treated with carboplatinum-based chemotherapeutic regimens have been reported.[133,134,135] In all three studies, platelet counts returned to baseline significantly faster, and in two of the three studies nadir platelet counts were higher in the patients given TPO, as compared with either those given placebo or the same patients during their first cycle of chemotherapy. However, the chemotherapy regimens administered in these two trials induced only modest thrombocytopenia (mean nadir platelet counts in the placebo groups of 60,000/mm^3 to 111,000/mm^3); in neither study was hospital discharge delayed because of thrombocytopenia nor were significant numbers of platelet transfusions required. In the third study,[135] compared to a first cycle of carboplatin-based therapy without the hormone, nadir platelet values and the number of days with ≤20,000 platelets/mm^3 were both significantly improved by the administration of a single dose of TPO following the second cycle of chemotherapy ($P < 0.001$). Moreover, 59% of the patients enrolled in this study required platelet transfusions after their first cycle of

chemotherapy, but only 26% needed them following the cycle of chemotherapy augmented with TPO ($P = 0.02$).

Several additional indications for TPO therapy have been envisioned. TPO increases the number of megakaryocytic and other hematopoietic progenitors *in vitro* and *in vivo*,[52,53] and it mobilizes stem and progenitor cells from the marrow into the circulation,[79,130,136] properties that might benefit patients receiving stem-cell transplants. In 75 patients with breast cancer undergoing autologous bone marrow transplantation, administration of PEG-rHuMGDF led to a 5 to 6 day earlier rise in platelet count to 20,000/mm^3 and a halving of the use of platelet transfusions as compared with placebo.[137] However, when tested in 64 patients in a phase III trial, the drug failed to accelerate platelet recovery. Likewise, the recombinant hormone was administered to 38 patients with delayed platelet recovery after peripheral blood stem cell or bone marrow transplantation; the use of rhTPO lead to platelet transfusion independence in only two individuals.[139]

TPO has also been tested as an aid in mobilizing stem cells for subsequent autologous transplantation. Somlo et al. found that compared to a group of autologous transplantation patients in whom stem cells were mobilized with G-CSF alone, the addition of TPO to the mobilization phase lead to a reduction from a mean of three to a mean of one apheresis procedure necessary to collect the requisite number of CD34$^+$ cells, and upon infusion the patients who received cells mobilized with G-CSF and TPO demonstrated accelerated red cell and platelet recovery, and required a small but statistically significantly reduced number of platelet transfusions compared to patients who received G-CSF mobilized stem cells.[146]

Another potential indication for the hormone is in increasing platelet yields in normal individuals who donate platelets by apheresis. At present a 2 to 3 hour apheresis of normal donors yields approximately 3×10^{11} platelets, which when transfused into a single donor results in a 10–40 $\times$ 10^9/L increment in platelet levels. By increasing the platelet count in a donor, the number of platelets recovered should increase, potentially increasing the mean platelet increment when the cells are subsequently infused into a single recipient; alternately, such enhanced platelet collections could allow a single donor pheresis to be used for multiple recipients. Despite initially encouraging reports of this strategy,[147] clinical trials were halted following a large study with healthy volunteers given PEG-rHuMGDF. Of the 538 subjects receiving the drug, 13 developed thrombocytopenia as a result of an antigenic reaction against PEG-rHuMGDF, which produced antibodies that also recognized endogenous TPO.[148,149,150] All clinical trials involving first generation thrombopoietic agents were subsequently discontinued.

Prior to the withdrawal of recombinant TPO for the immunologic effects noted above, it was clear that the hormone could increase reduced platelet numbers in patients with ITP.[151] Although the typical bone marrow examination of a patient with classic ITP demonstrates impressive megakaryopoiesis, it remains possible that the levels of the hormone attainable physiologically are less than necessary for maximal megakaryopoiesis, and that levels attainable pharmacologically could further increase platelet levels in this disorder.

Following the failure of recombinant peptides in clinical trials, the focus shifted to the development of novel thrombopoietic agents that could be easily produced and administered without producing an antigenic response. By isolating hormone binding site peptides from phage display libraries, Cwirla et al.[152] identified a 14–amino acid peptide (Ile–Glu–Gly–Pro–Thr–Leu–Arg–Gln–Trp–Leu–Ala–Ala–Arg–Ala) with a high binding affinity for human c-Mpl, but no sequence homology with endogenous TPO.[152] To increase longevity and activity in circulation, the linear peptides were either PEGylated or fused to human Fab or Fc scaffolds. Romiplostim, (also known as AMG-531 and marketed as N-plate; Amgen) described as a "peptibody," was generated by the covalent fusion of four TPO peptide agonists to the Fc fragment of the human IgG1 heavy chain.[153,154] Romiplostim competed with rhTPO for binding to c-Mpl, and also activated downstream signaling pathways and promoted megakaryocyte differentiation in a similar manner to rhTPO.[155] When administered subcutaneously, romiplostim increased platelet counts in healthy volunteers and in 80% of patients with ITP, peaking at days 12 to 16. Longer-term studies show that romiplostim is well tolerated and no cases of anti-TPO neutralizing have been reported (clinical data reviewed in[142]). In 2008, the FDA approved the use of romiplostim for patients with chronic ITP who have not responded to traditional treatments.

Large-scale screening of nonpeptide, orally available TPO mimetic compounds in cell-based assays has also generated a number of potentially valuable reagents, including the leading nonpeptide TPO mimetic, eltrombopag (also known as SB497115 and marketed as Promacta/Revolade; GlaxoSmith Kline).[156–160] Unlike peptide mimetics such as romiplostim, which compete for binding to c-Mpl in the same region as endogenous TPO, eltrombopag binds to the transmembrane domain of c-Mpl,[161] resulting in a conformational change and its subsequent activation, in a manner similar to activating mutations of c-Mpl. Clinical trials of eltrombopag report significant increases in platelet counts, although these are several times lower than the increases reported following romiplostim.[162] In >80% of chronic ITP patients, platelet counts above 50 $\times$ 10^9/L were achieved, maintained over a longer-term trial, and accompanied by decreased bleeding symptoms (reviewed in[143]). The FDA has also approved the use of eltrombopag as a second-line treatment for patients with ITP.

Following the success of the second generation thrombopoietic agents in treating ITP, the number and variety of thrombopoietic agents in development is constantly increasing. Some concerns remain whether long-term use of these agonists may increase the risk of developing other conditions, namely increased platelet activation and/or increased bone marrow fibrosis, although neither condition has been reported. The approval of both romiplostim and eltrombopag by the FDA makes the thrombopoietic agents a gold standard in the development of other hematopoietic growth factor mimetics.

ROLE OF THROMBOPOIETIN AND ITS RECEPTOR IN PATHOLOGY

Other than the remarkable insights into the pathogenesis of chronic myelogenous leukemia afforded by the t(9;22) translocation, as of 5 years ago little had been known about the pathogenesis of the chronic myeloproliferative neoplasms (MPNs) polycythemia vera, ET, and primary myelofibrosis. Early evidence suggested that the cellular response to hematopoietic growth factors, dysregulation of the growth factors themselves, or of their signaling networks might contribute to the etiology of these disorders. The concept of autocrine secretion of peptide growth

factors by tumor cells dates to the work of Sporn and Todaro.[163] Later work suggested that hematopoietic growth factors and their receptors might play an important role in the malignant transformation of marrow cells in other clinical settings.

MPNs share a common origin in a pluripotent hematopoietic progenitor cell. Consequently, if growth factors, growth factor receptors, or their signaling networks play a role in these disorders, then that cytokine receptor must be expressed in HSCs. As illustrated above, Mpl is present on the repopulating HSC, and genetic elimination of *Tpo* or c-Mpl lead to the conclusion that it plays a critical, nonredundant role in these cells.

Several lines of evidence support the notion that TPO and/or its receptor play a role in MPNs. First, mutations of Mpl can lead to autonomous cell growth and leukemogenicity in mice. Perhaps the best example of this is the mutant form of *c-Mpl* that lead to its discovery, the *v-Mpl* oncogene.[6] Following upon the characterization of a constitutively active mutant of the EPO receptor, Alexander et al. engineered constitutively active receptor mutants of c-Mpl by substituting cysteine residues into the dimer interface domain of the receptor. Factor-dependent hematopoietic cells transduced with the mutant receptor displayed constitutive phosphorylation of both Mpl and the signal transduction molecules implicated in Mpl function, suggesting a potential for contributing to the tumorigenicity of the mutant cells.[164] Onishi et al. identified an activating mutation in the transmembrane domain of Mpl by using a combination of retrovirus-mediated gene transfer and polymerase chain reaction-driven random mutagenesis. This mutation resulted in the abrogation of factor dependency in all IL-3-dependent cell lines tested. One IL-3-dependent murine cell line that expressed only the mutated form of Mpl induced autocrine growth and leukemogenesis when transduced into syngeneic mice, apparently by constitutive activation of two distinct intracellular signaling pathways.[165] Finally, our group has demonstrated that truncation of the membrane-distal part of the extracellular domain of c-Mpl induces autocrine growth in factor-dependent cell lines.[166] These studies appear to confirm that certain mutations of the TPO receptor may contribute to the malignant transformation of some hematopoietic progenitors *in vivo*, at least in the mouse. Second, expression of normal c-Mpl in all marrow cells, or TPO in hematopoietic cells leads to MPNs. For example, Cocault et al.[167] demonstrated that a retrovirus containing natural murine Mpl cDNA is erythroleukemogenic *in vivo* when injected into adult mice. And several groups have observed that transgenic overexpression of *Tpo* in mice induces megakaryocyte proliferation in the spleen and massive marrow fibrosis, a disorder bearing strong resemblance to agnogenic myeloid metaplasia and myelofibrosis in humans.[168–170] Third, as noted above, one of the most characteristic findings in patients with myeloproliferative syndromes is "autonomous" colony growth, which in reality represents growth factor hyperresponsiveness. Hematopoietic progenitor cells taken from blood and bone marrow that normally die in culture without exogenous growth factors survive and proliferate in the presence of serum alone. Li et al.[171] have demonstrated, for example, that with the introduction of an antisense oligonucleotide to Mpl, there is a reduction in spontaneous colony formation in serum-free assays and in plasma clot assays. Similar conclusions were drawn by Taksin et al.[172] using an inhibitory, soluble form of the c-Mpl receptor. This outcome suggests that the TPO receptor may influence autonomous growth of these cells in culture. Fourth, TPO levels and c-Mpl expression is altered in patients with several of the MPNs. For example, Pitcher has observed that circulating TPO levels are unexpectedly high in patients with ET[173] although this has not been a universal finding.[174] Moreover, c-Mpl expression on megakaryocytes from patients with MPNs has been abnormal,[175,176] and a signaling defect has been observed in cells derived from patients with polycythemia vera.[177] In addition to being of potential diagnostic benefit, these findings again point to an abnormality in the TPO/c-Mpl system in patients with MPNs.

In 2006, two novel somatic mutations of c-Mpl ($MplW_{515}L$ and later $MplW_{515}K$) were identified in a small population of patients with IMF and ET.[178,179] $MplW_{515}L$ stimulates cytokine-independent growth in hematopoietic cells, activating JAK2-STAT signaling in the absence of TPO. $MplW_{515}K$ was identified in granulocytes of 1,182 MPN patients, and is also an activating mutation, although its exact effects on signaling remain unclear. In murine *in vivo* studies, transplantation of bone marrow transduced with Mpl carrying the $W_{515}L$ mutation resulted in the rapid development of myeloproliferative disease.[179] Further studies suggest that this mutation affects the amphipathic region of c-Mpl (KWQFP), located at the junction of the transmembrane and cytoplasmic domains.[180] It would appear that the normal $MplW_{515}$ residue acts to prevent the conformational change required for activation when TPO is not present. Consequently, the W to L/K mutation results in the constitutive activation of the receptor. Pharmacologic JAK2 inhibitors, a number of which are currently in clinical trials for treatment of $JAK2V_{617}F$-positive MPNs, have been shown to significantly reduce the extent of myeloproliferation in $MplW_{515}L$ mouse models[179] and could potentially be used to treat patients with Mpl activating mutations.

Homozygous, or compound heterozygous nonsense, or severe missense mutations of *c-Mpl* are responsible for the majority of cases of congenital amegakaryocytic thrombocytopenia (CAMT).[69,70,71,72,181] Infants with this disorder usually present with excessive bleeding in the perinatal period, although occasionally the diagnosis is delayed a short time. A diagnostic bone marrow aspiration and biopsy usually reveals normal cellularity, although only a few megakaryocytes are initially found, and those present are unusually small and hypolobated. With the availability of plasma TPO measurements nearly every such patient has been found to display very high levels of the hormone, which is biologically active and such children fail to express c-Mpl on their platelet surfaces, due to nonsense or severe missense codons in the extracellular domain of the receptor. Although children with CAMT usually present with isolated thrombocytopenia, they almost always develop pancytopenia within a few years of birth, presaged by declining numbers of marrow-derived hematopoietic progenitors of all types.[72] At this point, their marrow morphology resembles that seen in aplastic anemia, and stem cell transplantation is their only curative option. Also of great interest in this regard is the recent observation that the development of anti-TPO antibodies can lead to pancytopenia.[148] As noted earlier, TPO plays an important role in stem cell biology, perhaps helping to explain the stem cell failure that characterizes the latter stages of CAMT and the aplasia associated with accidentally neutralizing endogenous TPO in patients. However, this finding also raises an interesting research question; mice nullizygous for c-MPL or TPO display similarly low platelet counts, but do not develop pancytopenia. Further study into the cause of this discrepancy is likely to reveal much about stem cell kinetics in mouse and man.

Although the majority of patients with immune thrombocytopenic purpura display enhanced megakaryopoiesis secondary to peripheral immune destruction of platelets, usually due to an autoantibody to platelet gpIIb/IIIa or gpIb, careful platelet turnover studies reveal that not all patients have rapid platelet elimination.[182] In such patients it is possible that reduced production of platelets might be due to antibodies against TPO or its receptor; an additional study has borne out this possibility, in both *de novo* disease, and in response to administration of a genetically modified form of TPO.[150,183,184] In both of these situations, the clue to the unusual pathophysiology was reduced megakaryopoiesis in the marrow of the patients with presumed immune thrombocytopenic purpura.

Soon after the *c-Mpl* protooncogene was identified, it became clear that it was expressed on a sizable proportion of blast cells from patients with myeloid malignancies.[185] Although there is little evidence that disorders of *c-Mpl* are involved in the generation of acute leukemia, a sizable body of evidence suggests that its expression on the surface of leukemic cells adversely affects the response characteristics of the cells and the overall survival of the patients who develop such leukemias.[186,187] The same is true of patients with myelodysplastic syndromes; the presence of c-Mpl on the surface of the dysplastic cells predicted enhanced likelihood of evolution to acute leukemia.[188] In contrast, however, the adverse prognosis of expression of c-Mpl did not extend to chronic myelogenous leukemia.[189]

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CHAPTER

26A

Major Platelet Glycoproteins: Platelet Glycoprotein Ib-IX-V

MICHAEL C. BERNDT AND ROBERT K. ANDREWS

In the past 5 years, understanding of the structure and function of the platelet glycoprotein (GP) Ib-IX-V complex[1,2] has advanced in a number of key areas that should facilitate future therapeutic targeting in thrombotic and other diseases. This chapter focuses on new experimental and clinical studies involving mechanisms regulating GPIb-IX-V expression, ligand binding and signaling, the role of GPIb-IX-V in hemostasis, and new nonhemostatic functions for this receptor. In hemostasis and thrombosis, engagement of GPIbα (the ligand-binding subunit of GPIb-IX-V) by von Willebrand factor (vWF) leads to platelet activation, secretion of platelet agonists such as ADP and thromboxane A_2, and initiation of intracellular signaling pathways resulting in activation of platelet integrins, mainly $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), the receptor that binds fibrinogen or vWF and mediates platelet aggregation (thrombus formation) (see Chapter 15B). Critical to the structure and function of GPIb-IX-V are specific adaptations enabling interaction between GPIbα and vWF or other ligands at high physiologic or pathologic shear rates in the bloodstream. This underlies much of the current interest in GPIb-IX-V as a target for antiplatelet therapy in heart attack and stroke.

GPIb-IX-V STRUCTURE

The GPIb-IX-V complex is made up of the ligand-binding GPIbα subunit, disulfide bonded to GPIbβ and noncovalently associated with GPIX and GPV.[1,2] Key structural domains are illustrated in FIGURE 26A.1. The stoichiometry appears to involve each GPIbα linked to 1 or 2 GPIbβ subunits *via* adjacent Cys residues in the extracellular domain of GPIbα nearby the outer face of the plasma membrane.[2,3] There are equivalent levels of GPIX and GPIb expression and approximately half as many copies of GPV;[4] GPIbα, GPIbβ, and GPIX are required for stable surface expression.[2]

GPIb-IX-V EXPRESSION AND SHEDDING

Expression of GPIb-IX-V is regulated by ectodomain shedding (FIGURE 26A.1). ADAM17 is one of the sheddases involved in release of the GPIbα ectodomain (glycocalicin).[5,6] GPIbα is constitutively shed from circulating platelets, and glycocalicin levels in plasma are 1 to 3 μg/mL. Shedding is increased upon platelet activation or aging *in vivo* or *ex vivo*.[7] Thrombin, ADAM10, or ADAM17 cleave the ectodomain of GPV within the same region.[5,8] Deficiency of GPV in mice enhances the capacity of thrombin to stimulate platelets *via* GPIbα.[9]

GPIb-IX-V: EXTRACELLULAR BINDING PARTNERS

The primary GPIb-IX-V ligand is vWF, a multivalent multimeric adhesive protein secreted from platelets and endothelial cells. Each mature subunit of vWF (~275 kDa) is composed of conserved domains (in order, D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2). The vWF-A1 domain, encompassing an intramolecular Cys509–Cys595 disulfide bond, contains the binding site for GPIbα. The vWF-A3 domain binding contains a binding site for collagen and the vWF-C1 domain contains an Arg-Gly-Asp (RGD) sequence that mediates binding to the activated integrin, $\alpha_{IIb}\beta_3$. Binding of vWF-A1 to GPIb-IX-V has been investigated using proteolytic fragments of native vWF or GPIbα,[10,11] crystal structures of GPIbα/vWF-A1 interactive domain complexes,[12,13] mutagenesis studies examining this interaction under shear conditions,[14] and *in silico* modelling of individual receptor/ligand bonds.[15,16] Together, these studies have provided detailed insight into how vWF can support platelet adhesion under shear conditions, by conformational shear-dependent activation of catch-slip bonds,[16] which enhance or decrease individual bond strength to regulate platelet attachment or detachment, respectively. It has also been demonstrated by structural studies how small molecule inhibitors that bind GPIbα within the *N*-terminal leucine-rich repeat domain can allosterically block vWF binding.[17]

Other adhesive ligands for GPIb-IX-V are the counterreceptors P-selectin,[18] expressed on activated platelets and activated endothelial cells, and the integrin $\alpha_M\beta_2$ (Mac-1)[19] expressed on leukocytes. Binding of P-selectin involves the anionic sulfated tyrosine sequence of GPIbα, analogous to an *N*-terminal sulfated sequence within the counterreceptor for P-selectin on leukocytes, P-selectin glycoprotein ligand-1 (PSGL-1). The α_M I-domain (homologous to vWF-A domains) contains the binding site for GPIbα.[20] This interaction is involved in recruitment of leukocytes to adherent platelets at sites of injury or infection (P-selectin on activated platelets binding to PSGL-1 on leukocytes leads to activation of $\alpha_M\beta_2$) and could also be involved in platelet clearance. In this regard, clustered carbohydrate structures of GPIbα on platelets refrigerated *ex vivo* are recognized by $\alpha_M\beta_2$ on hepatic macrophages, and provides at least one mechanism for the cold storage lesion that results in rapid clearance of transfused cold-stored platelets.[21]

There is also evidence that collagen can bind GPV,[22] although the physiologic importance of this interaction is not yet established. GPV deficiency in mice does not result in a bleeding phenotype[23] and there is at most a limited effect on hemostatic

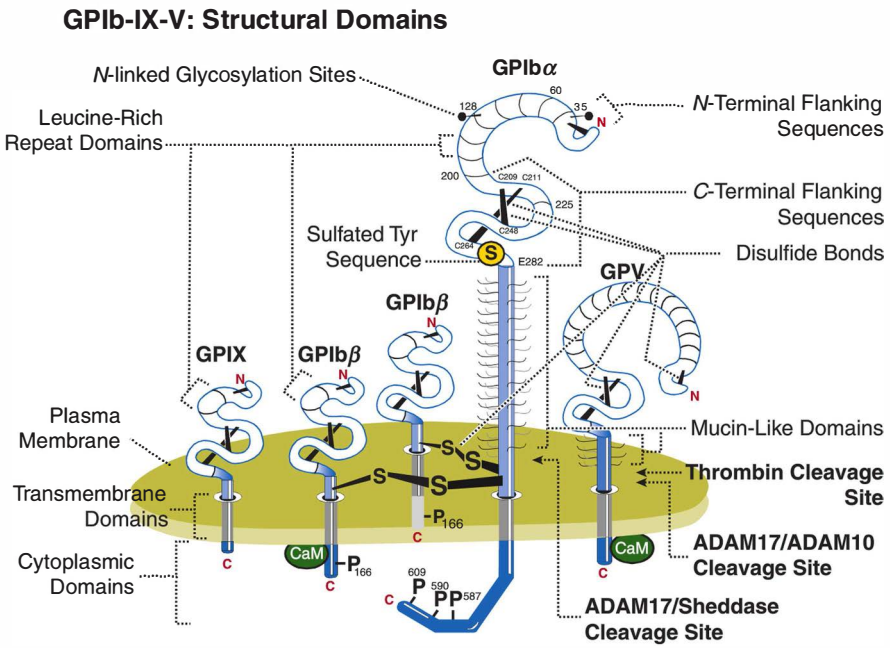

FIGURE 26A.1 GPIb-IX-V: Structural domains. Illustration of the GPIb-IX-V complex, consisting of GPIbα, GPIbβ, GPIX, and GPV subunits, showing leucine-rich repeats, disulfide-looped *N*- and *C*-terminal capping sequences, transmembrane domains, and cytoplasmic domains. GPIbα contains a mucin-like domain elevating the major ligand-binding domain (FIGURE 26A.2). The anionic sulfated tyrosine sequence (residues 269 to 282) contains three sulfated tyrosine residues (Tyr275, Tyr276 and Tyr278). ADAM17 or other sheddases cleave *N*-terminal to the disulfide bonds of GPIbα generating the soluble ectodomain (glycocalicin), and leaving a remnant membrane-associated fragment that remains disulfide linked to GPIbβ. Thrombin and ADAM10/ADAM17 cleaves the ectodomain of GPV at distinct sites, with loss of GPV enhancing the capacity of thrombin to stimulate platelets *via* GPIbα. The stoichiometry is not shown.

function.[24] Nevertheless, release of GPV by metalloproteinase-mediated ectodomain shedding in activated platelets or by thrombin (FIGURE 26A.1) has led to investigation of plasma GPV as a platelet-specific marker of platelet activation or coagulation *in vivo*.[25]

GPIbα also binds to coagulation factors from the intrinsic pathway (high molecular weight kininogen, FXII and FXI) and thrombin generated from activation of intrinsic or extrinsic coagulation pathways. Each of these factors bind to the *N*-terminal ligand-binding domain of GPIbα, whereas the extracellular mucin-like domain or other subunits may regulate the procoagulant activity of platelets by interacting with FVIIa and/or other factors.[26,27] Activation of platelets by engagement of GPIb-IX-V or other receptors can promote coagulation by inducing the expression of anionic phospholipids on the platelet surface, thereby leading to the activation of FX to FXa and secretion of polyphosphates or other factors that promote activation of FXII.[28]

In addition to these interactions, other extracellular or intracellular binding partners regulate GPIb-IX-V–dependent signaling. First, the ectodomain of GPIbα interacts with platelet collagen receptor, GPVI, of the immunoreceptor family. GPVI forms a noncovalent complex with Fc receptor γ chain that is required for GPVI surface expression and for downstream following of GPVI engagement.[29] GPIbα also associates with FcRγ in human platelets,[30] and acts with GPVI/FcRγas accessory signaling proteins that share common signaling pathways, as discussed below.[31] Second, the intracellular domain of GPIb-IX-V interacts with calmodulin that binds to membrane-proximal amphipathic/positively charged sequences of GPIbβ and GPV and with 14-3-3ζ that binds to phosphoserine-containing sequences at the *C*-terminal or upstream regions of GPIbα, or within GPIbβ (FIGURE 26A.2). Calmodulin dissociation regulates ectodomain shedding of GPIbα or GPV by ADAM17 or ADAM10/17, respectively.[5,32,33] The regulatory p85 subunit of phosphatidyl inositol 3-kinase (PI3-kinase) also binds to the *C*-terminal sequence of GPIbα, independent of phosphorylation status or 14-3-3ζ binding.[34,35]

GPIb-IX-V: INTRACELLULAR BINDING PARTNERS AND SIGNALING

Engagement of GPIb-IX-V triggers intracellular signals leading to activation of Src kinase (Lyn),[36] Syk, SLP-76, PI3-kinase (consisting of p85 regulatory and p110 catalytic subunits), Btk,[37] phospholipase Cγ, and protein kinase C. Activation results in elevation of cytosolic Ca^{2+}, secretion of α-granules and dense granules, and activation of integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) that binds vWF or fibrinogen to mediate platelet aggregation.[31] The calmodulin-binding sequence within the cytoplasmic domain of GPIbβ also binds TNF receptor-associated factor 4 (TRAF4) involved in assembly of the NADPH oxidase (Nox2) complex that generates ROS, enhancing platelet activation.[38,39]

GPIb-IX-V is also associated with the platelet cytoskeleton *via* an interaction between a sequence within the cytoplasmic tail of GPIbα and the actin-binding protein filamin-A.[40–43] Disruption of the filamin-binding sequence in transgenic

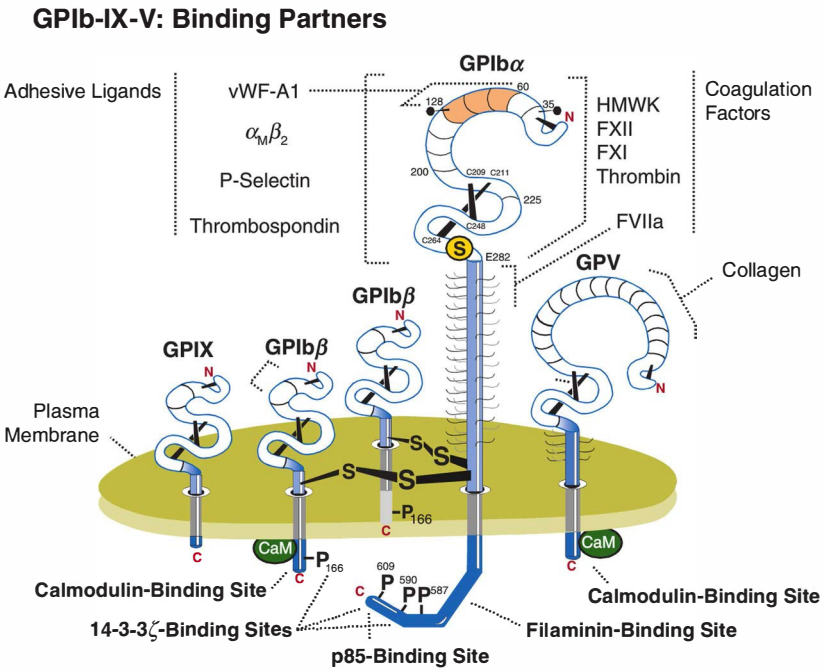

FIGURE 26A.2 GPIb-IX-V: Binding partners. Binding regions for adhesive or procoagulant ligands on GPIbα at the *N*-terminal globular domain (residues 1 to 282); collagen binds GPV. Binding of vWF under shear conditions depends on leucine-rich repeats (residues 60 to 128), and binding of P-selectin or thrombin is inhibited by antibodies against the sulfated sequence of GPIbα ("S"). The central region of the GPIbα cytoplasmic domain centered on Phe568/Trp570 binds the cytoskeletal protein filamin. Calmodulin binds membrane-proximal amphipathic, positively charged sequences of GPIbβ and GPV, and regulates surface expression/ectodomain shedding of GPIbα and GPV; this sequence in GPIbβ also binds TRAF4 of the Nox2 complex that generates ROS. Binding of 14-3-3ζ involves phosphorylated serine residues at the *C*-terminal penultimate residue (Ser609) and upstream residues at Ser587/Ser590 of GPIbα, and Ser166 of GPIbβ. Binding of p85 (regulatory subunit of PI3-kinase) also binds to the same *C*-terminal sequence of GPIbα, but is independent of phosphorylation/14-3-3ζ binding. See the text for details.

mouse models (wild-type human GPIbα or a mutant form of human GPIbα with Phe568Ala and Trp570Ala substitutions in the filamin-binding site) demonstrates that the link between platelet GPIbα and the cytoskeleton does not alter vWF binding or signaling by GPIbα, but results in unstable membrane tethers and defective platelet adhesion under high shear conditions.[44] This suggests that the GPIbα-filamin interaction regulates the membrane skeleton structure and mechanical stability of the plasma membrane under conditions of high shear.

HEMOSTATIC FUNCTIONS OF GPIb-IX-V

Platelet GPIb-IX-V plays a central role in hemostasis and thrombosis. First, in Bernard-Soulier syndrome, a rare human disease where there is a deficiency of functional GPIbα on platelets due to a variety of inherited mutations (see Chapter 65), patients have a mild to severe bleeding phenotype, typically associated with large platelets and low platelet count (macrothrombocytopenia).[45] The Bernard-Soulier syndrome supports a hemostatic role for GPIb-IX-V, but there may not be a precise correlation between the level of GPIbα expression and clinical bleeding due to other contributing factors.[46] The GPIbα-knockout mouse displays a comparable phenotype.[47]

In animal models, GPIbα is not only associated with arterial thrombosis or stroke,[48,49,50,51,52] but in recent studies, blocking GPIbα binding to vWF-A1 revealed that this interaction is involved in venous thromboembolism[53] and deep vein thrombosis.[54] Studies using mutant vWF expressed in vWF-deficient mice suggest that vWF binding to GPIbα and collagen, rather than to $\alpha_{IIb}\beta_3$, is critical in these thrombosis models.[55]

The role of GPIbα in arterial thrombosis may not be restricted to its interaction with vWF-A1,[48,51] and could also involve interactions with coagulation factors, interaction with activated leukocytes *via* $\alpha_M\beta_2$, and/or interaction with activated endothelium *via* P-selectin (FIGURE 26A.2). Therapeutic targeting of GPIbα in humans could potentially involve variations of fusion proteins consisting of GPIbα/vWF-A1 binding domains or humanized inhibitory antibodies used in experimental models.[49,52–54]

NONHEMOSTATIC FUNCTIONS OF GPIb-IX-V

In addition to the hemostatic roles described above, platelet GPIbα may also participate in some of the nonhemostatic functions of platelets.[56,57] In experiments involving injection of tumor cells into mice, depletion of GPIbα inhibited tumor metastasis and solid tumor formation.[58,59] Although the precise mechanism for these protective effects was not firmly established, they could be related to a role for adherent platelets in

regulating the angiogenesis required for solid tumor growth.[57] GPIb-IX-V is also critical in recruitment of leukocytes to sites of vascular injury through the interaction of GPIbα with $\alpha_M\beta_2$,[60] an important step in inflammation and wound healing, and potentially, in initiation of atherosclerosis depending on the model used or other variables.[61,62] Finally, platelet GPIb-IX-V also interacts with bacterial proteins and this can contribute to platelet activation during infection and/or promote the inflammatory response (reviewed in[63]).

CONCLUDING STATEMENT

Through interactions with adhesive proteins (vWF, thrombospondin), counterreceptors on other vascular cells (P-selectin, $\alpha_M\beta_2$), coagulation factors (kininogen, FXII, FXI, thrombin, FVIIa), and cancer cells or bacteria, platelet GPIb-IX-V plays a central role in initiating platelet dys/function in vascular biology. Downstream of GP-IX-V engagement, activation of platelets leads to secretion of bioactive molecules that can influence thrombus formation, inflammation, and other responses, and lead to activation of other platelet adhesion receptors such as $\alpha_{IIb}\beta_3$.

SUMMARY

The platelet GP Ib-IX-V complex of the leucine-rich repeat family is made up of GPIbα (the major ligand-binding subunit), GPIbβ, GPIX and GPV. In addition to its traditional role as a vWF receptor mediating platelet thrombus formation at arterial shear rates in hemostasis or thrombotic disease (heart attack or stroke), new evidence has shown that multiple extracellular ligands of GPIb-IX-V are involved in a plethora of new non-hemostatic functions for this unique receptor. New findings also show how intracellular interactions involving the cytoplasmic domain of GPIb-IX-V and binding partners such as filamin, 14-3-3ζ, p85 (regulatory subunit of phosphatidylinositol 3-kinase), and TRAF4 control activation of signaling pathways, cytoskeletal changes, and/or generation of reactive oxygen species (ROS) critical to platelet function.

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CHAPTER

26B

Major Platelet Glycoproteins: INTEGRIN $\alpha_{IIb}\beta_3$ (GPIIb-IIIa)

FENG YE AND MARK H. GINSBERG

The first insight into the involvement of the integrin $\alpha_{IIb}\beta_3$ in hemostasis came from the observation that two major glycoprotein bands were either abnormal or completely missing from the platelets of some patients with Glanzmann thrombasthenia (GT),[1,2] a rare inherited bleeding disorder characterized by the absence of platelet aggregation. The two glycoproteins, named glycoproteins IIb (GPIIb) and IIIa (GPIIIa), were later found to form a cation-dependent complex.[3,4] The Phillips lab then successfully purified and reconstituted a functional complex containing these two glycoproteins, demonstrating biochemically that GPIIb and IIIa are subunits of the same protein.[3,5]

In the mid-1980s, a family of functionally and structurally related adhesion receptors, including GPIIb/IIIa,[6–8] emerged from research in a variety of biologic disciplines including immune biology and vascular biology and development. This family of proteins functionally integrates their extracellular ligands with the intracellular cytoskeleton and was formally named integrin in 1987. GPIIb/IIIa was renamed as integrin $\alpha_{IIb}\beta_3$.[9]

Further research demonstrated that the interaction between $\alpha_{IIb}\beta_3$ and its ligands was a critical event for platelet aggregation, hemostasis, and thrombosis.[10–12] Biochemical and structural details of the interaction between $\alpha_{IIb}\beta_3$ and its physiologic ligands facilitated the development of $\alpha_{IIb}\beta_3$ antagonists that are currently used in the treatment of acute arterial thrombosis by percutaneous coronary intervention (PCI).[13–16] Work over the past two decades underscored that integrins, including $\alpha_{IIb}\beta_3$, are not merely adhesion molecules but also signaling receptors.[17–19] Signals originating from inside the cell can modulate ligand binding to integrin extracellular domains (inside-out signaling). Inside-out signaling is tightly controlled, both temporally and spatially, to achieve hemostasis and prevent unwanted vessel occlusion. At the same time, signals initiated by ligand binding to the integrin extracellular domains (outside-in signaling) can trigger intracellular signals to regulate anchorage-dependent cellular responses. In the case of platelets, these include full aggregation and granule secretion as well as clot retraction. In this chapter, we will discuss integrin $\alpha_{IIb}\beta_3$ and its role in platelet function and bleeding disorders.

EXPRESSION AND STRUCTURE OF INTEGRIN $\alpha_{IIb}\beta_3$

$\alpha_{IIb}\beta_3$ expression is limited primarily to cells of the megakaryocytic lineage. However, the β_3 subunit is widely expressed as the beta subunit of the integrin $\alpha_v\beta_3$. Therefore, restriction of $\alpha_{IIb}\beta_3$ expression is limited by the promoter activity of α_{IIb} in cell types other than megakaryocytes.[20,21] Only a few exceptions have been reported. For example, $\alpha_{IIb}\beta_3$ is detected in certain cancer cells.[22] $\alpha_{IIb}\beta_3$ has also been detected on early hematopoietic progenitors and on mast cells where it plays a role in adhesion and in the response of the latter cells to chronic inflammation.[23,24] Efficient surface expression of the $\alpha_{IIb}\beta_3$ complex requires α_{IIb} and β_3 in a ratio of 1:1.[25–27] Excess expression of one results in the accumulation of the excess in the endoplasmic reticulum where association of nascent α_{IIb} and β_3 subunits occurs. Indeed, a subset of patients with GT results from mutations that alter the biosynthesis of only one subunit. In turn, this impairs the surface expression of the complete $\alpha_{IIb}\beta_3$ complex.[28,29]

$\alpha_{IIb}\beta_3$ is the most abundant protein on the platelet surface: there are approximately 80,000 copies on the surface of unactivated platelets.[30] An intracellular pool of $\alpha_{IIb}\beta_3$, located in the membrane of α-granules, can be translocated to the cell surface following platelet activation, increasing the amount of $\alpha_{IIb}\beta_3$ by 30% to 50%.[30–32] This high level of expression is important for efficient platelet function as platelets need to strongly adhere to multiple adhesive proteins to form a stable clot.

β_3 is expressed as a single polypeptide of 762 amino acid that folds into 10 distinct domains: a plexin-semaphorin-integrin (PSI) domain at the *N*-terminal, a βA domain that is inserted into the hybrid domain, followed by hybrid, EGF-1, EGF-2, EGF-3, EGF4, and βTD domains, a transmembrane domain (TMD) embedded in the lipid bilayer and a cytoplasmic domain[33,34] (FIGURE 26B.1). Folding is facilitated by 28 disulfide bonds formed from 56 cysteine residues. The β_3 βA domain has three divalent cation-binding sites: a metal ion–dependent adhesion site (MIDAS), a site adjacent to MIDAS, and a ligand-induced metal binding site, also referred to as the synergistic metal ion binding site. The β_3 A-domain assumes a nucleotide-binding (or Rossmann) fold in which a central six stranded β-sheet is surrounded by eight α-helices[33,34] (FIGURES 26B.1–26B.3).

α_{IIb} has 1,008 amino acid and folds into six domains: β-propeller, thigh, calf-1, calf-2, transmembrane, and cytoplasmic domains.[33,34] α_{IIb} is initially synthesized as a single polypeptide chain. During protein folding and maturation, it is proteolytically cleaved at a site in the calf-2 domain into heavy and light chains by a furin-like enzyme in the Golgi complex. However, the heavy and light chains remain covalently associated via a disulfide bond.[35] Thus, in nonreduced sodium dodecyl sulfate gels, α_{IIb} has an apparent molecular weight of 130 kDa. In reducing gels, however, it consists of a 115 kDa fragment containing most of its extracellular domains and a 25 kDa fragment that contains part of the calf-2 domain, as well as the transmembrane and the cytoplasmic domains. At its *N*-terminus, α_{IIb} consists of seven β sheets containing approximately 60 amino acids each that fold into a seven-bladed β-propeller (FIGURE 26B.2A). Four divalent cation binding sites are found at the base of the

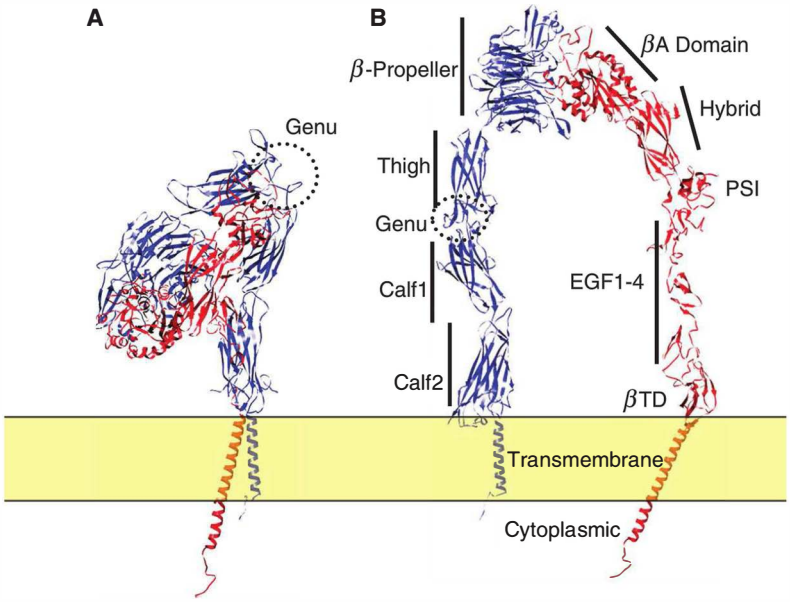


FIGURE 26B.1 Domain structures of $\alpha_{IIb}\beta_3$. **A:** Structures of $\alpha_{IIb}\beta_3$. The ribbon representation of $\alpha_{IIb}\beta_3$ was generated by combining a crystal structure of the $\alpha_{IIb}\beta_3$ extracellular domain (PDB file 3FCS), an NMR structure of $\alpha_{IIb}\beta_3$ TMD (PDB entry 2k9j), and an NMR structure of the β_3 cytoplasmic domain (PDB entry 2H7E). **B:** The $\alpha_{IIb}\beta_3$ molecule was arbitrarily extended to display individual domains as labeled in the figure. In both **(A)** and **(B)**, α_{IIb} was shown in *blue* and β_3 was shown in *red*.

propeller.[33,34] A flexible loop between the thigh and the calf-1 domain (often referred to as the "genu" region) then allows the molecule to undergo a functionally important global structural rearrangement[33,34,36,37] (FIGURE 26B.1).

α_{IIb} and β_3 associate to form a noncovalent heterodimer. Several interfaces hold the heterodimer together: a large interface between the β-propeller and βA domain that buries approximately 1,600 Å^2 of surface area and with Arg261 of the βA domain protruding off-center into the β-propeller's channel[33,34,38] (FIGURE 26B.2B); an interaction between the α_{IIb} and β_3 TMDs; and an interaction between the membrane proximal (MP) portions of each cytoplasmic domain.[39,40] Divalent cations are involved in this association because exposure to ethylenediaminetetraacetic acid causes the heterodimer to dissociate.[4,41]

Although no divalent cation is observed at the α and β interface, cations in the β-propeller and thigh–calf-1 interface might allosterically contribute to α and β interactions.[33] The Arg-Gly-Asp or RGD motif present in many integrin ligands inserts into a crevice between the βA and β propeller domains. The Arg side chain inserts into a narrow groove on the top of the β-propeller, and the side chain of Asp protrudes into a cleft in the βA domain. The Gly residue lies at the interface between the α and β subunits where it makes several hydrophobic contacts. Overall, 355 Å^2 of surface area or 45% of the total surface area of the peptide is buried, thereby contributing to a stable ligand–receptor interaction[42] (FIGURE 26B.3).

Structures of the $\alpha_{IIb}\beta_3$ TMD heterodimer, determined by nuclear magnetic resonance (NMR) spectroscopy, revealed that

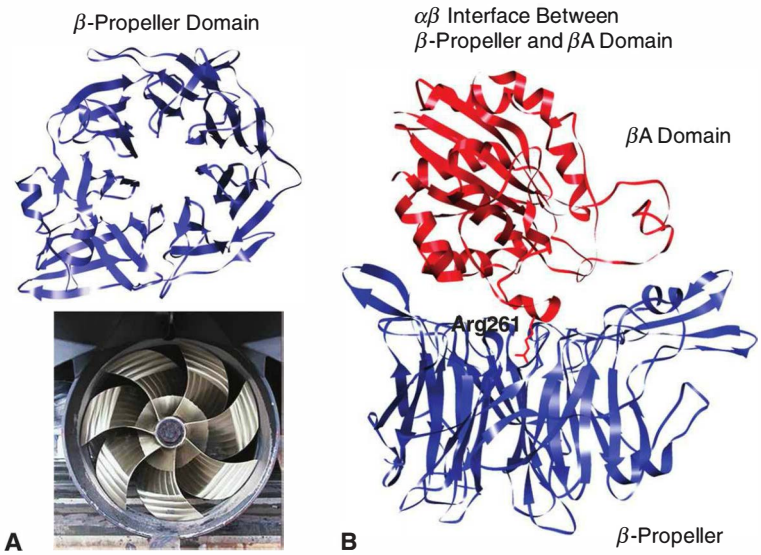


FIGURE 26B.2 The α_{IIb}-propeller domain and the interface between β-propeller and βA domain. **A:** The α_{IIb} β-propeller domain is formed by seven blades of β-sheet fold. **B:** At the interface, Arg261 of the βA domain protrudes off-center into the β-propeller's channel. From PDB entry 3FCS.

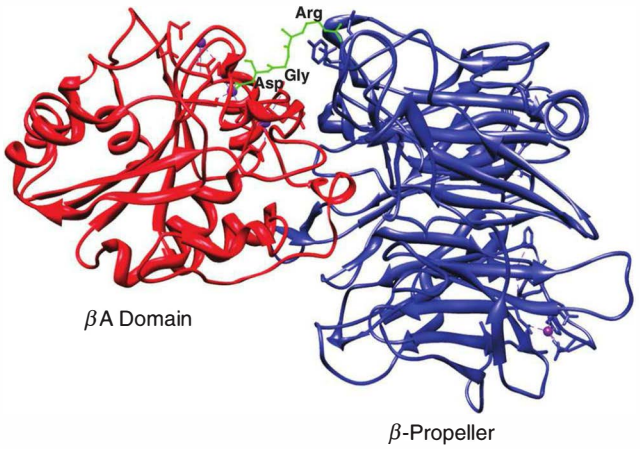


FIGURE 26B.3 Binding of the Arg-Gly-Asp (RGD) motif to the integrin $\alpha_v\beta_3$, a paralogue of $\alpha_{IIb}\beta_3$. The RGD peptide inserts into the interface between the β_3 βA and α_{IIb} β-propeller domains. The Arg points to the β-propeller whereas Asp make contacts with the βA domain. Gly lies at the interface. From PDB entry 3FCS.

the α_{IIb} and β_3 TMD pack together at two main interfaces with a vertical α_{IIb} TMD and tilted β_3 TMD.[39] At the outer membrane region, α_{IIb} and β_3 tightly pack against each other via a groove formed in the α_{IIb} $G^{972}XXXG^{976}$ motif and β_3 G^{708} (termed outer membrane clasp or OMC). The β_3 TMD forms a continuous helix that extends to the MP region of the cytoplasmic domain. A hydrophobic patch in the membrane-proximal region of β_3 is inserted into the membrane as a part of the TMD. Consequently, the β_3 TMD is long and crosses the lipid bilayer at an angle of 25 degrees. In contrast, the α_{IIb} TMD is a short and straight helix that ends at Gly991. Distal to Gly991, α_{IIb} makes a sharp turn toward β_3, enabling Phe992 and Phe993 to embed in the membrane and pack against β_3 residues Trp715 and Ile719. In addition, this places α_{IIb} Arg995 in close apposition to Asp723 of β_3, enabling an electrostatic interaction between them. The Phe packing interfaces and Arg995–Asp723 interaction completes the inner membrane interface (termed inner membrane clasp, or IMC)[39] (FIGURE 26B.4).

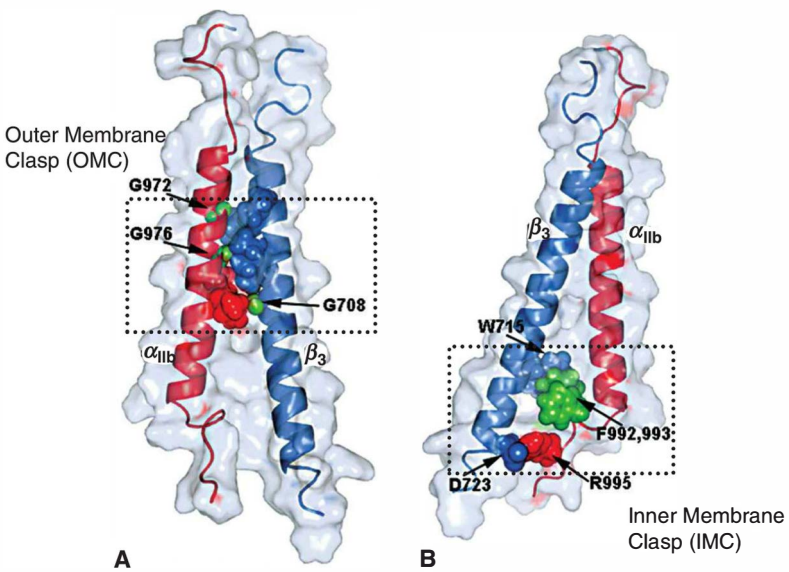
STRUCTURAL MECHANISMS OF INTEGRIN REGULATION

The combination of crystal and NMR structures and mutational studies has provided a detailed picture of integrin regulation. Integrin TMDs have two main interaction interfaces with three main contacts.[39,40] Mutations that disrupt these interactions favor the active form of the integrin. Charge reversal mutations of either α_{IIb} Arg995 or β_3 Asp723 disrupt the IMC and favor integrin activation, whereas combined charge reversals shift the equilibrium toward the inactive form.[43] Similarly, Phe to Ala mutations in the α_{IIb} MP region disrupt the IMC, inducing constitutive $\alpha_{IIb}\beta_3$ activity.[43]

Activating mutations are also found in the OMC. Most are substitutions of the Gly residues in both the α_{IIb} $G^{972}XXXG^{976}$ motif and β_3 Gly708 with residues containing bulky side chain, such as Leu, Ile, or Asn.[44–46] The side chains of these residues disrupt the tight packing interface between α_{IIb} $G^{972}XXXG^{976}$ motif and the β_3 TMD.[39,47] Furthermore, the 25-degree tilting angle of the β_3 helix is required to enable simultaneous interactions at the IMC and OMC.[39] Mutations that change the β_3 tilting angle, such as Lys716 to Ala, also result in disruption of the association of the β_3 TMD with α_{IIb}.[17] In short, integrins are kept in an inactive form by TM and cytoplasmic domain interactions. Following inside-out signaling, the α_{IIb} and β_3 TMD rearrange, causing integrin activation.

Integrin extracellular domains have multiple conformations, including those associated with inactive, active, and active ligand-occupied forms. The transition from one conformational state to another results in characteristic exposure of epitopes. A large inventory of conformation-specific $\alpha_{IIb}\beta_3$ antibodies has accumulated to study the relationship between $\alpha_{IIb}\beta_3$ conformation and function. How do the TMD and cytoplasmic domain rearrangements propagate to the integrin head

FIGURE 26B.4 Structure of the $\alpha_{IIb}\beta_3$ TMD heterodimer showing interactions at OMC and IMC. **A:** OMC interface. α_{IIb}(G972), α_{IIb}(G976), and β_3(G708) are shown as *green spheres*, with their β_3 and α_{IIb} packing residues shown in *blue* and *red*, respectively. **B:** IMC interactions. α–β TM complex stabilized by hydrophobic packing of α_{IIb}(F992, 993) (*green spheres*) against β_3(W715) (*blue sphere*) and α_{IIb}(R995) (*blue sphere*)–β_3(D723) (*red sphere*) electrostatic interaction. (From Lau TL, Kim C, Ginsberg MH, et al. The structure of the integrin alphaIIbbeta3 transmembrane complex explains integrin transmembrane signalling. *EMBO J* 2009;28:1351–1361.)



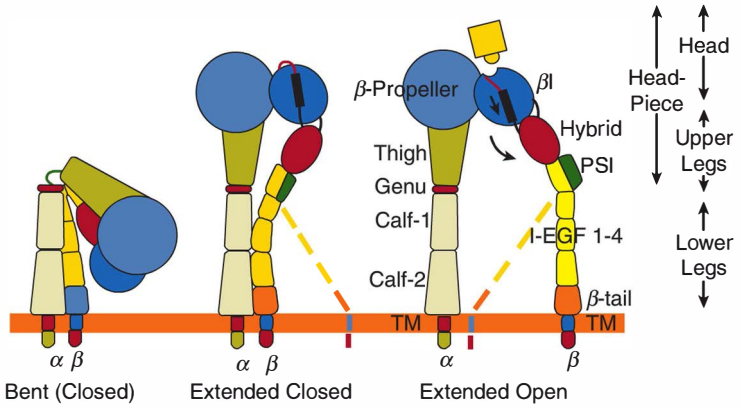


FIGURE 26B.5 Cartoon illustrating $\alpha_{IIb}\beta_3$ conformational changes. It has been proposed that $\alpha_{IIb}\beta_3$ has bent, extended closed, and extended open conformations that correspond to inactive, intermediate, and active state of the integrin molecule. (From Springer TA, Dustin ML. Integrin inside-out signaling and the immunological synapse. *CurrOpin Cell Biol* 2012;24:107–115).

piece to cause higher ligand binding affinities? One hypothesis proposes that a bent integrin conformation has a low affinity for ligand, while a more open and extended conformation is associated with high ligand affinity[37] (FIGURE 26B.5). It has also been proposed that TMD and cytoplasmic domain separation results in extension of integrin extracellular domain at the genu region and swing out of the β_3 hybrid domain.[48] The β_3 hybrid domain movement is coupled through the βA α7-helix to rearrange the ligand binding crevice at the interface between β-propeller and βA domains.[38] This global structural rearrangement increases the affinity of $\alpha_{IIb}\beta_3$ for its ligand[37] (FIGURE 26B.5). An alternative hypothesis posits that the bent conformation can be an active conformer, and that extension of the molecule is a post–ligand-binding event. This theory also has experimental support.[33,42,49,50] This is an area where there is likely to be continued debate.

INSIDE-OUT SIGNALING AND PLATELET AGGREGATION

At sites of vascular damage, platelets encounter agonists such as subendothelial collagen, ADP, or thrombin. Following agonist stimulation, signaling cascades are activated that eventually convert $\alpha_{IIb}\beta_3$ to its high affinity form. Activated $\alpha_{IIb}\beta_3$ binds tightly to multivalent ligands such as fibrinogen,[51,52,53] fibronectin,[54,55] and von Willebrand factor[56] each of which can contribute to platelet aggregation.

Among the earliest responses to agonist stimulation is activation of phospholipase Cβ (PLCβ) by Ca^{2+}. PLCβ then hydrolyzes the polar head group of phosphatidylinositol 4,5-bisphosphate to generate the secondary messengers diacylglycerol and inositol 1,4,5-triphosphate. This is followed by activation of protein kinase C (PKC), calcium- and diacyglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI), and phosphoinositide 3-kinase (PI3K). These signaling pathways can lead to Rap1B activation.[57,58,59–61] Rap1 activation is delayed in platelets lacking CalDAG-GEFI, and is completely abolished when both PKC and CalDAG-GEFI functions are absent.[62] Furthermore, knockout of Rap1B, the predominant Rap1 isoform in platelets, leads to reduced platelet aggregation, prolonged tail bleeding times, reduced $\alpha_{IIb}\beta_3$ activation, and defects in platelet spreading.[63] Thus, Rap1 has emerged as a possible common downstream effector for these signaling cascades. Rap1 interacts with a Rap effector, Rap1-GTP-interacting adaptor molecule (RIAM), which in turn binds to talin, forming a Rap1-RIAM–talin complex that targets talin to integrins[64,65,66] (FIGURE 26B.6). Talin, along with kindlin, forms the immediate $\alpha_{IIb}\beta_3$ activator.[67,68]

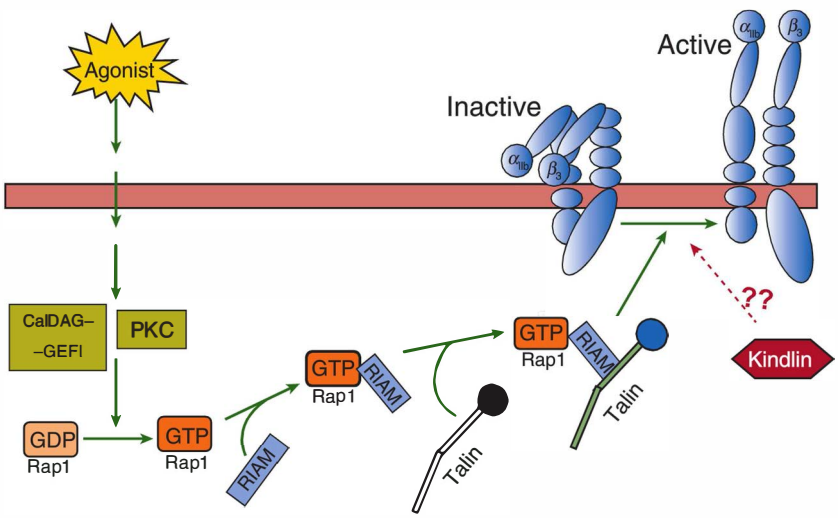


FIGURE 26B.6 Simplified representation of a signaling pathway from agonist stimulation to $\alpha_{IIb}\beta_3$ activation.

Talin, a 270 kDa cytoplasmic protein, can bind to integrin β tails through its 50 kDa head domain.[69,70] There are also multiple actin-binding sites in its 220 kDa rod domain.[71–73] Overexpression of the integrin-binding head domain in cells strongly activates $\alpha_{IIb}\beta_3$.[69] Tissue-specific deletion of talin in cells of the megakaryocyte lineage causes severe defects in platelet aggregation and agonist-stimulated $\alpha_{IIb}\beta_3$ activation. Consequently, mice that have lost talin in megakaryocytes and platelets have prolonged bleeding times and experience frequent pathologic bleeding.[74,75] The talin head domain binds to the β_3 cytoplasmic domain at two sites. First, it binds strongly to the β_3-W^{739}DTANNPLY^{747} sequence (membrane distal [MD] interaction) and then weakly to β_3 H^{722}DRKEFAKFEEER^{726} (membrane proximal [MP] interaction) that depends upon the presence of the first strong talin–integrin MD interaction.[76] In addition to the two integrin binding sites, the talin head domain has multiple positively charged residues on its surface that can interact with negatively charged phospholipids and to configure the talin–integrin interaction[77,78,79] (FIGURE 26B.7). The precise configuration of this integrin–talin-membrane interaction enables talin to alter the relationship between integrin α_{IIb} and β_3 subunits by both competitively disrupting α_{IIb} Arg995 and β_3 Asp723 electrostatic interaction pair and changing the tilting angle of the β_3 TMD.[77,79] As described above, the separation of α_{IIb} and β_3 results in integrin activation and higher affinity for ligands. *In vitro* reconstitution of inside-out $\alpha_{IIb}\beta_3$ activation using $\alpha_{IIb}\beta_3$-containing nanodiscs and liposomes demonstrated that all three interactions—distal and proximal talin binding to β_3 and talin interaction with the lipid bilayer—are necessary and sufficient for talin to activate $\alpha_{IIb}\beta_3$ *in vitro* and to favor an extended conformation.[80]

The kindlins, a second family of integrin β cytoplasmic domain-binding proteins, cooperates with talin to regulate $\alpha_{IIb}\beta_3$ activity.[67,68] There are three kindlin orthologues in mammals: kindlin-1, 2, and 3. Hematopoietic cells, including platelets, primarily express kindlin-3.[81] Loss of functional kindlin-3 in platelets results in severe bleeding, defective integrin activation, and defective platelet aggregation.[82] In model systems, coexpression of the talin head domain and kindlin-2 enhances talin-mediated integrin activation.[83,84] Thus kindlin and talin, together, regulate $\alpha_{IIb}\beta_3$ activation. The precise mechanism of kindlin function is a subject of intense investigation.

OUTSIDE-IN SIGNALING AND PLATELET FUNCTION

After $\alpha_{IIb}\beta_3$ engages ligands, intracellular signaling leads to events such as additional granule secretion, platelet spreading on extracellular matrices, stabilization of platelet adhesion and aggregation, and clot retraction that consolidates fibrin thrombi. Ligand engagement also induces further conformational changes in $\alpha_{IIb}\beta_3$ itself, as shown by exposure of new extracellular domain epitopes.[85] More importantly, multimeric macromolecular ligands, such as fibrinogen, fibrin, or fibronectin, induce $\alpha_{IIb}\beta_3$ clustering.[86–88] Conformational change and $\alpha_{IIb}\beta_3$ clustering together transmit the signals outside-in.

The earliest outside-in signaling event is activation of Src family kinases (SFK), particularly c-Src bound to the distal β_3 cytoplasmic domain.[89,90] Activated SFK rapidly phosphorylates ligand-occupied $\alpha_{IIb}\beta_3$ at β_3 residues Tyr747 and Tyr759. β_3 phosphorylation protects it from cleavage by proteases such as calpain,[91] and may enhance post ligand-binding events by generating docking sites for SH2-containing protein (Shc), an adapter in Ras signaling, and myosin, a motor protein involved in clot retraction and stabilization of platelet aggregates.[92] Mice in which both Tyr747 and Tyr759 have been mutated to Phe showed a tendency for rebleeding in tail bleeding time assays and impaired clot retraction.[93] Integrin clustering also brings

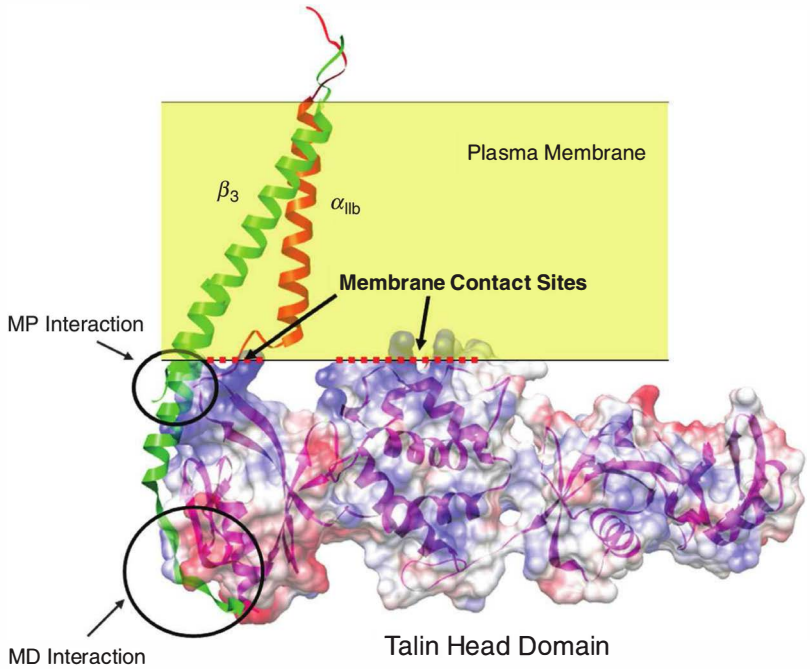


FIGURE 26B.7 Structural model of talin–integrin interaction at the plasma membrane. Talin binds to two sites in the integrin β_3 tail: the membrane distal (MD) interaction site centered on NPxY747 motif and a membrane proximal (MP) site. Talin also makes contact with the plasma membrane through the positively charged residues on its surface (*blue color* in the figure, *red dotted lines* indicate the membrane contact interfaces). Shown in *red* and *green* are the α_{IIb} and β_3 TMD from NMR structures (PDB entry 2k9j). The talin head domain (PDB entry 3IVF) is shown in surface representation. The ribbon diagram representation of β_3 was extended to the cytoplasmic domain by aligning the β_3 TM domain with the β1D cytoplasmic domain structure from a β1D-talin F2F3 (PDB entry 3G9W).

c-Src into clusters and result in dissociation of *C*-terminal Src kinase (CSK) from c-Src. Clustered c-Src is rapidly autophosphorylated at Tyr418 in the Src activation loop resulting in full activation of c-Src kinase activity[89,90] (FIGURE 26B.8). Activated Src phosphorylates immunoreceptor tyrosine-based activation motifs, which can then activate Syk.[94] Src and Syk together then phosphorylate and modulate the function of p190 Rho GTPase activating protein, SLP-76, Adhesion and Degranulation-Promoting Adapter Protein (ADAP), and Vav (a Rac exchange factor), all of which are implicated in further activation of platelets and contractile events that underlie clot retraction. For example, transient phosphorylation and activation of p190 Rho GTPase activating protein deactivate RhoA, which enables platelet to spread on $\alpha_{IIb}\beta_3$ ligands.[95,96] On the other hand, late and prolonged activation of mitogen-activated protein kinases and Rac-1 and myosin light chain phosphorylation result in platelet contraction and clot retraction.[97] Mutant mice in which the Src binding site in β_3 has been deleted manifest defective platelet spreading and reduced tyrosine phosphorylation of c-Src substrates. These mice are protected from carotid artery thrombosis after vessel injury with $FeCl_3$.[98]

$\alpha_{IIb}\beta_3$ AND BLEEDING DISORDERS

Given the important role of $\alpha_{IIb}\beta_3$ in platelet function and hemostasis, it is not surprising to find diseases resulting from mutations in $\alpha_{IIb}\beta_3$ and $\alpha_{IIb}\beta_3$ regulators. For example, loss of either α_{IIb} or β_3 expression due to nonsense mutations causes GT, a hereditary hemorrhagic disorder[1,2] (see Chapter 65). Another subset of GT patients carry mutations that disrupt folding, posttranslational processing, or intracellular transport of either α_{IIb} or β_3, resulting in reduced $\alpha_{IIb}\beta_3$ surface expression.[11] The most informative mutations have been those that cause GT without altering $\alpha_{IIb}\beta_3$ expression. These mutations perturb $\alpha_{IIb}\beta_3$ function and can be separated into three groups. One group of mutations is located in the extracellular portion of $\alpha_{IIb}\beta_3$ (e.g., β_3 Arg214 to Trp, β_3 Asp119 to Tyr) and perturbs the ligand binding pocket at the interface between α_{IIb} and β_3.[99,100] Another group is present in the $\alpha_{IIb}\beta_3$ cytoplasmic domains and disrupts the binding of $\alpha_{IIb}\beta_3$ regulators (e.g., β_3 Ser752 to Pro, β_3 Arg724 to Ter).[82,101,102] The third group locks $\alpha_{IIb}\beta_3$ in an activated conformation (e.g., β_3 Cys560 to Arg, β_3 Cys598 to Tyr).[103,104] In the latter group of mutants, patients exhibit GT-like symptoms because $\alpha_{IIb}\beta_3$ is constitutively occupied by ligand and may be internalized; thus it is not available to bridge $\alpha_{IIb}\beta_3$ from another platelet. An extensive list of $\alpha_{IIb}\beta_3$ mutations identified in GT patients has been compiled (available online at http://sinaicentral.mssm.edu/intranet/research/glanzmann/menu).

Patients with a variant of the leukocyte adhesion deficiency syndrome (LADIII) also display bleeding symptoms. These patients have mutations in kindlin-3,[105,106,107,108] the protein that together with talin regulates integrin activation. In platelets manifesting kindlin-3 mutations, $\alpha_{IIb}\beta_3$ activation in response to agonist stimulation is defective. Consequently, these platelets fail to aggregate or retract fibrin clots. A subset of kindlin-3–deficient LADIII patients were also reported to carry mutations in CalDAG-GEFI,[107,109] which might exacerbate the bleeding phenotype by impairing Rap1 activation.

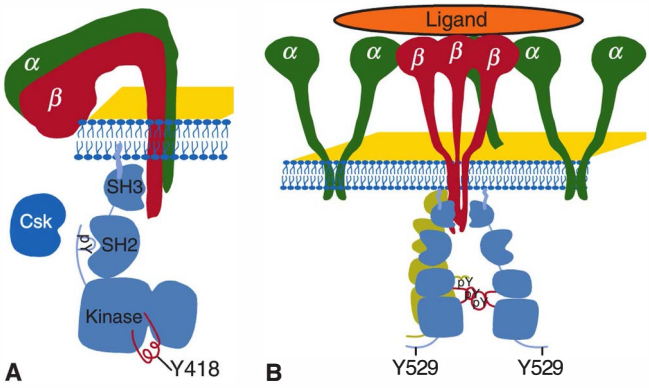


FIGURE 26B.8 Model for activation of β_3-associated Src. **A:** In platelets, a pool of c-Src (and several other SFK) is constitutively bound to the β_3 cytoplasmic tail through its SH3 domain. Because Tyr529 remains phosphorylated by Csk, Src is kept in a partially unclamped but not yet fully active state. **B:** Upon $\alpha_{IIb}\beta_3$ ligation and clustering, Src also becomes clustered and Csk dissociates from the integrin. This results in dephosphorylation of Tyr529, autophosphorylation of Tyr418 (Y418) in the Src activation loop, and full activation of Src kinase activity. (From Shattil SJ, Newman PJ. Integrins: dynamic scaffolds for adhesion and signaling in platelets. *Blood* 2004;104:1606–1615.)

$\alpha_{IIb}\beta_3$ ANTAGONISTS

The phenotype of patients with GT and of mice expressing $\alpha_{IIb}\beta_3$ mutants clearly shows that loss of $\alpha_{IIb}\beta_3$ function prevents platelet aggregation and protects against arterial thrombosis. Moreover, patients with GT rarely have spontaneous central nervous system bleeding despite suffering frequent mucocutaneous hemorrhages. These observations suggested that $\alpha_{IIb}\beta_3$ might be a therapeutic target for preventing arterial thrombosis. Three $\alpha_{IIb}\beta_3$ antagonists have been developed and approved for use in PCIs and as adjunctive therapy to prevent ischemic complications of coronary artery angioplasty in high-risk patients. The first clinically approved $\alpha_{IIb}\beta_3$ antagonist was abciximab, a humanized mouse monoclonal antibody 7E3 fab fragment containing murine variable regions and human IgG1 constant region.[110] In clinical trials, abciximab reduced all-cause mortality by approximately 20% during long-term follow-up after PCI.[111] This benefit outweighed the increase in absolute risk of major bleeding of approximately 7%. Furthermore, by reducing the dose of heparin used in combination with abciximab, the absolute increase in bleeding risk can be reduced to approximately 2% or less.[112]

Two low molecular weight $\alpha_{IIb}\beta_3$ antagonists, eptifibatide, a cyclic heptapeptide that selectively blocks $\alpha_{IIb}\beta_3$,[113] and tirofiban, a nonpeptide RGD-mimetic, were also approved for clinical use in patients with unstable angina and those undergoing PCI.[114,115] Eptifibatide and tirofiban confer a similar increase in risk of bleeding. Another complication common to all three $\alpha_{IIb}\beta_3$ antagonists is the occurrence of thrombocytopenia.[116,117] One possible cause of thrombocytopenia is the generation of antibodies against neoepitopes on $\alpha_{IIb}\beta_3$ exposed by the binding of the drugs to $\alpha_{IIb}\beta_3$. Use of abciximab resulted in even higher risk of thrombocytopenia probably due to the presence of alloantibodies against the murine components of the humanized antibody.

In contrast to the success of intravenous $\alpha_{IIb}\beta_3$ antagonists, chronic administration of oral $\alpha_{IIb}\beta_3$ antagonists to prevent recurrent thrombotic events has not been successful. Surprisingly, the oral $\alpha_{IIb}\beta_3$ antagonists were associated with increased mortality.[118,119] Several mechanisms have been proposed to explain this toxicity and the dissociation between benefits of intravenous and oral antagonists.[119,120,121] Compared with intravenous administration, fluctuation in plasma levels with oral dosing may provide suboptimal platelet inhibition for a portion of the treatment period. However, this explanation was undermined by the observation that higher-dose oral $\alpha_{IIb}\beta_3$ inhibition is associated with an even greater fatality risk.[118] It has also been speculated that the small molecule antagonists might act as ligand to trigger conformational change and outside-in integrin signaling, resulting in paradoxical thrombosis. In addition, the neoepitopes exposed by antagonists-triggered conformational change might be recognized by patient's antibodies, resulting in thrombocytopenia. Finally, these RGD-mimetic oral antagonists also have off-target effects on procaspase-3 activation in cardiomyocytes, resulting in increased cardiomyocyte apoptosis.[122] The exact mechanism of oral $\alpha_{IIb}\beta_3$ antagonist toxicity is still not known and may be a combination of these factors.

FUTURE OF $\alpha_{IIb}\beta_3$ RESEARCH AND MEDICAL APPLICATIONS

Research on the structure and function of $\alpha_{IIb}\beta_3$ has led to the development of the first generation intravenous $\alpha_{IIb}\beta_3$ antagonists. In the past 10 years, enormous progress has been made in understanding $\alpha_{IIb}\beta_3$ regulation. Novel therapeutic targets identified by these studies might offer superior clinical benefits with reduced risk of pathologic bleeding.[98,123] There is also an attempt underway to improve on the currently available drugs. For example, by designing drugs that inhibit $\alpha_{IIb}\beta_3$ without altering its conformation[124] or that only bind to and block active $\alpha_{IIb}\beta_3$,[125] both thrombocytopenia and antagonists-induced integrin outside-in signaling may be attenuated.

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CHAPTER

27

Platelet Glycoprotein Polymorphisms and Relationship to Function and Immunogenicity

THOMAS J. KUNICKI AND DIANE J. NUGENT

In order to place the effect of glycoprotein gene polymorphisms on platelet function and immunogenicity into proper perspective, a brief summary of key aspects of platelet function is helpful.

Following vascular damage, platelets interact with specific components of the extracellular matrix, particularly collagen, and a complex series of receptor–ligand interactions ensue that ultimately lead to the formation of a stable platelet plug or thrombus. This process is a continuum of at least three phases that can be described as *initiation*, *extension*, and *consolidation*, each requiring the orchestrated cooperation of a group of receptors.

In the *initiation* phase, plasma von Willebrand factor (vWF) binds to collagen via its A3 domain and becomes structurally altered, enabling its A1 domain to bind to the platelet membrane receptor complex glycoprotein Ib-IX-V (GPIb complex). It is GPIbα, the larger subunit of GPIb, that makes direct contact with vWF. This association is a requisite step in platelet adhesion to exposed thrombogenic surfaces at sites of vessel wall injury or in regions of atherosclerotic plaque rupture. This leads to the formation of a more stable platelet monolayer on the collagen surface, mediated predominantly by the platelet-specific receptor glycoprotein VI (GPVI) and the platelet integrin $\alpha_2\beta_1$.

The engagement of these receptors causes platelet activation leading to the *extension* phase, mediated largely by the conversion of prothrombin to thrombin on the activated platelet surface and the secretion of compounds from platelet granules (α-granules and δ-granules), which can further stimulate platelets. One of these compounds, adenosine diphosphate (ADP), augments platelet activation by binding to its cognate platelet receptors, the purinergic receptors $P2Y_1$ or $P2Y_{12}$. Activated platelets also produce and/or release additional agonists, including thromboxane A2 (TXA_2), which then binds to the platelet TXA_2 receptor. Most of the receptors involved in the events of the *extension* phase are members of the G-protein–coupled receptor family.

Other agonists that function during the extension phase include thrombin and epinephrine (EPI). Thrombin (also known as coagulation factor II) binds to a number of receptors, but of particular importance for this discussion is the protease-activated receptor 1 (PAR-1). EPI binds to the platelet α2A-adrenergic receptor (ADRA2A). EPI can contribute to the initiation phase and, in low doses, is thought to prime platelets for enhanced activation by other agonists. The ADRA2A is a G-protein–coupled receptor and activates heterotrimeric G proteins, including those containing the β3 subunit (GNB3).[1] Alternative splicing variants of GNB3 appear to enhance G-protein signal transduction[2,3] and could thus alter the effect of EPI on platelets. Thrombin contributes to the augmentation of platelet activation during the extension phase because the surface of activated platelets is a nidus for prothrombin conversion. Nonetheless, both thrombin and EPI, as well as ADP, are capable of binding to and activating the naïve platelet in an alternative initiation phase.

In the *consolidation* phase, platelet–platelet cohesion (aggregation), mediated by the binding of fibrinogen and/or vWF to the activated platelet integrin $\alpha_{IIb}\beta_3$ (also known as GPIIb-IIIa), together with the assembly of a fibrin network, results in the generation of platelet-rich aggregates or thrombi.

SINGLE NUCLEOTIDE POLYMORPHISMS AND GENE HAPLOTYPES

Receptor Glycoprotein Gene Haplotypes

This chapter is concerned with polymorphisms, defined as nucleotide sequence differences with a minor allele frequency (MAF) of ≥0.02 in any racial or ethnic group and that are detectable in unrelated normal subjects. Polymorphisms that are immunogenic and elicit a humoral response in normal subjects are defined as alloantigens and are designated by the prefix human platelet antigen (HPA-),[4] as shown in Table 27.1. Immunogenic polymorphisms that are unique to a single group of related subjects are considered familial or private antigens and are not discussed in this chapter. Single nucleotide polymorphisms (SNPs) in platelet glycoprotein receptor genes can give rise to differences in expression levels, activity, and/or immunogenicity. Genes harboring SNPs that create alloimmunogenic epitopes include the integrin α_2 subunit gene *ITGA2*,[5,6] the integrin αIIb subunit gene *ITGA2B*,[7,8] the integrin β_3 subunit gene *ITGB3*,[9,10,11–20] the GPIbα subunit gene *GP1BA*,[21,22] and the gene *CD109*.[23] In this chapter, protein and cDNA sequences are numbered from the ATG translation start codon, as recommended by the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

ITGA2. Polymorphisms in the *ITGA2* promoter and regulatory regions generate a fourfold range of $\alpha_2\beta_1$ expression on platelets and other cells. The six most common haplotypes are defined by three SNPs, C-52T (rs28095), C759T (rs1126643), and G1600A (rs1801106). The rs1801106 alleles result in the nonconservative amino acid substitution E534K, creating the clinically relevant HPA-5 alloantigen system, but these alleles do not affect $\alpha_2\beta_1$ binding to collagen.[5] G1600A is in linkage disequilibrium (LD) with C-52T that has a substantial effect on the rate of ITGA2 transcription. However, unlike C-52T, it is not associated

Table 27.1

Human platelet alloantigens

Antigen	Synonym	Gene	dbSNP	cDNA	Amino Acid	Reference
HPA-1	Pl^A, Zw	*ITGB3*	rs5918	T176C	L59P	9,10
HPA-2	Ko, Sib	*GP1BA*	rs6065	C482T	T161M	21
HPA-3	Bak, Lek	*ITGA2B*	rs5911	T2621G	I874S	7
HPA-4	Yuk, Pen	*ITGB3*	rs5917	G506A	R169Q	12
HPA-5	Br, Zav, Hc	*ITGA2*	rs1801106	G1600A	E534K	5
HPA-6bw	Ca, Tu	*ITGB3*	rs13306487	G1544A	Q515R	13
HPA-7bw	Mo	*ITGB3*	rs121918448	C1297G	A433P	14
HPA-8bw	Sr	*ITGB3*	nd	C1984T	C662R	15
HPA-9bw	Max	*ITGA2B*	rs74988902	G2602A	M868V	8
HPA-10bw	La	*ITGB3*	nd	G263A	Q88R	16
HPA-11bw	Gro	*ITGB3*	nd	G1976A	H659R	17
HPA-12bw	Iy	*GP1BA*	nd	G119A	Q31G	22
HPA-13bw	Sit	*ITGA2*	rs79932422	C2483T	T828M	6
HPA-14bw	Oe	*ITGB3*	nd	1909–1911 delAAG	L637del	18
HPA-15	Gov	*CD109*	rs10455097	C2108A	Y703S	23
HPA-16bw	Duv	*ITGB3*	rs74708909	C497T	T166I	19
HPA-17bw	Va	*ITGB3*	nd	C662T	T195M	11,20

nd, not designated.

with regulation of a2 expression. [24] However, selected gene association studies indicate that the minor allele of rs1801106 (1600A) is associated with recurrent stroke[25] and an increased risk for breast cancer in women.[26] Even though this SNP does not alter $\alpha_2\beta_1$ binding to collagen, it is possible that it modulates the binding of other physiologically relevant $\alpha_2\beta_1$-specific ligands, such as C1q, decorin, endorepellin, or perlecan.[27–30]

The *ITGA2* mutation T828M (rs79932422) produces the HPA-13bw alloantigen,[6] but has a MAF of 0.0025 and is not of clinical significance. Although T828M has no effect on platelet adhesion to collagen I, II, or IV, four individuals have been reported who are at least heterozygous 828M and whose platelets exhibit a diminished aggregation response to low-dose collagen (2.5 µg/mL) but a normal response to high-dose collagen (10 µg/mL).[6] Nonetheless, these reports must be interpreted with caution because platelet levels of $\alpha_2\beta_1$ in the affected individuals were not measured.

ITGA2B. Two major *ITGA2B* haplotypes are characterized by the SNP T2621G (rs5911) giving rise to the substitution I874S that defines the HPA-3 alloantigen system.[7] I874S is adjacent to the binding site of the murine monoclonal antibody PMI-1 that inhibits platelet adhesion and spreading on collagen,[31] suggesting that the region of α_{IIb} encompassing the HPA-3 and PMI-1 epitopes participate in aIIbb3-mediated platelet adhesion. The precise impact of the T2621G substitution on platelet function has not been determined, but homozygosity for the 2621G haplotype is associated with a fivefold increase in the risk of ischemic stroke among women with hypertension or diabetes.[32]

ITGB3. The β_3 SNP T176C (rs5918) results in an L59P substitution that defines the HPA-1 alloantigen system.[10] In whites, sensitization to HPA-1a is the leading cause of alloimmunization against platelets (discussed below). There has been considerable debate about the influence of *ITGB3* HPA-1b on platelet function and the risk of acute coronary disease or cerebrovascular disease. Several reports from one group of investigators indicated that HPA-1b confers either enhanced $\alpha_{IIb}\beta_3$ function or resistance to inhibitors of platelet function,[33–37] but not all studies have observed an increased biologic activity associated with the HPA-1b haplotype.[38,39] After considerable conflicting evidence from several gene association studies, most of which were seriously underpowered, subsequent meta-analyses failed to clarify the potential association of HPA-1b with risk for coronary artery disease: One meta-analysis[40] concluded that there is a weak but significant association of HPA-1b with overall cardiovascular disease in the general population and a stronger association in subgroups, such as younger cohorts or a restenosis subset with stents; the second meta-analysis[41] simply found that HPA-1b is not associated with an increased risk of developing myocardial infarction. As regards cerebrovascular disease, a recent meta-analysis found that HPA-1b does not represent a useful marker of increased risk[42].

GP1BA. The GPIb complex is a heptamer composed of four distinct gene products and consists of two molecules of GPIbα, two of GPIbβ, two of GP IX, and one of GP V. Each GPIbα molecule is disulfide-linked to a GPIbβ, whereas the interactions of GPIbα with GPIX and GPV are noncovalent. vWF binds directly to the *N*-terminal portion of GPIbα. Clinically relevant polymorphisms have been associated with the gene *GP1BA* encoding the GPIbα subunit, and two major haplotypes are distinguished by the SNP C482T (rs6065), giving rise to a T161M substitution within the GPIbα ligand-binding region that defines the HPA-2 alloantigen system.[21,43]

T161M is in LD with a variable number of tandem repeats (VNTRs) in the mucin-like macroglycopeptide region of GPIbα. The VNTR region results from duplication of a 13–amino acid sequence once (VNTR A), twice (VNTR B), thrice (VNTR C), or four times (VNTR D), leading to polypeptide lengths of 610, 623, 636, or 649 amino acids, respectively.[44,45] Because the repeats are rich in serine and threonine, they can be O-glycosylated, and each repeat adds 32 Å to the length of the GPIbα extracellular domain.[44] This progressively increases the distance of the vWF- and thrombin-binding sites on GPIbα from the plane of the platelet plasma membrane, increasing the accessibility of these binding sites and perhaps accounting for the reported increased risk for acute coronary artery disease associated with the GPIbα longer variants, VNTR C and VNTR D, which are in LD with M161.[46,47]

In addition, the SNP C-5T (rs2243093) at a position 5 nucleotides upstream from the GPIbα initiator codon ATG appears to enhance its translational efficiency.[48,49] Thus, the presence of the −5C allele increases the mean level of GPIbα on the platelet plasma membrane (roughly, a 50% increase in homozygous individuals and a 33% increase in heterozygous individuals).[48] The C–5T alleles and the M161T alleles are in complete LD.[50]

CD109. The *CD109* SNP C2108A (rs10455097) gives rise to the HPA-15 alloantigen system.[23,51] The resulting Y703S amino acid substitution is carried by the glycosylphosphatidylinositol-linked protein CD109, a negative regulator of the transforming growth factor (TGF)-β system in keratinocytes.[52,53] Currently there are no published data concerning the effect of anti-HPA-15 antibodies on platelet function.

PLATELET GLYCOPROTEIN IMMUNOGENETICS

Progress in understanding the biology of platelet membrane glycoproteins was stimulated by study of their immunogenicity because these glycoproteins figure prominently as alloantigens, autoantigens, and targets of drug-dependent antibodies. Platelet membrane glycolipids and phospholipids also represent autoantigenic components, but much less is known about the structure and immunogenicity of these platelet constituents. Thus, this review will highlight the immunogenetics of platelet glycoproteins.

Two clinically important syndromes, fetomaternal alloimmune thrombocytopenia (FMAIT), also known as neonatal alloimmune thrombocytopenia, and posttransfusion purpura (PTP), result from sensitization to platelet-specific alloantigens. The development of these alloantibodies is a major obstacle to platelet transfusion therapy in sensitized patients, such as those undergoing chemotherapy in conjunction with myelodysplastic syndromes or hematopoietic stem cell transplantation.

FMAIT is caused by maternal sensitization to paternal alloantigens on fetal platelets. The frequency of alloimmunization against each of the HPA varies according to race because of significant differences in frequencies of the minor alleles (Table 27.2).

In whites, sensitization to HPA-1a and -5b dominates. In one large study of 1,054 cases of clinically definable FMAIT, HPA-specific antibodies were detected in only 22% of cases,[54]

Table 27.2

Minor allele frequencies

	Brazil		Europe	Africa		North America		
	Amerindian	Black	White	Berber	Tunisian	Black	White	Indian
HPA-1	0	0.11	0.16	0.25	0.25	0.08	0.17	
HPA-2	0.04	0.17	0.09	0.18		0.18	0.09	0.12
HPA-3	0.36	0.34	0.39	0.32	0.31	0.37	0.41	
HPA-5	0.04	0.16	0.10	0.14	0.22	0.18	0.09	0.02
HPA-15	0.28	0.43	0.40				0.53	
References	99–102	99,100,102,103	50,56,104–113	114	115	116,117	23, 50,116	117

	Australia		Asia					Bahrain
	Aboriginal	White	Korean	Japanese	Chinese	Taiwanese	Thais	Arabs
HPA-1	0.005	0.14	0.008	0.002	0.004	0.001	0.002	0.24
HPA-2	0	0.07	0.08	0.14	0.03	0.05	0.06	0.23
HPA-3	0.07	0.38	0.44	0.46	0.43	0.39	0.48	0.43
HPA-4				0.008				0.07
HPA-5	0.20	0.09	0.02	0.06	0.02	0.18	0.04	0.13
HPA-6w				0.02				
HPA-15						0.46	0.52	
References	118,119	119	116,120	61,103,121,122	123,124	125,126	125,127,128	129

consistent with previous reports.[55–57] Among cases in which HPA-specific antibodies were detected, HPA-1a and -5b were the relevant target antigens in 95% of cases, while 5% of cases involve antibodies specific for antigens of the HPA-2, -3 or -15 systems. In another large study of 3,743 cases,[58] HPA-specific antibodies were detected in 31% of cases, with the distribution: 79% anti-HPA-1a; 9% anti-HPA-5b; 2% anti-HPA-3a; and 4% anti-HPA-1b. The HPA-4b allele is virtually nonexistent in white populations, and only two reports of FMAIT caused by HPA-4 incompatibility have been reported,[59,60] The remaining eleven HPA systems have been defined by at least one case of FMAIT but have been assigned the status "bw" since antibodies against the major allele have not yet been reported. These bw alleles are extremely rare, and antibodies specific for these were detected in only 8 of 1,054 cases in one comprehensive study of white women.[54]

In Asian populations, the HPA-1a frequency is so low as to be nonexistent (Table 27.2), and no cases of FMAIT associated with anti-HPA-1a have been reported.[61] On the other hand, the HPA-4b allele figures more prominently among Asians, and in a large study of pregnant Asian women (99% Japanese), the detected alloantibodies were most often specific for HPA-5b (75%) or HPA-4b (22%).[61]

The response to HPA-1a among individuals homozygous for HPA-1b is HLA restricted,[62–64] and responsive individuals are almost exclusively HLA DRB3*0101[63] or DQB1*02.[65,66] For DRB3*0101, the calculated risk factor is 141, a risk level equivalent to that of HLA B27 in ankylosing spondylitis.[65] On the other hand, the converse response of homozygous HPA-1a individuals to the more rare HPA-1b haplotype is not linked to HLA.[65,67] It is probable that T cells are responsible for the HLA restriction in this case, and Maslanka et al.[65] reported that in one case of FMAIT, T cells that share CDR3 motifs are stimulated by peptides that contain the same Leu59 substitution that is recognized by anti-HPA-1a alloantibodies.

PTP can result within 7 to 10 days after an immunogenic blood (platelet) transfusion and most often affects previously nontransfused, multiparous women. As with FMAIT, there is an increased risk to develop PTP among HLA-DR3–positive individuals, and HPA-1a is the antigen most often implicated in white populations.[62,68]

CANDIDATE GENE ASSOCIATION STUDIES

Prior to the genomic era, most attempts to identify genetic differences influencing platelet function in normal individuals relied upon a candidate gene approach. Target genes were rationally selected as candidates (Table 27.3) based on their established or logical involvement in any of the important molecular events that contribute to normal platelet function. Thus, studies focused on platelet surface receptors, for

Table 27.3

Candidate gene SNPs employed in association studies

Gene	Protein Identification	dbSNP or Chr. Location[a]	Cytoband	MAF
ADRA2A	ADRA2A	chr10: 112,839,580[b]	10q24-q26	0.41
F2R	Coagulation factor II (thrombin) receptor; PAR-1	rs168753	5q13	0.14
FCGR2A	IgG Fc receptor type IIa	chr1: 161,479,745[c]	1q23	0.44
GNB3	Guanine nucleotide-binding protein β-3 subunit variant	rs5443	12p13	0.45
GP1BA	Platelet GPIb, α subunit	rs6065	17pter-p12	0.10
GP6	Platelet GPVI	rs1613662	19q13.4	0.16
ITGA2	Integrin subunit α_2	rs1126643	5q23-q31	0.38
		rs28095		0.36
		rs1801106		0.08
ITGA2B	Integrin subunit α_{IIb}	rs5911	17q21.32	0.41
ITGB3	Integrin subunit β3	rs5918	17q21.32	0.17
P2RY1	Purinergic receptor $P2Y_1$	rs1065776	3q25.2	0.05
P2RY12	Purinergic receptor $P2Y_{12}$	rs6809699	3q24-q25	0.14
PTGS1	Prostaglandin-endoperoxide synthase 1; Cyclooxygenase-1 (COX-1)	rs3842787	9q32-q33.3	0.06
TBXA2R	TXA_2 receptor	rs1131882	19p13.3	0.13
		rs4523		0.30
		rs5758		0.45

[a]NCBI SNP database (dbSNP) identification number (Build 131) or flanking nucleotide sequence (when SNP has not been assigned a dbSNP number).
[b]CATTCCCAACTCTCTCTCTCTTTTT**(G/A)**AAGAAAAATGCTAAGGGCAGCCCTG.
[c]AATGGAAAATCCCAGAAATTCTCCC**(A/G)**TTTGGATCCCACCTTCTCCATCCCA.

example, (a) fibrinogen-mediated platelet aggregation via integrin $\alpha_{IIb}\beta_3$ [37,69–72]; (b) collagen-induced platelet adhesion and signal transduction via integrin $\alpha_2\beta_1$[73,74] or GPVI[75,76]; (c) vWF-mediated platelet adhesion via GPIbα[21,44,45,48]; or (d) platelet signaling induced by various physiologic agonists.[77–81]

The success of any candidate gene association study depends on four key factors: (a) an accurate, reproducible, and vetted measure of the disease phenotype or of platelet function; (a) the inclusion of adequate cohort sizes; (c) a consideration of ethnic homogeneity of the cohorts; and (d) the appropriate SNP selection. Most gene association studies are compromised by small cohort sizes or a poor selection of SNPs. The most appropriate SNP choices, or tagSNPs, are those that take into consideration linkage and reflect haplotype definitions.[82] However, only a few of the foregoing studies used well-defined tagSNPs. Consequently, although the reported associations for *ITGA2* C807T (rs1126643),[74] *GP1BA* C-5T,[48,83] and the *GP6* haplotype b[75] have been replicated, others were not.[39,84] These studies and the details of the factors used to qualify their success have recently been reviewed.[85]

Functional Genomics Approach

Through the comparison of a relatively large number of SNPs ($n = 1{,}327$) in a selected group of several candidate genes ($n = 97$), Jones et al.[86] have recently identified 17 independently associated SNPs that account for 48% of the variation in platelet reactivity induced by either ADP or the GPVI agonist cross-linked collagen reactive peptide (CRP-XL). The success of this study reflects careful attention to study design and establishes a paradigm for future genomic studies. The study used the Platelet Function Cohort (PFC), a group of 500 healthy individuals with a similar Northern European ancestry.[87] Careful consideration was given to the selection of indices of platelet function. Consequently, platelet function was compared by measuring fibrinogen binding to activated $\alpha_{IIb}\beta_3$, the requisite step for aggregation, and the expression of P-selectin, a marker of platelet degranulation after stimulation with either ADP or CRP-XL. These agonists represent two distinct signaling pathways. GPVI signaling is an early event that acts via an Fc receptor-gamma chain/immunoreceptor tyrosine-based activation motif (FcRγ/ITAM) pathway, and ADP amplifies platelet activation in later stages through the G-protein–coupled receptors $P2Y_1$ and $P2Y_{12}$. Based on *a priori* knowledge of the protein components of each of the signaling cascades, 97 candidate genes were selected. The exons of these genes were resequenced in 48 reference DNA samples from the Center d'Etude du Polymorphism Humain European Cohort to maximize the database of sequence variation, leading to the identification of 1,327 SNPs that were then genotyped in the PFC samples.

This association study confirmed that *GP6* is a strong quantitative trait locus (QTL) for platelet response to CRP-XL. But the success of this study lay in the analysis of a large number of genes each exerting a small effect. Using a *P* value <0.005 as a cutoff, to overcome the errors inherent in multiple testing, 68 SNPs encompassed within 15 genes showed an association with the platelet response to CRP-XL, ADP, or both (Table 27.4).

Table 27.4

Genes associated with platelet hyperreactivity via a functional genomics approach

				Functional Trait[b]			
Gene	**Protein Function**	**SNP[a]**	**Cytoband**	**PA**	**FA**	**PC**	**FC**
GP6	Platelet GPVI	rs1613662	19q13.4	X		X	X
FCER1G	Adapter protein for agonist receptor	rs3557	1q23.3			X	X
PEAR1	Platelet surface receptor PEAR1	rs3737224	1q23.1			X	X
		rs11264579		X	X		
RAF1	Protein kinase	rs3729931	3p25.1		X	X	X
JAK2	Protein kinase	rs10429491	9p24.1	X	X		
P2RY12	Purinergic (ADP) receptor $P2Y_{12}$	rs1472122	3q25.1	X	X	X	
GNAZ	Adapter protein for agonist receptor	rs3788337	22q11.22	X			
VAV3	Intracellular signaling molecule	rs17229705	1p13.3	X	X	X	
CD36	Platelet surface receptor	rs1049654	7q21.11			X	X
MAP2K2	Protein kinase	rs350916	19p13.3		X		
ITGA2	Integrin subunit α_2	rs41305896	5q11.2	X	X		
AKT2	Protein kinase	rs41275750	19q13.2			X	X
MAPK14	Protein kinase	rs851007	6p21.31	X	X	X	
MAP2K4	Protein kinase	rs41307923	17p12			X	X
ITPR1	Intracellular signaling molecule	rs17786144	3p26.2	X	X		

[a]SNP Exhibiting lowest *P*-value.
[b]PA, P-selectin expression in response to ADP; FA, Fibrinogen binding in response to ADP; PC, P-selectin expression in response to CRP-XL; FC, Fibrinogen binding in response to CRP-XL.
Data from Jones CI, Bray S, Garner SF, et al. A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. *Blood* 2009;114:1405–1416.

Variation in *GP6*, as anticipated, accounted for up to 40% of the variation in GPVI signaling.[87] This is consistent with our own *in vitro* results that the minor isoform of GPVI (GPVIb) is associated with decreased Fyn/Lyn binding and enhanced calmodulin binding to the cytoplasmic tail.[76]

For the remaining genes, 18 independent associations in 15 loci were identified. These included the cell surface receptor genes *CD36*, *GP6*, *ITGA2*, *PEAR1*, and *P2RY12*; adapter proteins *FCERG1* and *GNAZ*; kinases *JAK2*, *MA2K2*, *MAP2K4*, and *MAPK14*; and the intracellular signaling molecules *ITPR1* and *VAV3*.

Of note, an association was not found between *ITGB3* and any of the indices of platelet response. As noted above, there is conflicting evidence in the literature regarding the association of *ITGB3* rs5918 with variation in platelet function or risk for acute coronary artery or cerebrovascular disease.[37,39,40–42,69–71,84] One possible explanation is that the MAF is low (0.15), and the majority of studies were severely underpowered by small cohort sizes. In addition, the diversity of functional assays and platelet agonists employed in these studies certainly contributed to the discrepant findings.

Additional novel findings resulted from this study. Most notably, the platelet endothelial aggregation receptor 1, encoded by *PEAR1*, is now known to be a platelet receptor that undergoes phosphorylation upon platelet–platelet contact.[88] The promoter variant (rs2768759) is associated with increased platelet aggregation in PRP in response to EPI.[89] In this study, *PEAR1* was identified as a regulator of both CRP-XL and ADP signaling, with two distinct associations observed. One SNP, rs41299597, was associated with CRP-XL–induced pathways, while the more common SNP rs11264579 was associated with the ADP pathway.

GENOME-WIDE ASSOCIATION STUDY

The genomic era was ushered in by technological improvements that enabled massive genome-wide SNP association and deep sequencing studies. A genome-wide association study (GWAS) using a direct measure of platelet function was recently performed by Johnson et al.,[90] who tested the association of 2.5 million SNPs with platelet aggregation responses to three agonists (ADP, EPI, and collagen) in two cohorts of European ancestry (n = 2,753 in the Framingham Heart Study; n = 1,238 in the Genetic Study of Atherosclerosis Risk). Seven loci were associated with platelet aggregation (Table 27.5), the two most strongly associated lying near or within the platelet glycoprotein genes *GP6* ($P = 4.6 \times 10^{-13}$) and *PEAR1* ($P = 3.4 \times 10^{-12}$). The remaining five loci were near or within the genes *ADRA2A* ($P = 3.3 \times 10^{-11}$), *PIK3CG* ($P = 3.1 \times 10^{-9}$), *JMJD1C* ($P = 1.6 \times 10^{-8}$), *MRVI1* ($P = 2.0 \times 10^{-8}$), and *SHH* ($P = 4.5 \times 10^{-8}$). Six of these loci (save *PIK3CG*) replicated at $P < 0.05$ in an additional African American cohort (n = 840 in the Genetic Study of Atherosclerosis Risk).

Three loci were significant for ADP-induced aggregation: 1q23.1 (*PEAR1*, rs12566888, $P = 3.4 \times 10^{-12}$), 11p15.4 (*MRVI1*, rs7940646, $P = 2 \times 10^{-8}$), and 7q36.3 (sonic hedgehog homolog [SHH], rs2363910, $P = 4.5 \times 10^{-8}$). The *PEAR1* minor allele was associated with a decreased response, while the minor alleles of *MRVI1* and *SHH* were associated with increased responses. *PEAR1* was discussed above, and its presence here provides strong confirmation for the previous results of Jones et al.[86] *MRVI1* (a.k.a. *IRAG*) encodes a homolog of murine retrovirus integration site 1, which is a member of a signaling complex that influences smooth muscle relaxation through negative modulation of inositol triphosphate (IP_3)-induced calcium signaling.[91] In mice, MRVI1 is directly involved in the inhibition of platelet aggregation and thrombus formation in vivo.[92] Microvesicles containing SHH will induce differentiation along the megakaryocyte lineage, suggesting a relationship to platelet biology.[93]

Table 27.5

GWAS of platelet aggregation induced by ADP, EPI, or collagen

Locus	Nearest Gene	Effect of Minor Allele	dbSNP
1q23.1	*PEAR1*	↓ ADP, ↓ EPI	rs12566888 (G>T)
11p15.4	*MRVI1*	↑ ADP, ↑ EPI	rs7940646 (C>T)
7q36.3	*SHH*	↑ ADP	rs2363910 (G>T)
10q25.2	*ADRA2A*	↓ EPI	rs4311994 (C>T)
7q22.3	*PIK3CG*	↓ EPI	rs342286 (A>G)
10q21.2	*JMJD1C*	↑ EPI	rs10761741 (G>T)
19q13.42	*GP6*	↑ collagen lag	rs1671152 (G>T)

Data from Johnson AD et al. Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists *Nat Genet* 2010:42:608–615.

Four regions were strongly associated with EPI-induced aggregation: 10q25.2 (*ADRA2A*, rs4311994, $P = 3.3 \times 10^{-11}$), 1q23.1 (*PEAR1*, rs12566888, $P = 7.3 \times 10^{-10}$), 7q22.3 (*PIK3CG*, rs342286, $P = 3.1 \times 10^{-9}$), and 10q21.2 (*JMJD1C*, rs10761741, $P = 1.6 \times 10^{-8}$). In the case of *ADRA2A*, *PEAR1*, and *PIK3CG*, the minor alleles were associated with reduced EPI-induced aggregation, while the minor allele of the peak SNP nearest *JMJD1C* was associated with increased aggregation. *ADRA2A* encodes the major platelet α-adrenergic (EPI) receptor, and its association here is not surprising. *PIK3CG* (rs342293), which encodes a subunit of phosphoinositide-3-kinase, was recently shown to be associated with decreased mean platelet volume.[94] *JMJD1C* (jumonji domain containing 1C) is the only gene without a documented functional link to platelets, although it does exhibit enhanced expression in megakaryocytes relative to other hematopoietic cells.[95]

A single locus 19q13.42 including *GP6* (rs1671152) was significantly associated with lag time to collagen-induced aggregation response (European ancestry, $P = 4.6 \times 10^{-13}$; African ancestry, $P = 0.048$). This SNP creates a Y323K substitution in the longer isoform of GPVI but is alternatively H322N in the shorter GPVI isoform that we studied in an earlier report.[76] In either case, Johnson et al.[90] determined that the minor allele was associated with a decreased collagen response (increased lag time), while we showed that the minor allele attenuated binding of Fyn/Lyn to the GPVI cytoplasmic tail.[76] These observations provide a plausible explanation for the prolonged lag time to onset of aggregation, since the minor allele would attenuate downstream responses to collagen engagement. Our prior results thus provide a mechanistic explanation for the strong association of this SNP with platelet responsiveness to collagen in this GWAS.

The findings for the *GP6*, *PEAR1*, and *ADRA2A* regions provide robust validation of prior studies,[75,86,89,96–98] although with

a much larger sample size, while the associations with loci that include *MRV11*, *SHH*, *JMJD1C*, and *PIK3CG* are novel.

SUMMARY

Polymorphisms in platelet membrane glycoprotein genes have created a repertoire of human platelet alloantigens that can be clinically significant as causes of FMAIT and PTP. The six most commonly involved alloantigen systems are HPA-1 and HPA-4 on integrin subunit β_3; HPA-2 on platelet GPIb; HPA-3 on integrin subunit α_{IIb}; HPA-5 on integrin subunit α_2; and HPA-15 on CD109. Because of significant differences in allele frequencies between racial/ethnic populations, the relative importance of each differs. In white populations, HPA-1 and HPA-5 are implicated in the vast majority of cases of FMAIT; while in Asian populations, HPA-5 and HPA-4 figure most prominently.

SNPs in selected glycoprotein genes can have significant effects on receptor expression or function. Candidate gene associations studies have demonstrated that several SNPs are consistently associated with differences in platelet function between normal individuals. With respect to receptor genes, the most notable are *GP6*, *ADRA2A*, *P2RY12,* and *PEAR1*, but also *GP1BA* and *ITGA2*.

The importance of *ADRA2A*, *GP6*, and *PEAR1* has been validated in a genome-wide association study of differences in platelet responses to ADP, EPI, and collagen. Such studies have and will continue to provide new insight into regulators of platelet function that would otherwise remained unexplored. Platelet function QTL studies have already identified key regulators of signaling pathways. Further meta-analysis of platelet function in tens of thousands of subjects will increase the number of QTLs, and the completion of the ongoing one thousand genomes initiative (likely tens of thousands by 2014) will serve to identify rare coding and noncoding variants. Some of these may exert a large effect on platelet phenotypes.

Finally, it should be understood that knowledge of platelet QTLs for function should not be used in clinical practice to infer risk of thrombotic or bleeding events in individual subjects.

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CHAPTER

28 The Basis for Platelet Adhesion

BRIAN SAVAGE AND ZAVERIO M. RUGGERI

PERSPECTIVE

Mammalian platelets are circulating fragments of bone marrow megakaryocytes[1,2] that function primarily to maintain the integrity of the vasculature by stopping hemorrhage after vascular injury and tissue trauma.[3] Although platelets lack genomic DNA, they contain megakaryocyte-derived messenger RNA and are capable of protein synthesis.[4,5] During their 10-day lifetime in the human circulation, most platelets never participate in irreversible adhesion, and are cleared from the circulation by macrophages in the spleen and liver. However, in response to alterations of endothelial cells lining the vessel wall or exposure of subendothelial matrix components following vascular injury, platelet adhesion, activation, and thrombus formation occur,[3] events that are crucial for maintaining primary hemostasis and preventing posttraumatic blood loss. On the other hand, thrombus formation in diseased vessels can impede blood flow to vital organs, for example, in heart coronary arteries and cerebral arteries, leading to myocardial infarction and stroke, respectively. In addition to their role in hemostasis and thrombosis, platelets are thought to contribute to other processes, such as atherogenesis[6–8] and inflammation,[9–12] by maintaining normal vascular permeability and directing other effector cells to sites where microbial and viral pathogens induce inflammatory responses.[13,14] Evidence also suggests a role for platelets in tumor angiogenesis[15] since tumor cells can activate platelets and induce release of angiogenic factors that directly affect the tumor endothelium.[16]

To ensure normal hemostasis without the undesired thrombus formation, the adhesive properties of platelets must be tightly regulated to respond to a precise set of stimuli. A comprehensive review of the signaling mechanisms that regulate platelet adhesion is not presented in detail here, but can be found elsewhere.[17–20] In this chapter, we focus on the principal substrates and receptors that mediate platelet adhesion, activation, and aggregation, and the role of blood flow in modulating platelet adhesive functions.

INDUCTION OF PLATELET ADHESION RESPONSES

Platelets circulating in close proximity to intact, unperturbed endothelial cells do not attach to them, at least in part because endothelial cells actively maintain an antiadhesive phenotype.[21–23] However, disruption of the endothelium by mechanical trauma or pathogenic stimuli resulting from endovascular or extravascular disease processes leads to platelet adhesion at the injured site. Initiation of platelet adhesion in posttraumatic hemostasis involves platelet interaction with exposed extracellular matrix components, consisting of a large number of adhesive macromolecules including various types of collagen,[24,25] von Willebrand factor (vWF),[26,27] fibronectin,[28] laminin,[29] fibulin,[30] and thrombospondin.[31] In addition, platelets can interact indirectly with extracellular matrix proteins that upon exposure to blood bind plasma-derived proteins such as fibrinogen/fibrin[32] vWF,[26] and vitronectin[33] that become immobilized onto subendothelial surfaces at sites of vascular damage. Depending on the prevailing rheologic conditions and the types of matrix proteins exposed to the flowing blood, platelet adhesion requires the synergistic function of distinct platelet receptors, ultimately leading to platelet activation and aggregation.[34] Following activation, the platelet membrane presents a negatively charged phospholipid surface required for the assembly of activated coagulation factor complexes, which in turn leads to thrombin generation and the formation of a fibrin network stabilizing the growing thrombus.[35]

PLATELET ADHESION TO vWF

The Essential Role of Immobilized vWF

vWF is a glycoprotein (GP) that circulates in plasma as large multimers ranging in size from 500 to 20,000 kDa[36,37] that contain binding sites for collagen, as well as the platelet receptors GPIbα and the integrin $\alpha_{IIb}\beta_3$(GPIIb/IIIa).[38] The monomeric subunit of mature vWF consists of 2,050 amino acids and is composed of four distinct repeating domains that are crucial for the function of the molecule.[39,40] Megakaryocytes and endothelial cells are the only cells that synthesize vWF. Megakaryocyte-derived vWF is stored in α-granules and is only released during agonist-induced platelet secretion. By contrast, endothelial cell vWF is either constituently secreted or directed to Weibel-Palade body storage granules for agonist-induced secretion.[36] Plasma vWF is almost exclusively derived from endothelial cells.[41] The multimers secreted from endothelial cell Weibel-Palade bodies are extremely large (ultra large vWF [ULvWF]) and their ability to bind to extracellular matrix proteins[42] and to the platelet receptor, GPIbα in the GPIb-IX-V complex is enhanced compared to the smaller vWF multimers present in plasma.[43] The metalloprotease, ADAMTS13, cleaves the secreted ULvWF to functionally less active, smaller multimers.[44,45]

Under conditions of elevated shear stress, typically present in arteries and small arterioles, vWF plays an essential role in initiating platelet adhesion and sustaining platelet aggregation.[26,32] It is present as a constitutive component of the extracellular matrix of endothelial cells[46] where it is associated with collagen type VI filaments,[47–49] and can directly support platelet adhesion.[50–54] In addition, circulating plasma vWF can interact with exposed extracellular matrix components, particularly collagen types I

and III in deeper layers of the vessel wall[55] and microfibrillar collagen type VI in the subendothelial matrix.[49,56–58] Other potential binding sites for plasma vWF include heparin, sulfatides, and fibrin.[3] The main site for vWF binding to collagen types I and III is located in the vWF A3 domain,[59] with a minor role, but possibly an essential one under high blood flow conditions, attributed to A1 domain binding to collagen type VI.[55,60] Additionally, plasma vWF can become immobilized by self-association; a process involving the reversible interaction of circulating vWF multimers with matrix bound and endogenous subendothelial vWF.[61] vWF self-association is modulated by multiple domains,[62] although none have been identified as essential, including A1 and A3[61] (FIGURE 28.1).

Platelet Capture by Immobilized vWF

When circulating platelets encounter immobilized vWF under arterial blood flow conditions, that is, wall shear rates exceeding 800/second (value for the human circulation; approximately double in the mouse circulation), they are captured and tethered by a mechanism involving the transient interaction of platelet GPIbα with the A1 domain of immobilized vWF.[32] Although this interaction is short-lived and insufficient by itself to mediate stable platelet adhesion, it maintains platelets in close contact with the blood vessel wall as they translocate in the direction of blood flow. This reduces their velocity and facilitates their subsequent adhesion and activation[32] (FIGURE 28.2). It is during the translocation phase that platelets establish contact with collagen in the extracellular matrix via platelet glycoprotein VI (GPVI) and integrin $\alpha_2\beta_1$, proteins that are unable to mediate platelet adhesion by themselves under high flow velocity conditions. Engagement of GPVI and $\alpha_2\beta_1$ with collagen triggers intracellular signals that activate platelets and shift platelet integrins to a high-affinity state, as well as the release of the agonists ADP and thromboxane A_2 (TXA_2) that contribute synergistically to induce full platelet activation. Following platelet activation, stable adhesion is reinforced by the β_1 integrins, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, that bind to collagen, fibronectin, and laminin respectively, as well as by integrin $\alpha_{IIb}\beta_3$ that binds to fibronectin and collagen-bound vWF.

Role of Receptor GPIbα

There are two physiologically relevant platelet receptors for vWF[63,64]: GPIbα, a component of the GPIb-IX-V complex[65] that binds to the vWF A1 domain,[66,67] and the integrin $\alpha_{IIb}\beta_3$[68] that binds to the Arg–Gly–Asp motif located in vWF C1 domain.[69] A third receptor, the integrin $\alpha_v\beta_3$,[70] may participate in vWF-mediated platelet adhesion to endothelial cells.[71] GPIbα is a highly abundant, extensively glycosylated protein expressed exclusively on megakaryocytes and platelets. It is a component of the GPIb-IX-V complex composed of four gene products: GPIbα, GPIbβ, GPIX, and GPV.[72–75] Resting platelets express 25,000 copies of GPIb-IX and 12,000 copies of GPV. Human GPIbα has a 290-residue *N*-terminal domain containing 8 tandem leucine-rich repeats[73,76] that binds vWF[34] and α-thrombin.[77,78] GPIbα has also been reported to contain binding sites for coagulation factors XI and XII,[79] high molecular weight kininogen,[80] P-selectin,[81] and the leukocyte receptor Mac-1,[82,83] but the precise identity of these sites remains to be determined. The interaction between GPIbα and vWF under dynamic conditions of blood flow supports the activation-independent capture and tethering of platelets to immobilized vWF[32] and this occurs despite forces opposing platelet adhesion

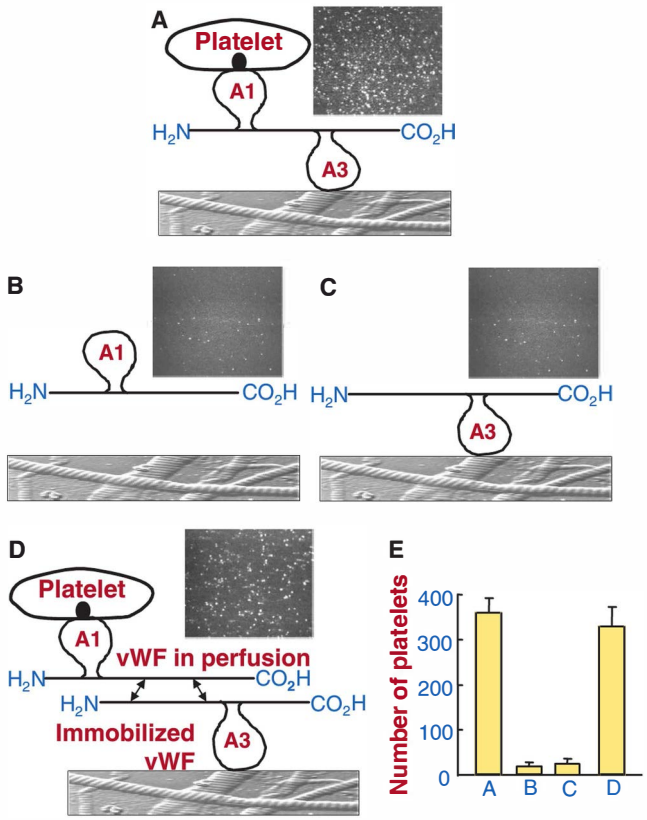

FIGURE 28.1 Functional self-association of vWF multimers. Suspensions of washed blood cells containing ethylenediaminetetraacetic acid (EDTA) to block integrin function and prostaglandin (PG) E_1 to block platelet activation were perfused over immobilized collagen type I fibrils at the wall shear rate of 1,500/s. **A**: Normal multimeric vWF was added to a cell suspension. Its A3 domain mediates vWF binding to collagen and the A1 domain interacts with platelet GPIbα. In the electron micrograph, tethered platelets are seen rolling on the collagen fibrils. **B**: vWF devoid of the A3 domain (ΔA3-vWF) which cannot bind to collagen or **(C)** vWF devoid of the A1 domain (ΔA1-vWF), which cannot interact with platelet GPIbα was added to the cell suspension. In neither case, were platelets tethered to the immobilized collagen. **D**: Collagen fibrils were precoated with ΔA1-vWF multimers and then exposed to the blood cell suspension containing ΔA3-vWF. Although the latter cannot bind directly to collagen, they can associate with the ΔA1-vWF multimers to support platelet tethering. The *two-sided arrows* between multimers indicate that the association is reversible. The images are single frames from a real time recording representing an area of 65,536 μm^2. The *bar graph* shows the number of platelets tethered to the surface under the different experimental conditions described above (mean ± SEM of two separate experiments). **E**: quantifies the results shown in **A–D**. (Modified from Savage B, Sixma JJ, Ruggeri ZM. Functional self-association of von Willebrand factor during platelet adhesion under flow. *Proc Natl Acad Sci U S A* 2002;99:425–430, and reprinted with permission. Copyright (2002) National Academy of Sciences, U.S.A.)

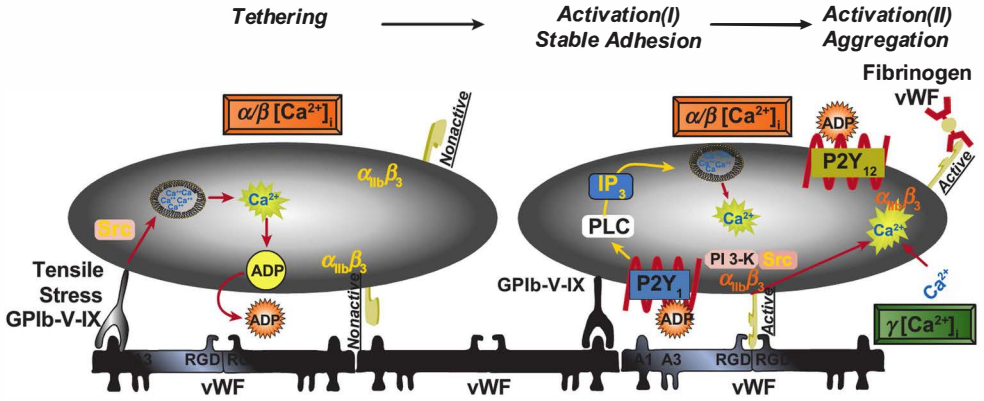

FIGURE 28.2 Schematic representation of the sequential signaling events induced by the interaction of platelets with immobilized vWF under high shear stress. On the left, a platelet is shown during the initial tethering to the A1 domain of immobilized vWF mediated by the GPIb-IX-V complex. An α/β $[Ca^{2+}]_i$ elevation is elicited as a consequence of this interaction and leads to ADP release from intracellular storage granules. Src family kinases may be involved at this stage and cAMP/cGMP levels modulate this and other downstream responses. Subsequent events are shown in the platelet on the right. Released ADP binds to the G_q-coupled $P2Y_1$ receptor, leading to PLC activation and enhanced Ca^{2+} release from internal stores during α/β oscillations. At this stage, there is a first level of localized $\alpha_{IIb}\beta_3$ activation that supports more prolonged platelet adhesion mediated by the RGD sequence in the vWF C1 domain. PI 3-K activation may enhance this response. Further PI 3-K activation, and possibly the involvement of Src family kinases, contribute to more generalized $\alpha_{IIb}\beta_3$ activation that permits soluble ligand binding and supports formation of platelet–platelet aggregates. This second level of $\alpha_{IIb}\beta_3$ activation is concurrent with or subsequent to a type $\gamma[Ca^{2+}]_i$ elevation dependent on a transmembrane ion flux. The second ADP receptor, $P2Y_{12}$, supports the formation of larger platelet aggregates through mechanisms that occur after the measured Ca^{2+} oscillations. The TXA_2 pathway inhibited by aspirin appears to have a very limited role in the successive stages of platelet adhesion, activation, and aggregation induced by the interaction with immobilized vWF. IP3, inositol-1,4,5-trisphosphate; Src, Src family tyrosine kinases; PLC, phospholipase C; PKC, protein kinase C; PI 3-K, phosphatidylinositol 3-kinase. (Adapted from Mazzucato M, Cozzi MR, Pradella P, et al. Distinct roles of ADP receptors in von Willebrand factor-mediated platelet signaling and activation under high flow. *Blood* 2004;104:3221–3227.)

at elevated flow rates.[32,84] GPIbα-mediated platelet adhesion to vWF is functionally similar to selectin-mediated leukocyte rolling on inflamed vascular surfaces,[85] and presumably shares similar biophysical characteristics, such as rapid on-rates[86–88] and rapid dissociation-rates.[84,86,88–90] In addition, both interactions support flow-enhanced adhesion[91] and are thought to involve "catch" bonds, rather than "slip" bonds.[92] Catch bonds are characterized by a lifetime that increases directly with applied shear force up to a maximum threshold value. As applied shear force increases above the threshold, the lifetime of the bond decreases, resulting in the transition to a more typical slip bond for which the lifetime decreases with increasing shear stress.[92] Recent studies have provided a better understanding of the biomechanical properties of vWF A1 domain binding to GPIbα.[93] A method that enables the repeated measurement of the binding and unbinding of interacting receptor and ligand domains engineered within a single molecule has revealed the existence of two states of the vWF A1 domain-GPIbα bond and has thus been named a flex-bond. One state is seen at low force, while the second state begins to engage at higher force (10 pN) with approximately 20-fold longer lifetime and greater force resistance.

Mutations in both vWF A1 domain and GPIbα can change the mechanical properties of the flex bond. A gain-of-function point mutation in GPIbα at Met239Val results in an approximately sixfold increase in affinity for the interaction with vWF A1 domain due to an increased on-rate.[94] By contrast, a gain-of-function point mutation in vWF A1 at Arg543Gln does not appear to form catch bonds with GPIbα, and has an increased affinity for GPIbα due mainly to a decreased off-rate, resulting from the formation of slip bonds even at low shear stresses.[95] Regulation of the GPIbα/vWF interaction appears to involve conformational changes in both the receptor and the ligand,[96] and phosphorylation of Ser166 of GPIbβ may negatively regulate vWF binding.[97]

GPIb-IX-V-Dependent Signaling

Until recently it was thought that the interaction of GPIbα with immobilized vWF only provides a mechanism for recruiting platelets to sites of vascular damage, thereby reducing their velocity to enable other platelet receptors to elicit intracellular activation signals. However, it is now clear that GPIb-IX-V, in addition to its role as an adhesion receptor, can mediate intracellular signals leading to activation of the integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$, allowing platelets to firmly attach, spread, and aggregate.[26,98–101] Several intracellular signaling pathways are involved in GPIb-IX-V-mediated platelet activation, including the Syk/phospholipase C (PLC) pathway,[101,102] the phosphatidylinositol 3-kinase (PI 3-K)-Akt pathway,[103–105] the cGMP[106] and mitogen-activated protein kinase pathways,[107] and the TXA_2 and ADP pathways.[108,109] Members of the Src family kinases appear to

play an important role because Src inhibitors prevent GPIb-IX-V-induced $\alpha_{IIb}\beta_3$ activation,[110,111] Ca^{2+} mobilization,[112] PI 3-K activation,[113,114] and TXA_2 synthesis.[108] The Src family tyrosine kinase Lyn has been shown to be crucial in GPIb-IX-V-mediated activation of the cGMP pathway.[111]

Engagement of GPIb-IX-V with the vWF A1 domain also leads to the tyrosine phosphorylation of adhesion and degranulation promoting adaptor protein (ADAP),[110] a potential linker between cell activation events at the plasma membrane and intracellular events. ADAP is required for the modulation of $\alpha_{IIb}\beta_3$ affinity induced by GPIb-IX-V interaction with the vWF A1 domain.[115] Despite the many signaling pathways attributed to the interaction of GPIb-IX-V with vWF, the relative importance of this interaction *in vivo* compared to other receptor/ligand signaling pathways remains to be established. Indeed, platelet translocation on immobilized vWF *ex vivo* may last for several minutes before stable $\alpha_{IIb}\beta_3$-mediated attachment occurs,[32] indicating that the activating signal is relatively weak compared with those induced by more thrombogenic surfaces like collagen.

As recently demonstrated, platelet adhesion and aggregation can be initiated independently of platelet or integrin activation through a mechanism that involves the interaction with immobilized as well as soluble vWF under high shear stress conditions[116] (FIGURE 28.3). A key feature of this mechanism is that sustained platelet attachment and aggregation only occur above a threshold shear rate of 10,000/second, a pathologically relevant shear rate in arterial thrombosis.[116] These studies have been extended using experimental mouse models of vascular injury, demonstrating that thrombi initially develop through the progressive stabilization of discoid platelet aggregates.[117] These discoid platelets adhere preferentially in low-shear zones downstream of forming thrombi, indicating that disturbed blood flow, creating gradients of increasing and decreasing shear stress, may play an important role in initiating the process of thrombogenesis in arteries.

The Role of vWF in Venous Thrombosis

Although the participation of vWF in arterial thrombosis has been repeatedly demonstrated, its role in platelet recruitment during the initiation of venous thrombosis has only recently been documented.[118] Unlike arterial thrombosis, thrombosis in deep veins is not initiated by injury to the endothelial layer,[119] but rather by blood flow disturbances resulting in complete or partial flow restriction.[120] Although increased procoagulant states are known risk factors for deep vein thrombosis,[121–123] disturbed blood flow due to long-term immobilization can also provoke deep vein thrombosis in the absence of hypercoagulability.[124,125] Flow disturbance–induced venous thrombosis in mice due to prolonged flow restriction in the inferior vena cava was significantly reduced in mice genetically deficient in vWF.[118] Furthermore, infusion of a mutant GPIbα–IgG chimera that inhibits the interaction of vWF A1 domain with platelets prevented thrombosis in wild-type mice with normal vWF levels. Although circulating vWF is coupled to factor VIII, infusions of recombinant factor VIII did not rescue thrombosis in vWF-deficient mice. These observations suggest that vWF–platelet adhesive interactions may play a key pathogenic role in initiating venous thrombosis following flow restriction.

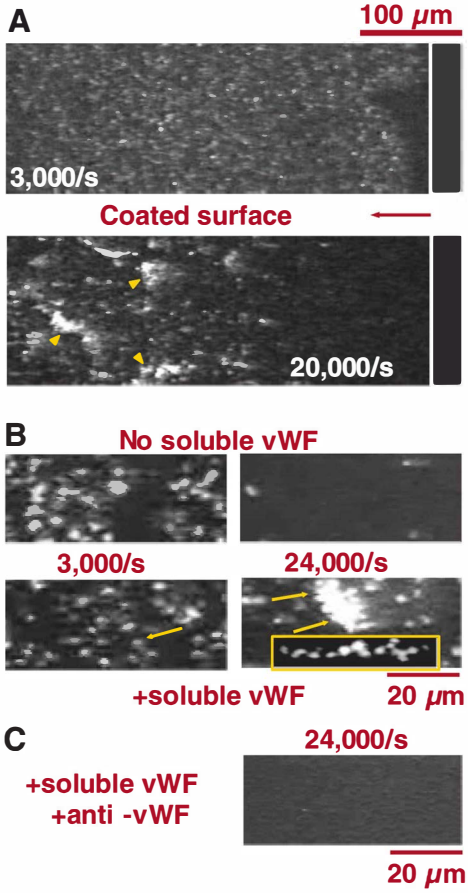

FIGURE 28.3 Activation-independent platelet adhesion and aggregation at the interface of immobilized and soluble vWF. **A**: Blood containing 93 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride to inhibit α-thrombin, the fluorescent dye mepacrine at 10 μM, 10 μM PG E_1, and 5 mM EDTA was perfused over immobilized vWF. The *white line* delimits vWF-coated (to the *left*) from uncoated glass. Single platelets adhere when the shear rate is 3,000/s **(top)**; rolling aggregates (some identified by *arrowheads*) form at 20,000/s **(bottom)**. **B**: Perfusion of washed blood cells suspended in buffer (20 mM Hepes, 150 mM NaCl, pH 7.4) over immobilized vWF. In the absence of soluble vWF, single platelets adhere when the shear rate is 3,000/s **(upper left)**, and fewer single platelets adhere at 24,000/s **(upper right)**. After adding 20 μg/mL soluble vWF, single platelets adhere at 3,000/s (**lower left**; an *arrow* points to a single platelet shown for reference), but aggregates form at 24,000/s (**lower right**; *arrows* point to a rolling aggregate and an *inset* highlights a stretched aggregate during stationary adhesion). **C**: Perfusion of washed blood cells with added soluble vWF and anti-vWF A1 domain monoclonal antibody (NMC-4, 20 μg/mL) over immobilized vWF. No platelet adhesion is detected. (From Ruggeri ZM, Orje JN, Habermann R, et al. Activation-independent platelet adhesion and aggregation under elevated shear stress. *Blood* 2006;108:1903–1910.)

PLATELET ADHESION TO COLLAGEN AND PLATELET COLLAGEN RECEPTORS

The vessel wall contains several different types of collagen, particularly the fibrillar collagens type I, III, and VI. The collagens can associate with each other and with other matrix proteins,

and play an active role in the initiation of platelet adhesion and aggregation in response to injury.[126] Experimental studies with collagens can be complicated by changes in collagen structure introduced during extraction of the protein from extracellular matrix. For example, acid-insoluble collagen type I fibers display a characteristic banded pattern due to the regular staggering of collagen monomers[126,127] and react with platelets bearing GPVI even when $\alpha_2\beta_1$ is inhibited. By contrast, pepsin-solubilized acid soluble collagen type I microfibrils lack the banded pattern and form spiral structures[127] and optimally interact with platelets bearing functional $\alpha_2\beta_1$, in addition to GPVI. As collagen is an insoluble matrix protein, preparations used in *ex vivo* experimental studies are often treated with proteases, such as pepsin, for solubilization resulting in functional behavior distinct from that of the native protein. Thus, the presence of spiral fibrils in pepsin-treated acid soluble collagen can lead to an overestimate of the role of $\alpha_2\beta_1$ in the interaction of collagen with platelets.

Collagen activates platelets primarily through the membrane proteins GPVI and $\alpha_2\beta_1$[55,128] (FIGURE 28.4); several other potential collagen "receptors" have been described, but none has been shown to convincingly play a role in platelet adhesion and aggregation. Even the relative contribution of GPVI and $\alpha_2\beta_1$ to these processes remains a topic for debate. Ambiguities introduced during the isolation of collagen from extracellular matrices, as discussed above, as well as poorly understood differences between human *ex vivo* and mouse *in vivo* experimental systems may explain the lingering uncertainties.[55,128] According to the prevalent current model, GPVI is essential for platelet activation and aggregation on collagen, whereas $\alpha_2\beta_1$ is required for firm platelet adhesion with only a relatively minor role in platelet activation.[128] More recent evidence, obtained studying platelet–collagen interactions under flow,[129,130] supports the existence of a functional synergy between $\alpha_2\beta_1$ and GPVI. Indeed, it was found that $\alpha_2\beta_1$ can signal independently of GPVI or $\alpha_{IIb}\beta_3$ (GPIIb–IIIa), and that GPVI function is facilitated by preceding intracellular Ca^{2+} elevations resulting from $\alpha_2\beta_1$ engagement. This does not exclude the possibility that the ligand-binding affinity of $\alpha_2\beta_1$ is enhanced following GPVI interaction with collagen and/or $\alpha_{IIb}\beta_3$ activation. Accordingly, the functional activities of $\alpha_2\beta_1$ and GPVI may be viewed as concurrent, or mutually reinforcing, rather than sequential, a concept supported by the finding that variations in the amount of $\alpha_2\beta_1$ on the platelet surface influences the extent of GPVI-dependent Ca^{2+} signaling.[129]

Glycoprotein VI

Approximately 10% of collagens I and III consist of a repeating GPO (glycine–proline–hydroxyproline) motif, also known as collagen-related peptide[131] that is the strongest platelet stimulus within the collagen molecule[132] and that reacts specifically with platelet GPVI.[133] The smallest collagen motif that recognizes and activates platelet GPVI consists of two GPO triplets.[134] On the platelet surface, GPVI is present in a noncovalent complex with the Fc receptor-γ chain, an interaction that is essential for both GPVI expression and function.[128,135] Functional GPVI is thought to be a dimer. Each GPVI monomer contains two extracellular Ig-C2-like domains, a mucin-like domain, a transmembrane region, and a short 51-residue cytoplasmic tail.[136,137] The cytoplasmic tail has distinct sites

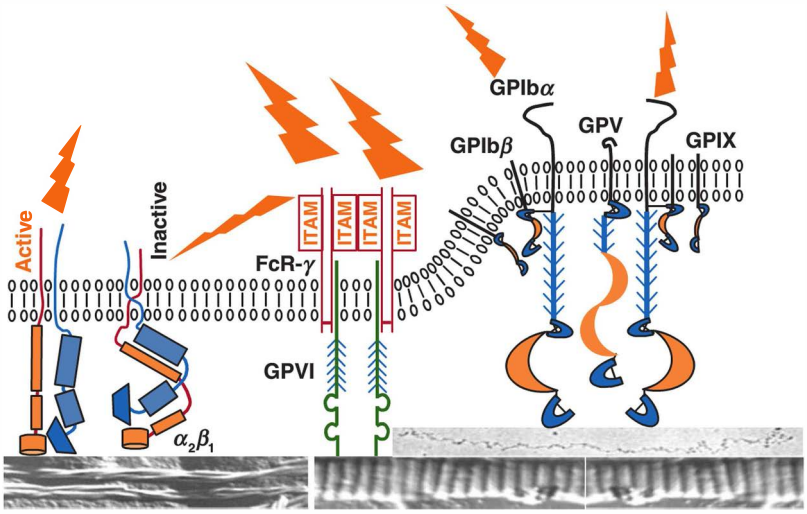

FIGURE 28.4 Schematic representation of the mechanisms of platelet adhesion to collagen. The collagen receptors, $\alpha_2\beta_1$ and GPVI, are depicted along with the GPIb-IX-V complex. Collagen and vWF are represented by electron micrographs (not in scale). Two forms of collagen type I are shown, "native" acid-insoluble fibers (on the **right**) and pepsin-treated acid-soluble "spiraled" fibrils (on the **left**), with a vWF multimer shown as if adhering to the native collagen fiber. On a surface in which collagen and vWF form a functional unit, initial platelet tethering is mediated primarily by GPIbα binding to vWF-A1 under high flow conditions, allowing subsequent GPVI binding to collagen, specifically to the prototypic repeating sequence Gly–Pro–Hyp (where Hyp is hydroxyproline), typical of the fibril-forming collagens. Both receptors can signal, but the role of GPVI is predominant in this regard. The integrin $\alpha_2\beta_1$ requires activation by an inside-out signal for high affinity binding to collagen; however, on partially degraded "spiraled" fibrils, it may be essential for the initial tethering through recognition of the sequence Gly–Phe–Hyp–Gly–Glu–Arg. The fact that GPVI is not sufficient for adhesion to this substrate attests to the conformational disruption of its recognition site in partially degraded collagen.

that interact with calmodulin to regulate Ca^{2+} signaling pathways and with the Src family tyrosine kinases Fyn and Lyn that signal independently of the Fc-receptor. GPVI can also signal in response to collagen or other ligands by an Fc-receptor-dependent pathway involving activation of the tyrosine kinase Syk.[99] Signaling through GPVI upregulates platelet integrins, including $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, events essential for shear-resistant platelet attachment to collagen.

A mechanism for regulating GPVI function is based on the metalloproteinase-mediated shedding of the GPVI ectodomain. This process involves the calmodulin binding site in the GPVI tail and results in the disappearance of GPVI from the platelet surface and the release of a 55 kDa GPVI fragment.[138] Unlike $\alpha_2\beta_1$, GPVI is a platelet-specific protein that has a unique and indispensable function in platelet activation and thrombus formation.[139–141] Studies of mice lacking GPVI expression due to targeted Fc-receptor-γ gene deletion confirmed the essential role of GPVI for collagen-induced platelet activation and adhesion to the subendothelial matrix.[139,140,142]

Impaired thrombosis on injured carotid arteries in mice rendered GPVI deficient by specific antibody treatment implies that GPVI plays an essential role in thrombogenesis under arterial flow conditions *in vivo*.[143] Barring unknown effects of the antibody used, these results suggest that the adhesive defect of platelets lacking GPVI is the consequence of reduced activation. Indeed, in *ex vivo* perfusion experiments, mouse platelets congenitally-deficient in GPVI exhibit essentially normal initial platelet contact with insoluble type I collagen fibrils, but no activation-dependent spreading and a complete defect in platelet aggregation.[140] However, the extent of the defect caused by the absence of GPVI appears to depend on the nature of the surface to which platelets are exposed. In *ex vivo* experiments, thrombus formation is normal when the blood of $GPVI^{-/-}$ mice is perfused over the extracellular matrix deposited by mouse skin fibroblasts.[26] Such a finding indicates that the vessel wall contains substrates for thrombus formation independent of collagen. The results of *in vivo* studies have confirmed the influence of additional variables on the role of GPVI in hemostasis[144] and thrombosis.[26] By measuring the tail bleeding time and the occlusion of the carotid artery following a ferric chloride-induced injury, it was apparent that $GPVI^{-/-}$ mice present a dichotomous behavior, with one group showing normal results and another displaying marked prolongation of the bleeding time and absent arterial thrombosis (FIGURE 28.5). Of note, each phenotype was a hereditary trait. A possible explanation for these results is the existence of one or more modifier genes that, directly or indirectly, influence the thrombogenic potential of GPVI-deficient platelets. One candidate gene has been postulated to exist within the modifier of hemostasis locus on the mouse chromosome four, suggesting that conditions may exist at a site of vascular injury that indeed make the role of GPVI more or less relevant for platelet adhesion and aggregation.

It is noteworthy that inhibition of α-thrombin has been shown to have a more profound antithrombotic effect in mice deficient in GPVI/FcR-γ than in normal controls, indicating that thrombin contributes to overcoming the functional defect of platelets with an impaired collagen-induced activation response. These results suggest that the pathways of platelet adhesion/stimulation in injured arteries likely to influence the extent of thrombosis are determined by the nature of the vascular lesion and of the substrates exposed to blood, including those leading to α-thrombin generation.

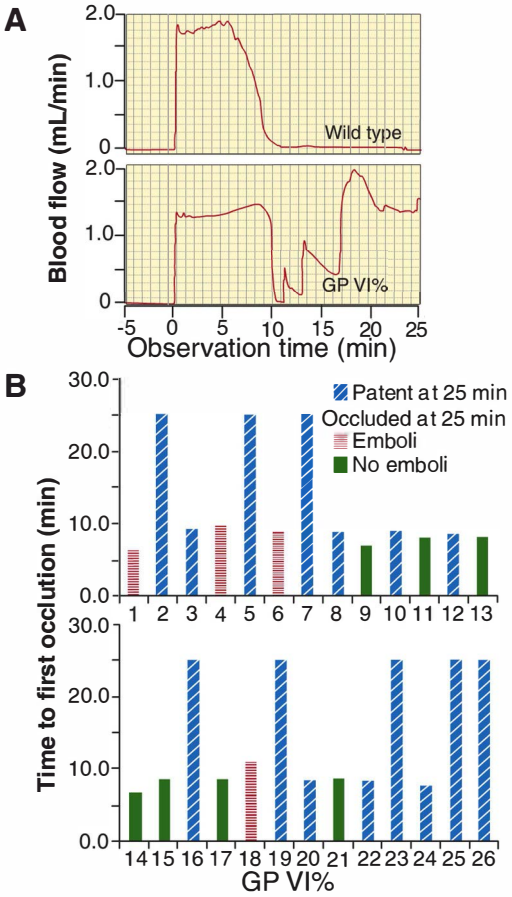

FIGURE 28.5 Ferric chloride-induced carotid artery thrombosis in GPVI deficient mice. **A**: Doppler tracings of blood flow in the carotid artery of wild type and $GPVI^{-/-}$ mice following $FeCl_3$ injury. In the wild type control shown, flow begins to decrease approximately 6 minutes after application of $FeCl_3$ to the artery and a complete occlusion occurs 3 minutes later with no resumption of flow. In the $GPVI^{-/-}$ mouse, flow reduction begins between 8 and 9 minutes after $FeCl_3$ application and complete obstruction develops over the next 1 to 2 minutes. However, blood flow resumes with alternating cycles of reocclusion and reopening of the vessel, indicating thrombus instability. Arterial flow is restored to normal at the end of the observation period in the $GPVI^{-/-}$ mouse. **B**: Bar graph representation of the time of first occlusion, occurrence of embolization, and presence or absence of occlusion at the end of the observation period in 26 $GPVI^{-/-}$ mice. (Modified from Konstantinides S, et al. *J Thromb Haemost* 2006;4:2014–2021).

$\alpha_2\beta_1$

The integrin $\alpha_2\beta_1$, also known as GPIaIIa, very-late-activation antigen-2, or CD49b/CD29, was the first collagen receptor to be identified and functions mainly as an adhesion receptor.[24,145–147] $\alpha_2\beta_1$ is composed of 150-kDa α_2 and 130-kDa β_1 subunits.[148] Its expression on normal platelets can vary up to 10-fold,[149] depending on a specific α_2 genotype. A polymorphism in the α_2 gene (C807T) was found to be a genetic marker of $\alpha_2\beta_1$ expression

levels, with platelets carrying the TT genotype expressing the highest levels, the TC genotype intermediate levels, and the CC genotype the lowest levels.[150] The amount of $\alpha_2\beta_1$ expressed on the platelet surface appears to correlate positively with the initial rate of platelet adhesion to collagen type I.[149,151] In addition to platelets, $\alpha_2\beta_1$ is expressed on fibroblasts where it functions as a collagen receptor and on epithelial and endothelial cells where it functions as both a collagen and laminin receptor.[152–154] The α_2 subunit contains an I-domain (amino acids 140 to 349) that serves as the primary ligand binding interface[155] when Mg^{2+} bound to the I domain interacts with a glutamate residue in the collagen molecule.[156] Recombinant α_2-I domain fusion proteins bind specifically to collagen in a divalent cation-dependent manner[157–160] and inhibit the interactions of platelets with collagen under flow.[157] Furthermore, antibodies that recognize epitopes in the I-domain inhibit $\alpha_2\beta_1$ binding to collagen.[161–163] Collagen-related peptides that contain the high affinity binding motif for $\alpha_2\beta_1$, Gly–Phe–Hyp–Gly–Glu–Arg,[164] have been used extensively in the study of platelet–collagen interactions.[24,156] In one study, $\alpha_2\beta_1$-mediated platelet adhesion to collagen-related peptides was enhanced by suboptimal stimulation of the thrombin receptor PAR4,[164] emphasizing the importance of multiple synergistic pathways in the adhesion process. Thus, while some receptors are constitutively active in a high affinity ligand binding state, others like platelet $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ require cellular activation and a conformational change to transition from a low to a high affinity binding state.[165,166]

The requirement that "inside-out" signaling precede high affinity $\alpha_2\beta_1$ binding to collagen has been confirmed using mouse platelets lacking GPVI.[128,139] Moreover, $\alpha_{IIb}\beta_3$ activation appears to be involved in platelet $\alpha_2\beta_1$ activation.[167] $\alpha_2\beta_1$ activation can also be induced by ligation of GPVI, as demonstrated by defective adhesion to matrix proteins of platelets lacking GPVI, or in the presence of GPVI inhibitors.[139,142,168] It should be noted that even in its low affinity state, $\alpha_2\beta_1$ might be capable of mediating platelet adhesion to collagen preceding GPVI-induced activation[24] in a manner akin to the binding of nonactivated $\alpha_{IIb}\beta_3$ to immobilized fibrinogen.[169] Indeed, evidence has been provided that $\alpha_2\beta_1$ can signal independently of GPVI and $\alpha_{IIb}\beta_3$.[129] The high affinity binding of $\alpha_2\beta_1$ to collagen induces a series of "outside-in" signaling events similar to those induced by GPVI, involving Src, Syk, SLP-76, and PLCγ_2.[128,170] Thus, despite their structural differences, the two main collagen receptors may share common signaling pathways to reinforce their interaction with collagen.[24,128,171] On the other hand, there are also differences in the signaling events following GPVI and $\alpha_2\beta_1$ ligation, as shown by the occurrence of distinctive intracytoplasmic Ca^{2+} transients following platelet adhesion to collagen/vWF.[129] It is now generally accepted that adhesion, activation, and aggregation are linked by an interplay of different receptors that integrate these functions according to the nature of the underlying vascular lesion.[26,172]

Deficits in the interaction of α_2-deficient murine platelets with collagen *ex vivo* have been detected, ranging from relatively mild decreases when platelet adhesion is measured under static conditions[173] to severely decreased adhesion when adhesion is measured under flow.[174] In the latter case, when blood is perfused over fibrillar collagen type I at shear rates between 400 and 1,300/second, isolated $\alpha_2\beta_1$ deficiency or the deficiency of the GPVI/Fc receptor γ-chain complex caused an equivalent but partial defect in platelet thrombus formation, whereas deficiency of collagen receptors caused complete inhibition.[174] These results suggest that although the two receptors can independently mediate platelet adhesion to collagen, they operate synergistically for optimal function. This contrasts with the alternate opinion that GPVI mediates all aspects of collagen-dependent platelet adhesion and activation, even in the absence of $\alpha_2\beta_1$.[128] Experimental conditions may tip the balance in favor of one view over the other, but it is likely that both receptors contribute to platelet thrombus formation depending on the nature of the vascular lesion and the presence of as yet unknown factors that can influence platelets and extracellular matrix components. It is noteworthy in this regard that congenital deficiency of either $\alpha_2\beta_1$[175,176] or GPVI[177] in humans results in a mild bleeding diathesis.

Conflicting results have been reported when $\alpha_2\beta_1$ function was studied *in vivo* using a variety of murine thrombosis models. Following mechanical injury to the mouse right common carotid artery, multiple integrin–ligand interactions appear to synergize to promote initial platelet adhesion, but there was no significant contribution by $\alpha_2\beta_1$. These results are consistent with previous *ex vivo* experiments using human blood and inhibitory antibodies[26] that indicated important roles for the integrins $\alpha_5\beta_1$ and $\alpha_6\beta_1$ as well as a contribution by $\alpha_{IIb}\beta_3$.[178] By contrast, when the right common carotid artery was injured photochemically, it was found that $\alpha_2\beta_1$ plays a role in subsequent thrombosis, although it was seemingly not involved in the intravascular thrombi and pulmonary emboli that occur after an intravenous injection of collagen.[179] These contradictory conclusions cannot be reconciled at present, but likely reflect differences in the nature of the vascular lesion, including severity of the endothelial damage and exposure of extracellular matrix components. Thus, similar to *ex vivo* studies, different thrombogenic surfaces, whether because of the composition or relative proportion of their constituents, seem to elicit distinct pathways of platelet adhesion and activation *in vivo*.

PLATELET ADHESION TO FIBRONECTIN

Fibronectin is a dimer composed of two covalently-linked 250 kDa subunits that is present as a soluble protein in plasma (at a concentration of 300 μg/mL), in platelets, and in other body fluids, as well as in insoluble form in the extracellular matrix of the vascular wall.[180,181] Each fibronectin subunit contains three types of homologous repeats, designated types I, II, and III,[182] and exists in multiple forms due to alternative splicing of a single pre-mRNA. This generates up to 20 variants, some with distinct functional properties.[183–185] A number of integrins interact with fibronectin including three present in platelets: $\alpha_5\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$.[186,187,188,189] Fibronectin binds to platelet $\alpha_{IIb}\beta_3$ in an activation-dependent manner,[190] but its interaction with $\alpha_5\beta_1$ is activation-independent.[191] Fibronectin contains distinct integrin binding motifs, the best known being the Arg–Gly–Asp (RGD) motif located in repeat III_{10}.[180] A second motif is present in repeat III_9 and has been designated a "synergy site" because it promotes $\alpha_5\beta_1$ binding by interacting with the α_5 subunit. The *N*-terminal region of fibronectin, containing repeats $I_{1–9}$ and $II_{1,2}$, also promotes $\alpha_5\beta_1$-mediated platelet adhesion.[180]

Fibronectin has other functional properties related to platelet adhesion, including binding to collagen/gelatin, heparin, and fibrin/fibrinogen.[192] Plasma fibronectin can bind to fibrin, fibrinogen, and self-associates through a mechanism that involves

cross-linking by activated factor XIII,[193,194] events that could be important for the incorporation of fibronectin into fibrin clots and for stabilizing platelet thrombi.[192] A role for fibronectin in thrombosis has been studied *in vivo* using intravital microscopy to measure thrombus formation in ferric chloride-injured arterioles of live mice.[195] In mice genetically deficient in plasma fibronectin, initial platelet adhesion was similar to that seen in control mice, but thrombus formation was delayed and thrombi were less stable. These results suggest plasma fibronectin plays a role in the formation of arterial thrombi. It is noteworthy in this regard that mice deficient in both vWF and fibrinogen display a normal bleeding time, and thrombi form at sites of vascular damage, although the platelet aggregates are unstable and embolize.[196] On the other hand, residual thrombus formation in mice deficient in vWF and fibrinogen is actually enhanced when plasma fibronectin is also deficient, implying that fibronectin is not essential for fibrinogen and vWF-independent thrombosis and that soluble fibronectin could function as an inhibitor of platelet aggregation.[197] Fibronectin isoforms containing the extra domain A are present in platelet α-granules, but absent in plasma fibronectin, and have been shown to have increased prothrombotic activity.[198] Further, soluble plasma fibronectin can assemble into fibrillar structures on the surface of fibroblasts and adherent platelets,[186,199,200] providing a potential substrate for the stable attachment of platelet aggregates.[201] Thus, although fibrinogen and vWF are the major ligands for platelet adhesion and aggregation, fibronectin may play a supplementary role and strategies that target fibronectin could have antithrombotic benefits.

PLATELET ADHESION TO THROMBOSPONDIN-1

Thrombospondins are a family of multidomain, multifunctional cell adhesive GPs[202] that is present in many cell types, including vascular endothelial cells,[203] smooth muscle cells,[204] and platelets[205] and is incorporated into the vessel wall matrix.[206] Thrombospondin-1 (TSP-1), released from platelet α-granules during activation-induced secretion,[205] can associate in a Ca^{2+}-dependent manner[207] with the platelet surface via specific thrombospondin receptors, and mediate platelet adhesion.[208–210] In general, TSP-1 is expressed in response to injury[211] and during growth and development[31] and has been found to be highly enriched in atherosclerotic plaques.[212,213] Although the physiologic function of TSP-1 is currently somewhat controversial, it can complex with many platelet adhesive proteins, including fibrinogen/fibrin, laminin, vWF, fibronectin, and collagen[214–217] and it may play a secondary role in platelet adhesion and thrombosis. TSP-1 has been reported to potentiate platelet activation and stabilize platelet aggregates *in vitro*,[218,219] although platelets from mice lacking TSP-1 aggregate normally.[220,221] TSP-1 has been identified as an alternative ligand to vWF for GPIb-dependent adhesion of activated platelets under high shear conditions *in vitro*[31] and has been reported to enhance thrombosis by protecting subendothelial vWF from cleavage by ADAMTS13.[221] It has also been reported to promote platelet aggregation by suppressing the production of the endogenous platelet inhibitor nitric oxide.[222,223] TSP-2, a constituent of extracellular matrices, but absent from platelets, has a high structural similarity to TSP-1, and interacts with a number of the same cellular receptors.[224,225] TSP-2 deficiency has been associated with impaired hemostasis in mice,[226] a finding that likely reflects the requirement for TSP-2 in normal megakaryocytopoiesis and the release of functionally competent platelets.[227]

PLATELET ADHESION TO LAMININ

Laminin, a major component of vascular basement membranes, interacts with collagen type IV and heparin sulfate proteoglycans to maintain the structural integrity of the basement membrane.[228] Purified laminin can mediate platelet adhesion and activation under static conditions by binding to the collagen receptor GPVI, an interaction that is facilitated by integrin $\alpha_6\beta_1$[229] in a manner analogous to the synergy between GPVI and $\alpha_2\beta_1$.[139,156,165,170] Under high shear flow conditions, laminin supports platelet adhesion by a mechanism that depends on the interaction of vWF with GPIbα,[230] reminiscent of the essential role of vWF and GPIbα for platelet adhesion to collagen at elevated shear rates.[26] Direct binding of soluble vWF to immobilized laminin has been detected by surface plasmon resonance spectroscopy, although the affinity of interaction was about 100-fold lower than that between collagen type I and vWF.[230] Studies to detect the direct binding of vWF to immobilized laminin using enzyme-linked immunosorbent assays were unsuccessful, possibly due to the low affinity or the reversibility of the interaction.[231] Although the relative contribution of subendothelial laminin to thrombosis remains to be established, the apparent redundant mechanism of platelet adhesion to laminin highlights a general paradigm for the interaction of platelets with the subendothelial matrices, involving GPVI, vWF, and GPIbα.

PLATELET ADHESION TO FIBRINOGEN AND FIBRIN

Whereas platelet $\alpha_{IIb}\beta_3$ cannot bind soluble fibrinogen unless platelets are activated, $\alpha_{IIb}\beta_3$ on nonactivated platelets can bind to surface-bound fibrinogen and to fibrin, thereby mediating platelet adhesion in a shear rate-limiting fashion.[32] Platelet adhesion to immobilized fibrinogen or fibrin in experimental flow systems becomes progressively less efficient with increasing wall shear rates, with relatively few platelet interactions at wall shear rates above 1,000/second.[32] Presumably the on-rate of bond formation is too low to permit platelet capture at high shear rates, or bonds may form, but their initial resistance to tensile stress may be insufficient to oppose the shear forces and maintain stable platelet attachment. However, most of the platelets that attach at low shear-rates do not detach when the flow rate is increased,[32] implying that the $\alpha_{IIb}\beta_3$-fibrinogen bond, once formed, may have a dissociation rate sufficiently low for sustaining stable attachment or that other factors such as $\alpha_{IIb}\beta_3$ receptor density and postadhesion platelet cytoskeletal rearrangements may influence the stability of platelet attachment. Platelets become activated and spread on immobilized fibrinogen and fibrin, but thrombus formation is noticeably absent unless other adhesive proteins are also present.[32] An explanation for this observation in not currently known, but it might reflect $\alpha_{IIb}\beta_3$ sequestration on the abluminal platelet

membrane surface resulting in a deficit in the amount of $\alpha_{IIb}\beta_3$ available to bind soluble ligands and support platelet–platelet cohesion.[232]

A ROLE FOR P-SELECTIN IN ARTERIAL THROMBOSIS

P-selectin, a GP present in the membranes of platelet α-granules and endothelial cell Weibel-Palade bodies, is rapidly translocated to the plasma membrane following cell activation. The P-selectin ligand, P-selectin glycoprotein ligand-1 (PSGL-1), is present on neutrophils and monocytes and provides a means of tethering these cells to activated platelets and endothelial cells.[233] By inducing tissue factor expression on circulating monocytes, P-selectin can promote fibrin formation since tissue factor is the major initiator of thrombin generation.[234] P-selectin also mediates the rolling interactions between leukocytes and the vessel wall that is required for leukocyte recruitment to sites of inflammation or infection.[235,236] Recent evidence indicates that P-selectin and PSGL-1 are involved in the process of thrombosis as well. Mice lacking either P-selectin or PSGL-1 exhibit reduced arterial thrombus formation, fibrin levels, and tissue factor accumulation following vascular injury.[237,238] Similar results were seen when wild type mice were treated with function-blocking P-selectin antibodies prior to the induction of arterial vessel damage.[239]

POSTADHESION EVENTS LEADING TO THROMBUS PROPAGATION

Whether platelet adhesion results in a monolayer of platelets or in thrombus formation depends on a number of factors, including the nature of the exposed substrate, the prevailing rheologic conditions, and the presence of functionally competent platelets. The initial adhesion and activation of platelets in response to changes in endothelial cells, or exposure to subendothelial matrices, promotes the binding of soluble adhesive proteins, mainly fibrinogen and vWF that form the immobilized substrate for platelet recruitment and thrombus propagation.[26,240] Since only the initial layer of adherent platelets are in contact with the subendothelial components, platelet accumulation into a growing thrombus depends on the formation of interplatelet bonds. However, platelet aggregation in this context should not be considered as a distinct process from platelet adhesion since it also involves the capture and arrest of free flowing platelets onto a reactive surface (the activated platelet membrane), and may even utilize common receptor/ligand interactions and signaling pathways leading to platelet activation. Because of these mechanistic similarities, both platelet adhesion and aggregation are subject to the same fluid dynamic influences generated by blood flow, a key modulator of thrombus formation and propagation.

Fibrinogen and vWF, present in circulating plasma or released from activated platelets, are the predominant adhesive molecules that link platelets to one another during thrombus development. On collagen type I fibrils, fibrinogen and vWF act cooperatively to support platelet aggregation since neither protein by itself can sustain the full development of a stable thrombi within the range of pathophysiologic flow conditions.[240,241] At high shear rates, vWF-mediated thrombi grow rapidly, but are unstable when fibrinogen is absent, whereas thrombi grow more slowly, but are more stable when both vWF and fibrinogen are present.[240] These observations are consistent with the clinical phenotype of patients with congenital deficiencies of either fibrinogen[242] or vWF.[243] However, studies with genetically modified mice have shown that sustainable thrombus formation can occur in damaged mesenteric arterioles, even when both vWF and fibrinogen are absent,[196] whereas both GPIbα and $\alpha_{IIb}\beta_3$ are absolutely required.[241,244] These studies suggest that other ligands for activated $\alpha_{IIb}\beta_3$ and GPIbα, such as fibronectin or TSP-1, might support the formation of stable platelet thrombi. During the thrombus propagation phase, the released secondary agonists ADP and TXA_2 reinforce the platelet activation by binding to the receptors $P2Y_1$, $P2Y_{12,}$ and the TpA receptors, evoking Ca^{2+} mobilization and the activation of intracellular signaling molecules such as protein kinase C and PI 3-K.

PERPETUATION OF ADHESION-INITIATED PLATELET ACTIVATION AND THROMBUS STABILIZATION

Platelet aggregation is a reversible process. Without stabilization, platelets disaggregate and embolization occurs under the shear forces produced by arterial blood flow. Although platelet agonists such as ADP, TXA_2, and thrombin initiate rapid integrin activation and platelet recruitment, signaling initiated in this manner is relatively limited in duration because their receptors become desensitized. Experiments indicate that sustained thrombus growth requires continuing function of ADP receptors, as well as PI 3-K-dependent activation pathways, and is accompanied by sustained cycles of intracytoplasmic Ca^{2+} transients across whole platelet aggregates.[245–248] Therefore, additional signaling mechanisms are necessary to perpetuate platelet activation and prevent dissolution of the platelet thrombus, at least until a fibrin meshwork can be established.

Activated $\alpha_{IIb}\beta_3$ plays a critical role in transmitting outside-in signals that lead to irreversible platelet aggregation and clot retraction.[249] Phosphorylation of tyrosine residues in the cytoplasmic domain of β_3 appears to be a critical step in this process.[250–252] Additional outside-in signaling may be initiated by Eph kinases and their ligands, the ephrins.[253] Human platelets have been reported to express at least one ephrin (EphinB1) and two Eph kinases (EphA4 and EphB1) and inhibiting Eph kinase binding to EphrinB1 appears to enhance the reversibility of platelet aggregation-induced low dose ADP, without affecting the exposure of fibrinogen binding sites on $\alpha_{IIb}\beta_3$.[253] Further, thrombi formed on collagen type I are significantly smaller.[254]

A number of adhesive molecules contained in platelet α-granules are absent on the surface of nonstimulated platelets but present after platelet activation. Some of these adhesive molecules have been reported to play an important role in the secondary reinforcement of thrombus growth and stability. Released CD40L[255] binds to $\alpha_{IIb}\beta_3$ and propagates platelet activation through $\alpha_{IIb}\beta_3$-dependent outside-in signaling, a process that is important for the stability of arterial thrombi in murine models of arterial thrombosis.[256,257] Growth arrest-specific 6 (GAS6), a ligand for the Axl/Sky family of receptor tyrosine kinases, is also involved in the stabilization of platelet thrombi.[258] Gas6 has been shown to enhance the formation of stable platelet aggregates in response to various agonists, and Gas6-deficient mice are protected against thrombosis without abnormal bleeding.[258]

On the other hand, the presence of Gas6 in human platelets has not been clearly established.[259] Likewise, the signaling lymphocyte activation molecule (SLAM) family of receptors may be important secondary mediators of platelet aggregation[260] since mice deficient in SLAM display delayed thrombus formation, but have normal tail bleeding times.[260] Platelet endothelial aggregation receptor 1 is another membrane protein identified on the surface of activated platelets that appears to signal secondary to $\alpha_{IIb}\beta_3$-mediated platelet–platelet contacts and may be involved in maintaining sustained platelet activation.[261] It has also been reported that semaphorin 4D (Sema4D) and its receptors are expressed on the platelet surface and that Sema4D$^{-/-}$ mice display a selective defect in collagen-induced platelet aggregation and an impaired vascular injury response.[262] It appears that $\alpha_{IIb}\beta_3$ and Sema4D jointly promote collagen responses through two interdependent but distinguishable processes leading to amplification of Syk activation and collagen-induced responses; one process is the binding of Sema4D to its receptors facilitated by integrin-mediated contacts and the other process is the enhancement of integrin outside-in signaling supported by Sema4D-receptor interactions.[263] Of note, loss of sema4D expression reduces the platelet hyperactivity otherwise found in dyslipidemia and confers protection against the development of atherosclerosis.[264]

Along with positive feedback, molecules that appear on the platelet surface after activation can also exert a negative regulatory function on thrombus formation. One example is endothelial cell specific adhesion molecule (ESAM), which localizes to interplatelet contact sites following activation and limits thrombus growth and stability, as shown by the finding that ESAM-deficient mice achieve more stable hemostasis than wild-type mice following tail transection and develop larger thrombi following arterial injury.[265] These effects may be explained by the enhanced response of ESAM-deficient, as compared to wild-type platelets, to lower concentrations of G protein-dependent agonists, and to greater resistance to disaggregation.[265]

CONCLUSIONS

There have been major advances in our understanding of the mechanisms involved in mediating platelet adhesion and the bidirectional coupling with platelet activation and aggregation. The application of genomic and proteomic approaches has greatly supported this progress, as has the use of an expanding collection of animal models with targeted gene deletions or mutations. The advent of improved drug development technologies is likely to permit the translation of this fundamental knowledge into more efficient therapeutic approaches. For example, preventing sustained integrin activation—as can be achieved by removing ADP, blocking ADP receptors, or inhibiting PI 3-K—may prove to be a relevant target for antithrombotic intervention and provide a means of restricting thrombus growth while avoiding major bleeding complications.

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Full Reference Lists can be found online

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CHAPTER

29

Shear Effects on Platelets and the Vessel Wall in the Pathogenesis of Atherothrombosis

DAVID L. BARK, JR., WARWICK S. NESBITT, ANGUS K.T. WONG, AND SHAUN P. JACKSON

The hemodynamic effects of blood flow play a critical role in regulating the cardiovascular system from early prenatal development until death. Beginning with the first heartbeats, the embryonic primary vascular plexus responds to various hemodynamic forces that modulate plexus differentiation into arteries and veins. Blood flow is a critical requirement for ongoing vascular development, with wall shear stress (WSS) playing a significant role in developmental remodeling of the vascular endothelium. The plasticity of the vasculature in response to prevailing blood flow conditions allows it to rapidly adapt to hemodynamic perturbations, both physiologic and pathologic, with the purpose of maintaining vascular integrity throughout our lives.

Disturbances of blood flow perform an important role in the development of cardiovascular diseases, including atherothrombosis. Chronic disturbances in blood flow lead to vascular changes that promote atherosclerosis, whereas acute changes in blood flow at sites of atherosclerotic plaque disruption are important for the development of thrombosis. Therefore, temporal variations (seconds through to years) in flow are relevant to atherothrombosis. Early seminal work by the German physician, Rudolf Virchow (1856), identified an important role for blood flow disturbances in promoting venous thrombosis. However, an awareness of the central importance of hemodynamics in promoting atherothrombosis did not develop until the 1970s. Two major theories regarding the pathogenesis of atherothrombosis developed at this time: (a) that low and/or oscillatory WSS is important for atherogenesis and (b) that high WSS promotes arterial thrombosis. The purpose of this chapter is to expand on these concepts, highlighting the importance of disturbed "bulk" flow (macroscale flow disturbance) in regulating endothelial cell and platelet function. Furthermore, we focus on an emerging concept in the thrombosis field that localized microscale (1 to 10 μm) changes in blood flow may play a potentially important role in promoting thrombus formation.

ARTERIES RESPOND TO SPECIFIC HEMODYNAMIC FORCES

Blood flow in the arterial circulation is primarily driven by cardiac contractions that create a pressure gradient across the systemic and pulmonary vasculature. A pressure differential pushes blood from regions of high pressure to regions of low pressure, which is resisted by the frictional nature of the vascular wall (Box 29.1). As a result, blood flow creates three dominant stresses within the arterial wall: (a) circumferential stress, (b) axial stress, and (c) WSS, where stress is defined as the distribution of internal forces throughout a deformable body (force/area). Blood vessels are highly responsive to these stresses and modify their overall architecture and composition to maintain a near-constant stress environment that limits metabolic demand and preserves vascular integrity.

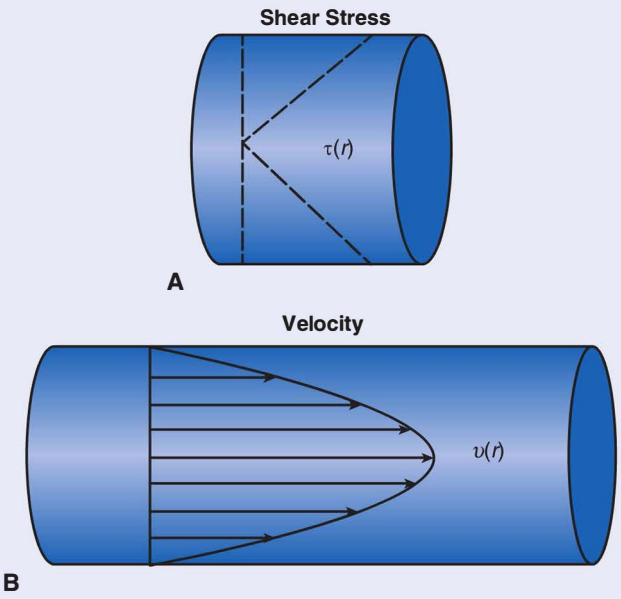
Circumferential Stress

Arterial pressure is a major source of stress in the vasculature. Pressure exerts an outward push on blood vessels, creating tension within the arterial wall. Tension results in circumferential stress, which is sensed by smooth muscle cells (SMCs) in the medial layer of arteries. These cells actively oppose this stress to prevent arterial expansion and bursting upon vessel pressurization. Furthermore, elastin and collagen provide additional passive structural support. Average circumferential stress remains at a near-constant value of 10^5 Pa in the arteries.[1] Hypertension creates chronic high pressure increasing circumferential stress and the vessel attempts to alleviate this stress by thickening the medial layer. Such changes to the vessel wall may promote atherosclerosis. The detailed effects of circumferential stress on vascular remodeling have been summarized previously[2,3] and will not be discussed further in this chapter.

Axial Stress

A stress that is indirectly linked to hemodynamics is the longitudinal (axial) stress, which exists because arteries are constantly stretched *in situ*. Axial stress remains near a value of 10^5 Pa, which is required to avoid vessel buckling (tortuosity) or tearing, and occurs through the modulation of the axial force.[3] Both endothelial cells and SMCs can sense axial stress and can signal for control of the vessel microstructure (collagen and elastin composition) in order to modify the internal axial force in response to changes in either the vessel radius or thickness.[2] It is interesting to speculate that microstructural changes from axial stress modulation may contribute to plaque formation or degradation, since axial stress is very sensitive to changes in wall thickness, which changes dramatically in the presence of plaque. However, little is known about the role of axial stress in atherothrombosis.

Box 29.1 Hagen-Poiseuille Flow

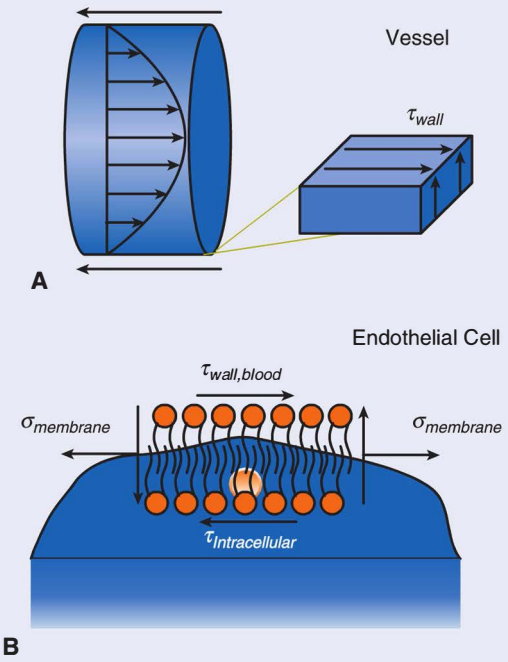


Blood flow is complex, but the general flow characteristics can be greatly simplified by some basic assumptions, leading to generalized Hagen-Poiseuille flow (see below). An important hemodynamic parameter to blood flow is the shear rate (velocity gradient), which is maximum at the vessel wall and linearly decreases to 0 at the central axis of the vessel for Hagen-Poiseuille flow. The shear stress, $\tau(r)$, is related to the shear rate by the dynamic viscosity (**A**). This flow exhibits a parabolic velocity profile, $v(r)$, and is driven by an axial pressure gradient, primarily generated by cardiac contractions (**B**).

Hagen-Poiseuille Assumptions:

- Newtonian fluid—valid for arteries with high shear and high flow—fluid where Newtonian constitutive equation can be applied
- Straight, circular pipe far from flow entrance (fully developed flow)
- Steady flow
- Laminar flow—valid, except in parts of the aorta
- There is no slip between fluid and the pipe wall.

Box 29.2 Wall Shear Stress



WSS, τ_{wall}, at the fluid–solid interface existing at the luminal side of the endothelium is equal between the shear stress in the blood, $\tau_{wall,blood}$, and shear stress on the endothelium (**A**). Therefore, the shear stress experienced by the endothelium can be defined by fluid properties, instead of the endothelial properties. Assuming a fluidic cytosolic interior for endothelial cells, the intracellular shear stress, $\tau_{intracellular}$, is negligible and the luminal shear stress is converted into a tension, $\sigma_{membrane}$, in the cell membrane and on the cytoskeleton (**B**). In addition to tension and luminal shear stress, there is also a vertical component to shear that resists cell rotation.

Wall Shear Stress

As blood flows through an artery, it creates a pulling force on the endothelial surface, similar to a frictional resistance. The force per unit area applied to and transmitted through the endothelium is equal to the hemodynamic shear stress at the surface (see Box 29.2). WSS is maintained at a near-constant arterial value of 15 to 20 dynes/cm^2 through alterations of the luminal radius.[1] The purpose, according to Murray's law, is to optimize the relationship between the flow rate and the arterial radius to reduce the metabolic work required from the body to maintain blood flow.[4] WSS is converted into a tensile force within the endothelial cell membrane[5] (Box 29.2) and cytoskeleton.

While the contributions of circumferential and longitudinal stresses to atherothrombosis remain poorly defined, the impact of WSS on the atherothrombotic process has been extensively investigated, as described below.

THE IMPACT OF WALL SHEAR STRESS ON ATHEROSCLEROSIS

It has long been recognized that certain areas of the vasculature, including the abdominal aorta, carotid bifurcation, coronary, renal, and femoral arteries, are prone to the development of atherosclerotic lesions. These vascular regions are more likely to exhibit chronic inflammatory changes that contribute to intimal thickening.[6–10,11] While systemic factors like hypertension, smoking, hyperlipidemia, obesity, and diabetes are associated with atherosclerosis propagation, a key factor determining plaque localization at specific arteries is the underlying regional hemodynamic environment, specifically the presence of low and/or oscillating WSS (FIGURE 29.1). Oscillating WSS refers to the temporal directional transition of WSS during the cardiac cycle, which occurs when near-wall blood flow reverses direction, a

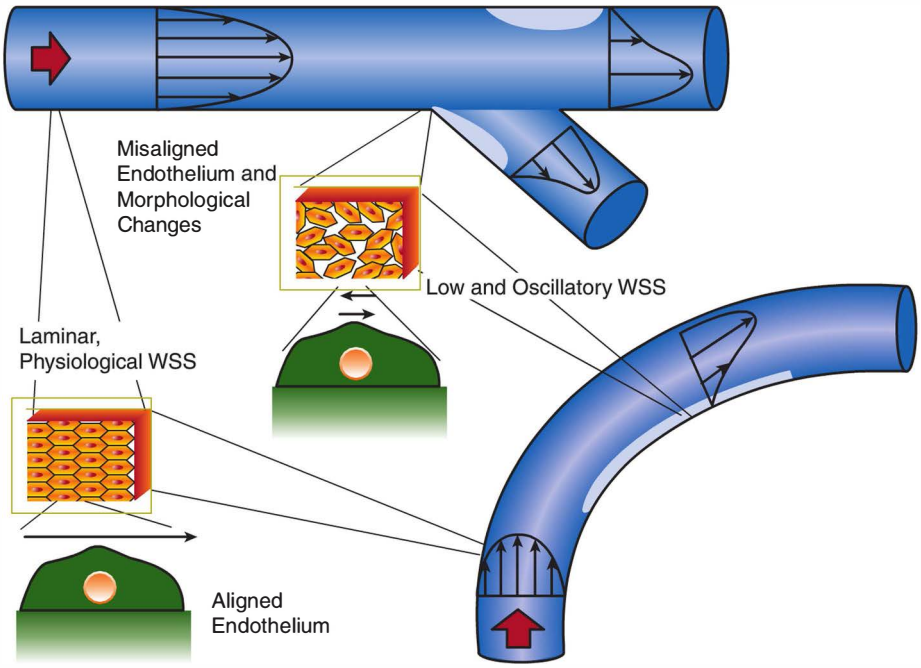


FIGURE 29.1 Regions prone to plaque formation include vessels with branches and sharp curvatures, corresponding to regions of altered velocity profiles. Areas of low and/or oscillating WSS can develop in these situations (*light gray regions*), which may promote atherogenesis. This process is promoted through numerous pathways, including endothelial cell morphologic and functional changes. One structural change is the altered shape and misalignment of neighboring ECs in regions of low and/or oscillating WSS (*arrows* representing WSS over ECs), resulting in increased endothelial permeability. Furthermore, these structural changes are associated with regions of chronic inflammation. Intimal thickening occurs most rapidly in regions of low and/or oscillating WSS, as determined by intravascular ultrasound.[11,12,13] Plaques found in these regions are commonly expansive, extending in the abluminal direction and preserving the luminal area. Expansive plaque generally has vulnerable characteristics, including a large lipid core, inflammatory infiltrate, and a thin fibrous cap.[14,15] Therefore, vessel geometries are closely tied in with atherosclerosis through the resulting effects on WSS distribution.

feature of proatherogenic arteries. Localized oscillating WSS promotes atherogenesis through multiple interrelated mechanisms including the tendency for foam cell formation and increased production and retention of proinflammatory mediators. These mechanisms promoting atherosclerosis have been most extensively associated with WSS-dependent altered endothelial structure and function (FIGURE 29.1).

Impact of Wall Shear Stress on Endothelial Cell Function

Endothelial cells play a key role in maintaining vascular integrity and in dynamically regulating vascular function. This occurs through several mechanisms including the regulation of vascular permeability, remodeling, synthesis, and secretion of vasoactive, prothrombotic, and antithrombotic molecules, as well as through the modulation of vascular tone through interactions with SMCs. The principle hemodynamic driver of endothelial cell function is the applied WSS. The response of endothelial cells to the WSS environment is complex and wide ranging, with changes in WSS triggering intracellular signaling pathways (FIGURE 29.2A) that modulate gene expression and cell morphology.[12]

Endothelial cells "sense" changes in blood flow, specifically oscillatory WSS (FIGURE 29.2A), and convert these mechanical signals into chemical signals (mechanotransduction) that modulate endothelial cell structure and function (FIGURE 29.2B). The luminal surface of the vascular endothelium contains several key membrane structures that are considered to be important for their mechanosensory function[13–15,16]:

1. **Caveolae**—these are small surface invaginations of the endothelial cell plasma membrane linked to the initiation and propagation of Ca^{2+} signaling in response to WSS. These functional lipid membrane compartments are important for shear dependent up-regulation of nitric oxide (NO) synthesis and represent a primary membrane component of the mechanosensory machinery.
2. **Primary cilia**—these are extensions of the endothelial cell plasma membrane that undergo mechanical deformation in response to blood flow. Bending of primary cilia is thought to lead to the opening of mechanically gated ion channels leading to Ca^{2+} influx and endothelial cell activation.
3. **The glycocalyx**—this is comprised of a network of glycosylated and sialylated transmembrane proteins protruding from the plasma membrane into the vessel lumen and has been found to directly respond to applied mechanical shear forces. Under low-flow conditions, this glycoprotein network exists in a tightly coiled state. With the application of blood flow, the glycocalyx uncoils in the direction of flow, leading to transmembrane conformational signaling. Significantly,

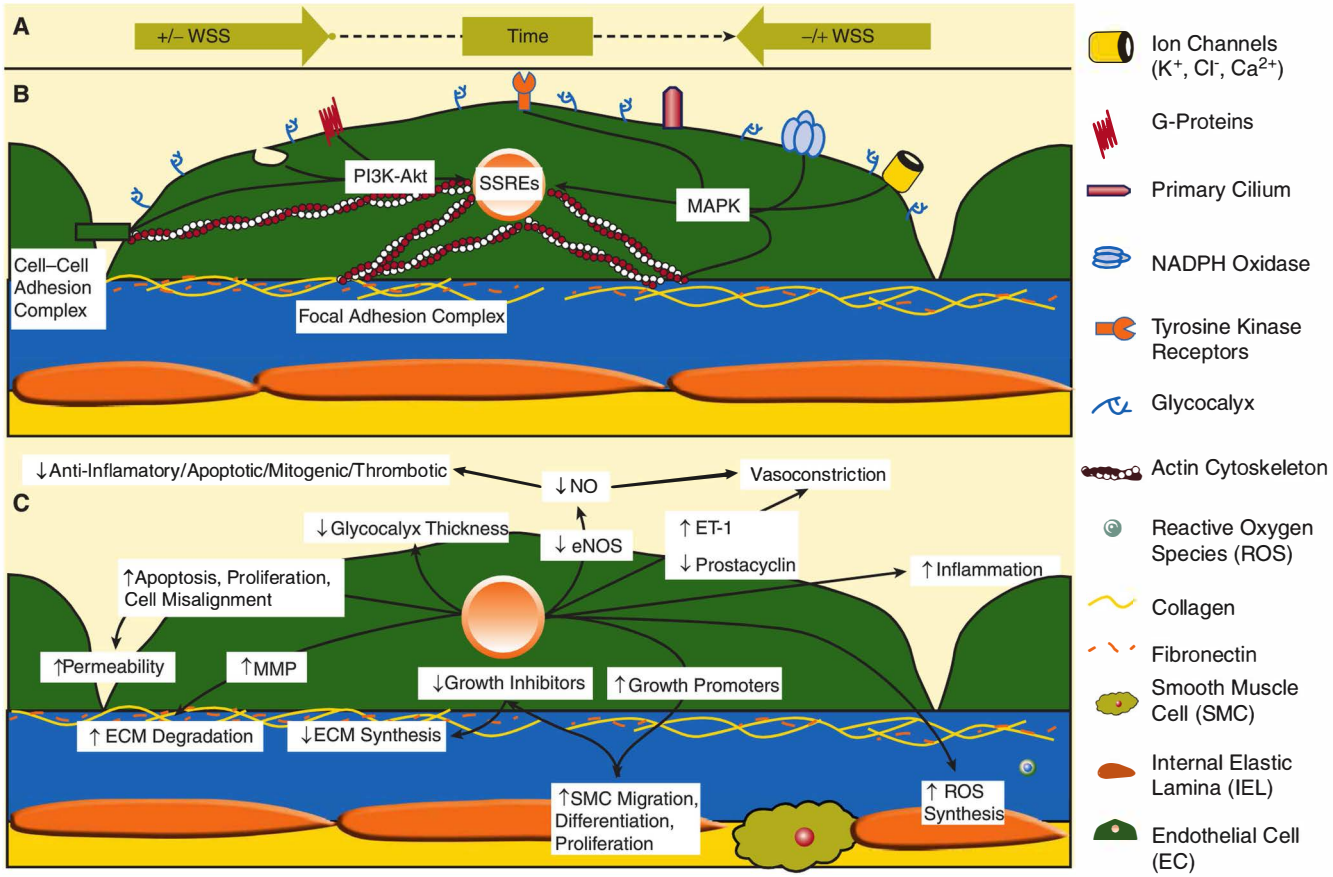


FIGURE 29.2 Endothelial cell responses at low and/or oscillatory WSS. **A:** Oscillatory WSS occurs in vascular regions where the near-wall flow changes direction during the cardiac cycle. These regions also typically correspond to regions of low WSS. When the near-wall flow changes directions, the overall WSS and drag force on the EC changes directions. Low/oscillatory WSS can occur even in regions where the bulk flow generally remains in monodirectional. These situations are possible in poststenotic regions and in the regions of a sinus, where the vessel expands abruptly. **B:** The luminal surface of the EC plasma membrane directly interfaces with blood flow and represents the sensing surface of the endothelium. On this surface are multiple mechanosensing elements, the caveolae, glycocalyx, and primary cilia. **C:** Multiple EC responses occur as a result of low/oscillatory WSS signaling, creating a proatherogenic environment. The extracellular matrix (ECM) is degraded by MMPs, while synthesis is down-regulated. SMC migration, differentiation, and proliferation are increased. The balance of vasomodulation is weighted toward vasoconstriction. Also, the overall EC morphology and alignment are disrupted, such that there are larger gaps between ECs. Figure is modified by permission from Chatzisisis et al.[23]

the glycocalyx serves to regulate vascular permeability, with increased permeability at oscillatory WSS resulting from an overall reduction in glycocalyx thickness.

In addition to mechanosensors on the luminal surface of endothelial cells, mechanical signals can also be transduced from the abluminal surface of endothelial cells, with integrin receptors on the basal plasma membrane playing a major role in transducing signals to the nucleus through the actin cytoskeleton (FIGURE 29.2B). The details underlying integrin mechanotransduction have been investigated in considerable detail and reviewed elsewhere[17] and will not be discussed further here.

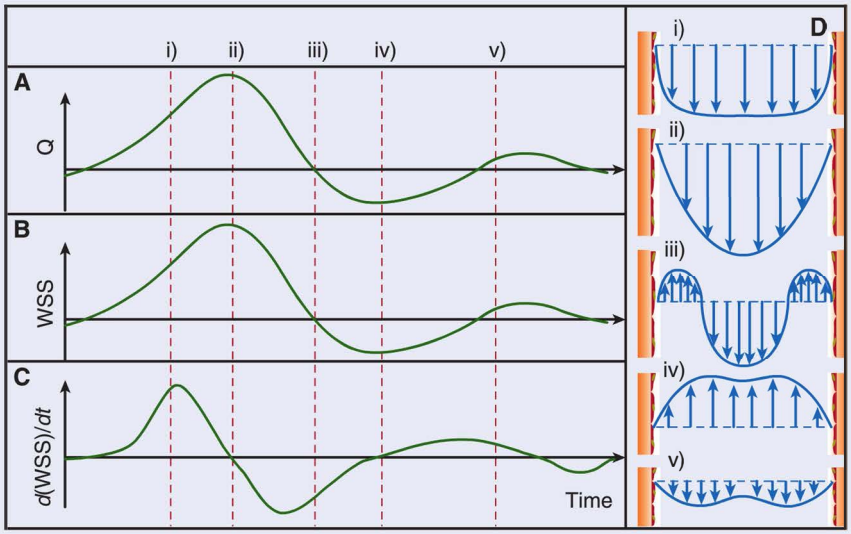
Atheroprotective Signals

Endothelial mechanosensors generally produce atheroprotective signals when the flow is unidirectional, laminar, and at a WSS that is >10 dynes/cm^2. A key antiatherogenic effect of endothelial cells is the up-regulated synthesis of NO and prostacyclin (PGI2), two major vasodilators that regulate local vascular tone and profoundly inhibit platelet activation and leukocyte adhesion.[18–20] Furthermore, such flow conditions also suppress endothelial cell proliferation and promote cell survival, through up-regulated expression of antiapoptotic genes.[21,22] In combination, these cumulative effects help reduce the proinflammatory and prothrombotic phenotype of endothelial cells, thereby inhibiting the development of atherosclerotic lesions.

Proatherogenic Signals

Atherosclerosis is promoted by localized disturbances in blood flow, specifically low oscillatory WSS (FIGURE 29.2A) and

Box 29.3 Temporal Wall Shear Stress Gradients



The cardiac cycle causes pulsatility in major arteries, including the coronary, carotid, femoral, and aortic arteries. Therefore, there is almost always a low temporal gradient in WSS for these arteries. Diseased arteries, specifically those with a stenosis, exhibit much larger oscillations in flow with respect to time, creating larger gradients in WSS.[39,40] In terms of cells in free flow (e.g., platelets) and cells at a vessel surface (e.g., ECs), both experience changes in WSS due to the pulsatility.

A) Flow rates through major atherosclerotic-prone arteries oscillate during the cardiac cycle. The waveform shown in this figure is an arbitrary waveform that has some characteristic features of flow through these arteries. Actual flow rate waveforms differ from artery to artery. During systole, flow rates accelerate (i) in most arteries, as pressure builds up in the aortic arch. Flow rates become the highest right before diastole (ii). During diastole (iii), the flow decelerates in most arteries. In the coronary arteries, the converse is true. During this cycle, flow can reverse direction (iv) in the coronary, femoral, and carotid arteries. Oscillatory WSS occurs when near wall blood reverses direction, such as the region consisting of sections (iii) and (iv).

B) WSS generally varies in a similar way as the flow rate, as illustrated by the proportionality between the two for Hagen-Poiseuille flow. However, it also depends on the velocity waveform, which changes throughout the cardiac cycle. The WSS presented here is an idealized case that is based solely on the flow rate and is independent of the velocity waveform, for the purpose of clarity.

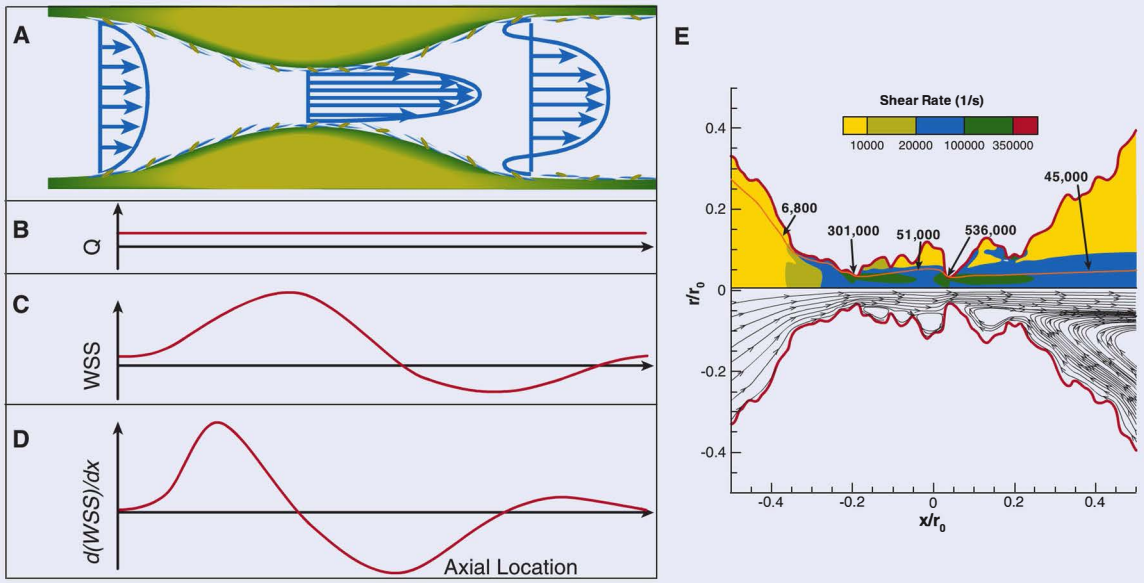
C) A temporal WSS gradient exists at a given location because of blood pulsatility. The gradient depends on local arterial conditions. It is maximum during flow acceleration and is minimum during flow deceleration. The temporal WSS gradient is the slope of the WSS versus time curve.

D) The pulsatility of blood flow creates an unsteady flow field. Velocity profiles do not remain parabolic, but instead can be characterized by the Womersley solution.[10,41,42] Velocity profiles are a commonly used method to visualize fluid mechanics but can be conceptualized as the amount a fluid moves for a given time and how that varies across the diameter of the blood vessel. The fluid velocity can partially (iii -not shown in B) or completely (iv) change directions during the cardiac cycle, depending on the artery and geometrical conditions, corresponding to the roman numerals over the flow waveform plot in (**A**).

temporal shear gradients (Box 29.3).[23,24–26] Spatial WSS gradients (Box 29.4) may also promote atherogenesis, although the absolute importance of these flow changes to the atherosclerotic process remains unclear.[27–29] The effects of shear gradients on endothelial function and structure are complex and wide ranging. For example, temporal shear gradients and low oscillatory WSS have been demonstrated to activate the MAPK[23,25,30] and PI 3-kinase signaling pathways (FIGURE 29.2B) leading to activation of the nuclear shear-sensitive response elements and the induction of proatherogenic transcription factors.[23,25] As a consequence, there is increased expression of ICAM-1 and E-selectin on the surface of endothelial cells[23,26] and increased production of the chemokine MCP-1.[23,24] These changes enhance monocyte recruitment. Local disturbances in blood flow also promote the synthesis of growth factors, such as PDGF-A, which has proatherogenic and proangiogenic properties (FIGURE 29.2C).[24,31]

Temporal WSS gradients, as well as low/oscillatory WSS, lead to enhanced endothelial cell proliferation,[27] while spatial WSS gradients promote changes in endothelial morphology and enhance motility,[28,29,32] leading to changes in the permeability of the endothelium.[33] Misalignment of endothelial cells (FIGURE 29.1) as a result of endothelial morphologic changes,[65,71,72,73,74] enhanced cell turnover, and apoptosis[17–19] favors recruitment of monocytes into the intima and promotes

Box 29.4 Spatial Wall Shear Stress Gradients



A shear gradient exists at locations where vessel geometry changes in some way (e.g., stenosis, aneurysms, curvature, and branches). Cells in the bulk flow (e.g., platelets) experience changing shear stress and velocity as they travel through a stenosis. However, cells on the surface (e.g., ECs) only experience the local WSS. Only cell–cell communication and the local WSS gradient would provide any information to an EC that a stenosis can be found in its region.

A) Flow entering a stenosis accelerates and then decelerates downstream. Flow recirculation and reversal can occur in the downstream region (as depicted by the *light gray color* and the *arrows*).

B) The flow rate through a stenosis is constant along the axial direction because of the conservation of mass, neglecting blood pulsatility, despite the changes in average blood velocity.

C) High WSS exists at the stenosis peak and low WSS is found downstream of the stenosis apex. WSS becomes negative in the recirculation region with zero WSS at the flow separation and reattachment point (*gray region* downstream of stenosis).[40,43–45]

D) The gradient in spatial WSS maximizes upstream of the stenosis apex and is minimal just downstream of the stenosis apex. The gradient becomes zero far downstream of the stenosis, as the flow becomes fully developed.

E) Microscale shear gradients exist over rough surfaces that can be found on atherosclerotic plaque, developing thrombus, and intravascular devices (e.g., stents), as opposed to macroscale gradients. An example rough surface is superimposed on a stenosis, illustrating the changes in shear that can occur along a streamline (platelet path). (Reproduced from Bark DL Jr, Ku DN. Wall shear over high degree stenoses pertinent to atherothrombosis. *J Biomech* 2010;43(15):2970–2977, with permission.) WSS is capable of changing by an order of magnitude, despite very small changes in geometry.

foam cell formation.[22,23,24] Moreover, the glycocalyx on the surface of endothelial cells, which normally acts as a barrier between leukocytes and endothelial cells, becomes less dense under the influence of low/oscillatory WSS,[20,21] facilitating leukocyte–endothelial interactions. In addition, under the influence of low/oscillatory WSS, endothelial cells also increase production of matrix metalloproteinases (MMPs),[9,10,11] leading to increased matrix degradation and enhanced SMC migration (FIGURE 29.2C). Extracellular matrix degradation of collagen and elastin reduces the passive structural support for the vessel,[5] leading to pathologic remodeling of atherosclerotic arteries.

In an attempt to return WSS to arterial physiologic levels (i.e., increase WSS to >10 dynes/cm^2), endothelial cells stimulate vasoconstriction by down-regulating the production of the potent vasodilators, NO, and prostacyclin and up-regulating the generation of vasoconstrictors, such as endothelin-1. Since NO is involved in a broad range of biologic processes, including anti-inflammatory, antiapoptotic, antimitogenic, and antithrombotic actions, decreased NO production is an important pathogenic event in the development of atherosclerosis.[34]

COMPLEX HEMODYNAMICS OF ARTERIAL THROMBOSIS

As is the case for atherogenesis, arterial thrombosis is critically influenced by local hemodynamic conditions. In contrast to the low and/or oscillating WSS environment of atherogenesis,

arterial thrombi develop under conditions of high shear stress where blood experiences relatively rapid and high magnitude changes in flow velocity. Encroachment of a developing plaque into the vascular lumen can introduce significant spatial and temporal gradients in blood flow velocity (Box 29.3). As blood enters a stenosis, it accelerates as the vessel hydraulic area decreases. Maximum fluid WSS occurs near the peak of the stenosis,[35,36] reaching over 100,000 s^{-1} for moderate–severe (>70% by diameter) stenoses.[35,37] Blood decelerates, downstream of the stenosis apex leading to the generation of an adverse pressure gradient for moderate stenoses (20% to 98% for coronaries), which can produce flow separation and recirculation. Flow may eventually transition into turbulent flow, depending on both the flow rate and stenosis severity,[35,38,39] although it remains controversial whether turbulent flow actually occurs in coronary or carotid arteries.

Flow disturbances at sites of arterial thrombosis have been demonstrated to enhance platelet deposition and thrombus growth,[40,41] particularly at the apex,[41–43] but have also been found in recirculation regions[44,45] and near flow reattachment points.[46,47] This may in part be attributable to the trapping of platelets at recirculation sites,[45] as well as enhanced platelet transport at the reattachment point.[22]

In the remaining sections of this chapter, we focus on the mechanisms by which high shear stress promotes platelet deposition and thrombus growth at sites of atherosclerotic plaque disruption. In particular, we discuss emerging evidence that platelet tether formation, shear microgradients (SMGs), and von Willebrand Factor (vWF) elongation plays a potentially important role in promoting high shear-dependent thrombus formation.

Platelet Adhesion Under Shear Flow

The molecular mechanisms by which platelets adhere and form aggregates under high shear conditions have been extensively investigated and are discussed in detail in Chapter 28. They will only be briefly discussed here. The three factors having the greatest impact on the efficiency of platelet accumulation at sites of atherosclerotic plaque disruption include (a) the nature of the thrombogenic substrates, (b) thrombin generation, and (c) flow-directed transport of platelets.

Thrombogenic Substrates

The adhesive substrates mediating platelet adhesion under high shear at sites of atherosclerotic plaque disruption include collagen, vWF, fibronectin, laminin, thrombospondin, and potentially fibrinogen/fibrin and vitronectin. Of these substrates, type I and III fibrillar collagens are typically enriched in advanced atherosclerotic lesions and are the most reactive substrates toward platelets, playing a major role in promoting platelet activation and thrombus growth. Surface binding of vWF to exposed subendothelial collagen is fundamental to the initiation of platelet adhesion under high shear.[48] The vWF macromolecule consists of A1 domains that engage the GPIbα component of the GPIb-V-IX complex.[48,49,50] These bonds are characterized by rapid on/off rates and by "catch" or "flex bond" mechanisms.[21,51] Thus, they cannot mediate stabilized adhesive interactions but rather, support a "flip-flop" rolling or sliding form of platelet translocation. The dynamics of platelet–vWF interaction are influenced by multiple factors including the number, lifetime, and tensile force of vWF–GPIb bonds.[51,52] Collagen binding to platelet receptor GPVI and integrin $\alpha_2\beta_1$ is important for more stable platelet adhesion after a platelet decelerates through vWF–GPIb binding.[53] More stable adhesion occurs primarily through platelet activation *via* the GPVI pathway.[54–57]

Thrombin Generation

α-Thrombin is one of the most potent inducers of platelet activation and plays an important role in regulating the thrombogenicity of atherosclerotic plaques.[58,59] Disrupted plaques are particularly efficient at activating blood coagulation due to the presence of relatively large amounts of tissue factor (TF) in the plaque's necrotic core. TF is primarily derived from apoptotic macrophages and T cells and is expressed on the surface of procoagulant microparticles.[60,61] TF-stimulated thrombin generation, in combination with fibrillar collagen, subsequently promotes the platelet procoagulant response. This local generation of thrombin on the surface of platelets further enhances thrombus growth and stability by activating neighboring platelets and generating fibrin within the developing thrombus. Coagulation reactions typically occur in regions of low (stagnant) blood flow, such as within the confines of a developing thrombus or in downstream low shear pockets, leading to the formation of a "fibrin-tail."[62–65] Flow-dependent removal of activated coagulation factors is an important mechanism preventing excessive thrombin generation and thrombus propagation.[66] Efficient platelet activation at sites of vascular injury is also dependent on the localized generation and release of endogenous platelet agonists, including TxA_2 and ADP. Platelets respond rapidly to soluble agonists by undergoing shape change, calcium mobilization, granule secretion, and activation of one or more surface integrins, leading to the development of stable platelet aggregates that can resist the detaching effects of blood flow.[53,67] The efficiency of platelet activation is partially dependent on the rate and extent of soluble agonist generation, as well as the impact of blood flow on the clearance of agonists from the site of injury.

Flow-directed Platelet Transport

The rate of platelet thrombus growth is also influenced by the efficiency of the flow-directed accrual of platelets to the site of vascular injury. Two of the important effects of shear on platelet transport include (a) the influence of flow on the distribution and localization of blood cells within the vessel lumen and (b) the enhanced rate of platelet collision with the vessel wall (FIGURE 29.3). Blood flow selectively partitions erythrocytes to the center of flow, while platelets are displaced laterally, increasing their effective concentration (up to 15× average concentrations) at the vessel wall.[68,69–71] Supplementing margination under flow is enhanced lateral motion due to platelet collisions, fluid forces, and RBC rotation.[72,73,74] The rate of lateral platelet movement may be four orders of magnitude larger than that predicted for thermal motion alone (diffusion), based on platelet studies at wall shear rates (WSR) below 600 s^{-1} and extrapolations from other solutes (e.g., ferricyanide) for shear rates approaching 5,000 s^{-1}.[72] Significantly, reductions in platelet transport at relatively low WSR (<1,000 s^{-1}) can considerably limit the overall rate of thrombus growth.[75] In contrast, flow-based transport effects within a stenosis can considerably amplify platelet accumulation.

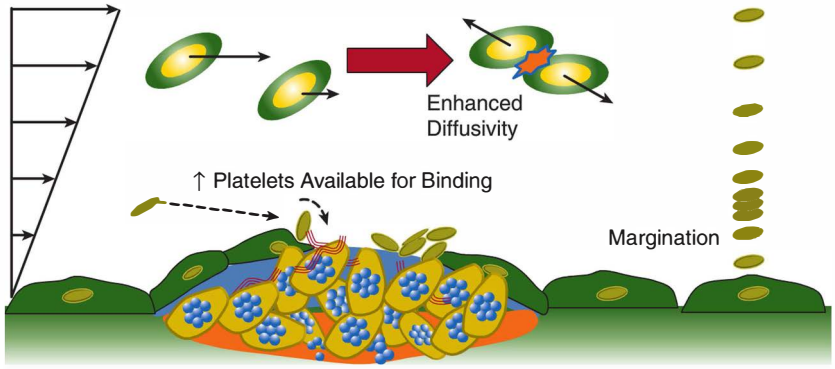


FIGURE 29.3 Platelet transport to a site of vascular injury is enhanced through the presence of red blood cells and shear. Hematocrit increases the number of cell–cell collisions because the number of cells in a given area is increased. Meanwhile, shear increases cell–cell collisions because the relative speed of cells in neighboring flow laminae is enhanced. Increased collisions contribute to enhanced lateral motion (*enhanced diffusivity*), which consequently supports the ability of blood to provide a consistent supply of platelets for hemostasis/thrombosis. Furthermore, platelets are also radially displaced to the vessel walls under flow by red blood cells. This phenomenon of platelet *margination* increases platelet concentration near the vessel wall, further supporting platelet collisions with the vessel wall, allowing platelets to reconnoiter the vessel for injury. (Maxwell MJ, Dopheide SM, Turner SJ, et al. Shear induces a unique series of morphological changes in translocating platelets: effects of morphology on translocation dynamics. *Arterioscler Thromb Vasc Biol* 2006;26(3):663–669.)

Localized Disturbances in Blood Flow and Discoid Platelet Aggregation: the Role of Shear Microgradients

In vivo studies have demonstrated that many of the platelets initially incorporated into the body of forming thrombi maintain their discoid morphology and[76,77] do not elicit a substantial cytosolic calcium response[76,78] or release their granule contents,[79] raising the possibility that platelet stimulation by soluble agonists is not essential for initial thrombus growth. Adhesive interactions between discoid platelets appear to be highly sensitive to local (microscale) changes in shear flow.[80] Thus, discoid platelets adhering to the surface of a growing thrombus experience an initial phase of shear acceleration, followed by a phase of shear deceleration (FIGURE 29.4) that facilitates the development of sustained discoid platelet adhesion contacts with the thrombus surface.[80] Notably, this SMG-dependent platelet aggregation mechanism is insensitive to the effects of ADP, TXA_2, and thrombin antagonists. SMGs are likely to develop on the surface of disrupted plaques and may contribute to the initial formation of platelet thrombi.[41,42,81]

Membrane Tethers Facilitate the Adhesion and Aggregation of Discoid Platelets

The mechanism by which discoid platelets adhere and aggregate under the influence of mechanical forces is primarily dependent on the development of membrane tethers. Investigations of *in vitro* and *in vivo* thrombosis models have demonstrated that discoid platelet capture at the surface of thrombogenic substrates and during platelet–platelet capture is mediated by the formation of specialized membrane adhesion structures that respond reactively to the local shear conditions.[53,82] These studies demonstrate that initial platelet impact with adhesive surfaces results in the pulling of thin filamentous membrane tubules or tethers that form the initial anchoring structure, denoted as a discrete adhesion point (DAP) by Reininger et al.[52] These thin membrane tethers can range in length from 0.91 to 47.90 mm.[52,83] A key observation from these studies has been the demonstration that platelet tether extraction becomes more prevalent with increasing levels of applied shear force.[82] While this may partially be due to the viscoelastic nature of the membrane, it may also suggest that tether generation conveys some

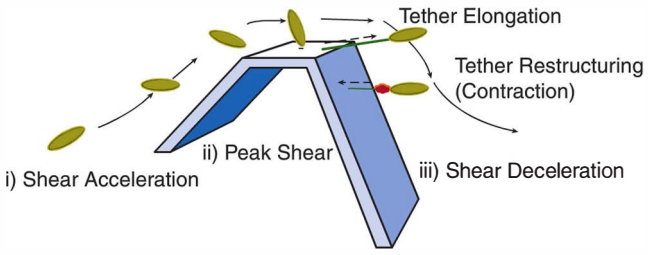
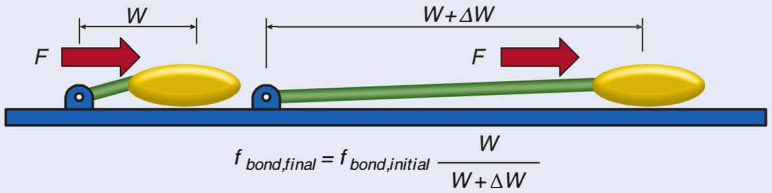


FIGURE 29.4 Platelets in free flow may experience microscale changes in the shear environment within stenoses that prime and subsequently trigger surface contact and subsequent tether formation. Microscale changes in shear following initial tether adhesion triggers localized restructuring of the membrane tether characterized by tether thickening and contractile shortening opposing blood flow leading to semistable platelet aggregate formation. Free-flowing platelets experience a rotational force directed perpendicular to their major axis forcing them toward the adhesive surface. Compressive force directed along the platelets' major axis leads to DAP contact formation forming the site of initial "touchdown" adhesion. Shear and elongational force due to blood flow leads to membrane–cytoskeletal failure initiating viscoelastic tether pulling. Elongation of the forming tether stabilizes the initial adhesive interaction.

Box 29.5 Platelet Tethers Reduce the Required Bond Strength of an Adhered Platelet



Platelet tether formation can reduce the required surface binding force by elongating the tether.

- First, the viscoelastic nature of the tether avoids a high impulse force (sudden acceleration or deceleration of an object) by extending the collision time.
- Secondly, a tether reduces the required strength of adhesive bonds by reducing the vertical component of the tension/force experienced by the bond as the tether extends (**A,B**). The overall bond force, *f*, required to maintain adhesion for a given drag force, *F*, reduces proportionally, if the tether elongates, by the ratio of the initial tether length over the new tether length, based on a simple static analysis.

adhesive advantage as the blood flow environment begins to destabilize adhesion contacts. Indeed, tensile force experienced by adhesion receptor bonds under fluid flow can be reduced by effectively changing the length of the tether, minimizing the tensile force in the normal direction to the surface (Box 29.5).

Platelet Tethers are Reactive Shear Sensors

In general, the biophysical mechanisms underpinning platelet tether formation are not fully understood. Within the context of a generalized cell model (FIGURE 29.4), the extraction of a membrane tether by a sufficiently large pulling force has been proposed to occur *via* a two-phase process characterized by (a) the formation of an initial viscoelastic protrusion at relatively low pulling forces and (b) a secondary phase in which progressive increases in pulling force lead to extraction of a viscous tether at the end of the initial membrane protrusion.[53] Initial filamentous platelet tether formation may be a result of membrane to cytoskeleton association failure and represent a passive phase of viscoelastic membrane pulling. While the membrane properties of the initial extension phase are relatively well understood, the material properties of the secondary phase are poorly defined and complex, involving active cytoskeletal reorganization and receptor-based outside-in signaling, localized to the site of initial adhesion contacts. Significantly, recent studies examining the response of platelet membrane tethers to perturbations of blood flow velocity demonstrate that filamentous platelet tethers undergo dynamic contractile remodeling when the applied shear rate falls below 300 s^{-1}.[80] Thus, dynamic restructuring of tethers may represent an important mechanosensory element of platelet adhesion in response to microscale changes in the hemodynamic environment.

von Willebrand Factor as a Shear Sensitive Thrombogenic Substrate

An important emerging concept in the field is that not only do platelets respond to rapid changes in the shear environment but that immobilized ligands such as vWF also react dynamically to elevated shear stress and more complex shear environments. vWF exhibits a reversible globular transition to a stretched chain-like state at moderate-to-high shear rates (3,181 to 5,000 s^{-1} in solution phase or ~1,000 s^{-1} in whole blood), as determined by atomic force microscopy and epifluorescence microscopy.[84,85] Significantly, flow-based experiments suggest that this transition is reversible upon reduction of the applied shear stress, suggesting that temporally varying shear could result in vWF undergoing multiple rounds of globular-extended transitions. Such large transitions can exist along a rough stenotic surface.[35] Thus, vWF may act as a hemodynamically triggered biopolymer that may undergo conformational activation at sites of acutely elevated shear stress.

The shear-responsive nature of both platelets and vWF may operate in concert to facilitate thrombus formation under pathologic shear conditions. Under conditions where WSS exceeds 100 dynes/cm^2 (5,000 s^{-1}), such as at the apex of stenoses, vWF A1 domains become exposed on plasma vWF multimers that can bind to GPIbα to promote platelet recruitment.[52,84,85,86] Significantly, these high shear aggregates can form in the absence of soluble agonists. Aggregates formed in this way are unstable until the shear stress approaches 400 dynes/cm^2 (20,000 s^{-1}) at which point adherent platelets at the interface of surface-immobilized and membrane-bound vWF are stretched into elongated "beads on a string" -like structures.[48,52,86] These high-shear vWF–platelet aggregates may persist for minutes in the absence of platelet activation and may be a key determinant responsible for acute thrombotic occlusion in stenotic arteries. Thus, the shear-responsive nature of platelets and vWF has evolved to enable efficient thrombus formation under pathologic hemodynamic conditions.

CONCLUDING REMARKS

The impact of hemodynamic parameters on the atherothrombotic process is complex and wide ranging. In addition to the well-defined role of macroscale perturbations of flow in promoting the formation of atherosclerotic lesions, growing bodies of evidence suggest that microscale changes in flow may also play a major role in promoting disease progression and thrombus growth. Multidisciplinary approaches applied to the

atherosclerosis and thrombosis fields are starting to uncover some of the fundamental cellular mechanotransduction mechanisms translating microscale flow changes into single-cell adaptive responses. An increased focus on the interplay between the macro- and microscale effects of blood flow on cardiovascular pathology will be important for a more complete understanding of disease progression and may ultimately lead to the identification of new approaches to limit atherothrombotic complications.

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CHAPTER

30 Platelet Collagen Receptors

STEPHEN P. WATSON, RICHARD W. FARNDALE, MASAAKI MOROI, AND STEPHANIE M. JUNG

Platelets, small anucleate circulating blood cells, are known to be activated by several physiologic substances, including ADP, thrombin, collagen, and adrenaline (epinephrine), as well as some nonphysiologic compounds. Collagen is thought to be one of the important physiologic activators because it powerfully induces platelet aggregation and is abundantly present in subendothelial tissue, which becomes exposed to the blood stream when blood vessels are damaged. Thus, exposed collagen fibers contribute to initial thrombus formation by interacting with circulating platelets. The mechanism of thrombus formation on collagen surfaces under blood flow has been studied by many investigators using *ex vivo* flow chamber systems designed to approximate the physiologic environment in blood vessels. This section provides a historical context for the platelet collagen receptors and subsequent sections provide details about our present knowledge of how these receptors initiate and facilitate the transmission of signals to result in thrombus formation.

After the high-affinity ligand–receptor interaction concept was established in the 1960s, such a stimulus-response reaction was also hypothesized for collagen–platelet interactions. Because platelets react specifically with collagen, a specific receptor for collagen was expected to be present on the platelet surface, but its identity remained elusive for many years because its ligand, collagen, a complex and insoluble macromolecule, is unlike classical ligands. This means that collagen may have numerous possible interactions, many of which would be of low affinity. Because of these obstacles, the collagen–platelet interaction was assessed only by collagen-induced platelet aggregation, not an ideal parameter since this response is influenced by numerous other platelet activating reactions, not only those directly related to platelet binding of collagen. Consequently, many proteins have been reported to be the candidates for platelet collagen receptors, even though they do not meet all the criteria for a physiologic receptor.

The first hypothesis, proposed by Jamieson et al.,[1] suggested that the collagen receptor was an enzyme, a collagen glucosyltransferase that works as a receptor through an enzyme–substrate interaction. This hypothesis was supported by reports showing that a glycopeptide fragment of chicken collagen induced platelet aggregation just like native collagen fibers. Later, Chiang et al. purified a protein that specifically interacted with the chicken collagen fragment and proposed that this was the collagen receptor, but subsequent investigators could not verify that this was the case. During this period, other proteins were suggested to be receptors including, fibronectin, GPIIb/IIIa (integrin $\alpha_{IIb}\beta_3$), thrombospondin, factor XIII, propolypeptide of von Willebrand Factor (vWF), and CD36 (GPIV). Although these proteins interact with collagen, to date, none have been confirmed to contribute significantly to the platelet–collagen interaction, particularly under physiologic conditions. The major platelet collagen receptors were subsequently found by a combination of biochemistry and, in the case of glycoprotein VI (GPVI), careful studies of patient platelets deficient in the receptor, as discussed below.

INTEGRIN $\alpha_2\beta_1$ (GPIa/IIa)

The integrins are heterodimers composed of an α and a β subunit selected from 18 α subunits and 8 β subunits, each a separate gene product, combining in 24 permitted pairings.[2] Key species are $\alpha_2\beta_1$, the only integrin collagen receptor expressed on the platelet; the fibronectin and laminin receptors, $\alpha_5\beta_1$ and $\alpha_6\beta_1$; and the fibrinogen receptor, $\alpha_{IIb}\beta_3$. $\alpha_2\beta_1$ also has laminin-binding activity. The integrin subunits each have a short, conserved cytoplasmic domain, a single transmembrane span, and a long glycosylated stem supporting the head group, where the two subunits interact strongly and where ligand-binding occurs. The α subunit head is a 7-bladed β-propeller.

Integrins fall into two categories, defined by the presence within the α subunit of an I domain, so named because it is inserted between blades 2 and 3 of the β-propeller. This 200 amino-acid domain has a similar structure to the A domains of vWF, and is sometimes called the vWF A-domain. In these integrins, which include $\alpha_2\beta_1$, the α subunit I domain contains the ligand-binding site,[3] comprising an electronegative DxSxS motif that forms part of the Mg^{2+}-binding metal ion-dependent adhesion site (MIDAS). This MIDAS Mg^{2+} ion is in turn coordinated by an amino-acid carboxylate anion in the ligand.

In the second category of integrins, those lacking an α subunit I domain, such as $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$, the ligand-binding site is found in a very similar structure, namely the β subunit I domain. For $\alpha_{IIb}\beta_3$, the ligand carboxylate anion might be the aspartate found within the RGD motif of the α chain of fibrinogen or in the C1 domain of vWF. Cooperative binding of such extended, polymeric proteins may occur through secondary ligand, for example, KQAGDV in the γ chain of fibrinogen[4] binding to the α subunit β-propeller that forms the largest segment of the integrin head. Regulation involves the β subunit MIDAS, and two other cation-binding sites, the ADMIDAS (adjacent to MIDAS) and LIMBS (ligand-induced metal ion binding site).[5]

In 1985, Nieuwenhuis et al.[6] reported a patient who had defective collagen-induced platelet aggregation and a bleeding tendency in association with a marked decrease in GPIa.*

*The loss of collagen-reactivity in this patient was later shown to be due to a more generalized defect in adhesion molecules and was not specific to collagen.

Around the same time, Santoro[7] provided biochemical evidence suggesting that GPIa interacts with collagen. Later, GPIa on the platelet membrane was shown to be present as a complex with GPIIa, the GPIa/IIa complex.[8] Analyses of membrane proteins interacting with extracellular matrix proteins were performed for many different cells, leading to identification of very late activation antigens (VLA) of lymphocytes, cell adhesion receptors of leucocytes, and extracellular matrix receptors (ECMRs) of fibroblasts; the proteins interacting with collagen in these systems, VLA-2 and ECMR-II, were thus identified. It was subsequently discovered that GPIa/IIa, VLA-2, and ECMR-II were all the same protein, which was then designated as integrin $\alpha_2\beta_1$, establishing that integrin $\alpha_2\beta_1$ was a collagen receptor present in many cell types. The structural details of $\alpha_2\beta_1$ are shown in FIGURE 30.1 and are described below, although one defining characteristic of $\alpha_2\beta_1$, as related to its ability to interact with its ligand collagen, should be noted here. Since collagen is insoluble, the interaction of collagen and platelet $\alpha_2\beta_1$ is usually analysed by adhesion assay, a condition in which platelet activation and interaction of $\alpha_2\beta_1$ with collagen occur concomitantly and thus integrin activation cannot be differentiated from collagen adhesion. However, Stephanie Jung and Masaaki Moroi[9] at Kurume University succeeded in separating these reactions by kinetic analyses with soluble collagen III monomers and demonstrated that $\alpha_2\beta_1$ must be activated to exhibit high-affinity binding to collagen III, the resting state showing almost no binding activity toward this ligand.

GLYCOPROTEIN VI

Although $\alpha_2\beta_1$ was established as a collagen receptor on platelets, there was a report suggesting the presence of an approximately 60-kDa collagen-reactive platelet protein on platelets based on patient studies. In 1987, Sugiyama et al.[10] reported a patient

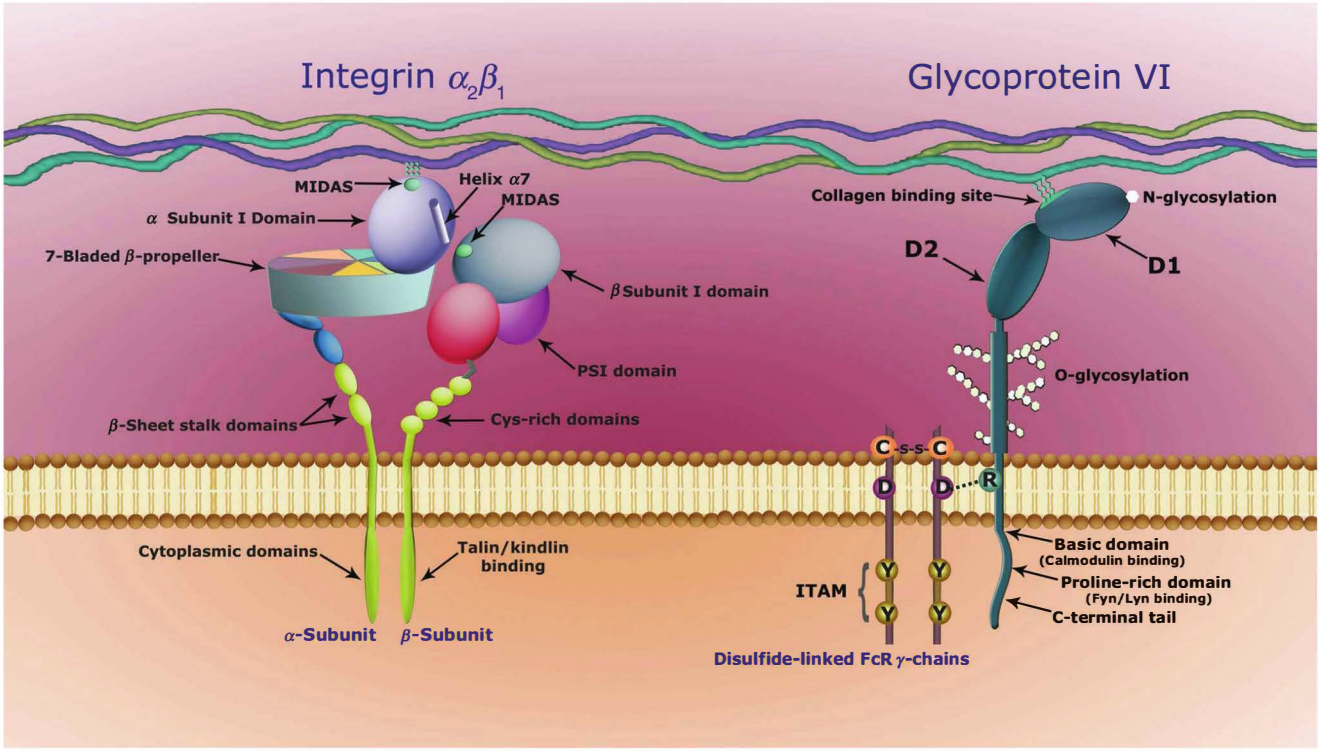

FIGURE 30.1 Structures of platelet collagen receptors. (Left) Integrin $\alpha_2\beta_1$: the extracellular domain of the α_2 subunit contains the I domain and its collagen-binding site. The I domain is located between blades 2 and 3 of the α subunit β-propeller. Helix 7, within the I domain, links the collagen-binding MIDAS with its counterpart in the β subunit I domain. The head of the β subunit also contains a hybrid domain (red), comprising polypeptide that is proximal to the I domain and a distal segment that leads into the plexin–semaphorin–integrin (PSI) domain, where the *N*-terminus is located. The β subunit stem contains cysteine-rich EGF domains. The cytoplasmic domains contain binding sites for numerous signalings, and adapter and cytoskeletal components, similar to focal adhesion components, and talin and kindlin binding regulate the activation of the integrin. (**Right**) Glycoprotein VI: The extracellular domain of GPVI contains two Ig-like domains (D1 and D2), and between the Ig-like domains and the transmembranes lies a mucin-like Ser/Thr-rich domain (O-glycosylation). A putative binding site for collagen is formed by interaction of two D1 domains when GPVI forms a dimer. The D1 domain may contain a glycosylation site (Asn72) for an *N*-linked carbohydrate chain. The presence of a charged residue (Arg252) in the transmembrane–membrane domain enables GPVI to associate with an FcR chain, which contains a negatively charged Asp residue. The cytoplasmic portion of GPVI consists of four regions: juxtamembrane portion; basic region, which contains a calmodulin binding site; proline-rich domain, to which Fyn and Lyn bind to through their SH3 domains; and the *C*-terminal region. Mouse GPVI is truncated, lacking the latter domain.

with idiopathic thrombocytopenic purpura (ITP) whose platelets were defective in collagen-induced aggregation. Her serum contained an antibody to a platelet protein of approximately 60 kDa, although her own platelets did not contain this antigen and lacked reactivity to collagen. These results suggested that the antigen, designated P62, may be a receptor for collagen or a protein involved in a collagen–platelet interaction. This was supported by studies of another patient whose platelets were deficient in GPVI. No one had studied the characteristics of this protein at that time, but it was named according to its mobility in nonreduced–reduced two-dimensional SDS gel electrophoresis according to the convention of Phillips and Agin.[11] The second patient's platelets also lacked collagen-induced aggregation, while showing no abnormalities in aggregation induced by other platelet agonists. The antibody from the serum of the first patient[10] did not react with platelets from the GPVI-deficient patient, indicating that P62 and GPVI were the same proteins. These results strongly suggested that GPVI was a newly defined platelet collagen receptor.

There were essentially no studies on GPVI for some years after its discovery because of the lack of tools to analyze this protein except for the patient autoantibody. While searching for platelet-reactive sites of collagen, Michael Barnes and Richard Farndale at Cambridge found that a collagen-mimetic peptide that was called collagen-related peptide (CRP), containing 10 repeats of the sequence Gly-Pro-Hyp (GPO), induced platelet aggregation and this reaction was independent of $\alpha_2\beta_1$.[12] Finally, the era of intense investigations on GPVI as platelet collagen receptor was initiated. Steve Watson's group in Oxford identified the Fc receptor γ chain as a critical component in collagen signaling in platelets that shortly thereafter was shown to associate with GPVI and to be essential for platelet activation.[13–15] CRP reacted specifically with GPVI[16] and could stimulate tyrosine phosphorylation in platelets in a manner similar to collagen.[17] A snake venom protein, convulxin, first identified in the late 1970s, was subsequently found to induce a pattern of tyrosine phosphorylation similar to that induced by collagen, including phosphorylation of the FcR γ chain. The identification of materials that specifically reacted with GPVI and activated platelets facilitated the purification and cloning of GPVI[18] and also the analysis of its function.

Human GPVI is a member of the immunoglobulin superfamily and is encoded in the leucocyte receptor complex on chromosome 19; the FcR γ chain is encoded on chromosome 1. GPVI possesses an *N*-terminal and a *C*-terminal extracellular Ig domain, designated D1 and D2, linked by a single peptide strand that allows some flexibility in the head of the receptor (FIGURE 30.1). A glycosylated stem connects D2 to the transmembrane domain and thence to the relatively short (50 amino acids) cytoplasmic domain that can bind calmodulin close to the plasma membrane, and the Src family kinases Fyn and Lyn through a proline-rich domain closer to the *C*-terminal cytoplasmic tail (see below). The transmembrane domain contains an arginine residue that forms a salt bridge with an aspartate residue in the equivalent location of FcR γ chain and supports the association between the two.[18] Coexpression of FcRγ with GPVI is required for cell-surface expression of the latter in platelets.

In 2006, the crystal structure of GPVI was described by Andrew Herr et al.[19] revealing interactions between two copies of the receptor that might form the basis for physiologic dimerization.[20] A collagen binding site, located largely within D1, was modeled onto the receptor, consistent with the effect of site-directed mutagenesis on ligand binding.[21] Whether this model properly reflects the interaction of GPVI with insoluble collagen has yet to be resolved. More recently, a crystal structure and binding study of LAIR1, a single Ig domain receptor that shares substantial homology with D1 of GPVI, led to the suggestion of a nearby but discrete ligand-binding site in GPVI.[22] These differing proposals will be fruitful areas for research.

GPVI has also been suggested to bind laminin, which is a major component of the basement membrane.[23] The interaction with laminin is dependent on integrin $\alpha_6\beta_1$ and leads to platelet spreading, but laminin is unable to induce aggregation of platelets in suspension, possibly because it has a 10-fold lower affinity for GPVI than collagen. Laminin is the major platelet-reactive component in the basal lamina, and, like collagen IV, is unable to activate platelets (see below). The physiologic significance of the interaction of platelets with laminin may be to mediate sealing and repair of small areas of damage to the vessel in the absence of thrombus formation.

Thus, platelets have two collagen receptors: $\alpha_2\beta_1$ that has higher affinity for collagen, but requires activation for its affinity to be fully expressed, and GPVI that activates platelets. Kinetic studies using recombinant GPVI indicated that the immunoglobulin receptor had a weaker affinity for collagen than $\alpha_2\beta_1$.[24] Under physiologic conditions, these two receptors would work synergistically, with $\alpha_2\beta_1$ supporting adhesion and GPVI supporting activation, and thus each contributing to effective thrombus formation. The use of GPVI- and $\alpha_2\beta_1$-specific peptides as surface coatings for *in vitro* flow studies reveals the need for both receptors along with vWF for full platelet aggregation to take place.[25]

There is also evidence in the literature that platelets may express one or more further receptors for collagen that may support adhesion and possibly activation. For the most part, this evidence is linked to a single study or a handful of studies that have implicated certain proteins, such as GPV, or have demonstrated signaling events that cannot be attributed to activation of GPVI and integrin $\alpha_2\beta_1$.[26] The overall significance of these putative receptors in platelet activation by collagen is uncertain and has been reviewed elsewhere.[27]

THE CLASSIC TWO-STEP, TWO-SITE MODEL AND THE CURRENT MODEL FOR PLATELET ADHESION

Any model proposed for how the platelet collagen receptors induce platelet activation must take into account the differential roles of $\alpha_2\beta_1$ and GPVI. In 1989, Michael Barnes reported that platelets could adhere to several specific cyanogen bromide (CB) peptides of collagen I and III, but only a subset of these peptides exhibited aggregatory activity.[28] This suggested that the collagen molecule may contain sites specific for platelet adhesion that are different from those that stimulate aggregation. Around this time, Samuel Santoro proposed the two-step, two-site model, based on chemical modification of collagen,[29] in which collagen has a higher-affinity binding site for $\alpha_2\beta_1$, for the adhesion reaction, and a lower-affinity binding site for a second receptor, presumably the one responsible for platelet activation. The development of peptide libraries (Toolkits)[30] based on the primary sequence of collagen type III by Richard Farndale's

group at Cambridge further advanced our understanding of the initial binding interaction between platelets and collagen. The various $\alpha_2\beta_1$-binding sites were defined,[31,32] and the activating site within CB4 of collagen III was identified as binding GPVI.[33] After GPVI was found to be a collagen receptor on platelets, this two-step, two-site model was applied to GPVI and integrin $\alpha_2\beta_1$ to explain their separate roles in the platelet–collagen interaction: $\alpha_2\beta_1$ would bind first, assisting the other collagen receptor GPVI to bind and initiate the activation of platelets. However, the finding that integrin $\alpha_2\beta_1$ exists in a resting state that exhibits low affinity for collagen III and an activated high-affinity state induced by platelet agonists mandated a reappraisal of this model: it is now thought that platelets first interact with collagen through GPVI, activate $\alpha_2\beta_1$, and the activated $\alpha_2\beta_1$ binds strongly to collagen, resulting in initiation of a cascade of reactions to activate platelets. However, it should be noted that the traditional two-step, two-site model and the currently accepted model are not mutually exclusive, since it may be possible that both types of receptor–collagen interactions may occur under physiologic conditions. Even in nominally "resting" platelets, the presence of a low level of the active, high-affinity form of $\alpha_2\beta_1$ may contribute to platelet adhesion to collagen and it is also possible that the resting receptor may be able to interact with the higher-affinity binding sequences in collagen type I (see below).

THE ROLE OF COLLAGEN IN THROMBUS FORMATION

The molecular events underlying thrombus formation in the high flow rates in arteries and arterioles are depicted in FIGURE 30.2. Platelets marginate along the vessel walls because of the large mass of red blood cells that flows in the centre of the vessels. Platelets are kept in a quiescent state by the continuous release of nitric oxide and prostacyclin from the vascular endothelial cells and by removal of low levels of ADP and ATP that have been released from damaged cells by apyrase-like activity of endothelial CD39. Damage to the endothelial surface exposes collagen fibers that capture vWF from the flowing blood through its collagen-binding A3 domain.

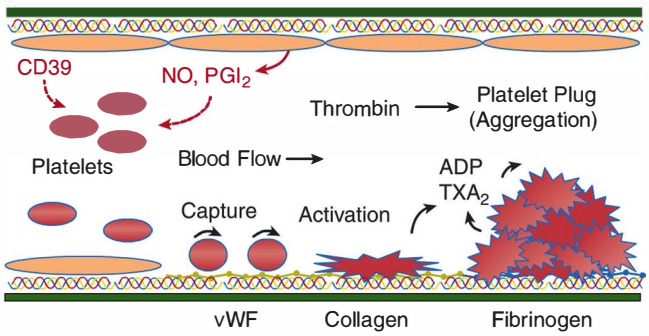

FIGURE 30.2 Role of collagen in platelet activation. Damage to the endothelial cell wall exposes subendothelial collagen fibers that bind to von Willebrand factor (vWF) in the bloodstream. Platelets are captured (or tethered) through the interaction of vWF and GPIb-IX-V complex on the platelet surface. This enables binding of collagen to GPVI, which stimulates activation of integrins $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$. In turn, the integrins bind to vWF and collagen, respectively, resulting in a stable adhesion. GPVI also promotes platelet spreading and secretion of the feedback agonists ADP and TxA_2 that activate circulating platelets. Cross-linking of platelets by fibrinogen mediates platelet aggregation.

vWF mediates platelet tethering through a very fast on-rate of association to the GPIb-IX-V complex on the platelet surface. The GPIb-IX-V complex is a unique receptor in the genome and mutations in GPIbα, GPIβ, or GPIX subunits give rise to the severe bleeding disorder Bernard Soulier syndrome due to loss of the capacity for platelet tethering, as well as variable thrombocytopenia. The interaction between GPIbα and the A1 domain of vWF, however, does not lead to rapid, stable adhesion because of a fast off-rate of dissociation. This can be illustrated experimentally by the rolling of platelets under flow conditions on an immobilized vWF surface.

In the subendothelial wall, platelet tethering facilitates the interaction of fibrillar collagen with the low-affinity GPVI receptor complex, stimulating powerful activation of the integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$. These two integrins mediate stable adhesion through binding to collagen and vWF, respectively. Additionally, GPVI stimulates platelet spreading, thereby strengthening the interaction with the subendothelial matrix. The action of GPVI is supported by much weaker signals from the GPIb-IX-V complex and integrins $\alpha_2\beta_1$ and $\alpha_{IIb}\beta3$. The relative contribution of each receptor to platelet activation is unclear as all four receptors signal through overlapping pathways with critical roles for Src and Syk tyrosine kinases, and phospholipase Cγ2 (PLCγ2).

Platelet activation by GPVI causes ADP release from platelet dense granules and *de novo* synthesis of TxA_2 from arachidonic acid. These secondary mediators interact synergistically to mediate activation of platelets that have become newly tethered to $\alpha_{IIb}\beta_3$-bound vWF. In turn, plasma fibrinogen, which is present in molar excess over other adhesion proteins, mediates platelet aggregation by cross-linking the integrin $\alpha_{IIb}\beta_3$. Thus, aggregate formation is critically dependent on the secondary mediators ADP and TxA_2 that signal through the $P2Y_1$ and $P2Y_{12}$ receptors and the TP thromboxane receptors, respectively. $P2Y_1$ and TP receptors signal through activation of PLCγisoforms downstream of the heterotrimeric G protein, Gq. The $P2Y_{12}$ receptor signals through the Gi family of heterotrimeric G proteins and undergoes a powerful synergy with signals from both Gq and tyrosine kinase-linked receptors.

Activated platelets provide a procoagulant surface that leads to formation of thrombin through the coagulation cascade. Thrombin reinforces platelet activation through the protease activated receptors (PARs), PAR-1 and PAR-4, which are also coupled to Gq. In addition, thrombin cleaves fibrinogen to fibrin, thereby further strengthening the growing thrombus, and this is further cemented by clot retraction mediated through actin–myosin contraction mediated downstream of integrin $\alpha_{IIb}\beta_3$. Together these interactions generate a robust thrombus that is able to withstand the high shear forces found within the arteriolar circulation.

In situations of mild injury, the roles of GPVI and integrin $\alpha_2\beta_1$ are redundant with those of other signaling receptors and adhesive integrins, respectively. The level of expression of $\alpha_2\beta_1$ on the platelet surface is approximately 1% to 2% of that of integrin $\alpha_{IIb}\beta_3$, which therefore dwarfs its role in supporting adhesion. This is illustrated by the absence of patients

with platelet-bleeding disorders that can be attributed solely to a reduction in functional $\alpha_2\beta_1$, whereas there are several hundreds of patients with function disrupting mutations in integrin $\alpha_{IIb}\beta_3$, a condition known as Glanzmann thrombasthenia. Very few patients have also been reported with functional defects in GPVI associated with a bleeding diathesis. Furthermore, in the majority of these cases, the loss of GPVI is mediated by an immune thrombocytopenia and is associated with a marked reduction in platelet count. There are currently just two patients with a bleeding diathesis in which the only known platelet defect is the loss of functional GPVI as a result of inheritance of function-disrupting mutations in the glycoprotein receptor. This very low number of patients can be explained by redundancy with other platelet signaling receptors such as those for thrombin, which is generated by exposure of tissue factor on the surface of subendothelial smooth muscle and other cells, and therefore also plays a role in the initial events that trigger activation.

Mice deficient in GPVI and/or the integrin α_2 subunit are viable and can be used to investigate the relative contributions of the two collagen receptors in platelet activation. The majority of results are in line with those in patients and confirm that neither GPVI nor integrin $\alpha_2\beta_1$ is essential for aggregate formation, with deficiency of either receptor giving rise to a mild bleeding diathesis or no apparent loss of clotting ability, dependent on the nature of the test.

Thus, neither collagen receptor is essential for platelet activation although both clearly make important contributions following severe injury where limiting the loss of blood as rapidly and effectively as possible is paramount. However, it should be emphasized that the mild nature of the bleeding diathesis in the absence of GPVI or $\alpha_{IIb}\beta_3$ should not be construed as evidence against a significant physiologic role of these proteins in response to major injury. Indeed, a similar situation applies to the receptors for TxA_2 and ADP, in that defects in either receptor gives rise to a mild bleeding diathesis with only a handful of patients identified world wide. The apparent paucity of patients with defects in these receptors and their mild bleeding symptoms can be attributed to the redundancy between receptors in regulating platelet activation.

COLLAGEN STRUCTURE AND COLLAGEN MIMETICS

In the healthy vessel wall, collagens are concealed beneath the vascular endothelium and are only exposed to circulating platelets in disease or upon injury. The endothelium rests on a basal lamina composed of a collagen IV network, with laminins, entactin, and perlecan as important co-constituents. Deeper in the vessel wall, in the tunica media, the fibrillar collagens I and III are the major forms of collagen expressed by vascular smooth muscle, along with several others, including the minor fibrillar collagen V and the nonfibrillar collagens VI, VIII, and XVI. A key feature of the fibrillar collagens is their ability to assemble as an organized, rope-like structure. The fundamental tropocollagen unit is a triple-helical (COL) domain of approximately 1,000 amino acids with short nonhelical *N*- and *C*-terminal telopeptide extensions. The COL domain has four complete D-periods of 234 amino acids, and a fifth, partial D-period. Tropocollagen monomers align side-by-side and end-to-end within a fibril, with a fixed, one D-period offset

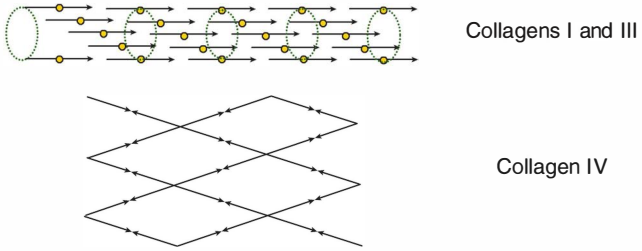

FIGURE 30.3 Structure of major blood vessel wall collagens. Each triple-helical collagen monomer is represented by an *arrow*, with the head at the *C*-terminus. In collagen fibers (I and III), monomers are aligned end-to-end with a gap between them, and each monomer is approximately 300 nm long. Cylindrical packing leads to a lateral separation between monomers of approximately 3 nm. Binding sites (*blue dot*) are aligned (*dashed circles*), and must appear on the fiber surface at least on every fifth monomer and perhaps more frequently. (Herr AB, Farndale RW. Structural insights into the interactions between platelet receptors and fibrillar collagen. *J Biol Chem* 2009;284:19781–19785). In collagen IV, monomers are arranged head-to-head and tail-to-tail forming a square-mesh network. Lateral separation between any binding motifs in collagen IV is approximately 800 nm. (Diagram after Ricard-Blum S. The collagen family. *Cold Spring Harb Perspect Biol* 2011;3(1):a004978.)

between adjacent molecules, indicating that a gap between the ends of tropocollagen molecules is needed to complete the partial fifth D-period (FIGURE 30.3). This periodic structure gives rise to the regular striations observed in transmission electron micrographs of tendon and other collagen preparations.[34] Fiber diffraction studies indicate that the next level of organization is the microfibril, comprising five tropocollagen strands, each offset longitudinally by one D-period and each stepping across to an adjacent axial position at each, less tightly packed, gap region.[35] Thus, each microfibril adopts a twisting structure. The collagen fiber is assembled as concentric layers of microfibrils within a fiber, giving rise, in cross section, to ordered quasihexagonally packed sectors with disordered liquid crystalline regions between them.[36,37] A consequence of this ordered fiber structure is that binding sites for platelet receptors and other relevant molecules such as vWF are not uniformly distributed on the fiber surface, but may be presented as coherent bands around the circumference of the fiber, allowing platelet receptors to be recruited in closely packed clusters that may be required to induce signaling.[38]

Receptor clustering is not readily achievable by collagen IV or other nonfibrillar collagens where triple-helical collagen monomers are not in close juxtaposition. Although the primary sequence of such collagens may contain motifs that can bind to platelet receptors, for example, GPO-rich regions in collagen IV that might in principle bind GPVI, these may be too far separated to support receptor clustering and signaling. Whilst recognition sequences in adjacent collagen monomers in a fiber lie <10 nm apart, in native collagen IV, they will be separated by approximately 800 nm, the effective mesh size of the collagen IV network. These considerations are depicted in FIGURES 30.3 and 30.4.

It is not yet clear which regions of the collagen monomer are available on a fiber surface. Some recognition motifs may

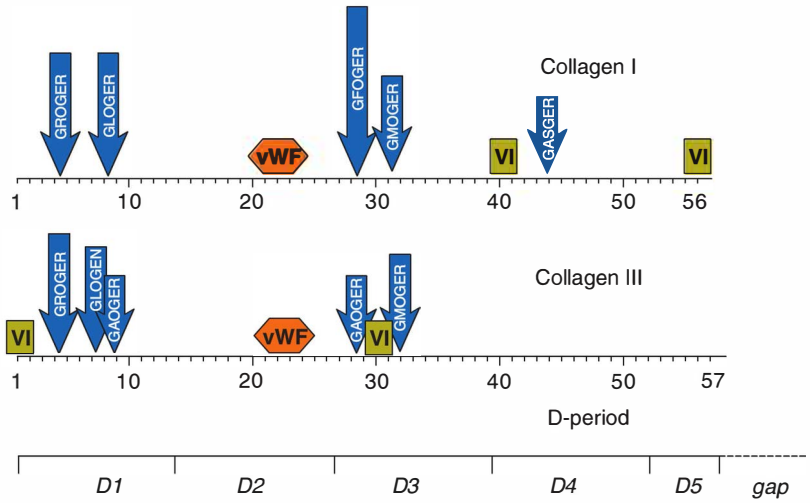

FIGURE 30.4 Receptor binding sites within collagens I and III. Peptide Toolkits allowed binding sites for GPVI (*squares*) and vWF (*hexagons*) to be mapped onto collagen monomers. Sites for $\alpha_2\beta_1$ are shown as *arrows*, where the length increases with intrinsic affinity of the site, and the motif is provided within the *arrow*. Numbers indicate the Toolkit peptide number. (Farndale RW, Lisman T, Bihan D, et al. Cell-collagen interactions: the use of peptide Toolkits to investigate collagen-receptor interactions. *Biochem Soc Trans* 2008;36:241–250.) Sites for collagen I are deduced from the highly-conserved collagen III Toolkit. Alignment of sites with D-period is indicated below.

be systematically concealed within the fiber by its precise architecture,[37] although this is debated.[38] However, a consequence might be that increased cell reactivity will result as fibers begin to disassemble, which might occur through limited proteolytic cleavage during inflammation or other disease.

Synthetic collagen-mimetic peptides, notably CRP,[39] are useful laboratory reagents because they contain receptor-binding motifs. GPO-rich peptides with more than 8 or 10 triplets will spontaneously adopt a triple-helical conformation, essential for interaction with the collagen receptors. If they contain suitable terminal residues, for example, Cys or Lys,[12] they can be chemically cross-linked to introduce polymeric structure that can support signaling through GPVI. In this cross-linked form, the peptide is known as CRP-XL, but without cross-linking, the peptide serves as an antagonist or partial agonist at GPVI.[12,40] The peptide can be made more potent by increasing its length,[41] though such materials remain to be developed for routine use.

The identification of motifs that bind $\alpha_2\beta_1$, typically GxOGER motifs,[42,43] has allowed the construction of triple-helical ligands of differing affinities for $\alpha_2\beta_1$. The most widely used and highest-affinity motif is GFOGER, derived from the α_1 chain of collagen I, but also found elsewhere including the α_1 and α_5 chains of collagen IV. This motif binds resting platelet $\alpha_2\beta_1$, but lower-affinity motifs, widespread in the collagens, such as GLOGER, GMOGER, and GAOGER, show increased binding upon platelet activation.[31] These motifs may be synthesized within GPO triplets that confer GPVI-binding activity on the peptide,[44] or within GPP triplets, that have sufficient propensity to form stable triple-helices but with little or no ability to bind GPVI. These peptide reagents are useful as surface coatings for research or diagnostic use, and have helped to establish the role of $\alpha_2\beta_1$ as necessary but not sufficient for thrombus deposition *in vitro* at low-to-moderate shear, and as contributing to the adhesion process at higher, vWF-dependent arterial shear rates.[25] It is noteworthy that collagens IV and V, although as replete with potential GPVI-binding sites (GPO triplets) as collagens I and III, are relatively poorly endowed with GxOGER motifs that bind $\alpha_2\beta_1$. Their lack of ability in native form to activate platelets no doubt results from a combination of inappropriate quaternary assembly of triple-helical monomers and scarcity of integrin-binding sites.

ANTIBODIES

Antibodies are important tools for basic research and may potentially have clinical applicability. An autoantibody from the blood of a patient with ITP was the first documented anti-GPVI antibody. Since IgG is bivalent, the patient's antibody induced platelet activation by cross-linking two GPVI molecules, but its monovalent Fab fragment inhibited platelet activation.[10] Most of the anti-GPVI antibodies developed subsequently also have these characteristics. A number of mAbs against GPVI were produced by hybridoma technology, including JAQ-1, 204-11, 9O12.2, and HY101, although OM4 was produced using knockout mice. Success has also been achieved using phage display that screens clones of Fab or scFv recognizing a specific epitope of GPVI. Examples of antibodies obtained in this way are 10B12, A10, and m-Fab-F, an antibody that recognizes GPVI dimer present on the platelet surface but does not bind to GPVI monomer and therefore is a powerful tool for detecting receptor dimerization (and activation—see below).

Many monoclonal antibodies against integrin $\alpha_2\beta_1$ have been developed, and unlike the GPVI antibodies, many are available commercially. These include activating antibodies such as TS2/16 that convert the inactive integrin to the active form.[45] Several sites on the β_1 chain have been identified as epitopes of the activating antibodies. Integrin $\alpha_2\beta_1$–specific inhibitory antibodies have also been reported. One of these antibodies, 6F1, developed by Coller et al.,[46] was reported to inhibit or delay platelet aggregation to collagen dependent on the conditions of the assay.

SNAKE VENOM AND OTHER TOXINS

Several toxins from venomous snakes have been found to activate platelets through GPVI. The best known is the C-type lectin convulxin from the South American rattlesnake *Crotalus terrificus*. Convulxin was proposed as a collagen mimetic in the late 1970s, but it was only after the identification of the GPVI-FcR γ chain complex that it was recognized to induce platelet activation through this glycoprotein receptor. Since then several other snake C-type lectin receptors, and also the metalloproteinase-disintegrin alborhagin, have been shown to activate platelets

through GPVI. GPVI-activating toxins have also been described in scorpion venom. In all cases, the toxin is believed to induce marked clustering of GPVI generating a powerful signal that leads to platelet activation.

The toxins are nonfibrillar and do not contain a collagenous GPO motif; thus they do not mimic collagen activation of GPVI. Several of the toxins may also bind to other platelet glycoproteins, thereby limiting their utility as GPVI-specific reagents. This includes convulxin that binds weakly to GPIb, although it is unclear if this influences its ability to induce platelet activation. The majority of the toxins, including the snake C-type lectins, are multimers of heterodimers, and cannot be readily expressed. They are available only as purified components, which brings the risk of contamination with other platelet-reactive agents.

GPVI SIGNALING

GPVI is the major signaling receptor for collagen on the platelet surface with the key functional motif being an immunoreceptor tyrosine-based activation motif (ITAM) in the associated FcR γ chain. ITAMs constitute the major signaling arm of many Fc and antigen receptors, including the low-affinity immune receptor, FcγRIIA, which is also expressed on platelets, and T- and B-cell antigen receptors. ITAMs are characterized by the sequence, $YxxLx_{6\text{-}12}YxxL$ with the two conserved tyrosines being critical for cell activation.

A related motif, known as a hemITAM and characterized by a single YxxL sequence downstream of a triacidic amino acid sequence, has recently been shown to signal in a similar manner to ITAM receptors. One of the founder members of the hemITAM family is expressed on platelets, namely the C-type lectin receptor, CLEC-2 (for review see[47]). The ligand for CLEC-2 is the transmembrane protein podoplanin, which is widely expressed with high levels on lymphatic endothelial cells, type 1 lung alveolar cells and kidney podocytes, but it is noticeably absent from vascular endothelial cells. The interaction between podoplanin on lymphatic endothelial cells and CLEC-2 on platelets plays a critical role in the separation of the lymphatic system from the cardinal vein.

The GPVI cytosolic tail contains a proline-rich region that confers binding to the Src kinases Fyn and Lyn through their SH3 domains.[48] This interaction places the two Src kinases in a "ready-to-go" position to mediate phosphorylation of the FcR γ chain ITAM, although deletion of this proline-rich region impairs but does not abrogate signaling by GPVI demonstrating the presence of an additional pathway of activation.[49] Clustering of GPVI leads to Src kinase–dependent phosphorylation of the two conserved tyrosines in the ITAM and subsequent binding and activation of the tandem SH2 domain-containing Syk tyrosine kinase. This leads to activation of Syk through autophosphorylation and phosphorylation by Src family kinases.

Syk initiates a downstream signaling cascade leading to formation of a signalosome that consists of a series of adapter and effector proteins (for review see[47]). Adapters are proteins with no enzymatic activity, but they serve as scaffolds for other proteins. The transmembrane protein, the linker for activation of T cells (LAT), plays a key role in GPVI signaling by localizing the signalosome to specialized cholesterol-rich regions in the membrane known as lipid rafts that are enriched in signaling proteins, including Src kinases with the exception of Src itself. LAT contains several conserved tyrosines that are phosphorylated by Syk, enabling binding to SH2 domains in the adapter proteins Grb2 and Gads, and also to PLCγ2. Gads is constitutively associated with the adapter SLP-76 and both proteins are recruited to the signalosome by phosphorylation of LAT. Several other effector proteins assemble into the signalosome including the tyrosine kinases Btk and Tec, the GTP exchange factors Vav1 and Vav3, the small GTPase Rac, and the PI 3-kinase α, β, and γ isoforms. Studies of mutant mice have shown that these proteins play varying roles in supporting activation of PLCγ2, which can range from relatively insignificant to being critical for activation of the phospholipase.[47]

At present, only two proteins have been found to be essential for activation of PLCγ2 by GPVI, namely Syk and SLP-76. LAT plays an important role in mediating activation, but its absence is overcome in response to high concentrations of GPVI ligands, including collagen. One explanation for the partial role of many of these signaling proteins is that of redundancy with other proteins in the same family. For example, while the absence of the GTP exchange factors, Vav1 or Vav3, has a minimal effect on the response to GPVI, mice deficient in both factors show a dramatic inhibition in response.[50] A related example is that of mice deficient in the tyrosine kinase Btk that have a marked reduction in platelet aggregation, whereas in mice lacking the related Tec family kinase, namely, Tec itself, aggregation is normal. On the other hand, the combined absence of Btk and Tec leads to abrogation of aggregation to GPVI ligands, including collagen.[51] Platelets from patients with the immunodeficiency disorder, X-linked agammaglobulinemia, which is caused by mutations in Btk, also exhibit a reduced response to collagen, but do not have a bleeding diathesis, presumably because of the redundancy with Tec.

PLCγ2 generates the two second messengers, inositol 1,4,5-trisphosphate (IP^3) and 1,2-diacylglycerol (DG) that release intracellular Ca^{2+} and activate protein kinase C respectively. The activation of PI 3-kinases generates a third second messenger, phosphatidylinositol 3,4,5-trisphosphate (PIP_3). These three second messengers synergize to mediate the majority of the responses associated with platelet activation including secretion, aggregation, procoagulant activity, and actin polymerization. Activation of platelets through GPVI undergoes a marked synergy with the platelet $P2Y_{12}$ receptor consistent with the feedback role of the Gi-coupled receptor in regulating activation.

Several other proteins play critical roles in GPVI signaling. In the absence of other agonists, the threshold for activation of the pathway is set by the membrane tyrosine phosphatase CD148.[52] Src family kinases are regulated through phosphorylation on stimulatory and inhibitory tyrosines. The phosphorylated inhibitory tyrosine residue (Tyr529 in Src and Tyr507 in Lyn) mediates intramolecular binding to the SH2 domain in Src kinases. Dephosphorylation of this residue is critical for activation of Src kinases and activation is further enhanced by phosphorylation of the stimulatory tyrosine. The tyrosine phosphatase CD148 is the major phosphatase that mediates dephosphorylation of Src kinases, thereby setting the threshold for platelet activation.

A second family of regulatory proteins belong to the immunoreceptor tyrosine-based inhibition motif (ITIM) family. This includes the immunoglobulin-gene family members PECAM-1 and G6b-B. In platelets, the ITIM in PECAM-1 is inducibly phosphorylated, while that in G6b is constitutively phosphorylated. Both proteins bind to the tandem SH2 domain-containing

tyrosine phosphatases, SHP-1 and SHP-2. It has been proposed that the recruitment of the two tyrosine phosphatases to PECAM-1 reduces signaling in platelets by GPVI and, to varying degrees, by other surface receptors, although the overall magnitude of this effect is small. By contrast, constitutively phosphorylated G6b-B has been proposed to play a critical role in inhibiting signals from GPVI and other platelet glycoprotein receptors in the absence of ligand engagement, thus preventing uncontrolled platelet activation.[53]

Platelet activation by GPVI is also regulated by other inhibitory proteins, including the SH2 domain-containing phosphatidylinositol 3,4,5-triphosphatase (SHIP), which removes the second messenger lipid PIP_3. It is noteworthy that signaling through GPVI is relatively insensitive to elevation of the cyclic nucleotides cAMP and cGMP, whereas they inhibit the action of the two G protein-coupled receptor feedback agonists, TxA_2 and ADP.

MODE OF ACTIVATION OF GPVI—DIMERIZATION

GPVI is expressed as a complex with the FcR γ chain that has a dual tyrosine phosphorylation site in its ITAM. The Src tyrosine kinases Lyn and Fyn are constitutively associated with GPVI. Thus, the clustering of GPVI by antibodies, snake toxins, or collagen induces colocalization of Lyn and Fyn with the FcR γ chain ITAM, allowing the ITAM to be phosphorylated.

Crystallographic studies using recombinant proteins indicated that GPVI has the potential dimerize, raising the possibility that this is required for formation of a collagen-binding site. This is supported by the observation that recombinant GPVI dimer can engage collagen under conditions where monomer cannot.[24] This suggests that dimerization or oligomerization of GPVI is an important event in platelet activation. At least some of the GPVI on resting platelets may be dimeric,[54,55] and an antibody against the GPVI dimer (GPVI-fc_2), m-Fab-F, has been developed that only recognizes the dimeric protein.[54] A model for the involvement of GPVI dimers in platelet activation is proposed in FIGURE 30.5: one GPVI molecule associates with the disulphide FcR γ chain as shown for other receptors in this class.[56] In this model, quiescent platelets have both GPVI dimers and monomers (upper left), which may exist in some type of equilibrium or with the number of dimers regulated. Upon vessel injury, the exposed endothelial collagen can bind to the pre-existing GPVI dimers, which may exist in small numbers but show high affinity for collagen. Upon collagen engagement, signaling is initiated and stabilization of dimers and/or

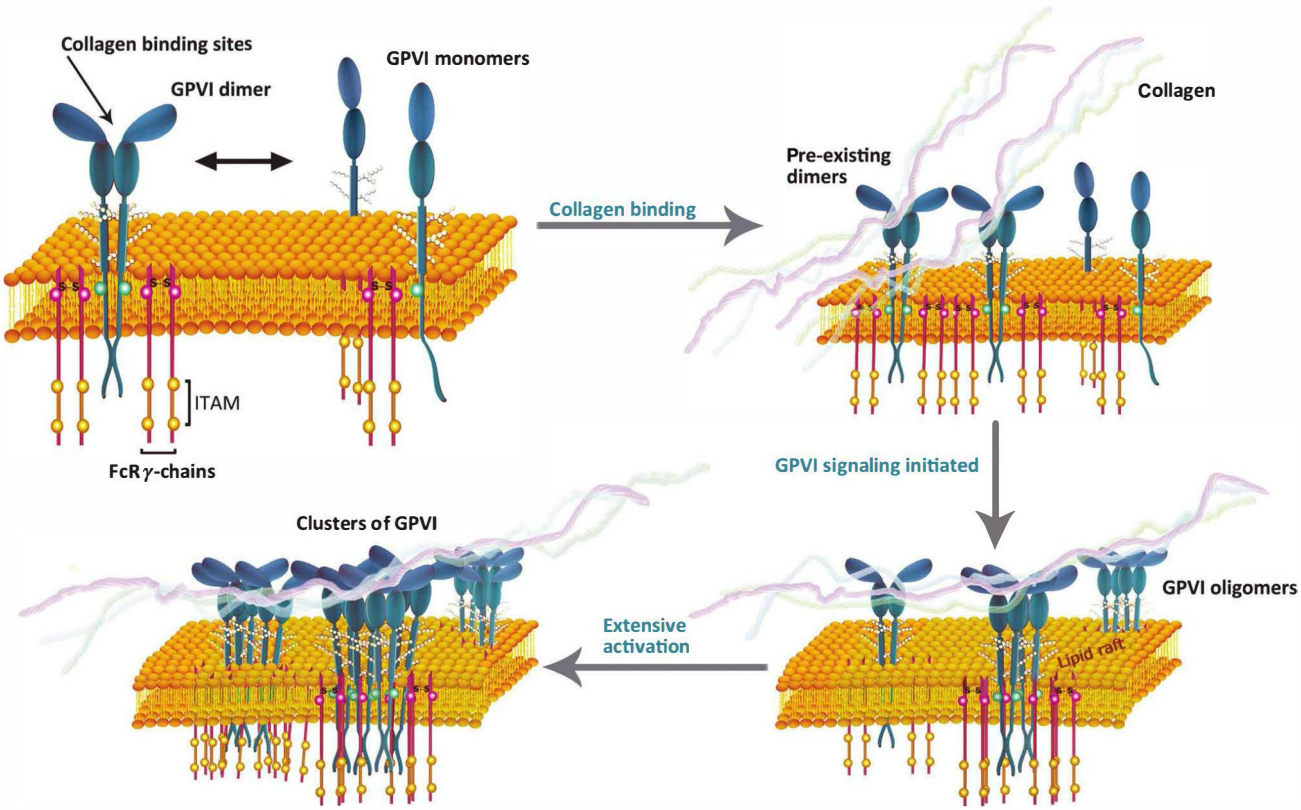

FIGURE 30.5 Mechanism of activation of GPVI by dimerization. **Upper left**: There is an equilibrium between the monomeric and dimeric forms of GPVI in resting platelets. A small amount of GPVI dimer is sufficient to bind to collagen, thus initiating the GPVI–signaling cascade (**upper right**). Collagen binding initiates signaling and stable dimers and/or oligomers of dimers form. This formation of stable dimers or oligomers of dimers may be mediated by translocation of GPVI molecules to lipid rafts and may involve reorganization of the cytoskeletal proteins (**lower right**). Activation results in further clustering of GPVI molecules, facilitating signaling reactions by juxtaposing the components of the GPVI signalosome at high local density (**lower left**).

oligomerization of dimers occurs (lower right). Because GPVI is present in lipid rafts in platelets,[57] newly formed GPVI oligomers would colocalize with the constitutive raft protein LAT, facilitating formation of the GPVI signalosome and platelet activation. Upon extensive activation, clusters of GPVI are formed, further increasing activation (lower left).

GPVI SHEDDING AND INTERNALIZATION

Programmed autologous cleavage of platelet receptors has emerged in recent years as an important mechanism for preventing the adhesive-signaling function of platelets, potentially acting to limit thrombus formation and thrombus stability (for review see[58]). Proteolytic activity can be mediated through metalloproteinase-induced removal of ligand-binding domains or by cleavage of cytoplasmic tails by intracellular proteases such as calpain. The former releases soluble ligand-binding domains, or ectodomains, and is a potential biomarker of platelet activation. Receptor shedding is mediated in platelets by two widely expressed members of the ADAM family of metalloproteinases, ADAM10 and ADAM17 (also termed TACE). These metalloproteinases are expressed on the cell surface in inactive forms but can be activated by a variety of stimuli including receptor activation.

GPVI is rapidly shed from the platelet surface *in vitro* following platelet activation by a variety of GPVI–specific ligands, including convulxin, collagen, and specific antibodies, releasing the 55 kDa ectodomain into the supernatant and retaining the 10 kDa membrane stalk. Shedding of GPVI can also be induced by activation of the low-affinity immune receptor, FcγRIIA, which also signals through an ITAM-dependent pathway, and by *N*-ethylmaleimide or mitochondrial damage (which activate ADAMs). By contrast, minimal shedding of GPVI is seen following stimulation by G protein–coupled receptor agonists.

GPVI shedding *in vitro* in response to GPVI ligation is mediated by ADAM10 and is believed to involve dissociation of calmodulin from a positively charged, amphipathic, juxtamembrane sequence in the cytoplasmic tail of the collagen receptor. Calmodulin dissociation can also be induced by W-7 in association with shedding of GPVI. By contrast, mitochondrial damage induces shedding of GPVI *in vitro* through ADAM17. Shedding of GPVI in response to GPVI ligands is not associated with loss of GPIbα, although shedding of the vWF receptor can be induced by W-7 through activation of ADAM17. Thus, the nature of the receptors that are shed and the protease responsible varies according to the mechanism of activation.

Bernard Nieswandt's group was the first to report that shedding, internalization, and transient thrombocytopenia can be induced *in vivo* in mice by antibodies to GPVI.[59] GPVI shedding can also be induced in human platelets that have been injected into a NOD/SCID mouse and treated with antihuman GPVI antibodies. Remarkably, shedding of GPVI *in vivo* in response to antibody challenge is independent of ADAM10 and ADAM17 and appears to be regulated by the intracellular level of cAMP.[60]

$\alpha_2\beta_1$ SIGNALING

There are approximately 1,500 to 2,500 copies of $\alpha_2\beta_1$ on platelets. In contrast, there are approximately 80,000 copies of the major platelet integrin $\alpha_{IIb}\beta_3$ on the resting platelet surface and another 40,000 copies on the δ-granule membrane that become exposed following platelet secretion. Integrin $\alpha_{IIb}\beta_3$ was the first glycoprotein receptor shown to stimulate tyrosine phosphorylation in platelets and has served as a major model for our understanding of integrin signaling in platelets and in many other cell types. It has also served as a model for our understanding of other platelet integrins, including integrin $\alpha_2\beta_1$ (for review see[61]).

$\alpha_{IIb}\beta_3$ signals through sequential activation of Src family kinases, notably Src and Fyn, and Syk kinase, independent of an ITAM. In turn, Syk initiates a downstream signaling cascade that culminates in activation of PLCγ2. Many of the proteins in this cascade are similar to those used by GPVI, including the adapter protein SLP-76, Vav and Tec family kinases, and PI 3-kinase α and β isoforms. On the other hand, the $\alpha_{IIb}\beta_3$ signaling cascade is independent of LAT as the integrin is excluded from lipid rafts where LAT is localized. The absence of an ITAM and a role for LAT in $\alpha_{IIb}\beta_3$ signaling may explain why it induces a much weaker signal than that of GPVI, despite having at least a 10-time higher level of expression.

$\alpha_2\beta_1$ generates a weak intracellular signal through Src and Syk family kinases and PLCγ2,[62] with the key events being similar to those of $\alpha_{IIb}\beta_3$. Relatively few studies have focused on $\alpha_2\beta_1$ signaling because of the powerful response initiated by GPVI relative to that of $\alpha_2\beta_1$-specific ligands, including antibodies and $\alpha_2\beta_1$-specific peptides. For example, mouse platelets only undergo partial spreading on an $\alpha_2\beta_1$-specific peptide, but undergo full spreading on immobilized CRP or in the presence of a G protein–coupled receptor agonist such as thrombin. Similarly, $\alpha_2\beta_1$-specific peptides do not induce aggregation even in the presence of threshold concentrations of G protein–coupled receptor agonists that induce integrin activation. Accordingly, signaling through integrin $\alpha_2\beta_1$ induces weak platelet activation.

It is unclear whether the weak signal from integrin $\alpha_2\beta_1$ is of functional relevance given the much more powerful signal elicited by GPVI. Its role may be restricted to supporting platelet adhesion and collagen binding to GPVI, thereby indirectly potentiating platelet activation as outlined above. The full significance of integrin $\alpha_2\beta_1$ signaling in hemostasis however is unclear because of the similarity in its signaling pathway to those of GPIb-IX-V and GPVI.

OUTSIDE-IN AND INSIDE-OUT SIGNALING THROUGH INTEGRIN $\alpha_2\beta_1$

The binding of collagen to $\alpha_2\beta_1$ and the conformational changes that mediate inside-out and outside-in signaling are now understood in some detail.[63] The α subunit MIDAS binds the glutamate of collagen GxOGER motifs described below. At its base, the α subunit I domain contacts and obscures the β subunit MIDAS, preventing direct ligand-binding activity of the β subunit.[2] Helix α_7 within the I domain moves up and down to regulate the binding affinity of the integrin for collagen, and the lower end of this helix contains a glutamate residue that engages the β subunit I domain. Thus, there is co-ordinated ligation of both integrin subunits by collagen, first, directly through the α subunit MIDAS, and then through the β subunit MIDAS by the Helix α_7 glutamate residue.[5,64] This molecular machinery is believed to mediate inside-out signaling, where cytoplasmic signals regulate the binding of species such as talin and kindlin to the β subunit tail.[65] This is proposed to separate the integrin

subunit stems, causing conformational change that culminates in the β subunit MIDAS pulling down on Helix α_7, opening up the α subunit MIDAS to allow collagen binding.[63] The binding of collagen then mediates outside-in signaling, with conformational changes upon ligation being transmitted through the β subunit stem to the cytoplasm.

The model integrin-binding motif within collagen is GxOGER. The highest-affinity motif found to date, occurring in the vessel wall collagens I and IV, is GFOGER.[32] Its phenylalanine residue binds snugly into a hydrophobic pocket on the α_2 I domain,[63] and GFOGER binds well to resting platelets, a capacity that does not increase upon platelet activation.[31] Other less-favorable sequences with hydrophobic residues in the x position occur elsewhere in collagen I and in collagen III.[31,41] For such sequences, there is a marked increase in binding upon platelet activation.

Thus, we envisage a situation where initial platelet binding to collagen, subsequent under high shear conditions to interaction of immobilized vWF with platelet GPIb/V/IX, will occur preferentially through high-affinity interaction with GFOGER, but after activation by collagen interaction with GPVI, other lower-affinity sequences become more important. Thus, an activated platelet will find a greater number of integrin binding sites within collagen, and so the tightness of the interaction will increase, facilitating weaker interactions with GPVI.

CONCLUDING REMARKS

Collagen is the most thrombogenic component in the vessel wall matrix and induces platelet adhesion through integrin $\alpha_2\beta_1$ and platelet activation through GPVI. The two receptors interact synergistically, with signals from GPVI activating the integrin and adhesion to the integrin increasing the binding of collagen to GPVI. Both receptors also work in combination with other platelet receptors to rapidly induce powerful platelet activation at sites of damage to the vasculature. Significantly, neither receptor appears to be essential for platelet regulation, as mice deficient in either GPVI or the integrin subunit α_2 present with mild defects in hemostasis and the two known patients with GPVI deficiency have a mild bleeding diathesis. There are no patients who are known to lack integrin $\alpha_2\beta_1$. These observations should not be taken as evidence against a significant physiologic role for the two collagen receptors in hemostasis as both almost certainly play vital roles in helping to prevent life-threatening bleeds following severe injury.

There has been considerable speculation as to whether GPVI and integrin $\alpha_2\beta_1$ are clinically relevant targets for development of novel antiplatelet agents that will have only minor effects on bleeding. Arterial thrombosis is frequently associated with rupture of atherosclerotic plaques in the vessel wall that leads to exposure of fibrillar collagen and powerful platelet activation. Several studies have shown effective platelet inhibition by targeting of GPVI in mouse thrombosis models using either a specific antibody to deplete GPVI from the platelet surface or recombinant dimeric GPVI to prevent binding of platelets to collagen. Antibody-induced depletion of GPVI, as pioneered by the Nieswandt group, is a particularly attractive form of prophylaxis as a single injection induces a sustained loss of the surface glycoprotein receptor with only a transient drop in platelet count.

It is becoming increasingly clear that platelets play additional roles beyond their established role in hemostasis including development of the lymphatic system and in angiogenesis. Collagen has a ubiquitous distribution in the body and platelets constantly come into contact with collagen at sites of damage to the vasculature and possibly via migration of platelet-leucocyte aggregates through the vessel wall. As we begin to learn more about the role of platelets beyond hemostasis in both health (e.g., lymphatic development[47]) and in disease (tumour metastasis[66] and rheumatoid arthritis[67]), it seems inevitable that we will identify new and further exciting roles for the interaction of collagens with platelet GPVI and integrin $\alpha_2\beta_1$.

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CHAPTER

31 Integrin $\alpha_{IIb}\beta_3$ and Platelet Aggregation

ANA KASIRER-FRIEDE, KAMILA BLEDZKA, JUN QIN, EDWARD F. PLOW, AND SANFORD J. SHATTIL

PERSPECTIVE

In 1977, Phillips and Agin[1] provided important insight into the hemostatic function of platelets by demonstrating that two major glycoproteins, subsequently named IIb (GP IIb) and β_3 (GP IIIa), were missing from platelets of patients with Glanzmann thrombasthenia, a rare inherited bleeding disorder characterized by the absence of platelet aggregation. Earlier, Nurden and Caen[2] had observed an abnormal gel pattern of membrane proteins of thrombasthenic platelets. Subsequently, it was found that α_{IIb} and β_3 are subunits of a noncovalent complex, $\alpha_{IIb}\beta_3$.[3,4] A second inherited disorder, afibrinogenemia, provided additional insight into the mechanisms of platelet aggregation. Platelets from patients with afibrinogenemia[5] or washed normal platelets suspended in defibrinated plasma[6] fail to aggregate; readdition of fibrinogen to the samples restored their aggregation response. In the late 1970s and 1980s, the connection between these observations was established when fibrinogen was found to associate with ADP-stimulated platelets,[7] bind specifically to $\alpha_{IIb}\beta_3$,[8–10] and mediate platelet aggregation.[10,11] In the mid-1980s, another series of observations impacted on our understanding of the biology of $\alpha_{IIb}\beta_3$ when it was discovered that $\alpha_{IIb}\beta_3$ is a member of a widely distributed family of structurally, immunologically, and functionally related adhesion receptors,[12,13,14] the integrins, so called because they *integrated* their extracellular ligands with the intracellular cytoskeleton.[15] Thus, discoveries from studies of one integrin apply broadly to other family members.

These seminal observations have provided the underpinnings for investigations in many laboratories into the structure of $\alpha_{IIb}\beta_3$ and its function in platelet adhesion, aggregation, and hemostasis. Molecular details of how fibrinogen and other ligands interact with $\alpha_{IIb}\beta_3$ to effect platelet aggregation and adhesion have been defined. This work ultimately led to the development of parenteral $\alpha_{IIb}\beta_3$ antagonists for use in the prevention and treatment of arterial thrombosis in specific clinical settings.[16] More recently, it has become apparent that integrins, including $\alpha_{IIb}\beta_3$, function not only as adhesion receptors but also as conduits for *inside-out* signals to regulate ligand binding to their extracellular domains and *outside-in* signals to regulate anchorage-dependent cellular responses, which in platelets include full aggregation and granule secretion as well as clot retraction. This chapter focuses on $\alpha_{IIb}\beta_3$ and its role in platelet aggregation and signaling.

EXPRESSION AND BIOSYNTHESIS OF $\alpha_{IIb}\beta_3$

$\alpha_{IIb}\beta_3$ synthesis is restricted primarily to megakaryocytes and consequently protein expression is limited to these cells and to platelets. However, α_{IIb} expression has also been detected on early hematopoietic progenitors and on mast cells where in the latter it plays a role in adhesion and in the response of these cells to chronic inflammation.[17,18] Restricted $\alpha_{IIb}\beta_3$ expression is imposed by the α_{IIb}-subunit because its promoter limits the expression to these hematopoietic cells.[19,20] This is not the case for β_3 since the integrin $\alpha_V\beta_3$ is expressed by many cell types, including platelets, endothelial cells, and smooth muscle cells. The genes for α_{IIb} and β_3 reside in close physical proximity on chromosome 17.[21] α_{IIb} and β_3 must associate in a 1:1 noncovalent complex for efficient cell-surface expression,[22] and naturally occurring mutations that alter the biosynthesis of one subunit affect surface expression of the other (e.g.,[23–25]). The association of nascent α_{IIb} and β_3 occurs in the endoplasmic reticulum.[24,26] Although α_{IIb} is synthesized as a single polypeptide chain, it is proteolytically cleaved into heavy and light chains by a furin-like enzyme in the Golgi complex. However, proteolytic cleavage is not required for $\alpha_{IIb}\beta_3$ expression on the cell surface nor is it required for $\alpha_{IIb}\beta_3$ function.[27] $\alpha_{IIb}\beta_3$ is the most abundant cell-surface glycoprotein on platelets, present at approximately 80,000 copies per cell.[28,29] In addition, an intracellular pool of $\alpha_{IIb}\beta_3$ is present in the membranes of α-granules and can become surface expressed upon granule secretion, increasing the number of functional $\alpha_{IIb}\beta_3$ receptors by approximately 30% to 50%.[28,30,31] Rapid shuttling of $\alpha_{IIb}\beta_3$ between the cell surface and intracellular pools has been observed[32] and may be involved in the import of fibrinogen into platelet α-granules.[33]

STRUCTURE OF $\alpha_{IIb}\beta_3$

α_{IIb} and β_3 consist of 1,008 and 762 amino acids, respectively.[34,35] The extracellular domains of both subunits are glycosylated.[36] As assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions, the apparent molecular weights of the α_{IIb}- and β_3-subunits are approximately 130 kDa and 95 kDa, respectively.[22] Upon reduction, the α_{IIb}-subunit separates into heavy and light chains with molecular weights of 115 and 25 kDa, respectively. The α_{IIb} light chain contains a 20–amino acid cytoplasmic tail (CT), a transmembrane (TM) region, and an extracellular segment that is disulfide linked to the heavy chain. The β_3-subunit is a single polypeptide chain and contains 56 cysteine residues that form an extensive array of intrachain disulfide bonds.[4] A schematic model depicting the domain organization of $\alpha_{IIb}\beta_3$ is shown in FIGURE 31.1. There are 16 domains in $\alpha_{IIb}\beta_3$: 6 in α_{IIb} and 10 in β_3, including their TM and CT domains.

Structure of α_{IIb}

The *N*-terminus of α_{IIb} consists of seven approximately 60–amino acid repeats that fold to form a seven-bladed β-propeller. At the base of the β-propeller are four divalent cation-binding

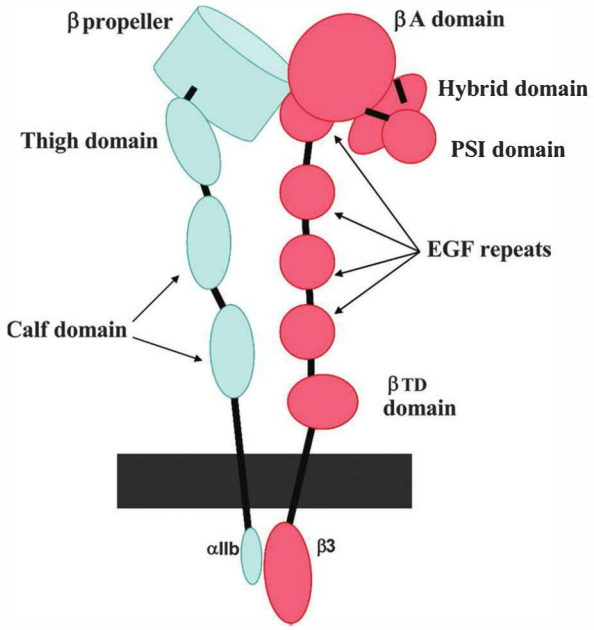

FIGURE 31.1 Schematic model showing the organization and structural domains of $\alpha_{IIb}\beta_3$. The α-subunit is in blue and the β-subunit is in red. PSI, plexin–semaphorin–integrin; EGF, epidermal growth factor, β-TD.

motifs in which oxygenated residues within short hairpin loops coordinate divalent cations. The remainder of the α_{IIb} extracellular region consists of a "thigh" and two "calf" domains. Between the thigh and the first calf domain is a "genu," a flexible segment that allows the molecule to bend and compact. The α_{IIb} TM domain is primarily helical.[37,38] Two available nuclear magnetic resonance (NMR) structures of the TM domain differ in the vicinity of the inner-membrane leaflet[37,38]: one indicates that the helix is followed by an inverse turn, the other indicates a continuous helix. These differences will be important to resolve since several regulators of $\alpha_{IIb}\beta_3$ function are purported to bind to this region of α_{IIb}.[39] Similarly, there are at least two different NMR structures for the α_{IIb} CT. In one structure, the membrane proximal region of the CT is composed of a short helix which terminates in a turn midway in the α_{IIb} CT and an acidic *C*-terminus.[40,41] A cation-binding site is present in this acidic *C*-terminal region, and occupancy of this site stabilizes the folding of the α_{IIb} CT.[40,42] In a second structure, the region following the membrane proximal helix is intrinsically disordered, consistent with a lack of hydrophobic residues to facilitate folding.[43]

Structure of β_3

The *N*-terminus of β_3 consists of two tandem nested domains. The first is the βA-domain whose fold is homologous to the I-domains present in the α-subunits of β_2-containing integrins, as well as the α-subunits α_1, α_2, α_{10}, α_{11}, and α_E (see FIGURE 31.1).[44] The βA-domain is inserted in the "hybrid" domain whose fold is similar to I-set Ig domain. The hybrid domain is itself inserted in the PSI (*p*lexin–*s*emaphorin–*i*ntegrin) domain that contains the *N*-terminus of β_3. The *C*-terminus of the PSI domain is contiguous with a protease-resistant region composed of a series of disulfide repeats arranged into four EGF-like domains. The final structural motif in the β_3 ectodomain is the βTD which extends to the outer leaflet of the plasma membrane.

The β_3A-domain contains three divalent cation-binding sites: a metal ion–dependent adhesion site (MIDAS) motif that is involved in ligand binding, a site adjacent to MIDAS termed ADMIDAS that when occupied by Ca^{2+} inhibits ligand binding, and a ligand-induced metal-binding site, also known as the synergistic metal ion–binding site or SyMBS.[45] The β_3A-domain is folded into an α-β-α sandwich in which six central β-sheets are surrounded by seven α-helices. A loop on its upper surface termed the "specificity-determining loop" or SDL, in conjunction with a "cap" composed of four loops on the upper surface of α_{IIb} β-propeller, forms a surface for ligand binding. The *C*-terminus of the PSI domain is contiguous with a protease-resistant region composed of a series of disulfide repeats arranged into four EGF-like domains. The final structural motif in the β_3 ectodomain is the βTD which extends to the outer leaflet of the plasma membrane.

The β_3 TM domain is a typical α-helix that extends into the CT. The helix is followed by a loop containing a kink,[38,46,47] a turn, and then by another short helix.[46] Noteworthy features of the β_3 CT are two NXXY motifs, NPLY747 and NITY759. In general, NXXY motifs bind proteins containing phosphotyrosine binding (PTB) domains and this is also true of the two motifs in $\alpha_{IIb}\beta_3$.[48,49] The interaction of these NXXY motifs with the PTB domains of binding partners has been implicated in regulating $\alpha_{IIb}\beta_3$ activation. The significance of the distal helix is demonstrated by the effects of the naturally occurring S752P mutation invariant Glanzmann thrombasthenia which disrupts the *C*-terminal helix, prevents binding of the kindlin-3 FERM domain to the β_3 CT, and impairs $\alpha_{IIb}\beta_3$ activation.[50,51]

Interdomain and Intersubunit Interactions of $\alpha_{IIb}\beta_3$

Each of the major regions of α_{IIb} and β_3, that is, the ectodomains, the TM helices, and the CTs, interact with each other to establish an inactive heterodimeric structure. While there are no disulfide bonds between the integrin α- and β-subunits, intra- and interdomain disulfide bonds play a significant role in maintaining the overall conformation of the integrin.[45,52,53]

The binding sites for fibrinogen and other adhesive ligands are located within a globular head formed by the interaction of the β-propeller in α_{IIb} with the β_3 A-domain.[54] Chemical cross-linking,[55,56] synthetic peptides,[57–59] and site-directed antibodies[60] have identified specific sequences as ligand-binding sites within $\alpha_{IIb}\beta_3$. Recent crystal structures of the $\alpha_V\beta_3$[53,61] and the $\alpha_{IIb}\beta_3$ extracellular domains complexed with peptides and small-molecule ligands[54] have further refined the location of the binding sites. Thus, fibrinogen binds at the interface between the α_{IIb} β-propeller and the β_3A-domain with β_3 contacts that include not only the MIDAS, but also a water-mediated interaction with the ADMIDAS, and with α_{IIb} contacts in a groove between loops that connect the 2nd and 3rd and 3rd and 4th blades of the propeller. It is noteworthy that some of the drugs that inhibit ligand binding to $\alpha_{IIb}\beta_3$ do so through allosteric mechanisms, that is, they change the conformation of the receptor, rather than binding directly at the site that engages macromolecular ligands.[54]

Two smaller discontinuous interactions between α- and β-subunits in the stalk and between the head and the stalk further stabilize the heterodimeric structure of the inactive ectodomain.[54,62] The α- and β-subunit TM helices also interact with each

other through multiple residues and cross at a 25- to 30-degree angle.[37,38] The membrane-proximal portions of CTs of the two subunits also interact weakly through electrostatic and hydrophobic bonds.[41,50] On the basis of mutagenesis experiments, it has been proposed that a salt bridge formed between α_{IIb} residue R995 and β_3 residue D723 is critical for maintaining $\alpha_{IIb}\beta_3$ in an inactive state,[63] but a structure of sufficiently high resolution to directly visual the salt bridge is lacking. Nonetheless, based on mutational analyses,[37,38] it is clear that changes in the interactions between the α_{IIb} and β_3 TM and CT domains regulate the ability of $\alpha_{IIb}\beta_3$ to bind extracellular matrix ligands.

PLATELET AGGREGATION IS MEDIATED BY $\alpha_{IIb}\beta_3$

The primary function of $\alpha_{IIb}\beta_3$ is to serve as a receptor for macromolecular ligands[22,64] including fibrinogen, fibrin, von Willebrand factor (vWF), fibronectin, vitronectin, and thrombospondin (reviewed in [65]). All of these ligands are constituents of extracellular matrices where their interactions with $\alpha_{IIb}\beta_3$ can support platelet adhesion under a variety of conditions.[66–68] Each ligand can also interact with at least one additional receptor on platelets. For example, fibrinogen can bind to $\alpha_V\beta_3$,[69] and $\alpha_5\beta_1$,[70] vWF to GPIb/IX/V,[71] fibronectin to $\alpha_5\beta_1$,[72] vitronectin to $\alpha_V\beta_3$,[73] and thrombospondin to GPIV (CD36).[74,75] Platelet aggregation requires engagement of $\alpha_{IIb}\beta_3$ by soluble fibrinogen[8,10,11] and/or vWF.[68] At high shear rates, such as those attained in stenosed arteries, vWF binding to $\alpha_{IIb}\beta_3$ is necessary for productive platelet aggregation.[68,76–78]

Ligand Recognition Specificity of $\alpha_{IIb}\beta_3$

Fibrinogen binding to activated $\alpha_{IIb}\beta_3$ is divalent cation dependent, rapid, and initially reversible, with a dissociation constant (K_d) of approximately 300 nM.[9,79,80,108] Stabilization then occurs, and the bound fibrinogen becomes essentially nondissociable.[9,79,80,81–83] This transition stabilizes platelet aggregates and presumably depends upon the formation of additional contacts between fibrinogen and $\alpha_{IIb}\beta_3$.[81,84] Stabilization may be promoted by multiple factors, including ligand-induced structural changes in the receptor,[82,85] receptor clustering, cytoskeletal reinforcements,[86] fibrinogen self-association, association of fibrinogen with other adhesive proteins, such as thrombospondin,[87,88] and receptor internalization.[89] Although the dimeric structure of fibrinogen and the multimeric structure of vWF may permit these ligands to bridge between receptors on adjacent platelets and thereby mediate aggregation,[90] such cross-bridging may not be the only mechanism that determines the size of platelet aggregates.[91,92] Postreceptor occupancy events occur upon binding of fibrinogen to $\alpha_{IIb}\beta_3$,[93] and are important for maximal aggregation. One direct manifestation of receptor occupancy is the induction of ligand-induced binding sites, which serve as epitopes for monoclonal antibodies that react with the occupied, but not with the resting or the activated but unoccupied conformers of $\alpha_{IIb}\beta_3$.[94–96] Occupancy of $\alpha_{IIb}\beta_3$ also leads to a series of intracellular responses, referred to as *outside-in signaling* (see section on Regulation of Platelet Function by $\alpha_{IIb}\beta_3$).[97,98,99]

Two synthetic peptides of four to six amino acids define the recognition specificity of fibrinogen for $\alpha_{IIb}\beta_3$.[100] The first of these peptides corresponds to the extreme *C*-terminus of the fibrinogen γ chain.[101] The *C*-terminal six amino acids of the γ chain are KQAGDV, and peptides corresponding to this sequence, or variants thereof, collectively referred to as the γ chain peptides, interact with $\alpha_{IIb}\beta_3$.[102] This sequence is present in fibrinogen but not in other $\alpha_{IIb}\beta_3$ ligands. Nevertheless, the γ chain peptide does inhibit the binding of other adhesive proteins to $\alpha_{IIb}\beta_3$.[91,103] A naturally occurring minor fibrinogen variant (the γ' variant) that results from alternative splicing of the γ chain mRNA alters the *C*-terminal sequence of the γ chain and neither binds to $\alpha_{IIb}\beta_3$ nor supports platelet aggregation.[104,105] Further, mutational studies conducted *in vitro*[106] and *in vivo*[107] confirm that the native γ chain sequence is essential for the productive interaction of fibrinogen with $\alpha_{IIb}\beta_3$.

The second peptide, referred to as the RGD peptide, has Arg-Gly-Asp-X (where X includes most amino acids) as its minimal sequence. Two RGD sequences are present in the Aα chain of human fibrinogen, and RGD-containing peptides inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ and platelet aggregation.[108,109] Indeed, several of the $\alpha_{IIb}\beta_3$ therapeutic antagonists were designed from the RGD sequence.[16,110] However, mutation of either RGD motif in the fibrinogen α chain does not alter fibrinogen recognition by the receptor.[106] However, the RGD motifs that are present in several other $\alpha_{IIb}\beta_3$ ligands mediate their interaction with $\alpha_{IIb}\beta_3$. The RGD motifs that are present in vWF[111] and vitronectin[112] are required to enable these proteins to interact with $\alpha_{IIb}\beta_3$. Fibronectin contains an RGD sequence as well as several alternative sequences that interact with $\alpha_{IIb}\beta_3$.[113,114] RGD functions as a major recognition sequence for several other integrins, including $\alpha_V\beta_3$.[115] Secondary roles for the RGD or other sequences in stabilizing $\alpha_{IIb}\beta_3$ interactions with its ligands have not been excluded. Roles for still other sequences, unrelated to the RGD and γ chain peptides, in the $\alpha_{IIb}\beta_3$-dependent retraction of fibrin clots by platelets have also been demonstrated.[116,117]

In contrast to $\alpha_{IIb}\beta_3$, there are only a few hundred molecules of $\alpha_V\beta_3$ per platelet,[118] and its physiologic significance is unclear. $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ share the same β-subunit, and their α-subunits are approximately 40% identical. Glanzmann thrombasthenia can arise from mutations in either the α_{IIb} or β_3 genes. Thus, β_3 mutations produce $\alpha_V\beta_3$ as well as $\alpha_{IIb}\beta_3$ deficiency.[23] However, phenotypic differences have not been reported between individuals lacking one or both β_3 integrins. $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$ bind many of the same ligands, including the two ligands that can mediate platelet aggregation, fibrinogen, and vWF,[119] but ligands that bind to one receptor and not the other have also been identified; for example, osteopontin[120] and adenovirus penton base[121] bind to $\alpha_V\beta_3$ but not to $\alpha_{IIb}\beta_3$, whereas the snake venom barbourin binds to $\alpha_{IIb}\beta_3$ but not to $\alpha_V\beta_3$.[122] This pattern is recapitulated at the level of the fine recognition specificities of the two integrins; RGD peptides bind with much higher affinity to $\alpha_V\beta_3$ than to $\alpha_{IIb}\beta_3$, and γ chain peptides bind with much higher affinity to $\alpha_{IIb}\beta_3$ than to $\alpha_V\beta_3$.[123] Like $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$ can undergo activation,[124–126] even in platelets.[127] The breadth of functions ascribed to $\alpha_V\beta_3$ in cells other than platelets, including endothelial cells, has stimulated the development of specific $\alpha_V\beta_3$ antagonists, primarily for use as antiangiogenic drugs which would be useful in inhibiting tumor growth and metastasis,[128] as well as for other diseases in which angiogenesis plays a prominent role.

$\alpha_{IIb}\beta_3$ Antagonists

Platelet adhesion is mediated by many receptors and platelet aggregation can be induced by many agonists, but aggregation itself is absolutely dependent upon $\alpha_{IIb}\beta_3$. This funneling of function onto $\alpha_{IIb}\beta_3$ provided a firm rationale for blockade

of its ligand-binding activity as an antithrombotic strategy and stimulated an intensive effort to develop specific and potent $\alpha_{IIb}\beta_3$ antagonists throughout the 1990s. In some respects, this effort was successful, but the hope that these drugs would provide both short-term and long-term protection against cardiovascular disease proved to be overly optimistic.[129] Three $\alpha_{IIb}\beta_3$ antagonists, abciximab (ReoPro), eptifibatide (Integrilin), and tirofiban (Aggrastat), were approved by the US Food and Drug Administration as intravenously administered antithrombotics for specific indications. These drugs continue to be utilized to prevent acute coronary events in association with percutaneous coronary interventions (PCIs).[130] Abciximab, the first $\alpha_{IIb}\beta_3$ antagonist approved for human use, is an Fab fragment of a humanized mouse monoclonal antibody to $\alpha_{IIb}\beta_3$.[110,131,132] Abciximab also reacts with $\alpha_V\beta_3$ and $\alpha_M\beta_2$, and some of the positive effects of the drug may be attributable to this cross-reactivity.[133] Integrilin contains a K(Lys)GD within a structure rigidified within a disulfide loop.[122] The RGD peptide was the starting point for tirofiban, but ultimately peptide bonds were designed out of its structure.[134] Following on the early successes of these drugs in the setting of PCI, it was anticipated that they would also be efficacious in reducing acute coronary syndromes in general. However, clinical trials demonstrated only modest or no benefits, and ultimately they did not measure up well against other platelet antagonists, such as clopidogrel, in terms of cost and efficacy. Despite the anticipation that orally active $\alpha_{IIb}\beta_3$ antagonists would provide a long-term benefit to reduce the risk of thrombotic events,[135] in clinical trials, these drugs were found at best to have little clinical benefit and, at worst, to be detrimental.[136] As a result, the development of oral $\alpha_{IIb}\beta_3$ antagonists was abandoned. Thus, although the principle that $\alpha_{IIb}\beta_3$ blockade should prevent thrombus formation is mechanistically sound, the therapeutic window is narrow, meaning that underdosing would be ineffective and overdosing could cause bleeding. At this juncture, parenteral $\alpha_{IIb}\beta_3$ antagonists are only used for limited indications.[129] The development of inhibitors that directly target $\alpha_{IIb}\beta_3$ continues to be explored. Antagonists that block ligand binding without engaging the β_3 MIDAS metal ion or disrupt interactions of the β_3 CT with its activating binding partners may be the targets of future antithrombotic drugs.[98,137]

REGULATION OF $\alpha_{IIb}\beta_3$ ACTIVATION (INSIDE-OUT SIGNALING)

Key to the ability of $\alpha_{IIb}\beta_3$ to bind fibrinogen and vWF and support platelet aggregation is its ability to undergo a transformation from a *resting* low-affinity state to an *activated* high-affinity state as depicted in FIGURE 31.2. The overall shape of $\alpha_{IIb}\beta_3$ depicted in this figure is based on cryogenic electron micrographic images of the isolated receptor,[138] crystal structures of the homologous $\alpha_V\beta_3$ extracellular domain (Protein Data Bank: 1JV2, 1M1X)[53,61] and the $\alpha_{IIb}\beta_3$ extracellular domain (Protein Data Bank 3FCS),[45] as well as NMR analyses of the TM and CT (Protein Data Bank 1M8O)[41,46] (Protein Data Bank 2K9J).[37,38,139] In this model, the globular head of resting, unactivated $\alpha_{IIb}\beta_3$ rests on two stalks. By bending at the genu of the α_{IIb} and β_3 ectodomains, the head approaches the cell membrane[140,141] (see FIGURE 31.2). When $\alpha_{IIb}\beta_3$ is activated, its height increases as its legs separate and straighten.[142] In association with leg separation, one of the helices in the β_3A-domain and the hybrid domain swings away to render the ligand-binding site formed between the β-propeller in the α_{IIb}-subunit and the β_3A-domain accessible to macromolecular ligands.[142–144] While these

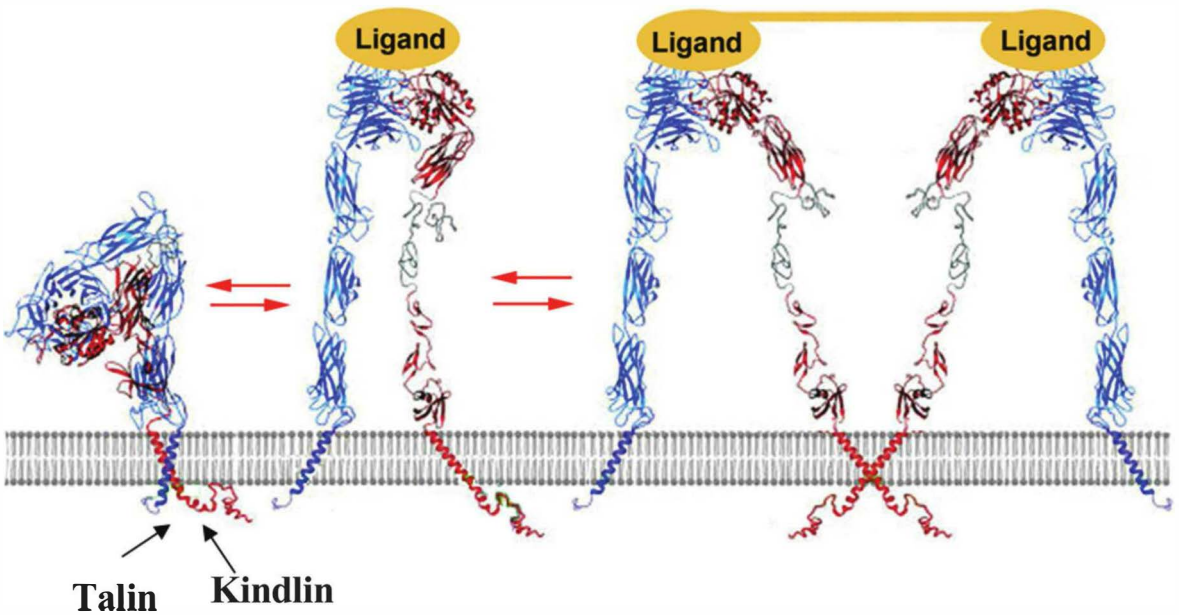

FIGURE 31.2 Model of a pathway for activation of $\alpha_{IIb}\beta_3$. Talin and kindlin binding to the CT of the β_3-subunit can lead to unclasping of integrin subunit tails and a membrane-associated structural change of the cytoplasmic face. This change may entail a transition from a bent conformation in its resting state to an extended conformation in which it becomes competent to bind soluble ligands. The α-subunit is in blue and the β-subunit is in red. (Reproduced from Qin J, Vinogradova O, Plow EF. Integrin bidirectional signaling: a molecular view. *PLoS Biol* 2004;2:e169, with permission.)

changes reflect the behavior of the ectodomains of the majority of $\alpha_{IIb}\beta_3$ molecules on the platelet surface during activation, it appears that some $\alpha_{IIb}\beta_3$ molecules are capable of binding ligands while still in a bent or partially unfolded conformation,[145] implying that extension of the ectodomain may also occur as a postactivation event.[146] The inside-out signals that induce these activation events may also promote clustering of $\alpha_{IIb}\beta_3$ heterodimers into heterooligomers[147]; clustering would be expected to increase $\alpha_{IIb}\beta_3$ avidity or valency.[148] However, conformational change and affinity modulation appear to be the dominant mechanism by which the ligand-binding activity of $\alpha_{IIb}\beta_3$ (and $\alpha_V\beta_3$) is regulated.[125,149]

Substantial progress has been made in understanding how integrin activation and the consequent movements of the ectodomain are triggered. In platelets, agonist signaling (inside-out signaling) eventuates in protein binding to and alterations in the interactions of the resting $\alpha_{IIb}\beta_3$ CT domain complex. A PTB domain located in the 270 kDa cytoskeletal protein talin binds to the β_3 CT domain and disrupts the hydrophobic and electrostatic bonds that maintain it in complex with the α_{IIb} CT domain. In turn, dissociation of this CT clasp affects insertion of the β_3 CT domain into the platelet plasma membrane and disrupts the association between the α_{IIb} and β_3 TM helices. Disengagement of the TM helices then provides the force needed to trigger the activating events in the $\alpha_{IIb}\beta_3$ ectodomain.[37,38,41,139,140,150–152] Once the CT and TM domains have dissociated, the propensity of the subunits to homooligomerize may also increase to influence the activation process.[147] With the ready availability of $\alpha_{IIb}\beta_3$ ligands in plasma, tight control of $\alpha_{IIb}\beta_3$ activation is essential. This regulation is achieved through the binding of cytosolic proteins to the α- and β-subunit CT domains. For integrin β CT, approximately 40 binding partners have been identified.[153] Among these, talin, filamin, kindlin, and integrin-linked kinase (ILK) are known to influence $\alpha_{IIb}\beta_3$ activation. Exactly how each does so remains unclear in many cases. Some of the proteins appear to induce or enhance integrin activation while others act as negative regulators.[37,154,155,156]

Proximal Regulation of $\alpha_{IIb}\beta_3$ Activation

A schematic identifying some of the intermediates in $\alpha_{IIb}\beta_3$ activation is shown in FIGURE 31.3. Talin binds directly to the CT of $\alpha_{IIb}\beta_3$ and is essential for integrin activation.[157] Talin is composed of an approximately 50 kDa head domain (talin-H) and a approximately 220 kDa rod domain (talin-R). The talin-H resembles a FERM (4.1 ezrin radixin moesin) domain and contains F1-, F2-, and F3-PTB subdomains.[158] In the resting platelet, talin exists in an autoinhibited conformation in which the talin-H interacts with talin-R.[159,160] The β_3 CT-binding site in talin-H can be exposed by calpain cleavage,[161] RIAM (Rap 1 interacting adaptor molecule) association,[162] or phosphoinositide binding.[163] Once unfolded, talin can interact with the $\alpha_{IIb}\beta_3$ CT.[155,164] The talin-H binds to two sites in the β_3 CT: the membrane-proximal NPLY747 motif with high affinity[165] and a membrane proximal region near the plasma membrane[41,166] with lower affinity. It is the second interaction that disrupts the clasp between the β_3 CT and the α_{IIb} CT, triggering the activation process. Talin-R also contains an integrin-binding site.[167]

Although it is clear that talin is necessary, and in model cell systems sufficient, to induce at least partial integrin activation,[155,168,169] in mice[170,171] and humans,[172,173] at least one additional molecule is essential for robust integrin activation. In platelets and other hematopoietic cells, this molecule is kindlin-3, and in other cells, the other two kindlin family members, kindlin-1 and kindlin-2, cooperate with talin to fulfill this *coactivator* role.[174,175,176,177,178] The coactivator role was originally demonstrated for kindlin-2, where the combination of kindlin-2 with talin-H–induced robust activation of integrins compared to talin-H alone.[168,179] The three mammalian kindlins have molecular weights of approximately 75 kDa. Like talin-H, kindlins also have a FERM domain. In kindlins, the F2 subdomain is interrupted by a pleckstrin homology (PH) domain. Four kindlin-binding partners have been identified: ILK,[179–181]

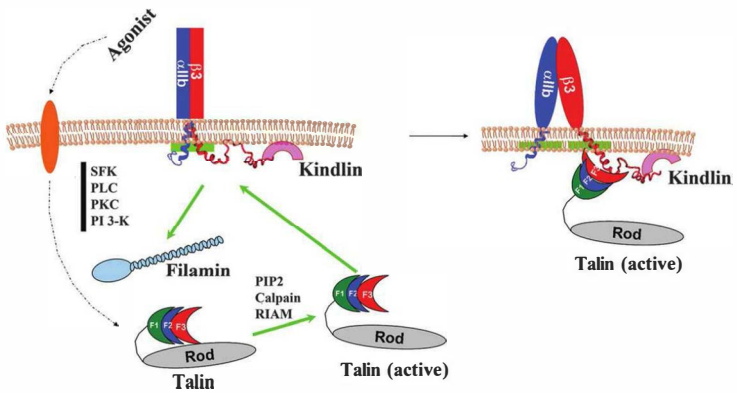

FIGURE 31.3 Model of a pathway for inside-out signaling to integrin $\alpha_{IIb}\beta_3$ in platelets. $\alpha_{IIb}\beta_3$ equilibrates between resting and activated states, the resting state predominating in unstimulated platelets and the activated state in stimulated platelets. Agonists induce activation of signaling intermediates, which leads to cytoskeletal changes, filamin dissociation, talin activation, affinity modulation of integrin $\alpha_{IIb}\beta_3$, and soluble ligand binding. The model depicted is incomplete and represents only some of the many molecules involved in this very complex process. Readers are referred to the text for a limited discussion. SFK, Src family kinase; PLC, phospholipase C; PKC, protein kinase C; PI-3K, phosphatidylinositide 3OH-kinase; PIP-2, phosphatidylinositol-4′5′-biphosphate; PIP-Kγ, phosphatidyl–inositol–phosphate kinase, gamma isoform.

migfilin,[181,182] and β_1-integrin and β_3-integrin CT.[179,183,184] Like talin, kindlins bind to β CT through their F3-PTB, but unlike talin, they bind to the membrane distal NXXY759 motif (NITY in β_3).[168,185] Phosphorylation of the Y in this sequence dissociates kindlin from the β_3 CT.[186] Kindlin-3 deficiency in humans gives rise to the leukocyte adhesion deficiency-III syndrome, LAD-III, also referred to as integrin activation deficiency disease[173] that is characterized by episodic bleeding of variable severity, increased susceptibility to infections, and sometimes osteopetrosis[172,173] and results from an inability to activate both β_2- and β_3-containing integrins. ILK, a 59 kDa cytoplasmic protein, can interact with the cytoplasmic domain of β_1, β_2, and β_3 integrin subunits.[187] Deficiency in mice or knockdown of ILK leads to defective platelet function,[188,189] although not as severe as that associated with kindlin-3 deficiency.

Filamin is a large cytoskeletal protein that binds to and stabilizes actin filaments.[190,191] Filamin contains an *N*-terminal actin-binding domain and a long rod-like domain of 24 immunoglobulin-like repeats. Filamin binds to a site in between the two NXXY motifs in integrin β-subunit CTs and overlaps sufficiently with the talin-binding site to dampen the talin–integrin interaction.[156,190,192] The cytoplasmic protein migfilin binds to filamin at the same site as integrin β CT. It is possible that by virtue of this competition, migfilin enhances integrin activation by blocking the inhibitory effect of filamin binding.[192]

REGULATION OF $\alpha_{IIb}\beta_3$ AND PLATELET ACTIVATION

Recent studies using novel inhibitors, proteomics, and gene-targeted mice harboring mutated and deleted proteins are leading to a new understanding of how signaling intermediates regulate, assemble, and relocalize molecules to drive $\alpha_{IIb}\beta_3$ activation in particular, and platelet activation in general. Platelets are transformed from a resting to an activated state when agonist stimulation of TM receptors induces intracellular signal transduction pathways. In addition to $\alpha_{IIb}\beta_3$ affinity modulation, platelet activation entails changes in platelet morphology, as well as centralization and exocytosis of storage granule. Inside-out $\alpha_{IIb}\beta_3$ activation can be triggered when any of a diverse array of agonists binds to their cognate receptors in the platelet plasma membrane (see Chapter 33). There are two principal receptor classes, based on their molecular associations with signal transducers: either G-protein–coupled or tyrosine kinase–coupled receptors.

G-proteins are heterotrimeric proteins consisting of α-, β-, and γ-subunits. They function as molecular switches that exchange GDP for GTP when they sense a conformational change in their associated receptor, resulting in dissociation of the α- from the β- and γ-subunits. The platelet-stimulating activity of thrombin is mediated by the protease-activated receptors PAR1 and PAR4 in humans and PAR3 and PAR4 in mice that are associated with Gαq, Gαi, and Gα12/13, whereas the thromboxane prostanoid receptor is associated with Gαq and Gα12/13. Full signaling by ADP requires that it bind to two purinergic receptors, the Gαq-associated P2Y1 and the Gαi-associated P2Y12. Epinephrine binds the β2 adrenergic receptor and signals through Gαz which couples to pathways similar to those coupled to Gαi.[193] Because ADP is released from platelet-dense granules during platelet secretion, ADP signaling constitutes a significant component of the total platelet stimulus initiated through multiple other receptors.[194,195] Deletion of receptors for each of these four major agonists results in abnormal thrombus formation in mouse models,[196] confirming their roles in platelet activation *in vivo*. The various G-proteins are associated with unique functions to promote platelet activation and aggregation: (a) Gαi inhibits adenylyl cyclase, the enzyme that catalyzes the formation of cAMP to maintain platelets in a resting state[197], and promotes phosphoinositide 3-kinase (PI-3K)–dependent Rap1 activation;[198] (b) Gαq stimulates release of membrane-bound phospholipase Cβ (PLCβ) to increase intracellular calcium levels and activate protein kinase C family members[199]; (c) Both Gα12 and Gα13 are present in platelets, although Gα13 is the primary mediator of thrombin-induced RhoA activation, shape change, and platelet aggregation.[200–202]

Tyrosine kinase–coupled adhesion receptors may signal independently or in conjunction with immunoreceptor tyrosine activation motif-containing receptors for optimal platelet activation. These adhesion receptors include GPVI which binds collagen and whose expression is regulated by the associated FcRγ,[203] GPIb-IX-V which binds vWF and signals alone[204] or together with FcRγchain in human and mouse platelets or with FcγRIIA in human platelets,[205,206] and $\alpha_{IIb}\beta_3$, a pool of which may associate with FcγRIIA.[207] Ligation of these adhesion receptors initiates a signaling cascade involving Src family kinases, activation of PLCγ2 for regulation of intracellular calcium levels,[208] and $\alpha_{IIb}\beta_3$ activation. Pathologically high shear stress stimulates signaling pathways activated by vWF binding to GPIb-IX-V.[209]

Platelet Phospholipases and Calcium Regulation

Platelets contain at least five isoforms of phospholipase C (PLC), including PLCβ and PLCγ2.[210] All PLC isoforms are activated by Ca^{2+} *in vitro*, while PLCγ2 may also be regulated by interactions of its PH domain with phosphoinositides.[211,212] These phospholipases hydrolyze the polar head group of phosphatidylinositol 4,5-bisphosphate (PIP2) to produce diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). The latter binds to the IP3 receptor to effect release of calcium from platelet-dense tubules.[213] DAG can in turn activate classic and non-classical members of the protein kinase C family. Both PLCβ and PLCγ are essential for normal intracellular calcium oscillations, whether in response to ADP and thrombin (PLCβ)[213] or collagen (PLCγ2).[214,215] The PLC-mediated increase in intracellular calcium is detected by stromal interaction molecule 1 (STIM1),[216] a stimulator of store-operated calcium entry, which can promote opening of membrane calcium channel, Orai1,[217] allowing influx of extracellular calcium. Micromolar concentrations of calcium permit activation of a wide range of calcium-dependent proteins, including proteases, cytoskeletal proteins, protein kinase Cs (PKCs), and CALDAG-GEFI (also termed RasGRP2). CALDAG-GEFI functions as a GTP exchange factor (GEF) in platelets for Rap1b, a small Ras-like GTPase important for $\alpha_{IIb}\beta_3$ activation and platelet function.[218] Rap1b is activated in response to platelet agonists and by platelet adhesion to fibrinogen.[219] Both CALDAG-GEFI- and Rap1b-deficient mice show signaling defects in response to G-protein– and tyrosine kinase–coupled agonist receptors[220,221] and manifest partial loss of aggregation *in vitro*, as well as bleeding defects and reduced thrombus formation *in vivo*.[222] Other phospholipases, such as

PLD,[223] may be activated by platelet agonists and contribute to arterial occlusion and cerebral ischemia.[215]

Protein Kinase C Family

Members of the protein kinase C family are serine/threonine kinases that positively and negatively regulate $\alpha_{IIb}\beta_3$ and platelet activation in an isoform- and agonist-dependent manner.[204,224–226] Platelets contain conventional (α, βI/II and γ), novel (δ, ε, {in megakaryocytes}, η), and atypical (ζ and ι/λ) PKC isoforms that are classified according to the structure of their regulatory domains.[227] Phosphorylation of PKC on tyrosine residues induced by thrombin and collagen may affect its activity, localization,[228,229] and/or coupling to receptors for activated C-kinase (RACK) proteins.[230] The latter bind selectively to PKC isozymes,[231] for which they are themselves substrates.[232] The best studied of the PKCs, PKCα, has repeatedly been shown to be essential for platelet and dense granule secretion,[233,234] and platelet stimulation by the phorbol ester PMA or thrombin causes the phosphorylation of substrates that regulate the exocytotic process, including SNAPS and SNARES,[235,236] pleckstrin,[237] and myristoylated alanine-rich C kinase substrate.[238,239] In addition to its role in secretion, PKCα may positively regulate $\alpha_{IIb}\beta_3$ affinity by promoting Rap association and activation of talin,[240,241] or by phosphorylation and deactivation of a constitutively associated phosphatase, SHP-1, to prevent dephosphorylation of SHP-1 substrates such as Vav1.[242,243] Both *in vitro* models examining platelet adhesion and aggregation on collagen and *in vivo* mouse arterial injury studies reinforce the importance of PKCα in thrombus growth.[226,244] The PKCδ isoform, in contrast, is thought to be a negative regulator of GPVI-induced secretion, $\alpha_{IIb}\beta_3$ activation, and platelet aggregation,[245] although recent reports have suggested that PKCδ is needed for PAR-induced dense granule secretion.[245] The PKCθ isoform has been shown to participate in feedback loops, where PKCθ phosphorylation of Bruton tyrosine kinase (Btk; a member of the Tec family of kinases) on serine residues in GPIb-IX-V–stimulated platelets promotes Btk phosphorylation of tyrosines on PKCθ.[246] PKCβ and PKCθ appear to significantly modulate outside-in signaling and are discussed further below. PKCη may also become phosphorylated in platelets following ADP stimulation.[247]

Phosphoinositide 3OH-Kinase

PI-3K phosphorylate the 3′-OH group of the inositol ring of specific phosphoinositols (PI) including PI(4)P and PI(4,5)P2. Their products, PI(3,4)P2 and PI(3,4,5)P3, transduce signals in part by binding to specific PH and Src homology-2 (SH2) domains in relevant signaling proteins such as Akt (Protein kinase B) and PDK1,[248] thereby recruiting them to their locus of action at the membrane. At least four PI-3K isoforms are expressed in platelets that are classified based on the p110 catalytic subunit and mechanism of regulation, including the two best characterized isoforms, class Ia member p85/p110 PI-3Kβ and Class Ib member p110 PI-3Kγ.[249,250] Interestingly, 3-phosphorylated phosphoinositides can activate certain atypical and novel isoforms of PKC,[251] suggesting one possible link between PI-3K, PKC, and affinity modulation of $\alpha_{IIb}\beta_3$. General PI-3K inhibitors indicate a prominent role for PI-3K in platelet activation: (a) ligation of GPIb-IX-V induces dissociation of PI-3K from an α-actinin/PI(3,4)P2 complex,[252] PI-3K association with Src, an early upstream effector in the GPIb-IX-V signaling pathway,[253] and $\alpha_{IIb}\beta_3$ activation[204]; (b) PI-3K may amplify platelet secretion through an ADP-dependent feedback loop[254]; and (c) mice deficient in Akt1 and Akt2, downstream effectors of PI-3K, have impaired thrombin-induced fibrinogen binding to $\alpha_{IIb}\beta_3$ and impaired *in vivo* thrombus formation.[255] Recent studies using gene targeting in mice and isoform-specific PI-3K inhibitors have identified both overlapping and discrete roles for the PI-3K isoforms β and γ: ADP P2Y12 receptor–driven Akt phosphorylation, Rap1b activation,[256] and thromboxane generation[257,258] are mediated primarily by PI-3Kβ, but full $\alpha_{IIb}\beta_3$ activation and platelet aggregation also require PI-3Kγ.[259,260] In contrast, activation through GPVI appears to be entirely PI-3Kβ mediated.[261,262] Interestingly, the consequences of deleting the p85α regulatory subunit are restricted to the GPVI pathway and the defects mirror those seen with PI-3Kβ.[263] While chemical induced thrombosis is compromised in these mice, there is no bleeding defect.

Ras and MAPK Family of Proteins

Platelets contain members of the Ras (H-Ras, R-Ras, Rap1 and Rap2, Raf, RalA, and Rab)[264] and Rho (cdc42, Rac1, and RhoA) families of small GTPases,[265] and the guanine nucleotide exchange factors, guanine nucleotide dissociation inhibitors, and GTPase-activating proteins[266,267] that regulate their GDP/GTP content. Ras family members are known primarily for their mitogenic effects and activation of mitogen-activated protein kinase (MAPK) cascades in nucleated cells.[268] However, Ras and ERK may be activated independently of each other in platelets,[269] and activation of similar cascades has not been conclusively confirmed. The MAPK family of serine/threonine kinases is composed of three main subgroups: p38 MAPK, ERK 1/2, and c-Jun terminal kinases (JNK), and several isoforms of each are found within platelets. MAPK members are known to be phosphorylated by physiological platelet agonists, acting through either G-protein– or tyrosine kinase–coupled receptors,[270,271] ADP P2Y receptors,[272] or thrombin where ERK activation may be PKC dependent,[266,273] and to positively regulate $\alpha_{IIb}\beta_3$ activation.[274] JNK is often referred to as a stress response sensor,[275] and this appears to hold true in platelets: inhibition of JNK1 *in vitro* decreases thrombus growth on collagen and vWF at arterial shear rates *in vitro*,[276] and mice lacking JNK1 show hemostatic defects including increased tail bleeding times and delayed chemical induced venous and arterial thrombosis in a mouse model.[277]

Adapter Proteins as Platelet Signaling Intermediates

Adapter molecules are often regulated by platelet agonists and serve to scaffold interaction partners to enable signal transduction and $\alpha_{IIb}\beta_3$ activation. Adapters have modular domains through which large, combinatorially diverse complexes may be formed. In many cases, they are crucial for relocalizing proteins to their sites of action. For example, two adapters in platelets that enhance $\alpha_{IIb}\beta_3$activation and contribute to *in vivo* thrombus formation are SLP-76[278] and the adhesion and degranulation promoting adapter protein (ADAP; Fyb, SLAP130).[279–282] GPVI/FcRγ stimulation results in phosphorylation of three important tyrosine residues on SLP-76 that induce recruitment of PLCγ and additional adapters including ADAP, NCK,

and LAT[281,283–285] to promote increases in intracellular calcium. ADAP interactions may be mediated by a constitutive interaction with the Src family kinase member Fyn[204] or by inducible interactions with Src.[286] Other adapters have been implicated in thrombus formation *in vitro* and *in vivo*, such as Cbl, which negatively regulates platelet function.[287] Further studies are needed to reconstruct the interaction networks induced by diverse platelet agonist signaling pathways.

REGULATION OF PLATELET FUNCTION BY $\alpha_{IIb}\beta_3$ (OUTSIDE-IN SIGNALING)

Several platelet responses are dependent, in part, on initial binding of fibrinogen or vWF to $\alpha_{IIb}\beta_3$. These include (a) firm adhesion under high shear conditions[68,288,289]; (b) spreading on extracellular matrices containing fibrinogen or vWF[290]; (c) secretion from platelet α and dense granules in response to "weak" agonists, such as ADP or epinephrine; (d) conversion of small platelet aggregates into larger, more hemostatically effective ones; (e) fibrin clot retraction[291]; and (f) microparticle formation.[292,293] Some of these responses may be dependent on ligand binding to $\alpha_{IIb}\beta_3$ and others on platelet aggregation, thereby establishing a temporal–spatial hierarchy to outside-in signaling.[98] Graded degrees of cytoskeletal rearrangement occur from filopodial extension to actin organization into clusters,[294] lamellipodia formation, and full spreading, which depend on the matrix encountered, the degree of hydrodynamic stress,[295] and the presence of amplifying costimulatory agonists such as ADP and thromboxane A2.[296,297] Platelet outside-in responses are propagated by constitutively—or inducibly—associated $\alpha_{IIb}\beta_3$ CT-binding proteins.[39,298,299] These may serve to initiate signaling cascades for actin polymerization, cytoskeletal reorganization, and platelet spreading or serve to anchor platelets to substrata by direct or proximal coupling to actin[300–302,303,304] (FIGURE 31.4).

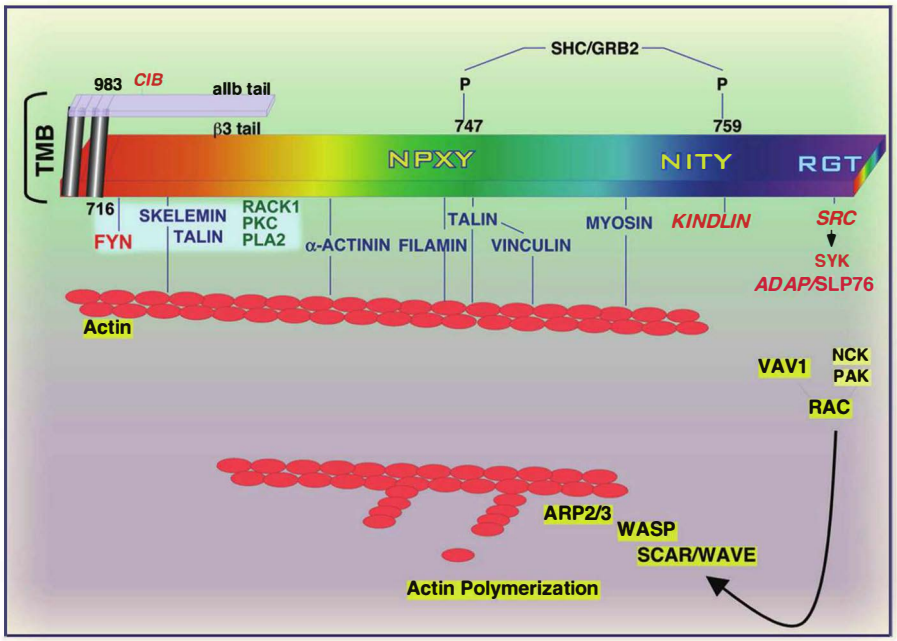

FIGURE 31.4 Model of integrin $\alpha_{IIb}\beta_3$ outside-in signaling mediators. Integrin $\alpha_{IIb}\beta_3$ is a type I TM heterodimeric protein composed of an α- and a β-subunit. Shown here is the intracellular platelet environment and highlights the α and β CTs (not drawn to scale) and several of the proteins that bind the tail subunits constitutively or inducibly upon ligand engagement. These proteins may function to anchor platelets to the substrate by direct or secondary linkage to actin, for example, talin-vinculin (labeled in *blue*), and/or may function to stimulate de novo actin polymerization, cytoskeletal reorganization, platelet spreading, or other outside-in signaling events by initiation and propagation of signaling cascades (labeled in *red*). Relevant sites proximal to the TM domains are, on α_{IIb} chain: CIB (amino acids (AA) 984--996), and on β3 chain: Fyn (AA 721–725), Skelemin (AA 716–730), a secondary talin-binding site, and an α-actinin-binding site. The region on the β3 chain between tyrosines 747 and 759 constitutes an important regulatory region: the talin-binding site spans AA739–752 and includes one of the two tyrosine-containing motifs, NPXY, and this site is partially overlapped and competed for by the actin-binding protein filamin. Kindlins recognize a region that includes the second tyrosine-containing motif, NITY. Phosphorylation of tyrosines 747 and 759 regulates binding of adapter proteins Shc and Grb2, and the cytoskeletal protein, myosin. The tyrosine kinase, c-Src, which is constitutively associated with the β3 chain three carboxy-terminal RGT residues, becomes activated upon integrin ligation and is critical for outside-in signaling. Src activation initiates a cascade of events including activation and recruitment of Syk, and other adapters such as SLP-76, ADAP, and NCK, which engage regulators of actin polymerization and reorganization (*yellow*). RACK1/protein kinase C family members may further promote significant integrin-proximal outside-in signaling (*green*).

Deletion of many of these proteins in mice decreases platelet spreading *in vitro* and thrombus stability *in vivo*.[196,305,306]

Talin was one of the first intracellular proteins found to provide a direct linkage between actin and integrins.[307,308,164] Cells lacking talin are unable to link integrins to the cytoskeleton[309] but are able to bind to the extracellular matrix.[310,311] Integrin binding to two other actin-binding proteins, α-actinin and filamin, has also been demonstrated.[312] Members of the kindlin family may play an important role as adaptor molecules linking the actin cytoskeleton to cell–extracellular matrix adhesion sites via a migfilin/filamin intermediary complex.[177] For example, cells that are defective in kindlin-2 expression have a severe spreading defect.[179] Kindlin-2 is also required to direct ILK to focal adhesions.[299] ILK deficiency disorganizes the actin cytoskeleton.[299] Many of these molecules are also implicated in integrin activation, and the distinction between their roles in inside-out and outside-in signaling becomes blurred as the processes are interconnected and cooperative.

Specific domains in $\alpha_{IIb}\beta_3$ serve to transduce signals subsequent to ligand binding. Phosphorylation of the β_3 tyrosines 747 and 759,[313] located in the NPLY- and NITY-binding motifs for talin and kindlin, respectively, promotes the association of β_3 with the Triton X-100-insoluble platelet cytoskeletal fraction, as well as binding of myosin and the MAPK cascade protein SHC (SH2 containing adapter protein) to the β_3 CT.[314,315] Phosphorylation of β_3 may also be induced by CD40 ligand[316] and necessitates mu-calpain.[317] Mutation of these tyrosines to phenylalanine in mice, peptides against this region, or truncation at β_3 at residue 724 affects outside-in signaling, as manifested by rebleeding after tail transection, a reduction in clot retraction, reduced $\alpha_{IIb}\beta_3$-dependent tyrosine phosphorylation of FAK, reduced platelet spreading on fibrinogen, and reduced $\alpha_V\beta_3$-dependent FAK and paxillin phosphorylation.[318–321] Interestingly, β_3 tail phosphorylation may advance downregulation of $\alpha_{IIb}\beta_3$ activation since tyrosine phosphorylation decreases the binding of two important $\alpha_{IIb}\beta_3$ affinity modulators, talin and kindlin.[186,322] Further, phosphorylation of β_3 serine 752 reduces platelet spreading.[323,324] In addition, structural changes in $\alpha_{IIb}\beta_3$ TM and extracellular domains induced by ligand binding may expose new CT epitopes for protein binding to promote spreading and clot retraction.[325,326]

$\alpha_{IIb}\beta_3$-mediated outside-in signaling initiates a cascade of events that result in cytoskeletal reorganization and are thought to begin with $\alpha_{IIb}\beta_3$ oligomerization.[125,327] $\alpha_{IIb}\beta_3$ oligomerization following fibrinogen binding was directly demonstrated in model cell systems using protein interaction-dependent fluorescence assays.[328,329] $\alpha_{IIb}\beta_3$ oligomerization (clustering) was found to promote cell adhesion to fibrinogen or vWF[125] and was followed by dynamic trafficking of intracellular kinases and adapters.[98] Oligomerization results in the activation of the c-Src that is constitutively associated with the RGT sequence located at the extreme *C*-terminus of the β_3 tail of resting platelets.[330–332] Src activation involves the dephosphorylation of Src residue Y529 by PTP-IB to relieve autoinhibition,[333,334] followed by autophosphorylation of Src at Y416.[334] Activated Src promotes the recruitment of the tyrosine kinase Syk to the Src-β_3 complex where Syk is activated.[335,329,330] The tyrosine kinase function of Src and Syk, as well as that of another Src family kinase Fyn, is required for full platelet spreading.[336] The Src–Syk complex can be seen in early membrane ruffles and lamellipodia where it is colocalized with cortactin.[329] Kinase activation promotes a molecular pathway of cytoskeletal rearrangement by activation of adapters including SLP-76, ADAP, Dok, Nck, Lnk, and the Cdc42/Rac GEF Vav1.[337,338,339] A small αIIβ-binding protein, CIB, associates with FAK to support focal adhesion and lamellipodia formation.[340] Overall, $\alpha_{IIb}\beta_3$-mediated outside-in signaling cascades promote actin polymerization. Consequently, branching actin polymerization may be effected by Rac-GTP activation of Scar/WAVE[341,342] to trigger WASP association and stimulate actin assembly[343] via the ARP2/3 complex.[344] In fully spread platelets, larger adhesive complexes are formed with PKC-dependent phosphorylation of α-actinin[345] and Src-dependent activation of vinculin.[346] Mutation or deletion of many of the above proteins in mice perturbs platelet spreading,[281,284,347,348] but regulation of the proteins, their intracellular localization, and how they are modulated by shear stress[295,349] are not fully understood.

Several studies have suggested that there are similarities between nonintegrin agonist-induced signaling pathways and $\alpha_{IIb}\beta_3$-mediated outside-in signaling. Thus, a pool of $\alpha_{IIb}\beta_3$ has been found to associate with Gα13 and modulate platelet contractility and shape change[200,350] as well as Src Y416 phosphorylation and platelet spreading.[351] Another pool has been identified that associates with FcγRIIA to control Syk- and PLCγ-mediated spreading.[207] These observations are supported by the participation of common signaling pathway intermediates. First, disruption of PLCβ or PLCγ2 and intracellular calcium elevations result in decreased platelet spreading and adhesion stability,[213,352,353] while a PLCγ gain-of-function mutation increases spreading and clot retraction.[215] Second, PI-3K can serve to stabilize platelet spreading,[262] clot retraction,[257] and adhesion stability under flow.[349,354,260] Third, certain PKC family members appear to selectively mediate outside-in signaling in platelets by partnering with isoform-specific RACKs and other signaling molecules. PKCβ can promote spreading through RACK1,[298] PKCδ attenuates filopodia formation through a constitutive interaction with VASP,[355] and PKC associated with $\alpha_{IIb}\beta_3$ promotes platelet spreading on fibrinogen through inducible interactions with Syk and Btk[356] but attenuates thrombus formation on collagen.[226] Therefore, it is possible that PKC/RACK and Src operate together, or may reflect distinct $\alpha_{IIb}\beta_3$-associated signaling pathways that converge on actin regulatory proteins to effect platelet outside-in responses. In support of this possibility, Dok1 and Dok3 undergo both Src-dependent and Src-independent phosphorylation following $\alpha_{IIb}\beta_3$ ligation[338] and mice lacking Group IVA cytosolic phospholipase A2, which releases arachidonic acid from membranes, have reduced PKCβ/RACK1 recruitment to β_3 and platelet spreading.[357]

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CHAPTER

32 Molecular Basis for Platelet Secretion

SHAOJING YE AND SIDNEY W. WHITEHEART

INTRODUCTION

Platelets are small, anucleate, discoid cell fragments produced by bone marrow megakaryocytes. Structurally, platelets are simple, containing plasma membrane with a specialized region known as the open canalicular system (OCS), microtubules, mitochondria, and three types of granules: dense granules, α-granules, and lysosomes. Upon encountering a vascular lesion, platelets become activated and initiate thrombosis. Engaging cell surface receptors initiates a number of intracellular signaling cascades leading to increased intracellular Ca^{2+} and inducing dramatic alterations in platelet shape mediated by actin cytoskeletal rearrangements. Platelet activation also leads to the release of their granular stores, and the resulting releasate affects the microenvironment at the site of damage. Released molecules attract and activate more platelets to propagate thrombosis and initiate *sequelae* of thrombosis such as wound healing and leukocyte recruitment. Thus, platelet secretion is not only important for hemostasis, but is also associated with many other vascular processes, both normal and potentially pathologic. Further, understanding the mechanism of platelet secretion has the potential to identify new targets for the development of novel antithrombotic therapeutics to control thrombosis and to affect pathophysiologic aspects of platelet function that participate in atherosclerosis, tumor angiogenesis, and inflammation.

At sites of vascular injury, platelets release cargo from three granular stores: dense granules contain small, bioactive molecules,[1–3] α-granules contain bioactive peptides and proteinaceous components,[4,5] and secretory lysosomes contain hydrolytic enzymes.[6] Release of dense granule cargo is clearly important to hemostasis, given the bleeding diatheses associated with genetic defects in dense granule biogenesis (i.e., Hermansky-Pudlak syndrome [HPS][7]). Released adenosine diphosphate (ADP) plays a key role in enhancing the responsiveness to other agonists generated at the site of vascular damage.[8] Release of α-granule cargo has a more heterogeneous impact. Patients who lack α-granules (i.e., gray platelet syndrome [GPS]) generally have more diverse bleeding phenotypes, ranging from mild to severe.[9,10] The role that lysosome release plays is unclear; it is plausible that the released hydrolytic enzymes could be important for thrombus remodeling. Nonetheless, while the physiologic importance of platelet secretion is clear, the nature of the machinery that controls and mediates platelet granule exocytosis is not. This chapter discusses our present state of knowledge of the molecular mechanisms of platelet secretion.

PLATELET GRANULES AND THEIR CARGO

As with other regulated secretory systems, for example, neurons, release of cellular stores occurs in response to external stimuli. The resulting signaling steps mobilize the secretory machinery that then mediates the fusion of cargo-containing granules with the plasma membrane to facilitate cargo release into the extracellular environment. Each of the classes of platelet granules is released upon stimulation, but to differing degrees, depending on the levels of platelet activation.

Dense Granules

Platelets contain approximately three to eight electron-dense organelles, 20 to 30 nm in diameter.[3,11] The electron density of dense granules, when viewed in unstained whole mounts, is due to the relative insolubility of calcium salts of the various compounds stored in dense granules (i.e., $CaPO_4$). Dense granules contain high concentrations of serotonin, which is taken up from plasma by a plasma membrane transporter and then concentrated in the granules.[3] ADP and adenosine triphosphate (ATP) are also highly concentrated in dense granules. There is more ADP than ATP in the dense granules, which is the reverse of their relative cytosolic concentrations.[12] Calcium, polyphosphates, sphingosine-1-phosphate, and epinephrine are also found in dense granules.[12–14] The concentration of serotonin, and perhaps other dense granule cargo, is the result of the lower pH (~6.1) maintained in dense granules due to the action of a H^+ pumping ATPase on the granule membrane.[3]

α-Granules

α-Granules are the most abundant granule type in platelets, numbering approximately 50 to 80 per platelet.[15,16] They are approximately 200 nm in diameter and show variations in electron density; often, they contain a central electron-dense area termed a nucleoid.[17] The more electron-lucent areas often contain tubular elements in which von Willebrand factor (vWF), multimerin, and factor V are preferentially localized.[18] Platelets may contain distinct subpopulations of α-granules that undergo differential release of α-granule cargo during activation. For example, some α-granules appear to contain proangiogenic proteins, such as vascular endothelial growth factor (VEGF), whereas others contain antiangiogenic factors, such as endostatin.[19] Alternatively, these factors may be distributed in distinct regions within the same membrane-bound granule.[20,21] This is yet to be resolved.

α-Granules acquire their cargo through both biosynthesis (predominantly at the megakaryocyte level) and endocytosis (at both the megakaryocyte and circulating platelet levels). Small amounts of many plasma proteins are found in α-granules; thus, the plasma concentrations of these proteins determine the amount present in platelets.[22,23] For example, the α-granule pool of immunoglobulins contains most of the platelet immunoglobulin;

therefore, total platelet immunoglobulin is more affected by changes in plasma immunoglobulin levels than by changes in surface immunoglobulin. Other proteins, such as VEGF, factor V, and fibrinogen appear to be specifically endocytosed and trafficked to α-granules for subsequent release. Consistently, clathrin-coated pits have been observed budding from the specialized region of the plasma membrane, the OCS.[24] $P2Y_1$ and $P2Y_{12}$ receptor desensitization and resensitization are also thought to occur through an endocytosis, re-expression pathway.[25] Though it clearly occurs *in vivo*, the mechanism, the extent, and the biologic importance of platelet endocytosis are largely unknown and understudied.

The pathways that regulate α-granule assembly are also not fully understood, but several studies suggest that multivesicular bodies (MVBs) play a crucial intermediary role in α-granule biogenesis.[26] These membranous compartments, containing numerous small vesicles, develop from budding vesicles in the Golgi complex within megakaryocytes and can interact with endocytic vesicles. They are abundant in immature megakaryocytes and decrease in numbers with cellular maturation, suggesting that they are the precursors of α-granules and/or dense bodies. MVBs may also function as a sorting hub to route proteins into distinct classes of α-granules.

Lysosomes

Lysosomes are a key component of the endosomal membrane system. Platelet lysosomes contain acid hydrolases (e.g., β-glucuronidase, cathepsins, aryl sulfatase, β-hexosaminidase, β-galactosidase, endoglucosidase [heparitinase], β-glycerophosphatase, elastase, and collagenase).[2] Similar to other hematopoietic cells, platelets secrete lysosomes upon activation, but lysosomal release is slower and less complete than that of α- and dense granules.[6] Thus, stronger agonists are required to induce lysosomal enzyme release. The elastase and collagenase activities released from platelet lysosomes may contribute to vascular damage at sites of platelet thrombus formation.[27] The heparitinase may be able to cleave heparin-like molecules from the surface of endothelial cells, and the resulting soluble molecules appear to inhibit growth of smooth muscle cells.[28]

Platelet Secretome

Present estimates suggest that fully activated platelets can secrete hundreds of different molecules.[4,5,29] While dense granules predominantly contain only small molecules, α-granules contain a myriad cargo that comprises the bulk of the platelet "secretome." Some of these proteins are produced at the megakaryocyte stage and are packaged into granules during their biosynthesis.[26] It is generally thought that patients with GPS have a defect in this process. Other α-granule cargo (e.g., fibrinogen and factor V) are not made by megakaryocytes, but are thought to be endocytosed by circulating platelets.[30] Once internalized, these cargo molecules are transported to α-granules.[26,31]

This "secretome" can be classified by granular source or proposed function.[32] Dense granules mainly contain nonpeptide, small molecules: for example, ADP, serotonin, and calcium, which are critical for platelet activation and vasoconstriction. As discussed below, failure to release these components leads to defects in thrombosis. α-Granules contain proteinaceous components which have a wide array of functions.

Functionally, the "secretome" can be divided into even more categories (reviewed in Ref.[33]). The most obvious class of proteins is the adhesive proteins such as fibrinogen, fibronectin, vitronectin, and thrombospondin that are released from α-granules and function in platelet–platelet cohesion and subsequent thrombus formation. Other proteins, such as P-selectin, are present on the α-granule membrane and are exteriorized to the surface upon granule–plasma membrane fusion. Platelets also release fibrinolytic agents such as PAI-1 and TAFI which are important for clot remodeling. Perhaps the most diverse category is the mitogens such as IGF-1, VEGF, and FGFβbFGF. These proteins are thought to promote wound healing and angiogenesis by stimulating chemotaxis, cell proliferation, and maturation of the cells surrounding the wound site. Finally, platelets release a number of chemokines and cytokines such as RANTES, IL-8, and MIP1α that promote activation of passing leukocytes and lead to a range of immune responses. This releasate catalog suggests that platelet secretion is pivotal in establishing the microenvironment at the wound site. Therefore, controlling release from the granule stores, either specifically or globally, may prove to be an effective strategy to manipulate this microenvironment.

Granule Heterogeneity

It is becoming increasingly clear that our old views of platelet granules may be overly simplistic. Recent studies have suggested that α-granules are heterogeneous in content and that heterogeneity may result in differential release of granule cargo. Italiano and colleagues have shown that α-granules can be distinguished based on the pro- or antiangiogenic factors that they contain.[19] Antiangiogenic factors, such as endostatin, reside in different granules than do proangiogenic factors such as VEGF. A similar degree of granule heterogeneity was initially reported by Storrie and colleagues who examined the localization of fibrinogen and vWF.[20,21] Functionally relevant granule heterogeneity is seen in other hematopoietic cells such as mast cells and neutrophils. In these cells, differential release in response to distinct agonists appears to be mediated by different SNARE proteins.[34,35]

The diversity of the platelet releasate suggests that platelet secretion could be pivotal in establishing the microenvironment at an injury site. However, it is unclear whether platelet secretion is a controlled, thematic process or a random event. Several studies found that some angiogenesis regulators are packaged into distinct α-granule populations. Immunofluorescence and immunoelectron microscopy show distinct localizations for pro- and antiangiogenic factors.[19] Other studies have also shown that stimulating platelets with different agonists (i.e., PAR1 and PAR4 agonists) causes differential release of certain cargo molecules.[19,36,37] This led to the proposal that platelet secretion is contextually thematic, that is, capable of releasing specific sets of cargo (e.g., pro- vs. antiangiogenic factors) in response to specific agonists. Other data suggest that cargo is spatially segregated into subregions of the same membrane-bound granules and that there is no "thematic" colocalization of factors with like functions.[20,21] Though these data are intriguing, it is yet difficult to ascertain the extent to which platelet secretion is heterogeneous. If there is heterogeneity in cargo packing and/or secretion, regulating the release of specific α-granule subpopulations may allow

specific manipulation of the microenvironment at an injury site without disturbing hemostasis.

PLATELET SECRETORY MACHINERY

SNARE Overview

Platelet granule–plasma membrane fusion is analogous to exocytosis in neurons where detailed studies have shown the importance of a core set of integral membrane proteins called soluble N-ethylmaleimide Sensitive Factor (NSF) attachment protein receptors (SNAREs)[38,39] (FIGURE 32.1). Since the original description of SNAREs in neurons,[40] there have been significant advances to our understanding of how SNAREs function. Recent models of SNARE function posit that vesicle/granule-target membrane fusion is governed, in part, by the matching of a vesicle SNARE (v-SNARE), with a heterodimeric or trimeric, protein complex in the target membrane (t-SNAREs). This transmembrane complex is the minimal element required for membrane fusion.[41] v-SNAREs are type II integral membrane proteins of generally low molecular weight (15 to 25 kDa) that contain a characteristic 60 to 70 amino acid SNARE motif.[38] At present, approximately 20 different v-SNARE isoforms have been identified in humans. t-SNAREs are of two types: the synaptosomal-associated protein 23/25 (SNAP-23/25) type (SNAP-23, SNAP-25, SNAP-29, Sec9p), and the syntaxin type (syntaxin 1-19, etc.). The SNAP-23-like proteins have two SNARE motifs, lack transmembrane domains (TMDs), and are generally anchored to the membrane through thioester-linked acyl groups attached to a central cysteine-rich domain. The type II membrane protein syntaxins contain the characteristic SNARE motif, an *N*-terminal regulatory domain, and generally, a *C*-terminal TMD.[42] SNAREs employ the SNARE motifs to form a four-helix bundle arranged as a parallel coiled-coil.[43] This structure is stabilized by core hydrophobic interactions running the length of the bundle. Based on this inherent stability, the temporal and spatial control of SNARE complex formation and disassembly represent important tasks for the secretory machinery. Three groups have contributed to our present knowledge of the function of platelet SNAREs (for reviews see Refs.[44,45]). To date, four vesicle-associated membrane proteins (VAMPs -2, -3, -7, -8), four syntaxins (-2, -4, -7, -11), and SNAP-23, SNAP-25, and SNAP-29 have been detected in platelets.[46–48,49,50,51,52,53,54]

Platelet v-SNAREs

In human and mouse platelets, the v-SNAREs are VAMP-2/synaptobrevin, VAMP-3/cellubrevin, VAMP-7/TI-VAMP, and VAMP-8/endobrevin, with the latter being most abundant[46,52,53,54] (FIGURE 32.1). Assigning roles for these v-SNAREs was initially ambiguous, perhaps because in the *in vitro* exocytosis assays used, the v-SNARE-effecting agents were being added to permeabilized platelets. Flaumenhaft et al.[49] found that P-selectin exposure (a metric of α-granule release) was inhibited by pretreatment of permeabilized platelets with Tetanus neurotoxin, a heterodimeric, zinc-dependent, endopeptidase specific for VAMP-1, -2, and -3.[55] Subsequently, Feng et al.[56] showed that an antipeptide antibody to the divergent, *N*-terminus of VAMP-3 could inhibit α-granule release, suggesting a role for VAMP-3. However, secretion by platelets from VAMP-3$^{-/-}$ mice was normal.[54] In fact, secretion for these platelets seemed faster.[53] Polgar et al.[52] showed that the cytoplasmic domains of VAMP-3 or VAMP-8, containing the SNARE motif, could inhibit release from dense and α-granules. Since the isolated cytoplasmic domains are promiscuous in their associations *in vitro*,[57,58] it was uncertain whether the inhibitory effects were illustrative of a v-SNARE role or of t-SNARE heterodimer binding preferences.

Ren et al.[53] showed that VAMP-8/endobrevin is the primary v-SNARE in mouse platelets. VAMP-8$^{-/-}$ platelets had a clear secretion defect, which was not seen in VAMP-3$^{-/-}$, VAMP-2$^{+/-}$, VAMP-2$^{+/-}$/VAMP-3$^{-/-}$ platelets or from either permeabilized human or VAMP-3$^{-/-}$ platelets that had been treated with the catalytic Tetanus toxin light chain. However, the secretion defect in VAMP-8$^{-/-}$ platelets was not complete. α-granule and lysosome release were significantly affected even at high agonist doses and upon extended incubation, but dense granule release was only attenuated and eventually approached wild-type levels. The VAMP-8–independent release could be eliminated by treatment of permeabilized VAMP-8$^{-/-}$ platelets with Tetanus toxin, suggesting

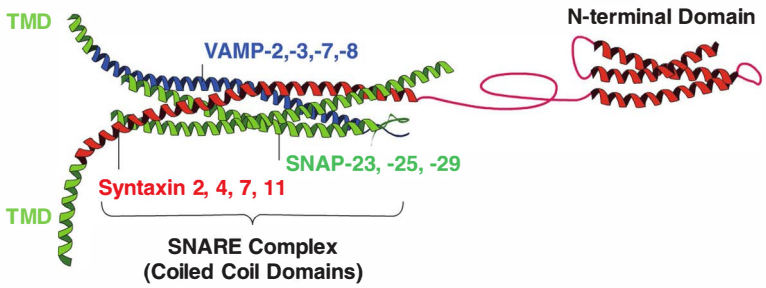

FIGURE 32.1 Platelet SNARES. SNARE proteins are membrane proteins that form a complex which bridges between the granule and plasma membrane. This complex is sufficient to mediate membrane fusion between the granule and plasma membrane to promote granule cargo release. SNAREs from the granules are known as v-SNAREs (vesicle SNAREs). They are small (~20 kDa) membrane proteins with a TMD and a cytoplasmically oriented SNARE motif. Platelets contain VAMP-2, -3, -7-, and -8. SNAREs from the plasma membrane (target or-t-SNAREs) come in two forms: the syntaxins (~35 kDa) are membrane proteins that generally contain a TMD, a SNARE motif, and an *N*-terminal regulatory domain. Platelets contain syntaxin 2, 4, 7, and 11. The syntaxins form a complex with the SNAP-23 family of t-SNAREs. The SNAP-23s (~30 kDa) lack TMDs and are anchored by thioester-linked acyl groups. They have two SNARE motifs. Platelets contain SNAP-23, -25, and -29.

that the secondary mechanism employed either VAMP-2 or -3. Experiments using permeabilized human platelets treated with Fab fragments prepared from VAMP-8-specific immunoglobulins showed that the role of VAMP-8 in mouse and human platelets is similar (Ren Q and Whiteheart SW, unpublished data, 2008).

The redundancy in v-SNARE usage is analogous to that seen in chromaffin cells[59] where there is a primary v-SNARE (VAMP-2) required for rapid and efficient, agonist-induced release, and a secondary v-SNARE (VAMP-3) which functions, albeit less efficiently, in the absence of the primary v-SNARE. Full ablation of release from chromaffin cells requires deletion of both VAMP-2 and VAMP-3. The redundancy in VAMP usage observed in *ex vivo* platelet secretion assays is consistent with the *in vivo* thrombosis phenotype of the VAMP-8$^{-/-}$ mice. Upon laser injury, VAMP-8$^{-/-}$ mice have a delay in thrombus formation and an increase in embolization, although the overall extent of platelet accumulation is not statistically different from wild type.[8]

In light of the role of VAMP-8 in platelets, it is noteworthy that a SNP (single nucleotide polymorphism) in the human VAMP-8 gene strongly correlates with early onset myocardial infarction (MI).[60,61] The authors of these studies suggest that this SNP might affect VAMP-8 expression levels, given its location in the 3'-untranslated region of the gene. It is conceivable that increased amounts of VAMP-8 could increase the likelihood that a platelet could release its granule cargo and thus be hyperthrombotic. Consistent with this possibility, expression of a microRNA controlling VAMP-8 expression correlated with platelet hyperactivity.[62] Normal donors, whose platelets were hyperresponsive to epinephrine, showed increased expression of the micro-RNA. Transfection studies suggested that increased VAMP-8 might make platelet hypersecretory, thereby explaining their hyperresponsiveness. Further analysis will be required to determine if VAMP-8 levels are a thrombotic risk factor.

Platelet t-SNAREs

There are two classes of t-SNAREs: the SNAP-23/25/29 type and the syntaxin type (FIGURE 32.1). Human and murine platelets contain syntaxins-2, -4, -7, and -11[49,50,63] as well as SNAP-23, -25, and -29.[49,50,64] All three SNAP-23/25-like t-SNAREs have been detected in platelets, although SNAP-25 and SNAP-29 levels appear to be low. Experiments introducing specific antibodies, Fab fragments, and inhibitory peptides have demonstrated that SNAP-23 is required for all three granule secretion events.[47,48,49,50,52,64,65] SNAP-23 is phosphorylated upon activation[64,66] and preliminary data from Karim et al. (*manuscript in preparation*, 2012) suggest that SNAP-23 might be an important link between platelet activation and membrane fusion. Polgar et al.,[64] using ion trap mass spectrometry, showed that human platelet SNAP-23 could be phosphorylated at Ser23/Thr24 and Ser61 after activation by thrombin. In murine mast cells and platelets, SNAP-23 phosphorylation sites are at Ser95 and Ser120.[66] SNAP-23 is important for full assembly of fusigenic SNARE complexes and, in mast cells, phosphorylation appears to contribute to that role.[66]

SNARE complexes may also be affected by calpain cleavage of SNAP-23. Calpains are calcium-dependent, nonlysosomal, neutral, cysteine proteases present in most mammalian tissues. Several inhibitors, such as calpeptin, calpastatin, and E64d, inhibit calpain activity and these inhibitors block SNAP-23 cleavage in activated platelets.[67,68] It is not clear if SNAP-23 cleavage plays a role in platelet exocytosis since the conflicting effects of the calpain inhibitors may reflect the assays used to monitor secretion.[68,69] Clearly, these data establish a role for SNAP-23 in platelet exocytosis and suggest that it may be regulated by several different mechanisms. Studies in knockout mice and/or humans with SNAP-23 mutations will be required to confirm this conclusion. Global deletion of SNAP-23 is embryonic lethal (at E3.5) and attempts to make conditional deletions have been unsuccessful thus far.[70]

Syntaxins-2 and -4 have been detected in platelets, but syntaxins-1, -3, and -5 have not.[49,50,65] Both syntaxins-2 and -4 had been shown to be involved in other secretion events at the plasma membrane.[71–74] Flaumenhaft et al.[49] first demonstrated a role for syntaxin-4 in platelet α-granule release using a permeabilized platelet assay system. Monoclonal antibodies against syntaxin-4 inhibited lysosome release but not dense granule release.[48] Chen et al.[47] demonstrated that an antisyntaxin-2 antibody could inhibit release of all three platelet granule stores.[48,51] Syntaxin-7 is localized to an intracellular compartment instead of the plasma membrane in nucleated cells[71] and was not considered a likely candidate to mediate platelet exocytosis. This was confirmed when specific syntaxin-7 antibodies were introduced into permeabilized platelets and had no effect on any of the three granule secretion events.[47,48,51] These early data suggested that syntaxins-2 and -4 are important for platelet secretion. As with the studies of the VAMPs, using antibodies as inhibitors can lead to ambiguous results. Recent studies have shown that deletion of both syntaxins from murine platelets causes no secretion defect despite a subtle effect on clot retraction (Ye et al., 2012, *Blood*, in press).

Proteomics studies were the first to show that syntaxin-11 is present in platelets.[63] Syntaxin-11 is a unique member of the syntaxin family, having an *N*-terminal, Habc, regulatory domain and SNARE motif, but lacking a classical TMD. Syntaxin-11 appears to be anchored to membranes by a, as yet to be identified, modification of its *C*-terminal cysteine residues.[75,76] Syntaxin-11 is associated with late endosomes and the *trans*-Golgi network in Hela cells.[76] Interestingly syntaxin-11 mutations are associated with Familial Hemophagocytic Lymphohistiocytosis type 4 (FHL4). FHL4 is a rare autosomal recessive disorder of immune dysregulation associated with uncontrolled T cell and macrophage activation (discussed below). Syntaxin-11–deficient NK-cells exhibit a defect in cytotoxic granules degranulation, indicating that syntaxin-11 could be involved in fusion with the plasma membrane. Analysis of platelets from an FHL4 patient suggests a role for syntaxin 11 (Ye et al., 2012, *Blood*, in press). Thus, loss of syntaxin 11 had no effect on syntaxin 2 or 4 levels, but significantly affected secretion of all three granule populations. Syntaxin 11 was also shown to form complexes with VAMP-8 and SNAP-23, further supporting its role in platelet exocytosis. Future studies should clarify how the various syntaxin proteins in platelets are used for secretion.

SNARE REGULATORS

Regulated platelet secretion only occurs in response to external signals, but how these signals are coupled to the SNARE machinery is unclear. Very few VAMP and SNAP-23/25 regulators have been identified. Based on the number of binding partners identified, it is thought that most SNARE regulation is mediated by syntaxins or syntaxin-containing complexes. Posttranslational modifications, such as phosphorylation of SNAREs or SNARE regulators could provide links from signaling to secretion.

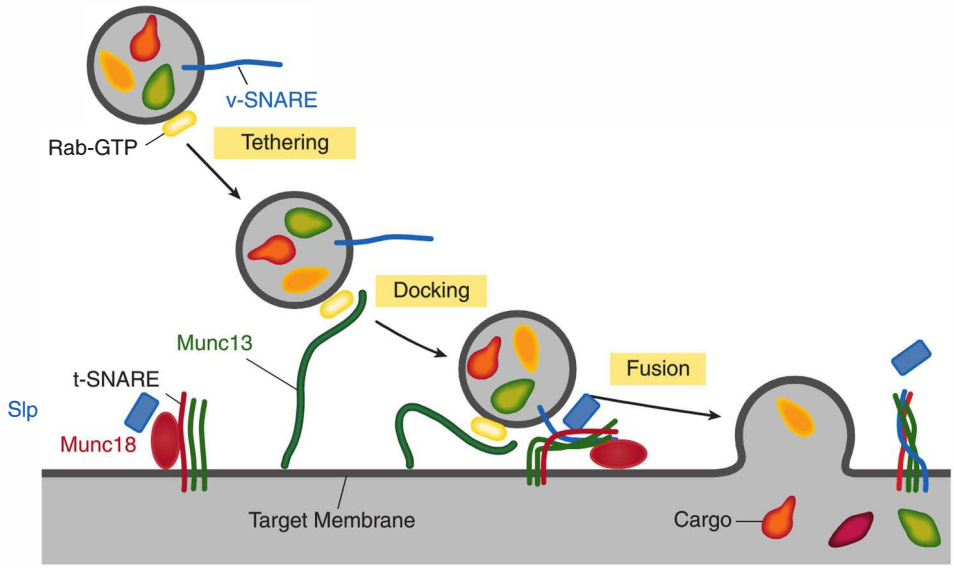

FIGURE 32.2 Platelet secretion pathway. The elements discussed in the text are arranged in a pathway of potential steps that could lead to cargo release. The SNARE proteins are proposed to mediate membrane fusion. The Munc18 proteins chaperone the t-SNAREs and perhaps participate in SNARE complex formation. The Munc13 and Rab proteins promote granule–plasma membrane tethering and docking. Docking facilitates the formation of the SNARE complexes. The role of the Slp proteins is less certain, though they do interact with Rabs and Munc18/syntaxin complexes. Tomosyn, not shown, somehow affects the t-SNARE complex before v-SNARE binding.

Several potential SNARE regulators have been identified in platelets and most are associated with t-SNARE heterodimers or t-SNARE–containing complexes. These include Munc18s, Rabs, Munc13s, Slps, and tomosyn (FIGURE 32.2).

While the SNARE proteins are sufficient for membrane fusion, they do so inefficiently and thus require accessory proteins to control where and when they interact. Many of these regulators appear to be sensitive to second messengers such as diacylglycerol (DAG) and Ca^{2+}, while others are potential kinase substrates. The Munc18 family (a, b, and c) control syntaxins and have been shown to be critical for platelet secretion.[65,77] Tomosyn may regulate t-SNARE heterodimers.[78] Munc13s bind to lipids and to SNAREs.[79] They have Ca^{2+} and DAG-binding sites and may be regulated when these secondary messengers are generated during platelet activation. Other potential SNARE regulators such as DOC2 and Slps have calcium-binding sites and thus could serve as calcium sensors.[80] The function of many of these proteins in platelets has yet to be studied.

Munc18s

Munc18s belong to the evolutionally conserved Sec1/Munc18 (SM) family, critical components of the secretory machinery[81] (FIGURE 32.2). There are three mammalian Munc18 isoforms (Munc18a, b, and c) and all have been detected in human and murine platelets by immunoblotting and/or polymerase chain reaction.[65,77,82] It has been thought that different SM family members are required for specific secretion steps through their interactions with specific syntaxins. Munc18a is almost exclusively expressed in neurons where it binds to syntaxin 1; Munc18c is expressed ubiquitously and appears to interact with syntaxin 4.[83] In platelets, Munc18a was shown to interact with syntaxin 2, Munc18c with syntaxin 4, and Munc18b was found associated with SNARE complexes[77] and with syntaxin 11 (Al Hawas et al., 2012, *Blood*, in press). Early studies suggested that Munc18c phosphorylation and Munc18c/syntaxin-4 complex formation could be important for platelet secretion.[65,82] Monoclonal antibodies that disrupted Munc18c/syntaxin-4 complexes had a positive effect on granule release from permeabilized platelets.[65] Introduction of peptides based on a conserved regulatory domain of Munc18s into permeabilized platelets inhibited Ca^{2+}-stimulated release from all three granule types.[77] Although these *in vitro* studies suggested that Munc18s are important for platelet secretion, platelets from Munc18c heterozygous mice exhibited normal release.[84] Double deletion of Munc18c is embryonic lethal.[85] Interestingly, the syntaxin-4 level is reduced approximately 50% in Munc18c heterozygous mouse platelets, which is consistent with Munc18c being the chaperone specific for syntaxin-4. The association between Munc18c and syntaxin-4 is required for the stability of both proteins.[77,84] Although *in vitro* studies suggest Munc18c plays a role in platelet secretion, the lack of secretion defect in Munc18c$^{+/-}$ platelets could be attributed to functional compensation by other Munc18s. Munc18a levels are low in human and murine platelets and although it is phosphorylated and binds syntaxin-2,[77] it is not clear if it plays any role in exocytosis. The most promising candidate for a functional Munc18 in platelets is Munc18b. Munc18b is the most abundant isoform and it was found to interact with syntaxin-11. Munc18b was also detected in pull-down experiments using SNARE complexes and t-SNARE heterodimers. Most importantly, Sandrock et al.[86] and Al Hawas et al. (2012, *Blood*, in press) showed that Munc18b-deficient platelets from FHL5 patients have a robust secretion defect, suggesting that Munc18b is required for platelet secretion. The fact that heterozygous patients have a partial

secretion defect might suggest that Munc18b is a limiting factor in the secretion process; this remains to be tested. Syntaxin-11 levels were affected in these patients.

Rabs

A pathway of protein–protein interactions has been proposed for platelet secretion in which granules are tethered and dock to the inner leaflet of the plasma membrane after which fusion of the two opposing lipid bilayers mediates cargo release.[87,88] Docking and tethering are thought to be, in part, mediated by small guanosine triphosphate (GTP)-binding proteins of the Rab family (FIGURE 32.2). The Rab subfamily of small Ras-like GTPase includes over 60 members in mammals and is thought to regulate most intracellular membrane traffic.[87,88] Platelets have been reported to contain at least 11 Rabs, although few have been shown to be functionally relevant. It is generally thought that different Rabs regulate different secretion routes through association with specific effectors. Rab6 and Rab8 are preferentially targeted to the plasma membrane and α-granules.[89] α-granule–associated Rab6 is phosphorylated upon thrombin stimulation in a protein kinase C (PKC)-dependent manner, and phosphorylation seems to increase its GTP loading.[90] Studies using a recombinant, dominant-negative mutant suggested that Rab4 regulates α-granule, but not dense granule secretion.[91] Biochemical studies have shown that the majority of Rab27 is associated with dense granules, while a small portion is located on the plasma membrane.[91] Rab27a/b is involved in both dense granule biogenesis in megakaryocytes and in platelet secretion. Deficiency of Rab27a in *ashen* mice causes "ghost" dense granules (a normal number but without contents),[92] while loss of Rab27b results in 50% reduction in both dense granule number and serotonin content[93] with normal α-granule contents and secretion. These data suggest that the Rab27 family is critical for dense granule biogenesis, but not for α-granule and lysosome biogenesis. Further, platelets from Rab27 double-knockout mice display more severe defect in serotonin secretion than knockout of Rab27b alone, suggesting that Rab27a can partially compensate Rab27b loss.[93] However, the exact role of Rab27 in dense granule secretion remains unclear. Presumably the distinct functions of Rab27 require different effectors. The fact that one of Rab27b effectors, Munc13-4, is required for platelet secretion[94,95] could explain why Rab27b plays a role in exocytosis. Further exploration of the mechanisms for Rab function in platelet secretion will clarify whether different granule secretion requires specific sets of Rab proteins, effectors, or even secretory machinery components in platelets.

Munc13s

The Munc13 family has four members: Munc13 1 to 3 are expressed in neuronal tissue; Munc13-4 is ubiquitously expressed.[96–99] Munc13s are widely believed to be vesicle priming factors (FIGURE 32.2). The strongest evidence for this comes from genetic ablation of both Munc13-1 and Munc13-2 in neurons. This abrogates synaptic vesicle priming and release.[100,101] Munc13-4 was first identified in platelets as a Rab27a-binding partner[95] and is essential for platelet secretion.[94] Mice lacking Munc13-4 have a severe bleeding diathesis, and release from dense granules is totally abolished, whereas release from α-granules and lysosomes is severely impaired. This implies that Munc13-4 mediates at least one step that is uniquely required for rapid, dense granule release, a step that is also critical for hemostasis *in vivo*. The residual secretion from α-granules and lysosomes could be due to compensation by another Munc13 family member since both Munc13-1 and Munc13-4 may be able interact with Munc18a/syntaxin-2 complex in platelets. Compared to other Munc13s, Munc13-4 does not contain a DAG-binding C1 domain, but does have two calcium/phosphatidylserine-binding C2 domains (C2A and C2B) and 2 Munc Homology Domains (MHD). Mutations in the MHD domain or deletion of C2B result in FHL3 in humans.[97] Recombinant Munc13-4 lacking C2B is a potent inhibitor of platelet secretion from permeabilized cells[94] and suggests that both the MHD domain and C2B domains are important for Munc13-4 function. It is possible that the MHD domain is required for interactions with important secretory machinery components, while C2B is important for anchoring to the plasma membrane or for regulating membrane fusion in response to Ca^{2+} signaling.

Munc13-4 is an essential component in dense granule secretion pathway. Identification of key Munc13-4 interacting partners will be an important step in understanding SNARE complex formation and membrane fusion upon stimulation. Clearly, Rab27 is a possible candidate, as are other Rab27 effectors such as members of the Slp family (see below). Another possible candidate effector is DOC2α because it has been shown to interact with other Munc13 isoforms.[102]

Synaptotagmin-Like Proteins (Slp)

Slps possess a *C*-terminal synaptotagmin-like domain with *C*-terminal tandem C2 domains (putative Ca^{2+}-dependent phospholipid binding sites) and an *N*-terminal Rab27-binding domain. There are five Slp isoforms (Slp1 to 5) in mammals.[103] Slp1 and Slp4 (or granuphilin) are present in platelets (Ye et al., *in preparation*, 2012 and Ref.[104]). Studies by Neumuller et al.[104] demonstrated that purified Slp1 can partially inhibit dense granule secretion from permeabilized platelets. Endogenous Slp1 forms a trimetric complex with Rap1GAP2 and Rab27 suggesting Slp1 is a Rab27 effector that regulates dense granule secretion (FIGURE 32.2).

Although little is known about the function of Slp4/granuphilin in platelets, it has been well studied in other secretory cells. Slp4/Granuphilin is abundantly expressed in beta cells and is considered to be essential for the stable attachment of insulin granules to the plasma membrane. There, it acts by linking Rab27a on the granule membrane with the syntaxin-1a/Munc18a complex on the plasma membrane,[105] and loss of Slp4/granuphilin causes a decrease in docked granules.[106] Slp4/Granuphilin can bind to both syntaxin-1a and Munc18a.[107–109] The enhanced insulin secretion from Slp4/granuphilin-deficient beta cells,[110] despite the docking defect, argues for it being a negative regulator. Granuphilin could have a negative effect on a step just prior to the final fusion event. It is possible that a local signal, such as increased intraplatelet calcium after stimulation, could cause dissociation of Slp4/granuphilin from the Munc18a/syntaxin-1a complex and allow SNARE assembly. Further analysis will be needed to determine what role this family plays in platelet secretion.

Tomosyn

Tomosyn-1 belongs to a family of WD-40 repeat–containing proteins associated with exocytosis, including Sro7/Sro77

in yeast, Tom1 in *Caenorhabditis elegans*, amisyn and lethal giant larvae (lgl) in mammals.[78,111–114] Two genes, tomosyn-1 and tomosyn-2, are present in mammals, but alternative splicing can produce a total of seven isoforms.[115,116] Little is known about the exact role of tomosyn-2 in secretion.[115] Tomosyn-1 is well studied in neuron and endocrine cell exocytosis. Tomosyn-1 has three isoforms (b-, m-, s-) and has several predicted phosphorylation sites. m-Tomosyn-1 was originally identified as a syntaxin-1 binding partner in rat brain tissue cell.[117] The s-tomosyn-1 and m-tomosyn-1 isoforms are enriched in synapse-specific domains while b-tomosyn-1 is ubiquitously expressed.[118] Structurally, mammalian tomosyn-1 contains an *N*-terminal WD-40 repeat, a small highly variable linker region, and a *C*-terminal v-SNARE motif.[116] Both the v-SNARE motif and *N*-terminal WD-40 repeat have been shown to inhibit secretion from PC12 cells[114] and neurons.[119] Thus, tomosyn-1 has been thought as a negative regulator for neurotransmitter release events. Consistently, deletion of tomosyn-1 enhances secretion.[119] Overexpression of tomosyn-1, in PC12 cells, reduces the ready-release pool and the resident time of secretory vesicles.[120] This observation is consistent with the hypothesis that tomosyn-1 functions by controlling vesicle priming.

It has also been suggested that the *N*-terminal WD-40 domain may catalyze the formation of nonfunctional SNARE complexes[121] or, through its loop region, by interacting with unknown factors.[122] Alternatively, the *C*-terminal v-SNARE domain could block v-SNARE binding to t-SNAREs, thus inhibiting membrane fusion and exocytosis. Posttranslational modifications, such as phosphorylation, have been suggested to regulate this process.[123] Despite these data, the exact mechanism by which tomosyn-1 functions as negative regulator remains unclear. Although data support a negative role for tomosyn, tomosyn also has been shown to have a permissive role. Overexpression of tomosyn in neurons enhances secretion,[123] and in chromaffin cells, it enhances the sustained component of exocytosis.[114,120] Moreover, depletion of both Sro7/Sro77 (yeast tomosyn) inhibits secretion, and knockdown of tomosyn in β-cell results in a secretion defect.[124] This suggests that tomosyn could function as place holder for v-SNAREs and positively regulate *trans*-SNARE complex formation.

The role of tomosyn in platelets has yet to be clarified. Tomosyn-1 was first identified in platelets by mass spectrometry as a specific binding partner of purified t-SNARE complexes (Ye et al., *in preparation*, 2012). The most abundant isoform of tomosyn in platelets is m-tomosyn, which is similar to that in β-cells.[124] Whether other isoforms of tomosyn-1 or tomosyn-2 are present in platelet is unknown. Platelets from tomosyn-1–deficient mice have a normal aggregation phenotype, but a partial secretion defect when compared to platelets from normal littermates. It is noteworthy that tomosyn-deficient mice manifest a robust bleeding phenotype in two *in vivo* thrombosis models, further suggesting that tomosyn plays an essential role for hemostasis (Ye et al., *in preparation*, 2012).

IMPORTANCE OF PLATELET SECRETION

In humans, defective platelet granule release presents as a mild to moderate bleeding diatheses. While generally not life threatening, this does increase the bleeding risks following traumatic and iatrogenic vascular damage. The two major class of storage pool deficiencies (SPD), Hermansky-Pudlak syndrome (HPS, δSPD)[7,125–127] and gray platelet syndrome (GPS, αSPD),[10,128,129] are characterized by the loss of cargo from dense granules and α-granules, respectively. There are 15 HPS-like mouse strains that have similar defects. Cloning of the defective genes and the subsequent analysis of their products has shown that the phenotypes are related to dysfunctional sorting of dense granule proteins during biogenesis.[126,127] Most of the HPS genes encode proteins that participate in the sorting of cargo from and through endosomal compartments. GPS patients present with more heterogeneous bleeding defects but generally have normal pigmentation. Less is known about the underlying molecular defects that cause GPS, though one knockout mouse strain (transcription factor Hzf$^{-/-}$) does display a loss of α-granule cargo.[130] In this strain, initial bleeding times are normal, but the incidence of rebleeding is increased. Mutations in the NBEAL2 gene have been identified as being associated with GPS.[131–133] This gene encodes a BEACH (beige-Chediak-Higashi)-domain-containing protein which is a member of a family that has been linked to other membrane trafficking events. Its function is unclear at present. One striking feature of GPS patients is that all have myelofibrosis.[10] This is perhaps due to the inability of the megakaryocytes to retain synthesized growth factors. Consistently, plasma levels of platelet factor IV (PF4) and β-thromboglobulin (both are α-granule cargoes) are elevated in GPS patients. Little is known about the consequences of the inappropriate distribution of α-granule cargo. Patients with loss of both types of granules are rare and do have bleeding defects.[134,135]

Platelet Secretion in Therapy and Diagnosis

The goal of antithrombotic therapies is to devise a regimen that dampens thrombosis without inducing excessive bleeding. The secretory machinery might prove an effective target to accomplish this goal. *In vivo* analysis of the VAMP-8$^{-/-}$ mice shows a clear defect in laser-induced arteriole thrombosis, but one not as complete as in an HPS mouse.[8] Thrombi in VAMP-8$^{-/-}$ mice display a significant delay in initiation as well as a lack of stability. However, thrombi do form, and there is no significant difference in tail-bleeding times in these animals. These data suggest that targeting the platelet's primary secretory machinery might be an effective way to manage thrombosis *in vivo* and thus limit clot formation without inducing excessive bleeding. Future analysis of other mouse strains with secretion-deficient platelets is ongoing to validate this concept and to probe the additional roles that platelet exocytosis might play in the vasculature.

FHL is a rare immune disorder characterized by proliferation of cells of the mononuclear phagocyte system (histiocytes) and hyperinflammation.[136–138] Typical clinical features include fever, hepatosplenomegaly, lymphadenopathy, jaundice, rash, and laboratory findings of lymphocytosis, histiocytosis, and hemophagocytosis. Five genes have been associated with FHL. FHL1 is associated with a potential gene locus (9q21.3-q22) but the specifically involved gene has not been identified.[139] The perforin gene (PFR1) mutations at 10q21-22 locus were described as the causative gene for FHL2.[140] Perforin is a toxin in cytotoxic granules that is essential for natural killer (NK) and cytotoxic T lymphocyte (CTL) cell-mediated killing. This pore-former plays a critical role in maintaining immune homeostasis.[141] Mutations in the genes encoding *Munc13-4* (FHL3), *Syntaxin-11* (FHL4), and *Munc18b* (FHL5) have been

shown to be involved in cytotoxic granule exocytosis from CTL and NK cells.[97,142,143] Detection of perforin, Munc13-4, syntaxin-11, and Munc18b expression in NK cells by fluorescence activated cell sorting (FACS) or immunoblotting analysis is a reliable method to screen for FHL2-5. However, these analyses may not be readily available to all patients. Although a consensus set of criteria for diagnosis of FHL was established in 2004 by the Histiocyte Society and was revised in 2007,[136] diagnosis is still difficult since most criteria are not specific. In reality, patients may be missed because some initial symptoms are rather nondescript,[137] may be disguised as a normal infection, or may overlap with some immune-deficient disorders. Thus, it is critical to find reliable methods for early screening in order to initiate therapy. Based on the studies of Munc13-4 (FHL3), syntaxin-11 (FHL4), and Munc18b (FHL5), it seems that analysis of platelet secretion might prove to be a valuable tool for diagnosing FHL.

Dysfunction of Munc13-4 (FHL3), syntaxin-11 (FHL4), and Munc18b (FHL5) all lead to a secretion defect that could be detected with simple platelet assays. Lumi-aggregometry to measure ATP release from dense granules or FACS analysis to measure P-selectin or LAMP-1 exposure from α-granules or lysosomes, respectively, are relatively simple and readily available assays that could be performed on small blood samples. A defect in secretion from platelets would suggest further tests and perhaps accelerate the diagnosis of FHL. Future analysis will be required to validate this approach. However, it should be noted that Murata et al.[144] have shown that FACS analysis of intraplatelet Munc13-4 protein is a sensitive and reliable method to screen for FHL3.

SUMMARY

Platelet secretion is clearly important for hemostasis and definitely contributes to the microenvironment at the site of vascular damage as well. Release of platelet granule cargo is mediated by SNARE proteins and regulated by proteins that control how SNAREs interact. In turn, these SNARE regulators integrate signals from the signaling cascades initiated by the numerous cell surface receptor proteins. Understanding how these signals control the secretory machinery has increased substantially over the last decade, and potentially relevant therapeutic targets are beginning to be uncovered, suggesting that directly modulating platelet secretion maybe a potentially useful way to affect thrombosis.

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CHAPTER

33 Platelet Signal Transduction

CHARLES S. ABRAMS AND LAWRENCE F. BRASS

Platelets become activated when agonists such as collagen or thrombin bind to receptors on the platelet surface, initiating intracellular signaling events that eventually lead to reorganization of the platelet cytoskeleton, platelet aggregation, and platelet granule secretion. This process can only occur when local barriers to platelet activation are exceeded or overwhelmed. Platelets are normally shielded from the connective tissue matrix within the vascular wall by a continuous barrier of endothelial cells which physically separates platelets from platelet activators in the vessel wall. When vascular injury occurs, platelets adhere to exposed collagen fibrils, forming a discontinuous carpet. *In vitro*, adhesion to collagen can occur without the help of accessory molecules, but *in vivo*, the shear forces and turbulence caused by flowing blood would strip platelets away from collagen were it not for the stabilizing effects of von Willebrand factor (vWF). The metalloprotease ADAMTS13 cleaves vWF to prevent the accumulation in plasma of ultrahigh molecular weight multimers that would otherwise cause spontaneous platelet clumping and arterial thrombosis.[1,2,3,4] vWF binding to collagen *via* its A3 domain exposes binding sites on the vWF A1 and C1 domains for the platelet adhesion receptors, GP Ib-IX-V and $\alpha_{IIb}\beta_3$ (GP IIb-IIIa), respectively.[5,6] Combined with the activating signals produced when collagen binds directly to the $\alpha_2\beta_1$ integrin and to glycoprotein VI on the platelet surface, platelets that have adhered to collagen change their shape, spreading along the collagen fibrils, and releasing into the circulation thromboxane A_2 (TxA_2) and adenosine diphosphate (ADP) (FIGURE 33.1).

Released TxA_2 and ADP recruit additional platelets, causing them to stick to each other and to the platelets directly adherent to collagen. The growing mound of activated platelets is eventually stabilized by a crosslinked fibrin clot, but the critical contact between adjacent platelets depends on the binding of fibrinogen or fibrin to $\alpha_{IIb}\beta_3$ on the platelet surface, and this occurs only after platelets have been activated. Since fibrinogen is a symmetrical molecule, it can bind to two platelets at the same time, as can fibrin. Repeated as many as 80,000 times per platelet, this allows platelets to stick to each other, a process that can be made to occur when platelets are in suspension, but should normally occur only at a site of vascular injury. Like collagen, soluble platelet agonists cause platelets to change their shape, losing the discoid appearance characteristic of resting platelets, and transform them into an irregular sphere with pseudopodia. Underlying this shape change is rapid reorganization of the platelet cytoskeleton as actin filaments are uncapped, severed, and rebuilt in response to intracellular signals that include a rise in the cytosolic Ca^{2+} concentration and the sequential activation of low molecular weight GTP-binding proteins such as Rac and Rho.

Collagen, ADP, and TxA_2 are not alone in their ability to activate platelets. Vascular injury and inflammation expose tissue factor as well as collagen, and formation of the tissue factor/factor VIIa complex leads to the local generation of thrombin from prothrombin. Thrombin is a potent agonist, activating platelets at concentrations in the pM range by interacting with receptors on the platelet surface. Activated platelets facilitate this process by providing procoagulant phospholipids that accelerate thrombin generation. As a result, platelet activation and fibrin deposition are intimately linked, maximizing the growth and strength of the hemostatic plug. Human platelets express two G-protein–coupled receptors (GPCRs) that can be activated by thrombin. Cleavage of these receptors accounts for the long-established observation that only proteolytically active thrombin can activate platelets.

The process of transforming $\alpha_{IIb}\beta_3$ on the platelet surface into a competent receptor for fibrinogen is one of the most fascinating aspects of platelet biology.[7] It is also the final common pathway in platelet responses to most agonists, making it a frequent target for drug development. Stated succinctly, the central issue is this: although circulating platelets are surrounded by plasma proteins, they normally do not bind fibrinogen to their surface and stick to each other unless they have been activated. The reasons for this are multiple, but are ultimately due to the inability of fibrinogen or fibrin to bind to the resting conformation of $\alpha_{IIb}\beta_3$. Platelet activation alters the conformation of $\alpha_{IIb}\beta_3$ and exposes the fibrinogen-binding site. Normally, this should occur only at sites of vascular injury. However, even in the absence of substantial vascular injury, it is likely that the buffeting that platelets withstand as they move through the circulation, perhaps encountering low concentrations of thrombin or fractured atherosclerotic plaques, pushes them toward inappropriate activation.

Working against this tendency are a number of internal and external controls that dampen intracellular signals that would otherwise promote platelet activation. In addition to providing a physical barrier and a source of vWF, endothelial cells release PGI_2 and NO, two molecules that globally depress intracellular signaling in platelets by raising cyclic AMP (cAMP) and cGMP levels.[8–14] The ability of cyclic nucleotides to inhibit platelet activation has been exploited in the development of antiplatelet agents such as dipyridamole (Persantine), which raises cAMP levels inside platelets by inhibiting cAMP phosphodiesterase.[15] Cyclic GMP also inhibits cAMP phosphodiesterase in platelets, contributing to the rise of cAMP levels. In turn, cAMP activates protein kinase A, which phosphorylates multiple platelet proteins including the β chain of GP Ib,[16,17] actin-binding protein (filamin),[18] myosin light chain,[19,20] vasodilator-stimulated phosphoprotein (VASP),[21,22] and Rap1B.[23] The net effect is a generalized inhibition of platelet activation. The importance of PGI_2

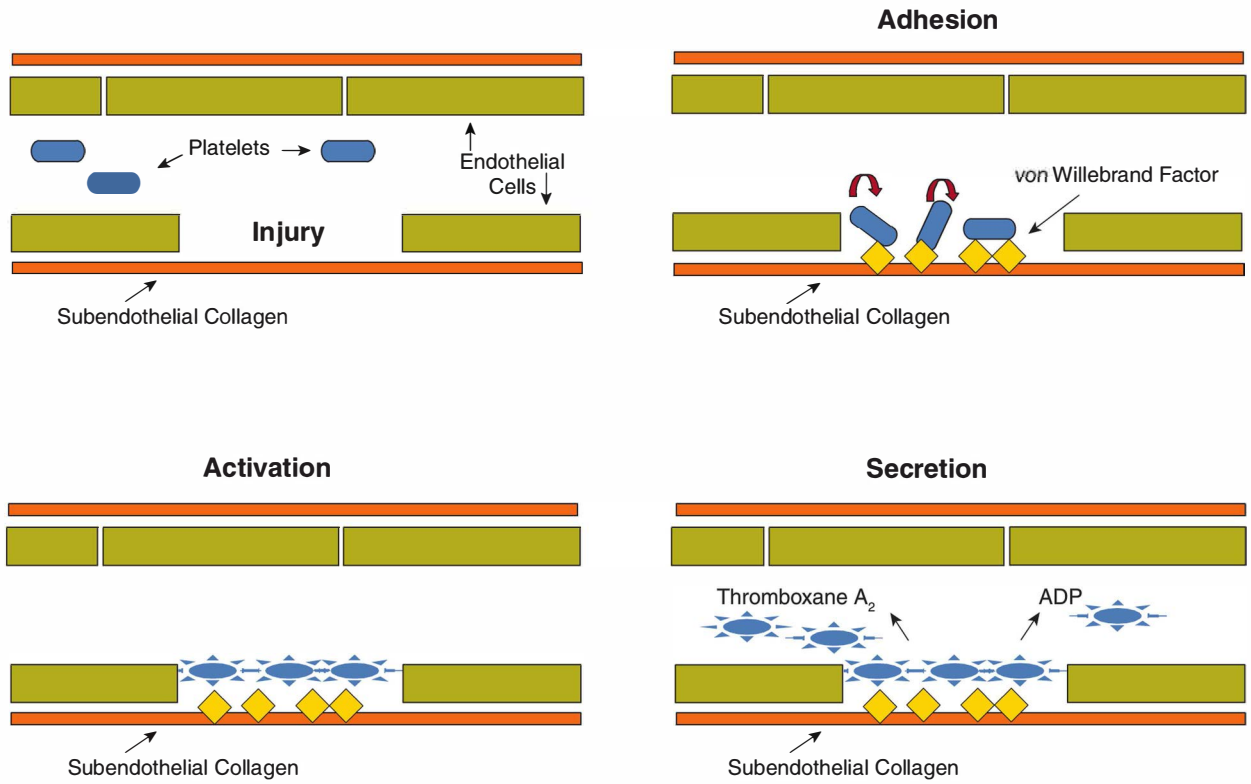

FIGURE 33.1 Stages in platelet plug formation. Prior to vascular injury, platelets are restrained from activation by the inability of plasma vWF to bind spontaneously to the platelet surface. The development of the platelet plug can be initiated by the exposure of collagen in the vessel wall or by the local generation of thrombin (or both). Rolling platelets adhere and spread on the collagen/vWF matrix, forming a monolayer of activated platelets that acts as a surface for subsequent recruitment of platelets by thrombin, ADP, and TxA_2.

and NO as barriers to platelet activation is indicated not only by the effectiveness of molecules that mimic PGI_2 as antiplatelet agents, but also by the prothrombotic effects in mice of deleting the gene encoding the platelet PGI_2 receptor (IP)[24] and by the decrease in basal cAMP levels found in $IP^{-/-}$ platelets.[25] In addition to releasing PGI_2 and NO, some endothelial cells express the *ecto*-ADPase CD39 on their luminal surface. CD39 can hydrolyze the small quantities of ADP released from damaged red cells and activated platelets, preventing the ADP from activating additional platelets.[26,27] Other barriers to unwarranted platelet activation include the diluting effects of continued blood flow, the presence of natural anticoagulants that limit thrombin formation, the short half-life of the platelet-derived agonist, TxA_2, and a variety of mechanisms that limit the duration of signaling by G proteins and GPCRs. This includes receptor desensitization, receptor internalization, and regulators of G-protein signaling (RGS) proteins. Collectively, these provide a threshold that helps to prevent platelet activation at inappropriate times and places.

THREE STAGES OF PLATELET PLUG FORMATION

One might consider the platelet surface as being crowded with receptors that support one or more of the phases of platelet plug formation. In fact, approximately half of the platelet's surface is occupied by $\alpha_{IIb}\beta_3$. Receptors that are directly involved in binding to adhesive proteins such as collagen, vWF, and fibrinogen are present in the greatest numbers. After recruitment of additional pools of receptors that are initially within the surface-connecting membrane system or in the membranes of α granules, there are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ and 15,000 copies of GP Ib on the surface of human platelets (these receptors are also discussed in Chapters 26A, 26B, and 31). In contrast, receptors that primarily serve as signaling response elements for platelet agonists typically range from a few hundred to a few thousand per platelet (Table 33.1). Although these numbers are not large in absolute terms, when placed in the context of the relatively small size of human platelets, the density of these receptors is high. In cells other than platelets, it has been shown that some signaling molecules tend to be concentrated within or near cholesterol-enriched microdomains within the plasma membrane.[28] There is growing evidence that this occurs in platelets as well, increasing the efficiency of platelet activation.[29–31]

The platelet monolayer that forms following the exposure of collagen and vWF initiates platelet plug formation but is insufficient to prevent bleeding. The extension or recruitment phase of platelet plug formation occurs when additional activated platelets accumulate on top of the monolayer.[32,33] Recruitment is mediated by the local accumulation of molecules that are released from platelets, such as ADP and TxA_2. Contacts between platelets are maintained by a variety of molecular interactions, of which

Table 33.1

GPCRs expressed on human platelets

Agonist	Receptor	Approximate Number of Copies per Platelet	G-Protein Families	Reference
ADP	$P2Y_1$	150	G_q	109,110
	$P2Y_{12}$	600	G_i	65
Thrombin	PAR1	2,000	G_q, G_{12}	73,88,89,213,214
	PAR4	Unknown	G_q, G_{12}	215
TxA_2	TPα and TPβ	1,000	G_q, G_{12}	129–131,216–218
Epinephrine	α_{2A}-adrenergic	300	G_i (particularly G_z)	121,122,219
PGI_2	IP	Unknown	G_s	220

the best described, and most critical, is binding of fibrinogen and vWF to activated $\alpha_{IIb}\beta_3$. Thrombin continues to participate in this phase of platelet activation and, in turn, activated platelets provide a procoagulant surface on which more thrombin can be generated *via* assembly of the tenase and prothrombinase coagulation complexes. Circulating or locally secreted epinephrine causes vasoconstriction, but also potentiates the effects of other platelet agonists. In contrast to platelet activation by collagen, platelet activation by most of the agonists involved in extension of the platelet plug is very rapid, with some responses occurring within a fraction of a second. Teleologically, this makes sense since circulating platelets would not be expected to linger at a wound site long enough for a slower process to occur. Videos of thrombus formation at sites of focal vascular injury show that initially most platelets move by the site of injury at speeds too rapid for activation to occur.[32] Tethering to vWF-decorated collagen or to previously deposited platelets allows new platelets to remain in place long enough to become activated.

Formation of the platelet plug following vascular injury can be thought of as involving three stages: adhesion, activation, and secretion (FIGURE 33.1). Adhesion begins with the rolling, arrest, and activation of moving platelets by the collagen/vWF complex to form a platelet monolayer. Activation refers to the recruitment of additional platelets through the stimulation by fast-acting agonists such as thrombin, ADP, and TxA_2, all three of which activate platelets *via* GPCRs on the platelet surface. Secretion of additional agonists and adhesive ligands perpetuates these events by recruiting additional platelets to participate in the formation of the platelet plug. These definitions are arbitrary, since the adhesion of platelets to collagen and vWF also initiates some of the early stages of activation. In addition to these functionally defined stages in platelet plug formation, stable contacts between platelets make possible an additional wave of intracellular signaling that reinforces platelet activation. This "contact dependent" signaling is mediated in part by adhesion molecules such as $\alpha_{IIb}\beta_3$ and in part by platelet surface ligands such as ephrinB1 and sema4D that bind to receptors on the surface of adjacent platelets.[34]

PLATELET ADHESION

The arrest and eventual activation of moving platelets by collagen is accomplished by receptors on the platelet surface that either bind directly to collagen (GP VI and the integrin $\alpha_2\beta_1$) or bind indirectly to collagen *via* vWF (the GP Ib/IX/V complex and the integrin $\alpha_{IIb}\beta_3$). The integrins require prior activation *via* intracellular "inside-out" signaling to engage collagen and vWF, after which they can contribute further platelet activation by generating outside-in signals. The presence of collagen and vWF-binding sites on GP VI and GP Ibα allows platelets to slow down long enough to become activated and fully adherent. Platelet activation by collagen is summarized in FIGURE 33.2. Intracellular signaling in response to collagen is mediated by at least three receptors: GP VI, GP Ib, and, after integrin activation has occurred, $\alpha_2\beta_1$. The structure of GP VI places it in the immunoglobulin domain superfamily. Its ability to generate signals rests primarily on its constitutive association with a second molecule, the Fc receptor γ-chain.[35] Platelets from mice that lack the γ-chain[36] have impaired responses to collagen. So do platelets from humans,[37,38] but possibly not mice,[39] with reduced expression of $\alpha_2\beta_1$. Using blocking antibodies or a depletion strategy, Nieswandt et al.[39] concluded that GP VI is required for platelet responses to collagen.

Signaling through GP VI can be studied in isolation with the snake venom protein, convulxin, or with synthetic "collagen-related" peptides. According to current models, collagen causes clustering of GP VI and its associated Fc receptor γ-chain. This promotes phosphorylation of the γ-chain by nonreceptor tyrosine kinases in the Src family, creating an immunoreceptor tyrosine-based activation motif recognized by the tandem SH2 domains of Syk. Association of Syk with the GP VI/γ-chain complex activates Syk and leads to the phosphorylation and activation of phospholipase C (PLC) γ_2. Loss of Syk impairs collagen responses.[36] PLCγ PLCg2 hydrolyzes membrane PI-4,5-P_2 to form the second messengers 1,4,5-IP_3 and 1,2-diacylglycerol (DAG). IP_3 opens Ca^{2+} channels in the platelet-dense tubular system (DTS), raising the cytosolic Ca^{2+} concentration and triggering Ca^{2+} influx across the platelet plasma membrane that is initiated when depletion of the DTS Ca^{2+} pool triggers a conformational change in the DTS membrane protein, STIM1, allowing it to bind to Orai1 in the plasma membrane.[40] DAG and Ca^{2+} activate the more common protein kinase C (PKC) isoforms that are expressed in platelets, making possible the regulatory serine/threonine phosphorylation events that are needed for platelet activation. At least one of those phosphorylation events regulates the exposure of fibrinogen-binding sites on $\alpha_{IIb}\beta_3$, but the full mechanism for activating $\alpha_{IIb}\beta_3$ remains obscure. Events

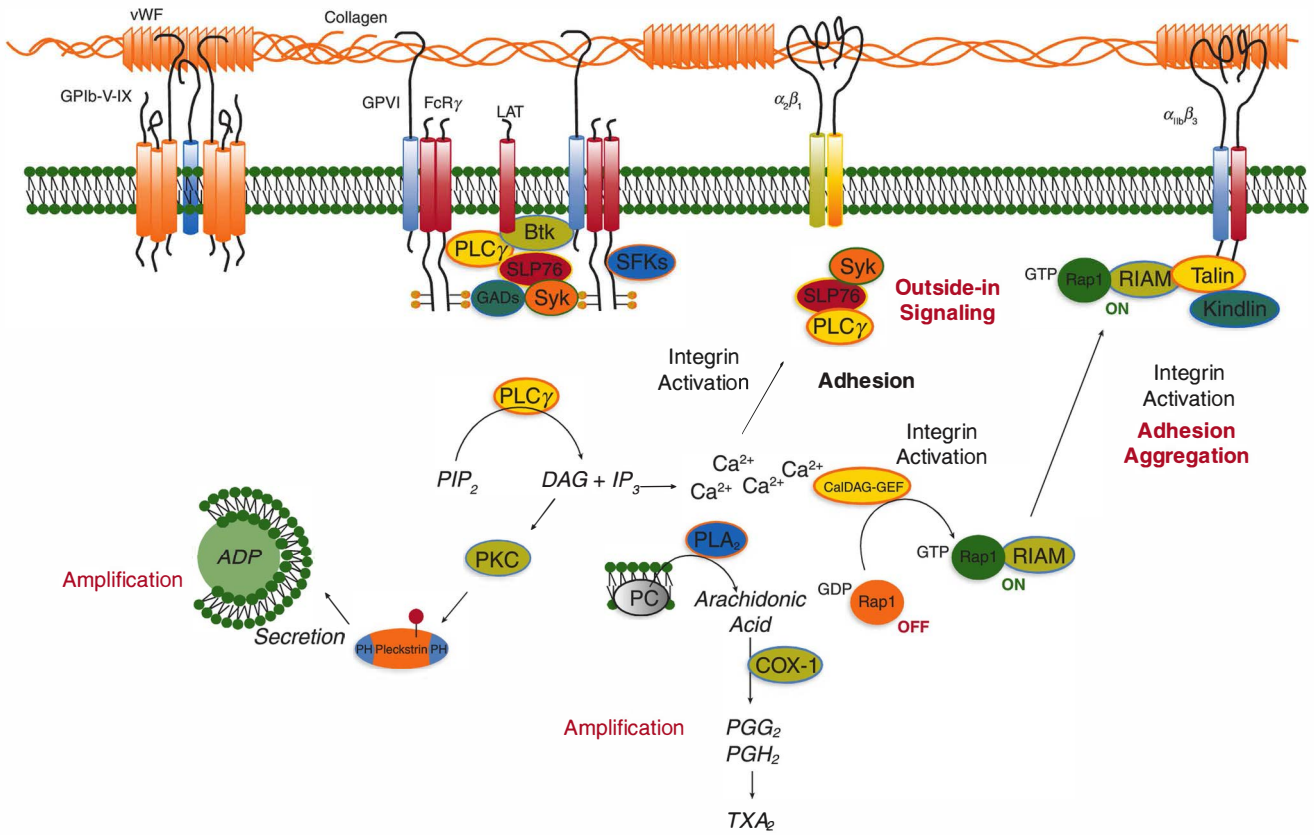

FIGURE 33.2 Platelet activation by collagen. Platelets use several different molecular complexes to support platelet activation by collagen. These include (a) vWF-mediated binding of collagen to the GPIb-IX-V complex and integrin $\alpha_{IIb}\beta_3$, (b) a direct interaction between collagen and both the integrin $\alpha_2\beta_1$ and the GPVI/FcRγ-chain complex. Clustering of GPVI results in the phosphorylation of tyrosine residues in the FcRγ cytoplasmic domain, followed by the binding and activation of the tyrosine kinase, Syk. One consequence of Syk activation is the phosphorylation and activation of PLCγ, leading to phosphoinositide hydrolysis, secretion of ADP, and the production and release of TXA_2. COX-1, cyclooxygenase 1; DAG, diacylglycerol; GP, glycoprotein; PG, prostaglandin; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLCγ, phospholipase Cγ; TXA_2, thromboxane A_2; vWF, von Willebrand factor.

triggered by GP VI lead to activation of $\alpha_2\beta_1$ as well as $\alpha_{IIb}\beta_3$. Engagement of the two integrins with their respective ligands initiates a further round of signaling that features some of the same molecules that are downstream of GP VI: Syk, SLP-76, and PLCγ_2.[41] This is a theme that repeats itself during platelet activation: different agonists with disparate receptors trigger signaling events that involve a common set of intermediates. Outside-in signaling is discussed at greater length in the last section of this chapter.

Finally, GP Ib serves both as an anchor point for the platelet cytoskeleton and as the focus for assembling signaling complexes in platelets that have become bound to vWF. The cytoplasmic domain of GP Ibα can bind to filamin and to the scaffolding protein, 14-3-3ζ. Src becomes associated with GP Ib following vWF binding. The p85 subunit of PI3-kinase appears to mediate this association. In turn, Syk is activated. Thus, signaling through GP Ib takes advantage of some of the same signaling molecules that are involved in GP VI- and integrin-dependent responses to collagen, with the same potential for causing protein tyrosine phosphorylation, PI-4,5-P2 hydrolysis, and recruitment of cytosolic proteins whose Pleckstrin homology (PH) domains can support associations with 3-phosphorylated phosphoinositides.[42–49]

PLATELET ACTIVATION BY SOLUBLE AGONISTS

The agonists involved in extension of the platelet plug typically cause platelet activation *via* GPCRs on the platelet surface. GPCRs are well suited for this task in a number of respects. First, most of these receptors bind ligands with high affinity. Second, GPCRs comprise a single subunit that does not require oligomerization (although recent studies have shown that some GPCRs exist as homo- or heterodimers even in the absence of their ligands).[50–52] Third, GPCRs are constitutively associated with G proteins, eliminating the time that would otherwise be required to recruit them into complexes.[50] Fourth, because they act as guanine nucleotide exchange factors, each occupied receptor can activate multiple G proteins and, in some cases, more than one class of G proteins. This allows amplification of a signal that might begin with a relatively small number of receptors. It also enables some receptors to signal *via* more than one

effector pathway. Finally, because several generic mechanisms exist that can limit the activation of GPCRs, platelet activation can be limited, a property that may be useful when platelet activation is inappropriate and needs to be contained.[34]

GPCRs comprise a single polypeptide chain with seven transmembrane domains, an extracellular *N*-terminus, and an intracellular *C*-terminus. Binding sites for agonists can involve the *N*-terminus, the extracellular loops, or a pocket formed by the transmembrane domains.[50] The G proteins that act as mediators for these receptors are heterotrimers comprised of α, β, and γ subunits. Within this complex, the β subunit forms a propeller-like structure that is tightly associated with the much smaller γ subunit. The α subunit contains a guanine nucleotide-binding site that is normally occupied by GDP. Receptor activation causes the exchange of GTP for GDP, altering the conformation of the α subunit and exposing sites on both G_α and $G_{\beta\gamma}$ for interactions with downstream effectors.[53–55] Hydrolysis of the GTP by the intrinsic GTPase activity of the α subunit restores the resting conformation of the heterotrimer, preparing it to undergo another round of activation and signaling.[56] A family of GTPase activating proteins known as RGS proteins help to accelerate the hydrolysis of GTP by G_α.[57] Prenylation of the γ chain and acylation of the α subunit helps to anchor the complete heterotrimer to the plasma membrane and continues to anchor G_α-GTP and $G_{\beta\gamma}$ to the membrane when they dissociate from each other to expose effector binding sites. Platelets express 10 forms of G_α (Table 33.2). Although there is little information about which types of G_β and G_γ are expressed in platelets, the forms of G_α identified have included at least one $G_{s\alpha}$ family member, four $G_{i\alpha}$ family members, three $G_{q\alpha}$ family members, and two $G_{12\alpha}$ family members. The roles of each have been defined through knockout studies in mice.[58] Based on the available evidence, the abundance of G-protein types in platelets appears to be necessary to support the actions of multiple dissimilar platelet agonists.[25]

GPCRs and Platelets

The GPCRs that respond to platelet agonists differ in their potency and their preferences for intracellular effector pathways. Some, such as the receptors for thrombin (PAR1 and PAR4), TxA_2 (TP), and ADP ($P2Y_1$), cause phosphoinositide hydrolysis and raise the cytosolic Ca^{2+} concentration by activating G_q (FIGURE 33.3).[59] Others, such as the $P2Y_{12}$ receptor for ADP and the α_{2A}-adrenergic receptor for epinephrine, are coupled by G_{i2} and G_z, respectively, to the inhibition of adenylyl cyclase and the activation of PI3-kinase and Rap1B.[25,60–62] Optimal platelet activation *via* GPCRs is thought to require activation of both a G_q-coupled receptor and a G_i-coupled receptor.[63] The ability of the four G_i family members that are expressed in platelets to inhibit adenylyl cyclase, thereby decreasing cAMP formation, is most relevant when PGI_2 secreted by endothelial cells has inhibited platelet activation by raising platelet cAMP levels.[25] Otherwise, it is likely that other G_i effectors account for the required G_i-coupled receptor activation during platelet activation. The biological relevance of the G_{i2}-coupled $P2Y_{12}$ receptor for ADP is supported by the phenotypes of G_{i2} and $P2Y_{12}$ knockout mice and by the efficacy of $P2Y_{12}$ antagonists such as clopidogrel and prasugrel as antiplatelet agents.[25,62,64,65,66] Thrombin and TxA_2 receptors can also cause the rearrangement of the actin cytoskeleton that underlies platelet shape change by coupling to guanine nucleotide exchange factors for Rho *via* G_{12} and G_{13}.[67] In cells other than platelets, the link from G_{12} family members to Rho activation is provided by proteins with RGS

Table 33.2

G-proteins in platelets

Family	G-Protein	Effectors	Function	Reference
G_q	$G_{q\alpha}$ (the G_q predominant isoform)	PLC	↑ IP_3/DAG	221,222
	$G_{11\alpha}$	PLC	↑ IP_3/DAG	221,222
	$G_{16\alpha}G_{15\alpha}$ in mice	PLC	↑ IP_3/DAG	223–226
G_{12}	$G_{12\alpha}$, $G_{13\alpha}$	p115-RhoGEF?	Actin cytoskeleton	67,132,222,227
G_i	$G_{i2\alpha}$>$G_{i3\alpha}$>>$G_{i1\alpha}$	Adenylyl cyclase, other effectors?	↓ cAMP	228
	$G_{z\alpha}$	Adenylyl cyclase, other effectors?	↓ cAMP	229–231
	$G_{\beta\gamma}$ from G_i family members	PLCβ	↑ IP_3/DAG	
		PI3K(γ)	↑ 3-PPIs	
G_s	$G_{s\alpha}$	Adenylyl cyclase	↑ cAMP	223

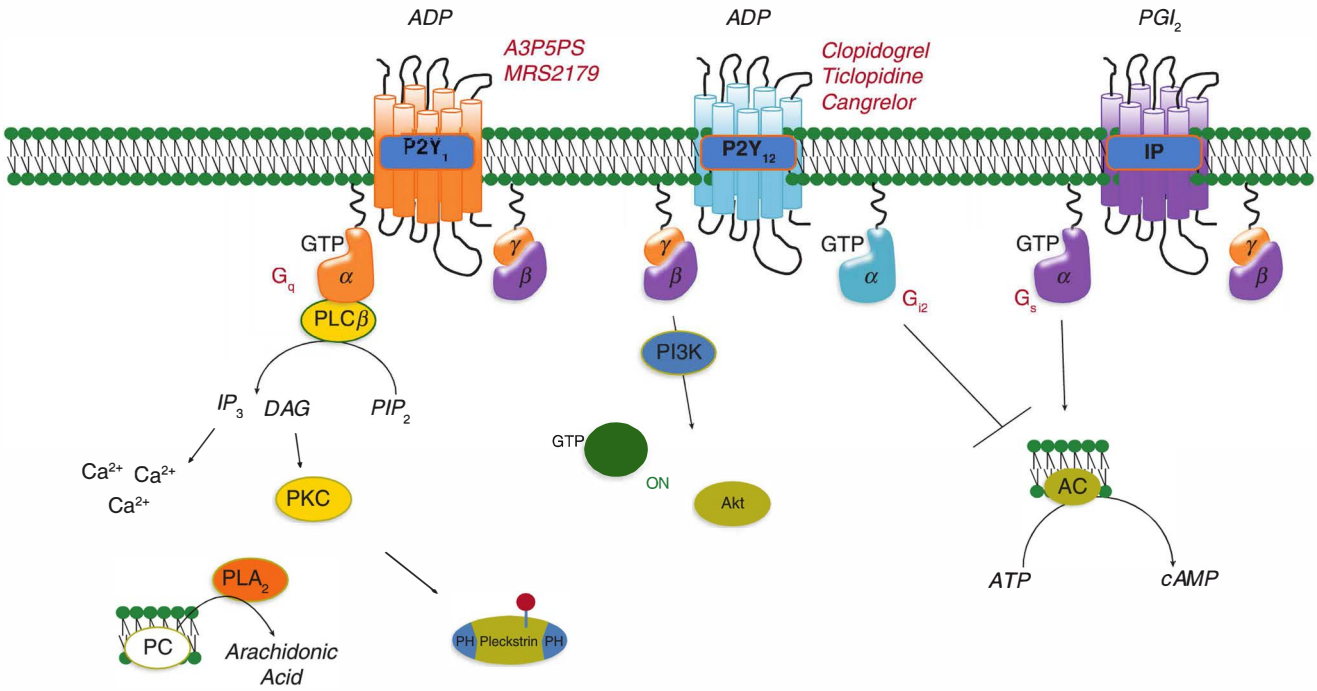

FIGURE 33.3 Platelet activation by ADP. Two receptors that can be activated by ADP have been identified in platelets. $P2Y_1$ and $P2Y_{12}$, which are coupled to different G proteins and therefore mediate different responses. G_q couples $P2Y_1$ receptors to the activation of PLCβ. The G_i family member, G_{i2}, couples $P2Y_{12}$ to the inhibition of cAMP formation (*via* $G_{i2\alpha}$) and to effector pathways that include PI3K and Rap1B (*via* $G_{\beta\gamma}$).

domains (for binding to G_α) and DBL-PH domains (for causing guanine nucleotide exchange on Rho family members)—proteins such as p115 RhoGEF, LARG, and PDZ-RhoGEF.[68] Which, if any, of these proteins plays a role in platelets remains to be determined.

Platelet responses to agonists such as thrombin, ADP, TxA_2, and epinephrine can be mediated by a single class of receptors coupled to different families of G proteins, by multiple classes of receptors each coupled to a single family of G proteins, or by the cumulative effect of two or more agonists, each of which evokes only a subset of the necessary G-protein–mediated responses.[69,70] The next section illustrates how this is thought to occur for several essential agonists, beginning with thrombin.

Platelet Activation by Thrombin

Thrombin activates platelets at concentrations as low as 0.1 nM (~ 0.01U/mL). Within seconds, the cytosolic Ca^{2+} concentration increases 10-fold to approximately 1 μM, triggering downstream Ca^{2+}-dependent events, including the activation of phospholipase A_2. Of these responses, platelet aggregation, phosphoinositide hydrolysis, and the increase in cytosolic Ca^{2+}, but not platelet shape change, are absent in platelets from mice lacking $G_{q\alpha}$.[59] Thrombin also activates Rac and Rho in platelets, leading to rearrangement of the actin cytoskeleton and shape change—presumably mediated by a combination of effector pathways coupled to G_{12} and G_q activation. Finally, thrombin is able to inhibit adenylyl cyclase activity in human platelets, either directly *via* a G_i family member coupled to a thrombin receptor[71] or indirectly *via* released ADP.[72]

Thrombin activates platelets *via* GPCRs of the protease-activated receptor (PAR) family, the first member of which, PAR1, was identified in the Coughlin[73] and Pouysségur[74] laboratories (reviewed at greater length in reference).[75] Prior work had shown that platelet responses to thrombin require the enzyme's proteolytic activity and are mediated by G proteins. Binding studies had identified high-affinity interactions with several sites on the platelet surface, including GP Ibα, but efforts to establish any of these as a receptor in the signaling sense were not entirely successful. Substrates for thrombin were identified on the platelet surface, including GP V. However, cleavage of GP V did not appear to be required for platelet activation by thrombin.

Four PAR family members have been identified, three of which (PAR1, PAR3, and PAR4) are thrombin receptors. Studies on PAR1 established a paradigm that applies to some of the other family members as well (FIGURE 33.4). PAR1 is normally activated when thrombin binds to the receptor *N*-terminus, cleaves it, and exposes a new *N*-terminus that serves as a tethered ligand. Given sufficient substrate recognition, proteases other than thrombin can also activate PAR1 or, by cleaving in the "wrong" place, render the receptor unresponsive to a subsequent addition of thrombin.[76] The contact sites for the newly exposed tethered ligand have been localized to the membrane-proximal region of the *N*-terminus and parts of the second extracellular loop.[77–79] Because the ligand is not free to diffuse away from the receptor, it is thought to effectively present a high local concentration at the receptor and perhaps maintain signaling longer than would otherwise occur. Peptides corresponding to the tethered ligand domain (SFLLRN) can also

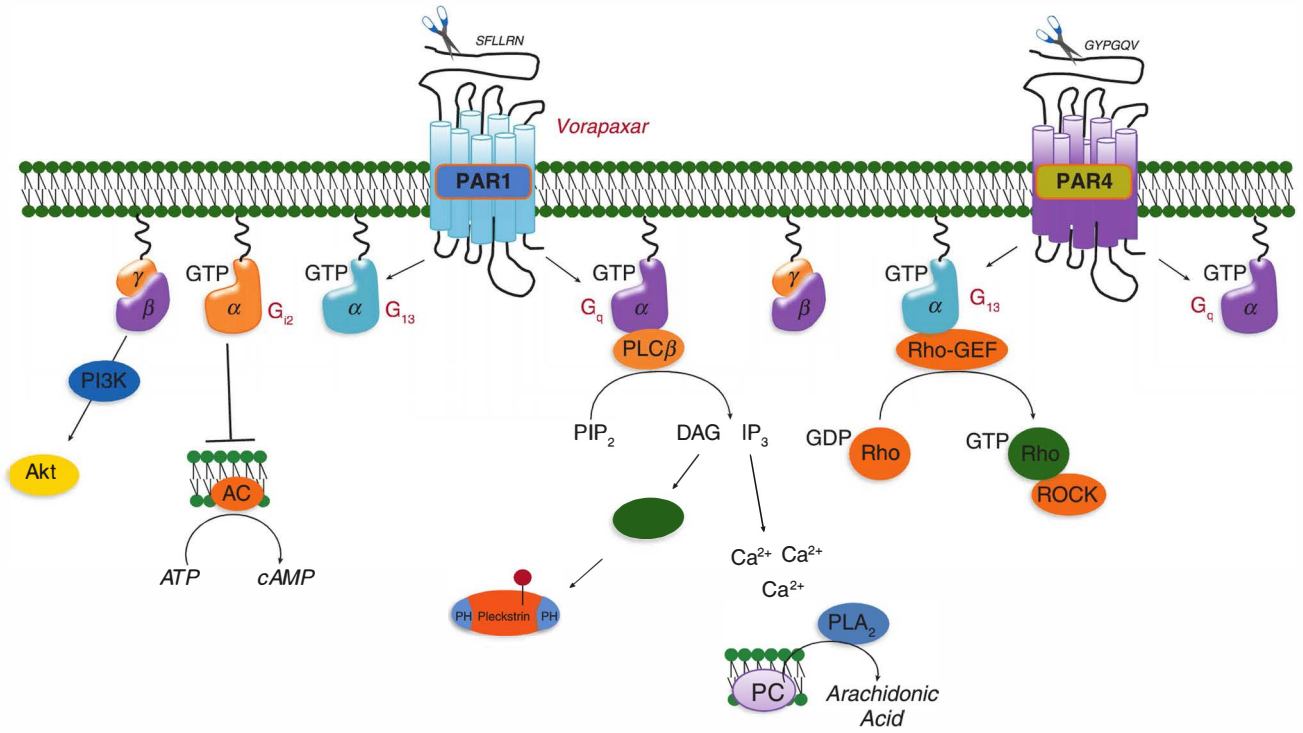

FIGURE 33.4 Platelet activation by thrombin. Platelet responses to thrombin are mediated by members of the PAR family. Human platelets express PAR1 and PAR4, which collectively are coupled to G_q- and G_{13}-mediated effector pathways. PAR1 can also couple to G_{i2}, but G_i-dependent signaling initiated by thrombin gets an additional boost from secreted ADP through $P2Y_{12}$ receptors. PAR1 (and presumably PAR4) are also subject to cleavage by proteases other than thrombin. Cleavage in the *N*-terminus by these proteases can activate PAR1 by exposing the tethered ligand domain or disable it by cleaving it elsewhere in the *N*-terminus that does not expose a competent tethered ligand.

activate PAR1, mimicking the effect of thrombin.[73,80] The same is true for PAR2 and PAR4, but not PAR3.

PAR1 was identified by expression cloning. The second PAR family member to be identified, PAR2, was cloned serendipitously. PAR2, expressed by endothelial cells, but not by platelets, can be cleaved and activated by trypsin, tryptase, TF/VIIa, and by factor Xa,[81,82] but not by thrombin.[83,84] The third family member, PAR3, was identified after gene ablation studies showed that platelets from mice lacking PAR1 were still responsive to thrombin.[85] PAR3 is a major regulator of thrombin responses in mouse platelets, but appears to do so solely by facilitating cleavage of PAR4.[86,87] PAR4 is expressed on human and mouse platelets and accounts for the ability of platelets from PAR3 knockout mice to respond to thrombin.[88,89] Simultaneous inhibition of human PAR1 and PAR4 with blocking antibodies or small-molecule antagonists completely abolishes platelet responses to thrombin,[90] as does deletion of the gene encoding PAR4 in mice.[91] Thus, the four PAR family members have some features in common, but there are differences among them as well. PAR1 and PAR3 have similar thrombin dose/response curves, while PAR4 requires 10- to 100-fold higher concentrations of thrombin, apparently because it lacks the hirudin-like sequences that interact with thrombin's anion-binding exosite and facilitate receptor cleavage.[87–89,92] Loss of PAR3 expression on mouse platelets shifts the thrombin dose/response curve to the right,[88] whereas loss of PAR4 abolishes thrombin responsiveness.[91]

Together, current evidence suggests that thrombin activates human platelets by cleaving and activating PAR1 and PAR4 (FIGURE 33.4). In turn, these receptors promote guanine nucleotide exchange on G_q, G_{12}, and G_i family members, leading to the activation of PLCβ, PI3-kinase, and the monomeric G proteins, Rho, Rac, and Rap1 and also cause an increase in the cytosolic Ca^{2+} concentration and inhibit cAMP formation. This process is supported, particularly at suboptimal thrombin concentrations, by released ADP and TxA_2. Since cleavage of human PAR4 requires a higher concentration of thrombin than does cleavage of PAR1, it is likely that PAR1 is the predominant signaling receptor at low thrombin concentrations. Once activated, PAR4 signals longer than PAR1.[93,94] By contrast, mouse platelets express PAR3 and PAR4, but signaling appears to be mediated entirely by PAR4, with PAR3 serving solely to facilitate the cleavage of PAR4 at low thrombin concentrations.[87,91]

One unresolved issue is the contribution of other thrombin receptors on platelets, particularly GP Ib (as discussed in Chapter 26A). GP Ib is a heterodimer comprised of α and β subunits that are disulfide linked.[95] There is a high-affinity binding site for thrombin at residues 268 to 287 on GP Ibα.[96] Deletion of the extracellular domain of GP Ibα or blockade of the thrombin-binding site decreases platelet responses to thrombin.[97–100] In theory, the binding of thrombin to GP Ibα could facilitate the cleavage of a PAR family member on human platelets, much as the binding of thrombin to PAR3 is thought to facilitate cleavage of PAR4 on mouse platelets.[100]

Platelet Activation by ADP

ADP is stored in platelet-dense granules and is released upon platelet activation. It is also released from damaged red cells at sites of vascular injury. When added to platelets *in vitro*, ADP causes TxA_2 formation, protein phosphorylation, an increase in cytosolic Ca^{2+}, shape change, platelet aggregation, and granule secretion. It also inhibits cAMP formation. These responses are half-maximal at approximately 1 μM ADP. However, ADP by itself is a weak activator of PLC in human platelets.[101,102] According to current models, human platelets express two distinct GPCRs for ADP: $P2Y_1$ and $P2Y_{12}$. Both are members of the purinergic class of GPCRs (Table 33.1 and FIGURE 33.3).[64,69,70,103,104] Optimal activation of platelets by ADP requires both receptors. A third "ADP" receptor, $P2X_1$, is an ATP-gated ion channel that allows a transient influx of Ca^{2+} before becoming desensitized.[105–108] All three proteins have now been cloned and the genes for each have been deleted in mice (Table 33.3).[65,109–111] In addition, $P2X_1$ has been overexpressed in mouse platelets by the introduction of a transgene driven by the α_{IIb} promoter.[112] The phenotype of the transgenic mouse includes increased responsiveness to agonists and increased susceptibility to lethal thromboembolism following the injection with collagen plus epinephrine.

In the absence of $P2Y_1$, ADP is still able to inhibit cAMP formation in mouse platelets, but its ability to cause an increase in cytosolic Ca^{2+}, platelet shape change, and platelet aggregation is greatly impaired, as was previously shown to be the case in platelets from mice that lack $G_{q\alpha}$.[59] The bleeding time in $P2Y_1^{-/-}$ mice is minimally increased and the mice are relatively resistant to disseminated thromboembolism following injection of ADP, but they show no predisposition to spontaneous hemorrhage. Primary responses to platelet agonists other than ADP are unaffected and when combined with serotonin, which is a weak stimulus for PLC in platelets, ADP causes the $P2Y_1^{-/-}$ platelets to aggregate. These results are consistent with earlier studies using receptor antagonists.[63,69] Taken together with observations made on $G_{q\alpha}^{-/-}$ mice, they indicate that platelet $P2Y_1$ receptors are coupled to G_q and responsible for activation of PLC. In view of the absence of ADP-induced shape change in $G_{q\alpha}$ knockout mouse platelets,[59] it is thought that ADP receptors do not couple to G_{12} family members in platelets.

The second platelet ADP receptor, $P2Y_{12}$, is also a member of the large family of purinergic GPCRs.[64,104] As predicted by inhibitor studies and by the phenotypes of individuals lacking functional $P2Y_{12}$,[113,114] platelets from $P2Y_{12}^{-/-}$ mice do not aggregate normally in response to ADP.[114] They retain $P2Y_1$-associated responses, including shape change and PLC activation, but lack the ability to inhibit cAMP formation. The G_i family member associated with $P2Y_{12}$ responses appears to be primarily G_{i2}, since platelets from $G_{i2\alpha}^{-/-}$ mice have an impaired ability to aggregate, inhibit cAMP formation, and activate Rap1B in response to ADP, while those lacking $G_{i3\alpha}$ or $G_{z\alpha}$ respond normally.[25,60–62]

The identification of the receptors that mediate platelet responses to ADP, the development of antagonists that target each of the known receptors, and the phenotypes observed in mice lacking the genes encoding $P2Y_1$, $P2Y_{12}$, $G_{q\alpha}$, or $G_{i2\alpha}$ have led to an increased appreciation of the contribution of ADP to platelet plug formation *in vivo*, as have studies performed *in vivo* with $P2Y_{12}^{-/-}$ mice in vascular injury and disseminated thrombosis models. The absence of $P2Y_{12}$ produces a hemorrhagic phenotype in humans, albeit a relatively mild one[64,113,115] (Table 33.3). Deletion of either $P2Y_1$ or $P2Y_{12}$ prolongs the bleeding time and impairs platelet responses not only to ADP, but also to thrombin and TxA_2, particularly at low concentrations.[65,110,116] Since platelet TxA_2 receptors do not couple directly to G_i family members, platelet aggregation induced by TxA_2 requires the secretion of ADP to inhibit adenylyl cyclase.[117] The identification of $P2Y_1$ and $P2Y_{12}$ has also made it possible to use the isolated receptors for the development of drugs to treat patients with cardiovascular and cerebrovascular disease. Metabolites of the thienopyridine compounds ticlopidine and clopidogrel irreversibly block $P2Y_{12}$. Other drugs, such as Cangrelor, are reversible $P2Y_{12}$ antagonists.

Table 33.3

Platelet phenotypes of GPCR knockout mice

Agonist	Receptor	Phenotype	Reference
ADP	$P2Y_1$	Impaired response to ADP. Absent increase in cytosolic Ca^{2+} and shape change in response to ADP. Normal inhibition of cAMP formation by ADP	109,110
	$P2Y_{12}$	Greatly diminished aggregation in response to ADP. Absent inhibition of adenylyl cyclase by ADP. Increased bleeding time	65
Thrombin	PAR1	None (not expressed on mouse platelets)	85,232
	PAR3	Higher concentrations of thrombin required to elicit responses, presumably because of the loss of the normal contribution of PAR3 to PAR4 activation	88
	PAR4	Complete loss of thrombin responsiveness	91
TxA_2	TPα and TPβ	Prolonged bleeding time, absent aggregation in response to TxA_2 agonists, and delayed aggregation with collagen	136
PGI_2	IP	Increased aggregation in response to ADP. Increased thrombosis following vascular injury	24

Platelet Activation by Epinephrine

In contrast to ADP and thrombin, epinephrine is a weak activator of human platelets and appears to serve primarily as a potentiator of platelet responses to other agonists. Nonetheless, there are reports of human families in which a mild bleeding diathesis is associated with impaired epinephrine-induced aggregation and reduced numbers of α_2-adrenergic receptors.[118,119] Platelet responses to epinephrine are mediated by α_{2A}-adrenergic receptors and are notoriously labile (Table 33.1).[120–122] The combination of epinephrine with a suboptimal concentration of ADP, thrombin, or a TxA_2 analog is a stronger stimulus for platelet aggregation than any of these agonists alone. Potentiation has traditionally been attributed to the ability of epinephrine to inhibit cAMP formation, but it is now clear that other effector pathways are involved as well, including pathways that lead to Rap1 activation.[61] Epinephrine has no detectable direct effect on PLC in platelets and does not cause shape change.[123] Taken together, these results suggest that platelet α_{2A}-adrenergic receptors are coupled to G_i family members, but not to G_q or G_{12} family members. Knockout studies show that the preferred partner is G_z.[60]

Of the four $G_{i\alpha}$ family members in platelets, $G_{z\alpha}$ is most distinct. Whereas $G_{i1\alpha}$, $G_{i2\alpha}$, and $G_{i3\alpha}$ are >90% identical to each other and ubiquitously expressed, $G_{z\alpha}$ is only 60% related to the others at the sequence level and has a restricted pattern of expression that includes some neurons and platelets. $G_{z\alpha}$ is also notable for having a relatively slow intrinsic rate of GTP hydrolysis[124] and for being a substrate for PKC [125] and p21-activated kinase (PAK).[126] One place where the $G_{z\alpha}$ sequence diverges from the other $G_{i\alpha}$ family members is at the *C*-terminus, a domain critical for receptor interactions. In particular, $G_{z\alpha}$ lacks a cysteine residue near the *N*-terminus that is the normal site for ADP-ribosylation by pertussis toxin. In reconstitution systems, overexpressed G_z will interact with a variety of receptors,[71,127] but studies using platelets from $G_{z\alpha}^{-/-}$ mice suggest that it normally couples only to α_{2A}-adrenergic receptors.[60] Aggregation in response to epinephrine, but not to ADP or a PAR4 agonist peptide, is reduced in the knockout at any concentration likely to be encountered *in vivo*. The diminished aggregation response is accompanied by a loss of epinephrine's normal ability to inhibit PGI_2-stimulated cAMP formation and by increased resistance to fatal thromboembolism following injection of epinephrine plus collagen, but not ADP plus collagen.[60] Taken together, these results suggest that epinephrine potentiates platelet activation by providing the type of G_i-dependent signaling that can also be seen with ADP, but does so *via* α_{2A}-adrenergic receptors coupled to G_z, rather than by $P2Y_{12}$ receptors coupled to G_{i2}.

Platelet Activation by Thromboxane A_2

TxA_2 is produced from arachidonate in platelets by the aspirin-sensitive cyclooxygenase pathway (see below). Once formed, TxA_2 can diffuse across the plasma membrane and activate other platelets (FIGURE 33.1).[128] Like ADP, this amplifies the initial stimulus for platelet activation and helps to recruit additional platelets. This process is effective locally, but is limited by the brief (≈30 seconds) half-life of TxA_2 in aqueous solution, helping to confine the spread of platelet activation to the original area of injury.

Only one gene encoding a TxA_2 receptor has been identified, but there are two splice variants (TPα and TPβ) that differ in their cytoplasmic tails (Table 33.1).[129] Human platelets express both forms of the receptor. Biochemical studies have shown that platelet TxA_2 receptors can physically associate with $G_{q\alpha}$[130] and $G_{13\alpha}$[131] and are able to activate G_{12} family members,[67] consistent with the observed ability of TxA_2 analogs to cause phosphoinositide hydrolysis and shape change in platelets. Loss of $G_{q\alpha}$ abolishes IP_3 formation and changes in cytosolic Ca^{2+} in response to the stable TxA_2 mimetic, U46619, but does not prevent platelet shape change.[59] U46619 continues to cause guanine nucleotide exchange on $G_{12\alpha}/G_{13\alpha}$ in platelets from $G_{q\alpha}^{-/-}$ mice.[132] Mice lacking $G_{13\alpha}$ have a defect in shape change, aggregation, and thrombosis, while mice lacking $G_{12\alpha}$ have no overt platelet phenotype.[133] In cells other than platelets, TP has been shown to couple to pertussis toxin-sensitive G_i family members.[134] However, in platelets, the inhibitory effect of U46619 on cAMP formation appears to be mediated by secreted ADP and is not seen when U46619 is added to platelet membranes.[135] Why this cell-specific difference occurs is unclear, but it suggests that platelet TP receptors are coupled to G_q and G_{12}, but not to G_i, family members. Platelets from mice lacking the gene encoding TP have a prolonged bleeding time and are unable to aggregate in response to TxA_2 mimetics (Table 33.3). They also exhibit delayed aggregation in response to collagen, presumably reflecting the role of TxA_2 in platelet responses to collagen.[136] A cohort of Japanese patients with impaired platelet responses to TxA_2 analogs, but normal ligand binding, were found to be either homozygous or heterozygous for a missense mutation replacing Arg60 with Leu in the first cytoplasmic loop of TP,[137] which presumably affects coupling, but not activation of the receptor.

Regulation of G-Protein Signaling in Platelets

RGS proteins in cells other than platelets have been shown to limit signaling intensity and duration by accelerating the intrinsic hydrolysis of GTP by activated G-protein α subunits.[138] The RGS family in mammals consists of at least 37 members, as defined by a conserved RGS domain.[139] As many as 10 have been identified in platelets at the RNA level, but only two, RGS10 and RGS18, have been confirmed at the protein level.[140–145] The evidence that RGS proteins are biologically relevant in platelets comes from studies on mice in which glycine 184 in the α subunit of G_{i2} was replaced with serine, rendering it unable to interact with RGS proteins, but without impairing its ability to interact with either receptors or downstream effectors. As would be predicted, this substitution produced a gain-of-function phenotype *in vitro* and *in vivo*, even in the heterozygous state.[146]

SECRETION

The platelet secretory response results in the release of the contents of intracellular storage granules. Granule constituents include substances that can stimulate circulating platelets and cause them to acquire new adhesive properties. Most of the substances that are actively and selectively secreted from platelets are packaged in preformed storage granules or are synthesized *de novo* from membrane phospholipids. Platelets contain three types of granules: (a) dense granules that contain small molecules such as ADP, serotonin, and Ca^{2+} which serve to amplify platelet activation and promote local vasoconstriction, (b) α-granules that contain numerous proteins that enhance the adhesive process and help regulate angiogenesis and wound

healing among other process, and (c) lysosomal granules that contain glycosidases and proteases which have unclear functions in platelet biology. Platelet secretion is triggered by a variety of strong agonists such as thrombin. Factor V, fibrinogen, thrombospondin, and other proteins secreted from the α-granules may be retained on the platelet surface by a transglutamination reaction.[147] This process is speculated to enhance the procoagulant activity of activated platelets.[148] Induction of secretion by weak agonists like ADP occurs when the cells are brought into close contact such as occurs during aggregation.[149,150] The latter secretory mechanism is clearly dependent on TxA_2 generated as a consequence of arachidonic acid release.

Granule Exocytosis

As noted previously, the two morphologically prominent platelet storage granules, α-granules and dense granules, contain a variety of substances important in platelet function. Since these granules have a limiting membrane, it would seem likely that the final secretory event involves exocytosis (i.e., the fusion of the secretory granule membrane with the plasma membrane).[151] This has been observed for dense body secretion.[152,153] Several families of proteins have been shown to be required for exocytosis in yeast and neuroendocrine cells. These include Rab GTPases and members of the SNARE-SNAP complex[154–157] (see Chapter 32 for a detailed discussion of the mechanism of platelet secretion).

Rab GTPases appear to be essential for both platelet α-granule and dense granule release. The SNARE-SNAP complex has also been shown to be critical for platelet secretion. SNARE proteins are docking proteins that are present on the inner face of the cell membrane, as well as on the outer face of vesicular membranes. The fusion of the vesicular membrane with the cell membrane requires the formation of a core complex created by the binding of SNARE proteins on the opposing membranes. Soluble NSF-attachment proteins (SNAPs) facilitate this process. Platelets have been shown to contain numerous members of these families, and blocking antibodies directed against several SNAREs and SNAPs impair granule secretion in permeabilized platelets.[154,157,158] PKC and its substrate pleckstrin are also essential components of the signaling cascade involved in platelet secretion. This will be discussed in the PKC section later in this chapter.

Eicosanoids and Arachidonate

In addition to the exocytosis of platelet granules, the passive release of TxA_2 from platelets is another mechanism that amplifies platelet activation. Eicosanoids are formed from the arachidonate released from membrane phospholipids by phospholipase A_2 during platelet activation.[159–161] Since the availability of arachidonate is the rate-limiting step in this process, phospholipase A_2 is tightly controlled. Platelet phospholipase A_2 activation is stimulated in part by the increase in the cytosolic Ca_{2+} that accompanies platelet activation. There is also evidence that MAPK pathway signaling activates phospholipase A_2, although not necessarily by direct phosphorylation of phospholipase A_2.[162,163] See Chapter 34 for a detailed discussion of platelet prostanoid metabolism.

Once released from membrane phospholipids, arachidonate can be metabolized to TxA_2 by cyclooxygenase–1 (COX-1).[164] Aspirin acetylates COX-1 causing it to be irreversibly inactivated.[165,166] Since platelets lack the ability to synthesize significant amounts of protein, inactivation of COX-1 by aspirin blocks TxA_2 synthesis until new platelets are released. Hence, aspirin is the most commonly used drug for antiplatelet therapy. However, there may be a subset of patients who are "aspirin resistant," although the molecular basis for this remains undetermined.[167,168] Nonsteroidal anti-inflammatory agents also inactivate COX-1, but without covalently modifying the enzyme.[169]

Once formed, TxA_2 can diffuse across the plasma membrane and activate other platelets through signaling pathways. Like ADP, TxA_2 amplifies the initial stimulus for platelet activation and recruits additional platelets. This process is effective locally, but is limited by the short half-life of aqueous TxA_2 in solution, confining the spread of platelet activation to the original area of injury.

INTRACELLULAR SIGNALING EVENTS

After platelet activation by an agonist, intracellular signaling is needed for cytoskeletal reorganization, fibrinogen receptor exposure, and granule secretion. Two pathways that are central to platelet activation are the phosphoinositide hydrolysis pathway and the eicosanoid synthesis pathway. The phosphoinositide hydrolysis pathway begins when PLC cleaves membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and DAG, both of which serve as second messengers. The eicosanoid pathway begins when phospholipase A_2 releases arachidonate from membrane phospholipids to form TxA_2. As mentioned above, most of the agonists that activate platelets do so *via* GPCRs, and it is the activation of G proteins that is the first step in the intracellular signaling pathway leading to platelet second messenger formation.

Inositol Signaling-Phospholipase C and PI3-kinases

One of the earliest responses of platelets to most agonists is the activation of PLC. Platelets contain β and γ forms of this enzyme. The β forms are activated by G proteins, while the γ forms (predominantly PLCγ_2) are regulated by tyrosine phosphorylation.[170,171] PLCβ is thought to be primarily responsible for the rapid burst of phosphoinositide hydrolysis that occurs during platelet activation by agonists such as thrombin and TxA_2.

In general, PLCβ_1 and PLCβ_3 respond best to G_α, particularly members of the $G_{q\alpha}$ family, while PLCβ_2 responds best to $G_{\beta\gamma}$. Coupling of specific heterotrimeric proteins to PLC was discussed earlier in this chapter. Once activated, PLC hydrolyzes PI-4,5-P_2 to DAG plus IP_3.[172,173] DAG activates PKC and contributes to protein phosphorylation, and IP_3 binds to receptors in the DTS and releases sequestered Ca^{2+}[174,175] (see below).

Phosphatidylinositol 3-kinases (PI3K) are a group of enzymes that phosphorylate the D-3 position of the inositol ring of phosphatidylinositol to produce phosphatidylinositol 3-phosphate (PI3-P), phosphatidylinositol 3,4-bisphosphates (PI3,4-P_2), and phosphatidylinositol 3,4,5-triphosphates (PI3,4,5-P_2 or PIP_3).[176] Four isoforms of PI3K that phosphorylate PI, PI4-P, and PIP_2 have been described in humans and are

classified according to their catalytic subunit: p110α, p110β, p110γ, and p110δ.

Using pharmacologic inhibitors, and platelets obtained from genetically modified mice, it has been possible to dissect the roles of different PI3K isoforms in platelets. Mice lacking the p110γ catalytic subunit of PI3K have a defect in ADP-mediated platelet aggregation,[177,178] whereas mice lacking the p85α regulatory subunit have a defect in signaling events initiated by the platelet collagen receptor, GPVI. No defect in platelet activation has been observed following stimulation by other platelet agonists such as ADP or thrombin.[179] Loss of PI3Kβ impairs Rap1B activation and thrombus formation *in vivo*, similar to PI3Kβ-selective inhibitors.[180] Most of the Akt expressed in platelets appears to be the Akt2 isoform, but Akt1 and Akt3[181] are present as well. All three appear to contribute to platelet activation, at least in mice. Deletion of the gene encoding Akt2 results in impaired thrombus formation and stability and inhibits platelet secretion.[182] Loss of Akt1 inhibits platelet aggregation[183] and affects vascular integrity.[184] Loss of Akt3 impairs aggregation and secretion.[181] Taken together, these results suggest that PI3K is involved in both the initial activation of $\alpha_{IIb}\beta_3$ and the subsequent stabilization of the $\alpha_{IIb}\beta_3$/fibrinogen interaction that leads to irreversible platelet aggregation. This may be due to irreversible effects of PI3K on the platelet's actin cytoskeleton.

Cytosolic Calcium Ions

Ca^{2+} ions serve as intracellular second messengers, and like protein kinases, affect enzyme activity and protein–protein interactions. Based on studies with intracellular probes such as Fura-2, the cytosolic free Ca^{2+} concentration in resting platelets has been found to be approximately 0.1 μM. Strong agonists, such as thrombin or collagen, cause an increase to >1.0 μM. Weaker agonists, particularly epinephrine, may have little or no effect on cytosolic Ca^{2+}.

The rise in cytosolic Ca^{2+} that accompanies platelet activation occurs in two steps, reflecting two sources of the Ca.$^{2+}$ The first surge in Ca^{2+} is due to the IP_3-mediated release of Ca^{2+} from the platelet DTS. The second surge occurs when depletion of the DTS Ca^{2+} pool triggers a conformational change in STIM1, a protein in the DTS membrane. This promotes the binding of STIM1 to Orai1 in the plasma membrane, triggering an influx of plasma Ca^{2+} driven by the steep Ca^{2+} gradient that normally exists across the plasma membrane.[40] These events can be separated by chelating extracellular Ca^{2+}, thereby precluding Ca^{2+} influx. The rise in $[Ca^{2+}]_i$ activates the GTP-binding protein, Rap1, which can then bind to and cause the translocation of RIAM and talin to the cytoplasmic domain of $\alpha_{IIb}\beta_3$ (FIGURE 33.2). Talin and kindlin binding to $\alpha_{IIb}\beta_3$ trigger a conformational change in the integrin extracellular domain, exposing a binding site for fibrinogen (discussed in Chapters 26B and 31).[7]

Protein Kinase C

PKC isoforms are a family of serine/threonine kinases that play an essential role in the signal transduction mechanism downstream of agonist receptor activation. PKC has been identified as the cellular receptor for the lipid second messenger DAG, and it is a key enzyme in the signaling events that follow activation of receptors coupled to PLC. PKC isoforms phosphorylate multiple cellular proteins on serine and threonine residues. Although some discrepancies between different studies on PKC isozyme expression exist, platelets probably express PKC α, β, δ, ε, η, θ, and perhaps ζ and λ.[185]

Because of the limitations of platelets as a system for genetic studies, studies on PKC in platelets have depended heavily on the use of pharmacologic activators (such as phorbol 12-myristate 13-acetate or PMA) and inhibitors. Those studies suggest that PKC isoforms control a variety of platelet functions, including aggregation, secretion, mobilization of intracellular Ca^{2+}, and regulation of cell shape. The δ and θ isoforms may be particularly important for these functions.[186,187] PKC isoforms also play an important role in megakaryocyte differentiation.

Recent evidence using genetically engineered mice has shown that the cytosolic protein pleckstrin is an essential component of PKC-mediated platelet secretion.[188] Pleckstrin constitutes approximately 1% of total cellular protein in platelets and contains two prototypic PH domains. PH domains are thought to mediate the association of proteins with phosphoinositides. Following platelet activation, pleckstrin is rapidly phosphorylated by PKC, inducing it to bind to membrane phospholipids such as phosphatidylinositol 4,5 bisphosphate. Although granules in platelets from mice lacking pleckstrin are able to move to the plasma membrane, the granule and plasma membranes fail to fuse so that the granules are unable to secrete their content into the extracellular environment.

Other Protein Kinases

In addition to other serine/threonine kinases such as p38 MAPK, platelets contain a large number of tyrosine kinases, some of which become active during platelet activation. These include receptors for extracellular ligands, such as growth factor receptors and ephA4, as well as a large number of nonreceptor (cytoplasmic) tyrosine kinases, including Src, Fyn, Lyn, Hck, and Syk. Broadly specific inhibitors of tyrosine kinases, such as genistein and the tyrphostins, can inhibit platelet activation.

Inhibitors of phosphotyrosine phosphatases, such as vanadate, promote platelet activation. In general, tyrosine phosphorylation serves two roles. It can have a regulatory effect on the phosphorylated protein, perhaps by causing a conformational change, or it can provide a binding site for modular domains located in other proteins, such as SH2 domains. Although studies of knockout mice have identified a role for some of these proteins in signaling pathways downstream of the GP VI collagen receptor, the function of most tyrosine kinases in platelets is still incompletely understood, as is the role of protein tyrosine phosphatases such as SHP-1 and SHP-2.

Cyclic AMP

Agents that raise the cAMP concentration in platelets inhibit platelet activation. In general, this effect is thought to be mediated by cAMP-dependent protein kinase A.[15] Platelet substrates for this enzyme include the 24 kDa β chain of glycoprotein Ib, actin-binding protein, and myosin light chain, VASP and Rap1B. Raising cAMP levels cause a number of specific changes in platelet function, including impaired phosphoinositide hydrolysis, attenuation of the increase in cytosolic free Ca^{2+} in response to agonists, and an accelerated uptake of Ca^{2+} into the DTS.

PGI_2 released from activated endothelial cells increases platelet cAMP levels by stimulating receptors on the platelet surface that are coupled to adenylyl cyclase *via* the G-protein G_s. This results in inhibition of platelet activation. Consequently, mice lacking the PGI_2 receptor manifest an increased thrombotic risk.[24] As described above, platelet agonists suppress cAMP formation by inhibiting adenylyl cyclase *via* one or more members of the $G_{i\alpha}$ family.

The drug dipyridamole (Persantine) exerts antiplatelet effects by inhibiting cAMP phosphodiesterase and increasing platelet cAMP.[15] However, the clinical efficacy of dipyridamole has been debated. Sildenafil has phosphodiesterase activity and decreases platelet cAMP levels. This has been speculated to contribute to the cardiovascular toxicity of this drug.[189]

Signals to and from $\alpha_{IIb}\beta_3$

A longstanding question in platelet biology is the mechanism by which inside-out signaling pathways produce integrin activation. Activation of $\alpha_{IIb}\beta_3$ is required for fibrinogen to bind to the platelet surface and for aggregation to occur (this is discussed in detail in Chapter 31). Human and mouse platelets lacking $\alpha_{IIb}\beta_3$ cannot aggregate. Normal platelets incubated with $\alpha_{IIb}\beta_3$ antagonists also show reduced or absent aggregation. The early (receptor-proximal) events have been identified. In general terms, the early events include the activation of one or more PLC isoforms yielding a rise in the cytosolic Ca^{2+} concentration and the activation of PKC. This leads to integrin activation *via* an intracellular pathway that includes a Ca^{2+}-dependent exchange factor (CalDAG-GEF), a switch (Rap1), an adaptor (RIAM), and proteins that interact directly with the $\alpha_{IIb}\beta_3$ cytosolic domains (kindlin and talin).[7] Multiple lines of evidence suggest that this is mediated by disruption of the interactions between the transmembrane regions of α_{IIb} and β_3.[190,191] This opening up of the transmembrane heterodimer induces a conformational change in the $\alpha_{IIb}\beta_3$ extracellular domain that exposes the fibrinogen binding site (FIGURE 33.2). The CalDAG-GEF/Rap1-dependent pathway accounts for the ability of Ca^{2+} ionophores to trigger platelet aggregation. Its biological relevance is reflected by the reduction of aggregation in platelets from Rap1 and CalDAG-GEF knockout mice.[192,193] That the CalDAG-GEF/Rap1 pathway is not the only way to accomplish integrin activation is reflected by the incomplete reduction of aggregation in $Rap1^{-/-}$ platelets and by the ability of PKC activators such as phorbol myristate acetate (PMA) to cause aggregation without causing an increase in cytosolic Ca^{2+}.[194] PKC isoforms phosphorylate multiple cellular proteins on serine and threonine residues. Activation of PKC by PMA is sufficient to cause integrin activation, granule secretion, and platelet aggregation in the absence of an increase in cytosolic Ca^{2+}. However, just as the ability of Ca^{2+} ionophores to activate platelets on their own does not fully account for platelet activation *in vivo*, neither does the response to PMA.

In addition to the inside-out signaling events that lead to activation of $\alpha_{IIb}\beta_3$, there are also outside-in signaling events that occur downstream of activated $\alpha_{IIb}\beta_3$ once fibrinogen binding has occurred.[195–197] Fibrinogen binding to and clustering of $\alpha_{IIb}\beta_3$ stimulates rapid increases in the activities of integrin-associated Src family and Syk tyrosine kinases. Studies of platelets from mice lacking these kinases suggest that these events are required for the initiation of outside-in signaling and for full platelet spreading, irreversible aggregation, and clot retraction.[198–201] Platelet aggregation results in the formation of large signaling complexes that include $\alpha_{IIb}\beta_3$. One of the proteins in these complexes is the kinase, FAK, which, when phosphorylated, can provide a binding site for, among other proteins, Src and the p85 subunit of PI3-kinase.[202,203,204] Proteins capable of binding directly to the cytoplasmic domains of $\alpha_{IIb}\beta_3$ include β_3-endonexin,[205] CIB,[206,207] talin,[208] Syk,[209,210] myosin[211] and Shc.[212] Shc binding requires phosphorylation of Y759 in the β_3 cytoplasmic domain.[212] Myosin binding requires phosphorylation of both Y747 and Y759.[211] The binding of talin, β_3-endonexin, and CIB is thought to be independent of β_3 tyrosine phosphorylation. Some of the protein:protein interactions that involve the cytoplasmic domains of $\alpha_{IIb}\beta_3$ (including talin) help to regulate integrin activation, others participate in outside-in signaling and clot retraction. Tyrosine phosphorylation of β_3 is thought to be integral to the later two events.

Tyrosine residues 747 and 759 of β_3 become phosphorylated following the onset of platelet aggregation.[211] Phosphorylation requires both activation of the integrin and its engagement with an adhesive protein. Inhibition of aggregation with an RGD-containing peptide or a peptidomimetic inhibits phosphorylation of β_3.[211] According to one model,[199,200] platelet activation and aggregation leads to the activation of an Src family member and the subsequent phosphorylation of β_3. Consistent with this hypothesis, phosphorylation is diminished in $Fyn^{-/-}$ mouse platelets.[200] Mutation of both Y747 and Y759 to phenylalanine produces mice whose platelets tend to disaggregate and manifest reduced clot retraction and a tendency to rebleed from tail bleeding time sites.[203] Clot retraction refers to the decreased volume of a clot that occurs when embedded platelets pull on fibrin strands. Loss of clot retraction is a hallmark of $\alpha_{IIb}\beta_3$-deficient platelets from patients with Glanzmann thrombasthenia, reflecting the dependence of clot retraction on the interaction of $\alpha_{IIb}\beta_3$ with extracellular fibrin and with intracellular actin/myosin filaments. The diminished clot retraction in the Y747F/Y759F mice is consistent with a reduction in the binding of myosin to β_3. Failure to phosphorylate β_3 in the Y747F/Y759F mice would also prevent the binding of the adaptor protein Shc,[212] abolishing any signaling pathways that lie downstream from that point.

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Full Reference Lists can be found online

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CHAPTER

34 Platelet Prostanoid Metabolism

TILO GROSSER AND GARRET A. FITZGERALD

The prostanoids, which include prostaglandins (PGs) and thromboxane (TX) A_2, are a series of lipid hormones that act on specific G-protein–coupled receptors expressed at, or close to, their site of formation. They are formed by the sequential action of the PGG/H synthases, commonly known as cyclooxygenases (COXs), and specific prostanoid synthases (FIGURE 34.1).[1] TXA_2 is synthesized by COX-1 in activated platelets and released as a local signal that amplifies activation and recruits additional platelets to the site of clot formation.[2,3] It is also a potent vasoconstrictor and stimulates mitogenesis and the proliferative response to vascular injury.[4–7] Patients deficient in TXA_2 formation or signaling have a mild bleeding disorder.[8–13] Suppression of TXA_2 formation by low-dose aspirin, which inhibits platelet COX, is sufficient to explain the reduction of the incidence of myocardial infarction and stroke by about 25% in patients who have had a previous arterial thrombotic event.[14,15,16,17] Conversely, the defect in platelet function caused by aspirin can also lead to bleeding complications—primarily gastrointestinal and less frequently intracranial hemorrhage.

The development of nonsteroidal anti-inflammatory drugs (NSAIDs) targeted specifically at inhibition of COX-2 has emphasized the importance of PGs in the physiologic restraint of thrombotic processes. All COX-2 selective NSAIDs approved by the U.S. Food and Drug Administration were shown in placebo controlled trials to cause thrombotic complications—myocardial infarction, stroke, and venous thrombosis. The underlying molecular mechanism—inhibition of COX-2–dependent formation of prostacyclin (PGI_2) and other PGs in the vasculature, the heart, and the kidney—has been elucidated in more detail than that of any other adverse drug effect.[18]

PGI_2 is the major platelet inhibitory prostanoid. It also elicits vasodilation close to the site of formation and has antimitogenic and antiatherogenic properties.[19] Augmented PGI_2 biosynthesis in syndromes of platelet activation serves to constrain the effects of stimuli to platelet activation and vasoconstrictors.[7] The increased incidence of myocardial infarction and stroke in patients receiving COX-2 selective NSAIDs, which is explained by inhibition of COX-2–dependent PGI_2 formation, supports this concept.[18,20]

The role in platelet function of other prostanoids is less established. PGD_2 produced cooperatively by platelet COX and plasma PGD synthase may afford a mechanism of feedback inhibition of aggregation.[21–23] High concentrations of PGE_2 may inhibit platelet function,[24] while lower concentrations activate platelets *in vitro*.[25] Deletion of the enzyme primarily mediating inflammatory PGE_2 formation did not affect thrombogenesis in mice.[26]

PROSTANOID SYNTHESIS

Arachidonic acid, the precursor of a complex array of more than 20 carbon lipid mediators including the prostanoids, is metabolized by three major groups of enzymes, the COXs, lipoxygenases, and epoxygenases, to form the prostanoids, the leukotrienes, and the epoxyeicosatrienoic acids, respectively.[27] A fourth group of arachidonic acid–derived molecules, the isoeicosanoids, is formed by nonenzymatic peroxidation. Arachidonic acid itself is released from membrane stores by the enzymatic activity of phospholipase (PL) A_2. Several PLA_2 enzymes that hydrolyze arachidonic acid from the sn-2 position of membrane glycerophospholipids have been identified in vertebrates.[28] Free arachidonic acid is either available for metabolism into one of the 20 carbon lipid mediators, or it can be reacylated in the so-called Lands cycle, in which it is converted to arachidonoyl-CoA by arachidonoyl-CoA synthetases and reincorporated into phospholipids by CoA-dependent acyltransferases.[29]

Many platelet agonists initiate TXA_2 generation by activating Group (G) IVA PLA_2, the main isoform expressed in the platelet cytosol, with a calcium signal.[30] GIVA PLA_2 possesses a regulative C2 domain that is thought to mediate its translocation to the phospholipid membrane in response to increments in intracellular calcium concentrations.[31] Other activation pathways, including von Willebrand factor–initiated adhesion of platelets through the GPIb-V-IX complex, are thought to involve mitogen-activated protein kinase-mediated phosphorylation of GIVA PLA_2 serine-505.[32–35] Vascular wall cells express multiple PLA_2 isoforms to release arachidonic acid for prostanoid formation, including calcium-dependent and calcium-independent mechanisms, and in inflammatory scenarios, such as atherosclerotic lesions, also secretory PLA_2s.[36,37]

COX-dependent metabolism of arachidonic acid results in the formation of the prostanoids. Two evolutionary conserved[38] COX isoforms, COX-1 and COX-2,[39] convert arachidonic acid to PGH_2. COX-1 expression is relatively constant in most cells. It is the only isoform expressed in mature platelets.[40] Megakaryocytes, and immature platelets released in clinical conditions of accelerated platelet turnover, also express COX-2,[41,42] although its role in platelet development and function has yet to be elucidated.[43]

COX-2, a member of the inducible immediate-early gene family, is highly regulated in various contexts such as inflammation, pain signaling, fever, renal salt handling, and cell proliferation and differentiation. Its promoter contains several transcriptional regulatory elements including cyclic AMP response elements, nuclear factor kappa B sites, AP-2 sites, an E-box, a TATA box, an SP1 site, and a CAAT enhancer binding protein site.[44] COX-2 expression is also regulated at the

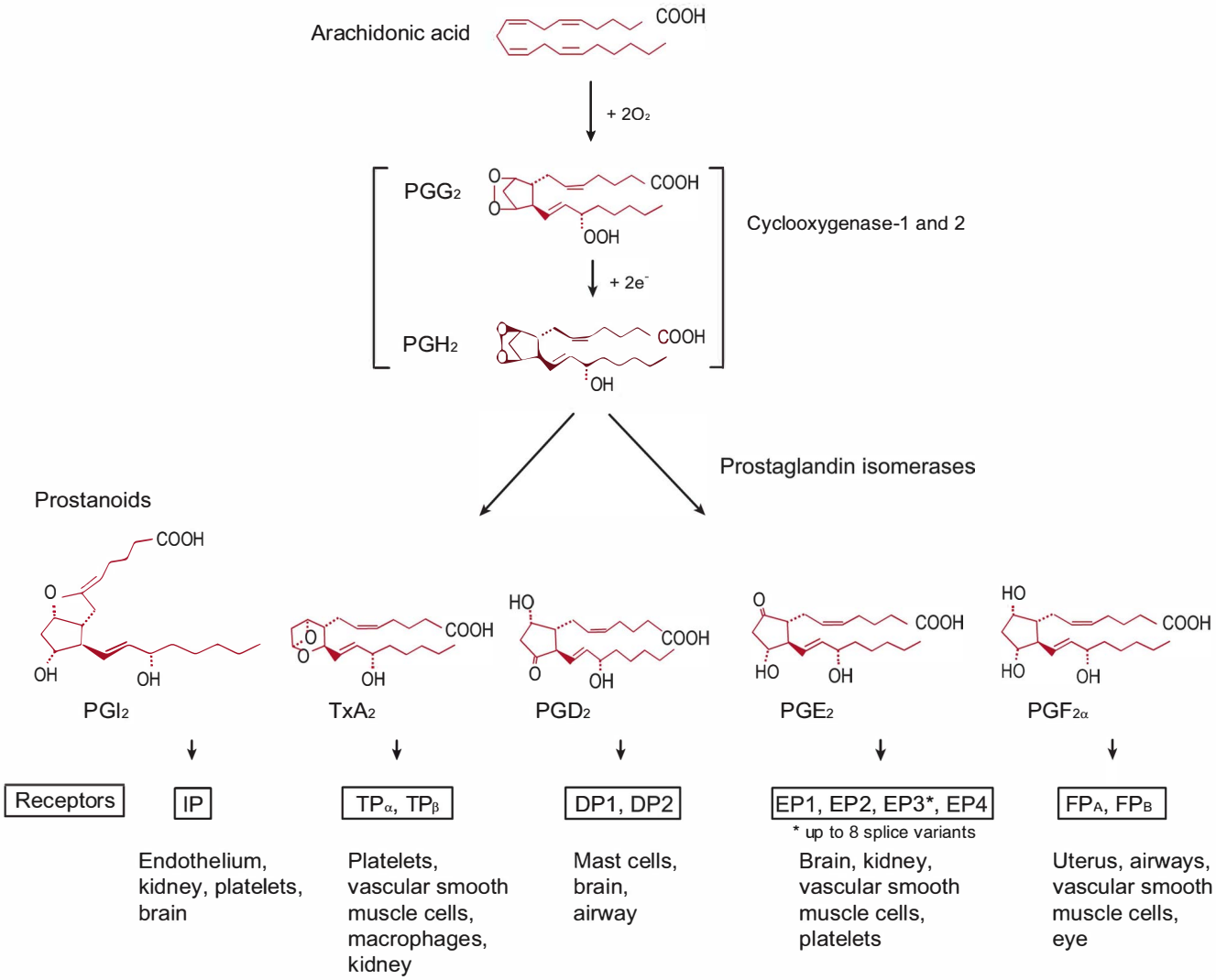

FIGURE 34.1 The cyclooxygenase pathway

posttranscriptional level by RNA-binding proteins and miRNAs forming RNA regulons.[45,46] While these influences on RNA stabilization, transport, and translation result in highly tunable gene expression, COX-2 is not only important during pathologic processes. It is developmentally expressed and has key functions in uninflamed tissues including the vasculature, kidney, gastric epithelium, spinal cord, and brain.[38]

The cyclooxygenase enzymes function as membrane-anchored dimers.[47] The two monomers act synergistically; inactivation of one monomer is sufficient to inhibit enzyme function.[47,48] Enzymatic PG synthesis involves both a COX and a peroxidase reaction. The COX activity incorporates oxygen into arachidonic acid that results in the formation of a labile intermediate peroxide, PGG_2. The peroxidase activity reduces PGG_2 to the endoperoxide product PGH_2.[39] This unstable compound is subject to further metabolism by at least nine terminal prostanoid synthases to form TX and the D, E, F, and I series PGs.[24,49]

While most human cells or tissues have the capacity to form an array of active prostanoids through tissue-specific expression of prostanoid synthases, the TX biosynthetic pathway predominates in platelets. The membrane-bound hemoprotein, thromboxane synthase (TXAS) that catalyzes the isomerization reaction of PGH_2 to TXA_2, also converts PGH_2 to 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and malondialdehyde (MDA).[50] However, the importance of HHT and MDA in the regulation of human platelet function is still poorly understood.

Despite its short half-life, PGH_2 released from platelets can be also metabolized by PG synthases expressed outside of platelets in the plasma or in vascular cells. For example, platelets can contribute to the production of PGI_2 in endothelial cells or in inflamed synovial cells *in vitro* through a transcellular shunt of PGH_2.[51,52] Platelet-derived PGH_2 is also the substrate for PGD_2 formation by plasma PGD synthase.[21]

PROSTANOID SIGNALING

Because of their short half-life, prostanoids do not circulate.[53] They modulate cell function close to the site of their formation acting via specific G-protein–coupled receptors (GPCRs) on the membranes of their target cells (Table 34.1).[24,49,54,55] A single gene

Table 34.1
G proteins associated with prostanoid receptors

Receptor	Primary Activated G protein
TP, IP, EP_2, EP_4, DP_1	G_s
TP, EP_1, FP	G_q
TP, EP_3, DP_2	G_i
TP, EP_3	G_{12}
TP	G_{16}

product receptor has been identified for TXA_2 (the T prostanoid receptor or TP), prostacyclin (the IP), and $PGF_{2\alpha}$ (the FP), while four distinct PGE_2 receptors (the EP_{1-4}) and two PGD_2 (the DP_1 and the DP_2) have been cloned. Additional isoforms of the TP (α and β), FP (A and B), and EP_3 (A–D) are generated through differential splicing.

The sequences of the TP_α and TP_β carboxy terminal splice variants diverge distal to the seventh transmembrane domain. Their expression is differentially regulated by two distinct promoters within the single human TP gene.[56] Differences in signal transduction pathways have been noted,[57] as have differential rates of agonist-induced desensitization.[58] While both transcripts are present in human platelets, only the TP_α protein has been detected.[59] The TP couples to multiple G proteins to stimulate small G protein signaling pathways and may activate or inhibit adenylyl cyclase via G_s (TP_α) or G_i (TP_β), respectively. In platelets, the TP interacts predominantly with G_q and $G_{12}/_{13}$. G_q activates protein kinase C-dependent pathways, which facilitate platelet aggregation, whereas G_{12}/G_{13}-mediated regulation of myosin light chain phosphorylation participates in receptor-induced platelet shape change. TXA_2-induced platelet activation beyond the site of vascular injury is thought to be restrained by its short half-life (about 30 seconds) and by rapid TP desensitization. Heterodimerization with other GPCRs, such as the IP, may afford another mechanism to modulate downstream signaling.[60] Additionally, endogenous inhibitors of platelet function, including PGI_2 and nitric oxide, serve to limit platelet activation.[20]

Both PGI_2 and PGE_2 acting via the IP, or theoretically, via the DP_1, the EP_2, and/or the EP_4 (all coupled via Gs to adenylate cyclase activation), may inhibit platelet function,[24] while lower concentrations of PGE_2 activate platelets via the EP_3 *in vitro*.[25] EP_3 isoforms can couple via G_i or G_{12} to elevation of intracellular calcium, inhibition of cAMP generation, and activation of the small G protein Rho.

PROSTANOID DEGRADATION AND ELIMINATION

Thromboxane

TXA_2 is rapidly hydrolyzed ($T_{1/2}$ ~30 seconds) to the stable and biologically inactive compound TXB_2 in biologic fluids. Quantitation of TXB_2 in serum or platelet-rich plasma permits assessment of the capacity of platelets to form TXA_2 *ex vivo*.[61–64] Typically, TXB_2 formation is detected in serum derived from whole blood allowed to clot under standardized conditions or in platelet-rich plasma after induced platelet activation.[63] These approaches have been used, for example, to validate the use of low doses of aspirin in cardiovascular prophylaxis[65] or to reveal drug–drug interactions between low-dose aspirin and NSAIDs.[66,67]

Attempts to use TXB_2 as an index of platelet activation *in vivo*, however, have proved unsuccessful. Platelets have the capacity to form TXA_2 in concentrations of approximately 300 to 400 ng/mL blood,[68] while maximal endogenous concentrations have been estimated to be three orders of magnitude lower, in the range of 1 to 2 pg/mL.[69] This divergence between the capacity to generate TXA_2 and the actual production rate *in vivo* is a feature of the feed forward, activation amplification role of TXA_2 in the propagation of platelet-mediated clotting.[70] Thus, the inevitable platelet activation that occurs during blood sampling, even under standardized conditions, results in TXB_2 bursts that exceed circulating levels and render the measurement highly variable and essentially meaningless.[71]

The quantitation of urinary TXB_2 metabolites, themselves not formed in whole blood, avoids this problem because TXB_2 is rapidly cleared from the circulation.[53,69] The first of two major pathways of TXB_2 metabolism results in the formation of the β-oxidation products, 2,3-dinor TXB_2, and 2,3,4,5-tetranor TXB_2.[72] The second involves the formation of molecules with a δ-lactone ring structure, of which 11-dehydro TXB_2 is the major urinary product and the most abundant TXB_2 metabolite in humans (2,3-dinor TXB_2 is the most abundant metabolite in mice).[72] Both, 2,3-dinor TXB_2 and 11-dehydro TXB_2 have been qualified as indices of TXA_2 biosynthesis *in vivo*.[69,71,73,74] Because neither metabolite is formed in the kidney, their detection in urine affords a noninvasive measure of systemic TXA_2. 11-dehydro TXB_2 itself undergoes extensive degradation, but its half-life in the circulation (~60 minutes) exceeds that of 2,3-dinor TXB_2 (15 to 17 minutes) and it is more abundant in urine.[75,76] Thus, this metabolite is thought to be the more robust index of TXA_2 formation *in vivo*.[76]

Experiments with low-dose aspirin revealed that roughly 80% of the systemic TX biomarker in urine derives from platelets under physiologic conditions.[77] Excretion of urinary TX metabolites is augmented in clinical syndromes of platelet activation, including unstable angina and myocardial infarction,[78] reperfusion following therapeutic thrombolysis,[79] severe peripheral vascular disease,[80] preeclampsia,[81] pulmonary hypertension,[82] sickle cell anemia,[83] systemic lupus erythematosus,[84] scleroderma,[85] and stroke.[86] Conversely, high levels of a urinary 11-dehydro TXB_2, in patients on low-dose aspirin therapy have been linked to elevated cardiovascular risk.[87]

Prostacyclin

PGI_2 undergoes a metabolic fate similar to that of TXB_2. It is degraded hydrolytically to 6-keto-$PGF_{1\alpha}$ and then enzymatically to 2,3-dinor-6-keto-$PGF_{1\alpha}$ (6.8% of infused PGI_2) and 15-keto-13,14-dihydro-2,3-dinor-6-keto-$PGF_{1\alpha}$.[53] While quantitation of 6-keto-$PGF_{1\alpha}$ allows for monitoring the formation of PGI_2 *in vitro*, urinary excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ has been qualified as an index of PGI_2 formation *in vivo*.[53] Quantitation of this metabolite revealed that the endogenous production of PGI_2 under physiologic conditions is very low.[53] The observation that its formation was markedly enhanced in clinical settings in which platelet–vascular interactions are increased, such

as unstable angina or myocardial infarction, suggested that PGI_2 may act as an endogenous platelet-inhibitory agent, perhaps to restrain the propagation of a thrombotic response beyond the site of injury.[88] Thus, concern was raised when clinical studies in healthy volunteers revealed unexpectedly that COX-2 selective NSAIDs depressed endogenous PGI_2.[89,90] These studies showed that COX-2 was the major source of PGI_2 biosynthesis in humans, suggesting—before the first compound within this class was granted marketing approval—that COX-2 inhibition might elevate cardiovascular risk.[18]

Other Prostaglandin Metabolites

While PGE_2 and PGD_2 are relatively stable in aqueous solutions, they are rapidly cleared from the circulation. PGE_2 is biotransformed by dehydrogenation, reduction, β-oxidation, and ω-oxidation, yielding the major urinary metabolite, 11α-hydroxy-9,15-dioxo-2,3,4,5-tetranor-prostane-1,20-dioic acid (PGE-M). PGD_2 is transformed to 1β-$PGF_{2\alpha}$, 2,3-dinor-11β-$PGF_{2\alpha}$, and 11,15-dioxo-9α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic acid (tetranor PGD-M).[91] Urinary PG metabolites of both PGE_2 and PGD_2 have been used to monitor COX activity during NSAID administration.

PROSTANOIDS IN THROMBOSIS AND HEMOSTASIS

Thromboxane

The primary role of TXA_2 signaling during thrombosis is the amplification of platelet activation and recruitment of additional platelets to the site of clotting. Thus, inhibition or disruption of TXA_2 signaling *in vivo* substantially limits the aggregation response, but it does not completely prevent clot formation. Consistent with the function of TXA_2 to provide rapid feed-forward activation, the relationship between the capacity of platelets to form TXA_2 and platelet function is nonlinear.[70] A residual capacity to generate 10% TXA_2 is sufficient to fully sustain TXA_2-dependent platelet aggregation.[92] Thus, only compounds that afford permanent inhibition of more than 95% to 98% of the capacity of platelets to generate TX would be expected to be effective antiplatelet drugs.[93] Currently, aspirin is the only compound in this category, as it inactivates platelet COX-1 irreversibly. Traditional NSAIDs, with the possible exception of naproxen in some individuals, which inhibit the enzyme reversibly, achieve a level of inhibition of TXA_2 synthesis that translates into inhibition of platelet function only transiently during the dosing interval.[66] Previous attempts to develop TXAS inhibitors failed in part because persistent and complete platelet inhibition was not achieved with the compounds that were selected for clinical development.

As mentioned previously, mutations in the human TXA_2 synthesis/response pathway may result in mild bleeding disorders, such as minor mucosal bleeding and easy bruising. Patients typically have prolonged bleeding times and defective aggregation responses to arachidonic acid/TXA_2 pathway agonists.[8–10,12,13,94–101] Genetic defects in this pathway can be modeled in mice. TXAS or TP deficient mice have reduced responses to thrombotic stimuli and prolonged bleeding times.[43,102] They also show altered hemodynamic responses to hypertensive stimuli,[102,103] consistent with the role of TXA_2 in renal function and vasoconstriction.[103,104] TXA_2 derived from both platelet and nonplatelet sources[105,106] may also accelerate remodeling of the blood vessel wall in response to vascular injury[7] and lesion development during initiation of atherogenesis.[107,108,109]

Prostacyclin

PGI_2, a dominant product of the vasculature,[110,111] is largely derived from COX-2 under physiologic conditions in humans.[89,90,112–114] Hemodynamic forces are thought to regulate vascular expression of COX-2 *in vivo*.[115] PGI_2 acts as a general restraint on stimuli that activate platelets such as collagen, thrombin, or TXA_2 by raising the cAMP concentration in the platelet through activation of its IP receptor. As mentioned above, local production of PGI_2 is thought to be a component of the mechanism that prevents thrombus formation beyond the site of injury and its close vicinity.

Following activation of the IP-G_s-adenylyl cyclase pathway, cAMP activates protein kinase A (PKA), which, amongst a number of target proteins, deactivates myosin light chain kinase leading to reduced myosin phosphorylation and decreases platelet aggregation. PKA also phosphorylates the vasodilator stimulated protein, which regulates fibrinogen binding by modulating the inside-out signaling of the integrin $\alpha_{IIb}\beta_3$, the major fibrinogen receptor in platelets. PGI_2 may also affect the hemostatic system by regulating the expression of thrombin receptors and thrombomodulin.[116]

Disrupting PGI_2 signaling in mice by deleting its specific receptor, the IP, predisposes to thrombosis *in vivo*[7,117] and various models have been used to demonstrate that perturbation of PGI_2 function augments the thrombotic response. Thus, IP deletion accelerated the thrombotic occlusion of large arteries in response to injury in a gene dose–dependent fashion.[26] Administration of a selective COX-2 inhibitor at doses that reduced PGI_2 formation also resulted in a shortened vascular occlusion time.[26] Similarly, genetic disruption of COX-2 or partial genetic disruption, which would be expected to mimic pharmacologic inhibition more faithfully than complete knockout, reduced PGI_2 formation and accelerated thrombosis.[26,118–120] COX-2–derived PGI_2 formation was mapped to the vasculature using tissue-specific deletion of COX-2 in endothelial cells and vascular smooth muscle cells. Indeed, tissue-specific deletion of COX-2 in vessel wall cells was sufficient to reduce PGI_2 biosynthesis and augment the thrombotic response in a microvascular laser injury model.[111] Finally, selective inhibition of COX-2 also depressed PGI_2 biosynthesis in rats and predisposed rats and hamsters to platelet activation and arterial thrombosis.[121,122] These observations are consistent with findings in studies of human genetics. Thus, polymorphisms in COX-2, PGI_2 synthase, and the IP have variously been associated with adverse cardiovascular outcome after angioplasty and hypertension.[123,124,125]

Other Prostanoids

The role of PGE_2 in platelet function *in vivo* remains less clear. *In vitro*, high PGE_2 concentrations (>10 μM), acting via the IP or, theoretically, the EP_2 or EP_4, inhibit platelet function.[25] Low concentrations of PGE_2, acting via the EP_3, augment submaximal platelet stimulation by other agonists. Consistent with the latter observation, mice lacking the EP_3 have an increased bleeding tendency and decreased susceptibility to thromboembolism.[126]

Deletion of the enzyme primarily mediating inflammatory PGE_2 formation, the microsomal PGE synthase-1 (mPGES-1), did not affect thrombogenesis *in vivo,* probably due to substrate rediversion and augmented formation of PGI_2.[26]

INHIBITION OF PROSTAGLANDIN FORMATION

Platelet Inhibition with Aspirin

The COX isozymes are configured such that the active site is accessed by the arachidonic acid substrate via a hydrophobic channel. Aspirin acetylates serine 529 of COX-1, located high up in the hydrophobic channel. Interposition of the bulky acetyl residue prevents the binding of arachidonic acid to the active site of the enzyme and thus impairs the ability of the enzyme to make PGs. Although covalent modification of COX-2 by aspirin (serine 516) also blocks the cyclooxygenase activity of this isoform, an interesting property not shared by COX-1 is that acetylated COX-2 synthesizes 15(*R*)-hydroxyeicosatetraenoic acid [15(*R*)-HETE]. This may be metabolized, at least *in vitro*, by 5-lipoxygenase to yield 15-epilipoxin A_4, which has anti-inflammatory properties.

As anucleate platelets have limited capacity to synthesize proteins, new platelets need to be released into the circulation to recover clotting function following aspirin exposure. Inhibition of platelet COX-1–dependent TXA_2 formation therefore is cumulative with repeated doses of aspirin as low as 30 mg/d. The unique sensitivity of platelets to inhibition by such low doses of aspirin is related to their presystemic inhibition in the portal circulation before aspirin is deacetylated to salicylate on first pass through the liver.[127] In contrast to aspirin, salicylic acid is a weak, reversible competitive inhibitor of cyclooxygenase. Full recovery from COX-1 inhibition takes platelets roughly 8 to 12 days—the platelet turnover time—once therapy has been stopped. However, even a partially recovered platelet pool—just a few days after the last aspirin dose—may afford recovery of sufficient hemostatic integrity for some types of elective surgery to be performed. However, partial platelet function may also predispose to thrombotic events in noncompliant patients.

Irreversible, complete, and permanent inhibition of platelet COX-1 is thought sufficient to explain cardioprotection by aspirin.[14] Traditional NSAIDs, with the possible exception of naproxen in some individuals, which inhibit the enzyme reversibly, achieve a level of inhibition of TXA_2 synthesis that translates into inhibition of platelet function only transiently during the dosing interval.[66] Aspirin reduces the incidence of fatal and nonfatal serious vascular events in patients, who already had a vascular occlusive event (i.e., secondary prevention), by roughly 25%.[16,17,128] The absolute incidence of serious vascular events in this population is typically reduced yearly by 10 to 20 per 1,000 patients.[16,17,128] Most of the clinical trials demonstrating cardioprotection from low-dose aspirin have used doses in the range of 75 to 81 mg/d. Regular use of aspirin, including aspirin at low doses, can cause serious gastrointestinal and intracranial bleeds. For example, aspirin for secondary cardioprevention increases the incidence of gastrointestinal bleeds approximately 2.7-fold.[17] The yearly increase in the absolute incidence of bleeding, however, is about an order of magnitude smaller (1 to 2 per 1,000 patients) than the reduction in serious cardiovascular events, so that the overall benefit outweighs the risk in secondary prevention.[17] Although aspirin has also been shown to reduce the risk of CV events when used in populations at lower baseline cardiovascular risk,[14] its use for primary prevention is controversial, because the absolute reduction of cardiovascular events is an order of magnitude smaller than for secondary prevention, while the gastrointestinal risk remains roughly constant.[17] Thus, only in adults with a cardiovascular risk >1% per year would the number of cardiovascular events prevented be similar to or greater than the number of episodes of serious bleeding evoked by aspirin.[129]

Aspirin Resistance

Given the potential redundancy in the process of platelet activation and aggregation, low-dose aspirin is remarkably effective in the secondary prevention of important vascular events; it is unsurprising that some patients should suffer thrombotic events while being treated with aspirin. While no precise definition has been widely accepted, the term "aspirin resistance" has been variably used for three observations: (a) treatment failure in patients on low-dose aspirin who have clinical thrombotic events; (b) failed inhibition of platelet function; and (c) biochemical evidence of failed inhibition of COX activity. The prevalence of "aspirin resistance" was reported to be up to 60% depending on the definition used,[130] although most studies have observed rates of 5% to 20%.[131–135] These estimates derive mostly from small patient populations (small in comparisons to the sizes of the studies that were necessary to demonstrate the therapeutic benefit of aspirin)[15,16,17] who were classified as responders and nonresponders based on the failure to meet arbitrarily selected response criteria in various platelet function tests. These criteria include adenosine diphosphate–, collagen-, or arachidonic acid–induced platelet aggregation, cartridge-based bedside whole-blood clotting assays, measurement of platelet TXB_2 formation *ex vivo*, and quantitation of urinary 11-dehydro TXB_2, alone or in combination.

Given the wide use of aspirin for cardioprotection, there is great interest in the discovery of reproducible sources of nonresponsiveness, and in the development of specific tests that would allow for the identification of individuals who would more likely benefit from prophylaxis with higher doses of aspirin or other platelet inhibitors. Many factors may result in an insufficient response to aspirin—and indeed to any medication. For example, the physicochemical properties of aspirin render it particularly susceptible to factors affecting its bioavailability, which is only roughly 40%. The bioavailability may be further reduced in enteric coated aspirin formulations by as much as twofold[136,137] that can translate into attenuated antiplatelet activity.[138,139,140] Given the redundancy in the pathways that result in platelet activation, situations are conceivable in which inhibition of the COX pathway has an insufficient effect on platelet function. For example, environmental factors, such as surgical stress,[141] may activate sympathoadrenal mechanisms of platelet activation resistant to inhibition by aspirin[142] and transcellular mechanisms of platelet activation involving interactions with red blood cells[143] or neutrophils[144] may also proceed, at least *in vitro*, unimpeded by therapy with aspirin. However, these are situations that would not be expected to be specific to aspirin, but might also affect purinergic receptor blockers (e.g., clopidogrel, prasugrel). "True" pharmacologic resistance to aspirin would result from failure of aspirin to inhibit platelet COX-1 either because of failure of aspirin to reach its molecular target,

by failure of aspirin to acetylate COX-1 despite sufficient concentrations in the platelet, or by failure of COX-1 acetylation to suppress TX formation.

Variation in the genes in the TXA_2 biosynthetic/response pathway may account for treatment failure in some individuals,[145,146] but these seem to be uncommon. However, several studies suggest that nongenetic causes are likely to play a prominent role in fluctuation in the response to aspirin.[138,147,148–154,155] Thus, "aspirin resistance" detected in clinical trials appears to be primarily an acquired phenotype. It may be caused by (a) failure to consume aspirin regularly;[147,148,156] (b) insufficient bioavailability; [138] (c) accelerated recovery from irreversible inhibition in situations of enhanced platelet turnover; [157] (d) reduced COX acetylation in the presence of elevated oxidant tone or cholesterol;[149–151] or (e) drug–drug interactions with reversible COX inhibitors and aspirin.[152–154,158,159] Traditional NSAIDs, which bind to both platelet COX-1 and COX-2—ibuprofen,[152] naproxen,[158] and indomethacin[159]—may interfere with the capacity of aspirin to inhibit platelet COX-1 irreversibly, most likely by impeding access of aspirin to its active site.[152] This mechanism may not be restricted to the compounds that have been studied so far and it would be expected to translate into clinical nonresponsiveness to aspirin—a lack of reduction of myocardial infarction and stroke.[160–162]

Cardiovascular Risk of COX-2 Inhibition

The importance of the prostanoids in hemostasis and thrombosis is also highlighted by the clinical experience with inhibitors of COX-2. Enthusiasm for COX-2 selective NSAIDs was initially high as they promised to reduce the likelihood of gastrointestinal complications, although this was actually established to be the case only for a subset of the "coxibs" in clinical trials after they had been introduced to the market. All FDA approved COX-2 selective NSAIDs, however, were shown in placebo controlled trials to cause cardiovascular complications—myocardial infarction, stroke, heart failure, and venous thrombosis—affecting approximately 10 to 20 per 1,000 patients in randomized controlled trials per year,[18,20] which led to the withdrawal of rofecoxib and valdecoxib.[163] It has been estimated that rofecoxib and celecoxib caused over 70,000 acute myocardial infarctions and strokes and 26,000 deaths in the first 5 years of their release on the US market.[164] The underlying molecular mechanism—depression of COX-2–dependent PGI_2 formation in the heart, kidney, and vasculature—has been elucidated in more detail than that of any other adverse drug effect-integrating evidence from cell biology, animal models, and human pharmacology.[18,20] It is likely that the response to COX-2 inhibition in the vasculature, the kidney, and the heart all contribute to the development of cardiovascular complications, either acutely or over time. Aspirin, which suppresses just a component of prothrombotic mechanisms, may be expected to reduce the risk, but not eliminate it.

Mechanistic studies in mice and humans do not support the notion that all NSAIDs would carry the same cardiovascular risk. They suggest that the toxicity profiles of compounds with respect to the major relevant organ systems are as diverse as their gastrointestinal toxicity profiles. NSAIDs are a chemically heterogeneous group of compounds, thus, it is unsurprising that individual compounds are distinguished by multiple characteristics with potential to impact on their risk/benefit profile, such as their half-lives, potency, and selectivity for COX-2. Indeed, it was suggested that the combination of all factors that determine drug exposure—including half-life, drug potency, selectivity, and dose—with baseline cardiovascular risk integrate to determine the likelihood of cardiovascular complications in a patient.[20]

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CHAPTER

35 The Role of Platelets in Blood Coagulation

PETER N. WALSH

The hemostatic response to vascular injury consists of a series of proteolytic reactions that occur on the surface of activated platelets and other intravascular cells including monocytes and neutrophils.[1–3] This localizes coagulation complex assembly to the hemostatic thrombus and protects coagulation enzymes from inactivation by plasma and cellular inhibitors of serine proteases.[1–3,4–6,7,8] The initial hypotheses explaining the normal coagulation mechanism, referred to as the *cascade*[9] and *waterfall*[10] *theories*, were proposed more than 45 years ago and have since been extensively revised.[11,12,13] The goal of this chapter is to describe our current understanding of the normal hemostatic mechanism. The contributions of leukocytes and monocytes to coagulation complex assembly and the role of cell membrane receptors in the assembly of the anticoagulant complexes involving activated protein C (APC), protein S, and thrombomodulin are discussed in Chapter 19. The interactions of thrombin with platelets and other cells is considered in Chapter 33.

SEQUENTIAL COAGULATION COMPLEX FORMATION

The cascade[9] and waterfall[10] theories of blood coagulation originally postulated a mechanism by which a minute initial stimulus was sequentially amplified through a series of enzymatic reactions to result in the local explosive generation of thrombin. These hypotheses postulated two alternative pathways for the initiation of coagulation, each leading to the activation of factor X (FX). One was termed the *intrinsic pathway* in which the "contact factors," FXII, prekallikrein (PK), high molecular weight kininogen (HK), and FXI, are assembled on negatively-charged surfaces,[14] and the other was termed the *extrinsic pathway*, initiated by FVIIa and by the exposure of tissue factor (TF).[13] It was subsequently shown that platelets make major contributions to each pathway, including an alternative, platelet-dependent pathway for the activation of FXI.[1,15–19] However, these hypotheses required revision because patients with deficiencies of FXII, PK, and HK do not experience abnormal bleeding,[14] whereas patients with deficiencies of factors VIII and IX (hemophilia A and B, respectively) experience severe, life-threatening, spontaneous, and posttraumatic bleeding,[20,21] and patients with FXI deficiency[22–29] and FVII deficiency[30] may bleed excessively after surgery or trauma. Nonetheless, because FIX activation can be catalyzed not only by FXIa[31–40] but also by FVIIa-TF,[37] it has been suggested that the TF pathway can initiate the intrinsic pathway directly and independently of the contact factor pathway. However, this does not account for the hemostatic defect observed in FXI deficiency,[22–29] and in 1991 an alternative mechanism for proteolytic activation of FXI by thrombin[11,12] was demonstrated. Thus, the initial event in hemostasis is likely the exposure of TF on cell membranes, resulting in the activation of FX by FVIIa-TF,[30] and leading to the generation of trace concentrations of thrombin because the TF pathway is rapidly shut down by tissue factor pathway inhibitor (TFPI).[41–46] The initial trace amounts of thrombin formed are sufficient to activate platelets, FXI, FVIII, and FV, permitting the intrinsic (or consolidation) pathway of coagulation to generate sufficient quantities of thrombin to promote hemostasis.[11,12] The intrinsic pathway is thought to occur on the surface membranes of activated platelets, where there are binding sites for FXI,[47–52] FXIa,[53–56] FIX,[57–60] FIXa,[57–73] FVIII,[74–78] FVIIIa,[74–78] FX,[75,79,80] FXa,[81–93] FV,[81–93] FVa,[81–93] prothrombin,[79,80] and thrombin.[2,94] These binding sites promote the assembly of coagulation enzyme–cofactor–substrate complexes that compared with solution-phase reactions, accelerate the kinetics of FXI activation by FXIIa,[95] of FX activation by FIXa,[57,58,62,64–66] and of prothrombin activation by FXa.[81–90] The assembly of these complexes is presented in schematic form in FIGURE 35.1, providing a point of reference for understanding the role of platelets and other cells in the initiation and consolidation pathways of blood coagulation.

INITIATION PATHWAYS

Assembly and Regulation of the Tissue Factor Pathway

As discussed in Chapter 11, the contact phase proteins can initiate intrinsic coagulation when foreign surfaces are introduced into the blood stream,[14] the initiating event in hemostasis is the expression of the transmembrane protein TF on cell membranes.[30] A number of cell types, including monocytes and leukocytes and those underlying the endothelium, can synthesize and expose TF to plasma proteins, but the specific cell type and mechanism of TF presentation has not been definitively identified.[30,96] Unactivated platelets do not constitutively express TF. However, TF-bearing microparticles,[97–103] derived from specialized membrane microdomains (i.e., lipid rafts) in monocytes, can fuse with platelet membranes, perhaps utilizing adhesive receptors such as P-selectin on activated platelets and PSGL-1 on microparticles to transfer TF to platelets by membrane fusion.

Platelets and the Contact Activation Pathway

The reciprocal proteolytic activation of FXII and PK on negatively charged surfaces in the presence of high molecular weight kininogen (HK) can lead to the limited proteolytic activation of FXI[14,104–112] and activation of the intrinsic coagulation pathway. However, the physiologic role of these reactions is uncertain

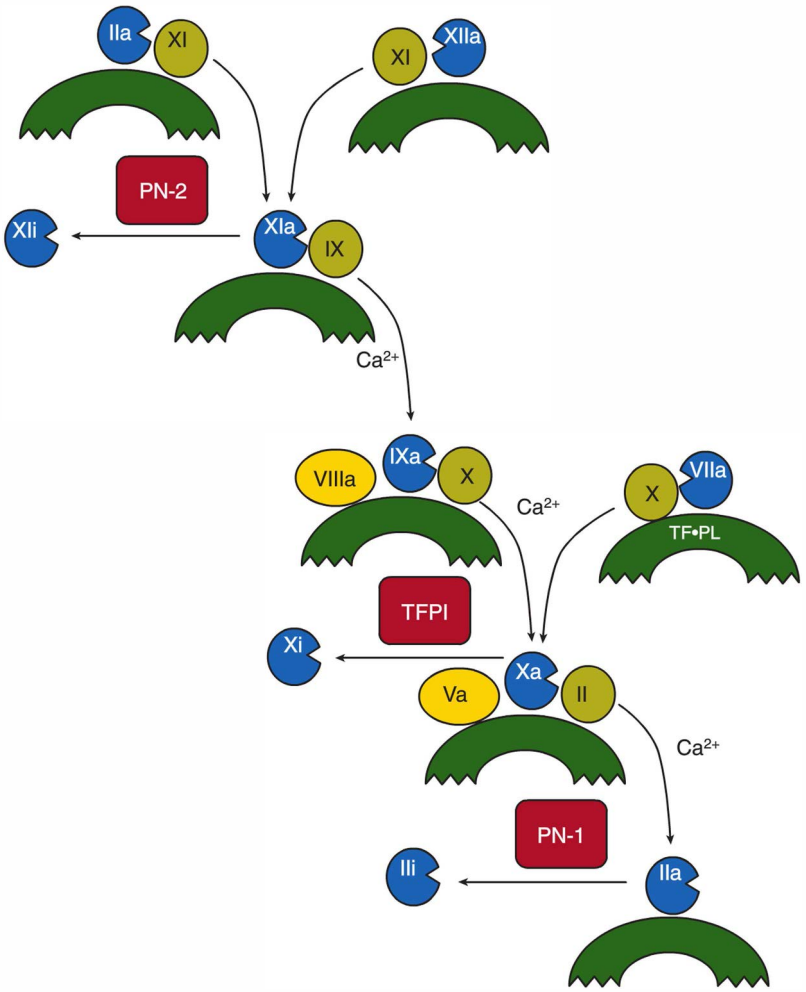

FIGURE 35.1 Schematic diagram representing a current hypothesis of the contribution of platelets to blood coagulation, and the postulated sequence of coagulation reactions that comprises the normal hemostatic mechanism. The crescentic forms represent the activated platelet membrane, except in the case of the form labeled **TF•PL**, which represents tissue factor expressed on a phospholipid membrane surface. The roman numerals represent the coagulation proteins including zymogens or substrates (*in circles*), cofactors (*in ellipses*), and enzymes (*in circles with segmental excisions*). TF, tissue factor; TFPI, tissue factor pathway inhibitor; PN2, protease nexin-2; PN1, protease nexin-1. The curved arrows represent conversions from zymogens (e.g., prothrombin, shown as II) to active enzymes (e.g., thrombin, shown as IIa), whereas the leftward pointing arrows represent the inhibition of these active enzymes (e.g., FXa, shown as Xa) to their inactivated forms (e.g., inactivated FXa, shown as Xi). Shown in boxes are the Kunitz-type inhibitors, PN-2 and TFPI, and the serpin, PN-1. The coagulation mechanism is initiated as the consequence of the assembly of the FVIIa/FX/TF/PL complex and is regulated by TFPI, whereas the consolidation phase of coagulation, regulated by PN-2 and PN-1, is activated by the resulting generation of low concentrations of thrombin sufficient to activate platelets, FXI, FVIII, and FV to produce thrombin in sufficient quantities to form a fibrin clot. (From Walsh PN. Platelets: yin and yang. *Blood* 2010;115:1–2, with permission.)

since patients deficient in contact activation pathway proteins do not bleed.[4,7,8,22–29,94,113,114] In fact, HK may downregulate platelet activation by impairing thrombin binding to the platelet glycoprotein (GP) Ib/IX/V.[115] Nonetheless, the contact activation pathway may contribute to the formation of pathologic thrombi, especially in patients with intravascular prosthetic devices.[14]

Activated platelets can participate in the activation of both FXII and FXI.[1,15–19] Thus, ADP- or collagen-stimulated platelets can promote the proteolytic activation of FXII by kallikrein and the subsequent proteolytic activation of FXI by FXIIa[95] by exposing high-affinity FXI binding sites that require the presence of either HK and zinc ions or prothrombin and calcium ions (Table 35.1).[47–55] Since there is evidence that activation of FXI on the platelet surface by thrombin can occur in the absence of FXII,[116] it is possible that under certain physiologic or pathologic conditions, assembly of contact pathway proteins on activated platelets can trigger intrinsic coagulation. For example, polyphosphates secreted from platelet dense granules[117–119] and RNA released from damaged vessels[120] can activate FXII. A recent systems biology approach, examining the kinetics of thrombin generation in blood treated with corn trypsin inhibitor (CTI) to inhibit contact activation, suggests that activated platelets support the initiation of coagulation via the combined activity of FXIIa and FXIa in the absence of any evidence for kinetically significant blood-borne TF.[121]

CONSOLIDATION PATHWAY

Interactions of Activated Platelets with FXI

FXI is a unique, homodimeric coagulation protein that circulates in plasma in a complex with HK. Although FXI can be activated by FXIIa, the physiologic relevance of this reaction is uncertain. On the other hand, thrombin has been implicated in feedback activation of FXI on negatively charged surfaces, either in the presence of HK and zinc ions or prothrombin and calcium ions.[11,12] Moreover, platelet activation appears to result in FXI binding to GPIb/IX/V, thereby colocalizing FXI with GPIb/IX/V bound in membrane microdomains or lipid rafts.[122,123] Although it was originally reported that platelet stimulation by thrombin promotes FXI activation,[124,125] these observations could not be confirmed.[126] On the other hand, activated platelets can enhance FXI activation when thrombin is generated by low concentrations of TF-FVIIa[116] and thrombin propagates further thrombin generation in CTI-treated plasma when FXI and platelets are present.[127] Nonetheless, the role of thrombin in FXI activation remains controversial. Although Pedicord et al.[128]

Table 35.1

Binding of coagulation zymogens, enzymes, and cofactors to activated platelets

Ligand	Conditions	K_D (nM)	# Sites/Plat	Function	References
FXI	HK + Zn^{2+} or FII + Ca^{2+}	10	1,500	FXI activation	47–52
FXIa	Zn^{2+}	1.7	250	FIX activation	53–56
FIX	Ca^{2+}	2.5	250	FIX activation	57–60
FIXa	Ca^{2+} FVIIIa + FX + Ca^{2+}	2.5 0.5	500 500	FX activation (k_{cat}/K_m ⇑ >10^8-fold)	57–73
FVIII		3.0	450	Unknown	74–78,157
FVIIIa	FIXa + FX	1.7 0.8	1,000 1,200	FX activation (k_{cat}/K_m ⇑ >10^8-fold)	74–78
FX	Ca^{2+} Ca^{2+} + FVIIIa	320 5.0	16,000 1,000	Shared with FII FX activation (k_{cat}/K_m ⇑ >10^8-fold)	75,79,80
FXa	Ca^{2+} + FVa	0.03	250	FII activation (rate ⇑ >10^5-fold)	81–93
FV		1.0	800	Unknown	81–93
FVa		1.0 0.1	4,000 800	FII activation (rate ⇑ >10^5-fold)	81–93
FII	Ca^{2+}	320	16,000	Unknown (shared with FX)	79,80

found no evidence that FXI activation by thrombin occurs in plasma or on the surface of activated platelets, others found that FXI activation by low concentrations of thrombin makes major contributions to the sustained generation of thrombin in plasma in the absence of FXII.[116,127,129–132] The latter observations are supported by evidence that activated platelets, but not endothelial cells, participate in the intrinsic pathway of blood coagulation by promoting the activation of FXI by thrombin and generating FXIa to promote the activation of FIX.[53,133]

Interactions of FXIa with Activated Platelets, FIX, and Protease Nexin-2

The zymogen FXI binds to approximately 1,500 sites on activated platelets via its A3 domain when HK and zinc ions are present, whereas FXIa binds to approximately 250 distinct sites via its catalytic domain in the absence of HK[56] (Table 35.1). Nonetheless, although both FXIa and FIX bind to activated platelets, the rate of FIX activation by FXIa is unaffected by the presence of activated platelets.[53,134,135] On the other hand, platelet-bound FXIa is protected from inactivation by plasma proteinase inhibitors including α_1-protease inhibitor.[135]

Protease nexin-2 (PN2), a truncated form of the Alzheimer β-amyloid protein precursor, contains a Kunitz-type protease inhibitor domain and is a potent inhibitor of FXIa whose activity is enhanced by heparin.[136–140] There is very little PN2 in normal human plasma, but the protein is present in sufficient amounts in platelet α-granules to achieve a plasma concentration of 2 to 5 nM, and possibly even as high as approximately 30 nM,[136,138,139] following platelet secretion. These concentrations, well above the K_i for FXIa inhibition, rapidly and completely inhibit FXIa in solution, whereas FXIa bound to activated platelets is completely protected from inactivation by PN2.[53,139] By contrast, FXIa bound to endothelial cells is not protected,[53] suggesting that platelets, by secreting PN2 and protecting FXIa from inhibition, provide a nidus for localizing coagulation reactions to the hemostatic thrombus.[1,53,133,141]

Interactions of Activated Platelets with FIXa, FVIIIa, and FX

In the early 1970s, it was found that human platelets, activated with collagen but not ADP, provided a membrane surface for the proteolytic cleavage of FX by FIXa in the presence of FVIII.[15,19,142] Subsequently, it was shown that activation of platelets by a variety of physiologic and nonphysiologic agonists, as well as activation of FVIII by thrombin, were required for optimal rates of FX activation by the FIXa–FVIIIa complex.[142–146] At a minimum, aminophospholipids exposed on the outer leaflet of activated platelets participate in the assembly of the FX-activating complex.[147,148] A variety of experimental results suggest that specific classes of platelet receptors exposed by platelet activation also participate in the assembly of the FX-activating complex,[57–69,74–76,79,80,149–151] but such receptors remain to be definitively identified.

FIXa Binding to Activated Platelets

The initial evidence that platelets play a specific role in FX activation was the observation that FIXa binds to approximately 500 high-affinity specific sites on activated platelets, half of which can also recognize the zymogen FIX when calcium ions are present (Table 35.1).[57] The presence of the cofactor FVIIIa and the substrate FX increases the affinity of FIXa binding approximately fivefold, but has no effect on FIX binding.[57] The conclusion that the FIXa binding sites are functionally relevant[58]

is supported by studies demonstrating that thrombin-activated platelets decrease the K_m for FXa activation >200-fold and permit FVIIIa to increase the V_{max} by >50,000-fold, resulting in an increase in catalytic efficiency >2 × 10^8-fold.[66] The effects of platelets on catalytic efficiency are almost identical to those of phosphatidylcholine (PC)/phosphatidylserine (PS) vesicles,[66,152] but are absolutely dependent on platelet activation by thrombin or the thrombin receptor activation peptide (SFLLRN)[57,58,79,80]; ADP has no capacity to expose FIXa receptors on platelets.[57,58,79,80]

The observations that FIXa binds to twice as many sites as FIX and displaces all bound FIX, whereas FIX displaces only 50% of bound FIXa[57] raise questions about the structural relation of the FIX and FIXa binding sites. FIX binding to activated platelets is mediated by residues G4–Q11 in the Ω loop of the FIX Gla domain, whereas FIXa binding is mediated by the Gla domain Ω loop, as well as residues C88–C109 of the EGF-2 domain.[59,61,63,65,67–69,151] Because FIX displaces FIXa from its Gla-mediated binding site, it was surprising to find that FIX potentiates FIXa-catalyzed FX activation by increasing the affinity of FIXa for the intrinsic FX-activating complex.[60] It is possible that by displacing FIXa from their shared binding site, FIX facilitates higher-affinity EGF-2 domain-mediated FIXa binding within the FX-activating complex (Table 35.1).

The active site of FIXa is not involved in its high-affinity, FVIIIa-dependent binding to activated platelets,[58] although this binding requires the presence of D47.[65] Moreover, FIX cleavage at R145–A146 is essential for its specific, high-affinity interaction with FIXa binding sites, as evidenced by studies of the variant $FIXa_{Chapel\ Hill}$ (D145 → H substitution).[65] Further, the EGF-1 domain of FIXa has no role in these interactions,[64] whereas both the Ω loop (residues G4–Q11) within the Gla domain and residues C88–C109 within the EGF-2 domain are essential.[59,61,63,67–69,151] Residues G4, K5, L6, F9, V10, and Q11 within the Ω loop (i.e., residues G4–Q11) of the Gla domain have been implicated for FIXa binding, for the assembly of the FX-activating complex[63] and in binding to phospholipid membranes.[153–156] Although conformationally constrained synthetic peptides comprising residues G4–Q11 of the FIX Gla domain displaced FIXa from 50% and FIX from 100% of their respective binding sites, these peptides were relatively ineffective (K_i ~165 μM) in inhibiting platelet receptor–mediated activation of FX by FIXa. Thus, although residues G4–Q11 mediate FIX/IXa binding to their shared platelet receptor, additional FIXa binding sites are functionally important in platelet-mediated FX activation.[59] Additional studies have implicated two disulfide-constrained loop structures, comprising residues C88–C109 within the EGF-2 domain in the high-affinity binding site of FIXa, but FIX, to approximately 250 sites per platelet.[58,68,69,71,72,151] When either residues G4–Q11 or residues C88–C109 in FIXa are mutated, decreased binding is observed; when both motifs are replaced or mutated, FIXa fails to bind to platelets or to promote FX activation on the platelet surface. This suggests that residues G4–Q11 comprise the shared FIX/FIXa platelet-binding site, whereas FIXa also binds via residues C88–C109 for efficient assembly of the FX-activating complex.

FVIIIa Binding to Activated Platelets

Nesheim et al.[157] reported that the procofactor FVIII interacts with approximately 450 high-affinity sites on thrombin-activated platelets (Table 35.1). The active cofactor FVIIIa binds to an additional 300 to 500 sites per platelet with an enhanced affinity; in the presence of active-site–inhibited FIXa and FX, FVIIIa binds to a total of approximately 1,200 sites per platelet with a K_d of approximately 0.8 nM.[74] Although FVa and FVIIIa are homologous, FVa does not compete with FVIIIa for platelet binding. On the other hand, the phospholipid binding protein annexin V is a potent inhibitor of FVIIIa binding to activated platelets,[74] whereas vWF inhibits FVIII, but not FVIIIa, binding. FVIIIa binding to thrombin-activated platelets is closely coupled to the rate of FX activation by FIXa.[74]

FVIII binds to platelets via its C2 domain[78,158–160] and is facilitated by the presence of the A2 and A1 domains.[74] The importance of FVIIIa binding to activated platelets is underscored by the severe hemorrhagic diathesis observed in a patient with the platelet defect known as Scott syndrome that is associated with impaired binding of both FVIIIa and FVa and with a deficiency in cell-surface exposure of PS.[62,85,161]

FX Binding to Activated Platelets

Equilibrium binding studies have revealed the presence of approximately 16,000 FX binding sites with a K_d approximately 320 nM on platelets activated with thrombin or the thrombin receptor (PAR-1) activation peptide (SFLLRN) but not with ADP[79,80] (Table 35.1). The FX binding site is shared with prothrombin. Detailed kinetic studies of FIXa-catalyzed FX activation in the presence of activated platelets demonstrated that FX bound to the activated platelets is preferentially cleaved by platelet-bound FIXa.[80] In addition, FVIIIa binding to the surface of activated platelets resulted in the generation of approximately 1,000 higher-affinity sites (K_d ~5 nM) that do not recognize prothrombin and whose occupancy was closely coupled to the rate of FX activation and to the >10^8-fold increase in catalytic efficiency compared to solution-phase catalysis.[72,74,75] Little is known about the motifs that mediate the interaction of FX with the latter site, but FX and prothrombin binding to the low-affinity, high-capacity binding site is mediated by their Gla domains interacting with aminophospholipids exposed on the surface of activated platelets.[79]

CONTRIBUTION OF PLATELETS TO PROTHROMBIN ACTIVATION

Interactions of Activated Platelets with FXa, FVa, and Prothrombin

Negatively-charged aminophospholipids, exposed on activated platelets, provide a surface for the assembly of the prothrombinase complex[162] that accelerates the activation of prothrombin by approximately 300,000-fold compared with FXa-mediated prothrombin activation in solution.[163–185,186–194,195] Kinetic and equilibrium binding measurements of the interaction of FV, FVa, and FXa in both human[82–86,93,196] and bovine[81,88,90–92] systems show that human[83,84] and bovine[81] platelets bind FXa, and that platelet activation is required. Reduced FXa binding to platelets from patients deficient in FV,[86] platelets studied in the presence of FV alloantibodies,[84] and platelets treated with APC,[197,198] demonstrate that FVa is the receptor for FXa. FVa is downregulated by APC during prothrombin activation, but both FXa and prothrombin protect FVa in a multiplicative manner, either in the presence or absence of protein S.[199]

On unactivated bovine platelets, FVa binds to approximately 800 high-affinity (K_d ~0.1 nM) and approximately 4,000 lower-affinity (K_d ~1 nM) sites per platelet, whereas FV binds to approximately 800 low-affinity (K_d ~1 nM) sites.[90] FVa can displace all bound FV, but FV displaces only FVa bound to low-affinity sites. Since Factor V is present in bovine plasma at a concentration of approximately 10^{-7} M, FV is always present on the platelet surface.[90] Further, although there are approximately 2,500 platelet bound FVa–FXa complexes, only approximately 900 (K_d ~0.2 nM) function in thrombin generation.[88,90]

Coordinate FXa–FVa binding studies have not been carried out using human platelets in part because human platelet α-granules contain 4,000 to 14,000 FV molecules per platelet,[200] complicating measurement of FVa binding. Nevertheless, FVa binding to human platelets does not require platelet activation, is stimulated by the presence of FXa and prothrombin, and is nonsaturable with as many as approximately 3,000 binding sites per platelet,[82,196] although only approximately 250 of these sites represent the functional FXa binding sites important for prothrombin activation.[83,84] On the other hand, kinetic measurements of prothrombin activation indicate that FVa and FXa colocalize on approximately 3,000 sites on thrombin-activated platelets with a stoichiometry of 1:1, an apparent K_d of approximately 0.1 nM, and a k_{cat} of approximately 30 per second.[87,89]

Exposure of anionic phospholipids such as PS on the outer leaflet of the activated platelet membrane has been correlated with the expression of prothrombinase complex formation.[186,201–207] Because thrombin concentrations >20 nM, as well as other "strong platelet agonists" such as the calcium ionophore A23187, mixtures of collagen and thrombin, or the complement protein lytic complex, C5b-9 are required to generate maximal FXa binding, functional prothrombinase complex assembly, and aminophospholipid exposure; it has been suggested that the physiologic site for prothrombinase assembly consists of aminophospholipids, exposed either on the outer leaflet of the platelet membrane or on platelet microparticles (see below).

In human platelets, FV is stored in massive, disulfide-linked complexes with the protein, multimerin,[208–211] is secreted from platelets in a partially activated form that can be further activated by FXa or thrombin, and accounts for 20% to 25% of the total FV in normal blood.[180,212–215] Platelet FV appears to be physiologically unique because FXa activates platelet FV 50- to 100-fold more effectively than thrombin,[212] whereas both proteins exhibit equivalent activity toward plasma FV.[216] Further, platelet FV, but not plasma FV, is phosphorylated by a casein kinase II–like enzyme[217–219] and when platelet FV is bound to platelet membranes, it is more resistant to inactivation by APC.[220,221] Platelet FV appears to play a significant role in hemostasis because patients with FV_{quebec} with nearly absent platelet FV and near-normal levels of plasma FV, have a severe bleeding diathesis,[222–224] whereas substantially less bleeding was observed in a patient with normal platelet FV, but markedly reduced plasma FV because of an anti–FV autoantibody.[214] The recent demonstration that residual platelet FV is sufficient to support adequate thrombin generation and to protect against major bleeding in patients with severe plasma FV deficiency[225,226] emphasizes its unique role, as does the substantially greater effectiveness of platelet transfusion over plasma in the management of hemorrhage associated with congenital FV deficiency.[227,228]

The serpin protease nexin-1 (PN-1) is stored in platelet α-granules. When it is secreted into plasma, it rebinds to the platelet membrane where it inhibits thrombin and reduces thrombin generation by TF. Platelets from PN-1–deficient mice demonstrate increased sensitivity to thrombin and enhanced thrombus formation in a ferric chloride-induced injury model, suggesting that platelet PN-1 has important antithrombotic properties.[229,230]

PLATELET RECEPTORS FOR FX AND PROTHROMBIN ACTIVATION

In addition to aminophospholipids, it is possible that platelet components, such as yet unidentified membrane proteins, participate in the assembly of the FX and prothrombin-activating complexes. Evidence supporting this possibility includes the observations that the affinity of FIXa for activated platelets is approximately 30-fold greater than for phospholipid vesicles, annexin V displays different kinetics for inhibiting FX activation on activated platelets compared to phospholipid vesicles, the kinetics of FX activation in the presence of phospholipid vesicles is more sensitive to variations in salt concentration than are the kinetics of FX activation in the presence of activated platelets,[149,150] and the order of bond cleavage that activates prothrombin is different on platelets compared to phospholipid vesicles. In the latter case, prothrombin is initially cleaved on the surface of activated platelets at Arg271 to generate prethrombin-2, which is subsequently cleaved at Arg320 to generate α-thrombin. By contrast, the order of bond cleavage is different on the surface of phospholipid vesicles with meizothrombin being initially formed by cleavage at Arg320, followed by subsequent cleavage at Arg 271 to generate α-thrombin and fragment 1.2.[231]

Circulating human platelets are heterogeneous with respect to age, density, volume, metabolic characteristics, and lipid peroxidation potential.[232,233] Moreover, although all platelets respond to thrombin, collagen, and other agonists by undergoing morphologic shape changes, aggregation, and secretion, perhaps only approximately 10% respond to thrombin and PAR-1 stimulation by exposing binding sites for FIXa, FVIIIa, and FX and assembling the FX-activating complex.[70] In addition, it has been reported that only approximately 10% of platelets display a sustained increase in calcium transients following platelet stimulation by thrombin and collagen, and only this subpopulation has the capacity to bind FIXa and other components of the FX-activating complex in support of FXa generation.[70,73]

Platelet stimulation with low concentrations of thrombin and collagen or convulxin may also produce a subpopulation of platelets that bind FIXa, FVIIIa, FX, and FVa, thereby assembling the FX-activating complex, so-called COAT platelets.[234,235] It has been suggested that COAT platelets arise when serotonin, secreted from platelet- dense granules, cross-links platelet-derived FVa and other platelet proteins to their receptors via platelet-derived FXIIIa.[236,237] It has also been reported that thrombin and collagen act synergistically to promote platelet procoagulant activity by inducing a sustained elevation in cytosolic calcium in a subpopulation of platelets and that the synergistic effect of thrombin on collagen-induced platelet procoagulant activity is mediated through PAR-1.[238] Recent studies utilizing flow cytometry have demonstrated that at concentrations of thrombin eliciting maximal platelet activation, only a subpopulation of platelets were positive for FVa and FXa binding and for prothrombinase assembly and function.[239] Taken together, these observations support the

surprising conclusion that although all platelets can undergo shape change, aggregation, and secretion, only a small fraction have the capacity to respond to physiologic agonists by undergoing pronounced and sustained increases in cytoplasmic calcium and participating in blood coagulation reactions by exposing binding sites and/or receptors for the assembly of the FX-activating complex.

ORGANIZATION OF PHOSPHOLIPIDS IN PLATELET MEMBRANES

Approximately 57% of the phospholipids in platelets are located in the platelet plasma membrane[242,243] and consist of 38% PC, 27% phosphatidylethanolamine (PE), 17% sphingomyelin (SM), 10% PS, and 5% phosphatidylinositol (PI).[240,241] Studies utilizing phospholipases and the nonpenetrating membrane probe trinitrobenzene sulfonic acid indicate that PC and SM are primarily located on the extracellular face of the resting platelet, whereas the acidic phospholipids, PE, PS, and PI, are located in the inner membrane leaflet.[142,187,193,203,240,242–249] The asymmetric distribution of phospholipids has been confirmed by studies using spin-labeled aminophospholipids and annexin V, a protein that binds with high affinity to aminophospholipids.[244,250] Annexin V binding to activated platelets inhibits the binding of FIXa and the assembly of the FX-activating complex, implying that the components of the FX-activating complex utilize acidic phospholipids to interact with platelets.[149]

Functional Aspects of Lipid Asymmetry

The asymmetric distribution of phospholipids in platelet membranes has major implications for blood coagulation since resting platelets, whose aminophospholipids are sequestered on the inner membrane leaflet, are virtually inert in assembling active coagulation complexes on the platelet membrane. Following platelet stimulation by a variety of physiologic and nonphysiologic agonists, the membrane undergoes major alterations, resulting in the exposure of PS and PE on the extrafacial membrane surface. Activation of platelets with thrombin alone increases the exposure of PE, but not PS, on the platelet surface[193] without altering the fatty acid composition of PE.[251] However, platelet activation with combinations of collagen and thrombin induces significant hydrolysis of both PE and PS.[242] Thus the extent of PS exposure depends on the activator, the most effective activator being the Ca^{2+} ionophore, A23187, followed by the complement membrane attack complex (C5b-9), collagen plus thrombin, collagen, and thrombin.[145,189,252]

The conclusion that externalization of aminophospholipids on activated platelets is physiologically important is supported by a correlation of the extent of PS exposure with the development of platelet procoagulant activity. The exposure of PS on the platelet surface provides binding sites for the components of the FX- and prothrombin-activating complexes.[70,149,253] The discovery that Scott syndrome,[254] characterized by an isolated defect in platelet coagulant activity, is associated with a decrease in PS exposure on the platelet surface,[62,85,161,245] provides further evidence of a role for PS in the development of platelet procoagulant activity, as does the observation that incubating platelets with phospholipase A2, which hydrolyzes PS, inhibits platelet coagulant activities, whereas phospholipases that spare PS do not.[189]

Platelet activation with strong agonists such as the calcium ionophore A23187, thrombin plus collagen, or complement proteins C5b-9 is accompanied by the shedding from the platelet surface of microparticles that contain plasma membrane GPs, P-selectin, and cytoskeletal proteins, as discussed in Chapter 36.[252,255–257] It has also been demonstrated that cytoskeletal preparations obtained from activated platelets contain all the components of the prothrombinase complex including procoagulant acidic phospholipids.[258,259] This suggests that the association of the platelet cytoskeleton with the plasma membrane prevents the shedding of procoagulant microvesicles from unstimulated platelets and that disruption of actin–membrane interactions by calpain may lead to the shedding of platelet microparticles.[260] On the other hand, calpain inhibition by MDL28170 fails to prevent the formation of microparticles that are enriched in acidic phospholipids.[202,244] Formation of platelet microparticles may be important for the assembly of the prothrombin-activating complex[245,252,255,256] since microparticle formation is defective in platelets from a patient with Scott syndrome.[252]

Regulation of Lipid Asymmetry

Three related enzyme-mediated mechanisms have been implicated in the regulation of the asymmetric distribution of phospholipids in platelet membranes, based on experiments utilizing fluorescent and spin-labeled lipid analogs, including nucleotide-binding domain-PS (NBD-PS) and NBD-PC.[246] The three enzymes consist of an inward-directed aminophospholipid translocase, an outward-directed flippase, and a bidirectional lipid scramblase.

An adenosine triphosphate (ATP)–dependent aminophospholipid translocase is postulated to rapidly transport PS and PE from the outer to the inner leaflet of the plasma membranes of platelets and other cells.[202,246,261–264] An ATP-dependent flippase, with properties similar to an ATP-dependent translocase in red blood cells,[265] is postulated to slowly transport all phospholipids from the inner to the outer surface membrane,[266] whereas the predominance of PC in the outer leaflet of plasma membranes is probably a consequence of passive translayer movement that compensates for the active transfer of aminophospholipids in the inner leaflet.[246] A lipid scramblase[267] is postulated to induce rapid, random, bidirectional movement of all phospholipids in surface membranes resulting in the exposure of aminophospholipids on the platelet surface membrane,[247] a rapid loss of phospholipid asymmetry, and the generation of platelet coagulant activity. Deficiencies of lipid scramblase[268,269] and flippase activities[266] have been reported in platelets from patients with Scott syndrome as the basis for their defects in platelet procoagulant activity. Recently a transmembrane protein (TMEM16F) has been identified and characterized as an essential component for Ca^{2+}-dependent exposure of PS on the cell surface, and the defect in PS exposure due to phospholipid scrambling activity in the platelets of a patient with Scott Syndrome has been demonstrated to arise from a mutation at a splice-acceptor site of the gene encoding TMEM16F, causing the premature termination of the protein.[270]

CONCLUSIONS

The schematic diagram presented in FIGURE 35.1 summarizes a current view of the postulated sequence of biochemical events

that comprise the normal hemostatic mechanism, emphasizing the intimate connections between the events of platelet activation, aggregation, and secretion on the one hand and blood coagulation on the other hand. Information summarized in this chapter indicates that the assembly of virtually all the coagulation enzyme–cofactor–substrate complexes that comprise the coagulation mechanism normally occurs on the activated platelet surface.[1] The platelet membrane comprises a surface for receptor-mediated catalytic complex assembly associated with major rate enhancements at each step in the reaction pathway.[1,9,10] The activated platelet surface also provides a protective nidus where binding of coagulation proteases protects them from inactivation by protease inhibitors.[1,271] Recent revisions in the theory of blood coagulation[11,12] replace the view that the intrinsic and extrinsic mechanisms, initiated by contact activation and TF exposure, respectively, are separate and alternative pathways converging on the common pathway for FX and prothrombin activation, with the revised hypothesis shown in FIGURE 35.1. Therefore, although blood coagulation can be triggered by exposure of the "contact factors" (FXII, HK, and PK) to negatively charged surfaces, the predominant mechanism for effecting normal hemostasis is initiated by TF exposure at sites of vascular injury resulting in the generation of minute quantities of thrombin, insufficient to convert fibrinogen to fibrin but sufficient to initiate the consolidation pathway by activating platelets, FXI, FVIII, and FV, leading to FIX activation, FX activation, and prothrombin activation, with enormous amplification at each reaction step, to produce sufficient thrombin to form a consolidated platelet–fibrin thrombus. Also emphasized in the figure are the contributions of the Kunitz-type inhibitors, PN-2 and TFPI, and the serpin, PN-1, all secreted from activated platelets that regulate the assembly of the FVIIa/FX/TF/PL complex (TFPI), whereas the consolidation phase of coagulation is regulated by PN-2 and the common pathway is regulated by PN-1.[271]

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CHAPTER

36 Blood-Borne Microparticles

JOSÉ A. LÓPEZ AND SWAMINATHAN MURUGAPPAN

This chapter concerns a blood element that has not been specifically addressed in previous editions of this textbook: blood-borne cell-derived microparticles. Although microparticles have been recognized for many years as important components of the procoagulant function of plasma, it is only in the past decade or so that they have emerged as important actors in many other biologic processes. Their roles are many and diverse. Besides procoagulant activity, they include cellular activation, signaling, endocrine functions, immune modulation, and transfer of biologically important molecules, including proteins, lipids, mRNAs, and microRNAs. The area of microparticle biology is fraught with controversy, owing in large part to the varied methods used to define microparticles, the difficulty in accurately measuring them, and the many artifacts associated with that measurement. Nevertheless, none of these factors justify ignoring them. In this chapter, we attempt to provide a useful way to think about this interesting biologic phenomenon.

DEFINITIONS

Several types of subcellular, membrane-bound particles are found in the blood. The most prevalent are exosomes, apoptotic bodies, and microparticles (FIGURE 36.1).

Exosomes

These particles range in diameter from 50 to 100 nM and are derived from the release of granules containing multivesicular bodies, each multivesicular body producing several exosomes.[1] Multivesicular bodies arise by invagination of granule membranes, producing vesicles whose outer membrane leaflets correspond to the inner membrane leaflet of the granule. Exosomes from immune cells have been the best studied and function in antigen presentation.[1–3] Conversely, exosomes derived from tumor cells can inhibit the immune response to the tumors by presenting tumor antigens to tolerogenic dendritic cells.[4,5] Exosomes can also activate the immune response against infectious agents by presenting pathogen-specific antigens.[1]

Exosomes can be detected by staining with antibodies against such antigens as CD63, CD81, CD9, and lysosomal-associated membrane protein-1 (LAMP-1), some of which are only found on internal membranes in intact cells.[6,7] Exosomes are distinguished from microparticles by both their smaller size and their failure to express phosphatidylserine (PS) and are best isolated by a combination of differential centrifugation and sucrose density ultracentrifugation.[8] They have been studied by a variety of techniques, including transmission electron microscopy (TEM), mass spectrometry, and immunoblotting after isolation by centrifugation.[8]

Apoptotic Bodies

These are membrane-bound cell fragments that arise as cellular blebs from cells undergoing apoptosis and range in size from 1 to 5 μm in diameter, thus overlapping platelets in size.[9] Like microparticles, they usually express PS on their external membrane leaflets, but unlike microparticles, they often carry DNA derived from the cell of origin.[10]

Apoptotic bodies are most commonly identified by flow cytometry, based on both surface antigen expression and annexin V staining (the latter being indicative of surface PS expression) and on their size characteristics. Protocols to isolate apoptotic bodies are lacking, in large part due to the overlap in size and antigen expression they share with other blood components such as microparticles and platelets.

Microparticles

Microparticles, also called microvesicles, are defined as membrane-bound cellular fragments that arise by blebbing from the plasma membranes of cells and range in size from 0.1 to 1.0 μm, with the majority being in the range of 0.2 μm.[11,12] Blood contains microparticles from a wide variety of cellular sources, including tissue cells and tumors, but the most abundant microparticles arise from blood cells or blood vessel cells in direct contact with the blood, endothelial cells.[6,13]

The most common method for measuring microparticles has been with flow cytometry.[14,15] With this method, it is possible to gauge the number and certain phenotypic characteristics of the microparticles, such as their cells of origin (Table 36.1). However, as will be discussed extensively later in this chapter, it is difficult by conventional flow cytometry to accurately count microparticles <500 nm in diameter,[16] unless those particles are stained for phenotype. Most microparticles also express PS on their external membrane leaflet, and this surface characteristic has been used in determining their number.

ORIGINS OF MICROPARTICLES

Blood-borne microparticles are highly heterogeneous with respect to their size and composition, in addition to their cellular sources.[13] By definition, their sizes span a range of diameters that varies by a factor of 10, from 0.1 to 1 μm. If the shape of a microparticle is assumed to be spherical, this size range translates to a 100-fold difference in surface area and a 1,000-fold difference in volume between the smallest and largest microparticles.

In addition to heterogeneity in size, their cellular sources are extremely varied. In the most common order of abundance

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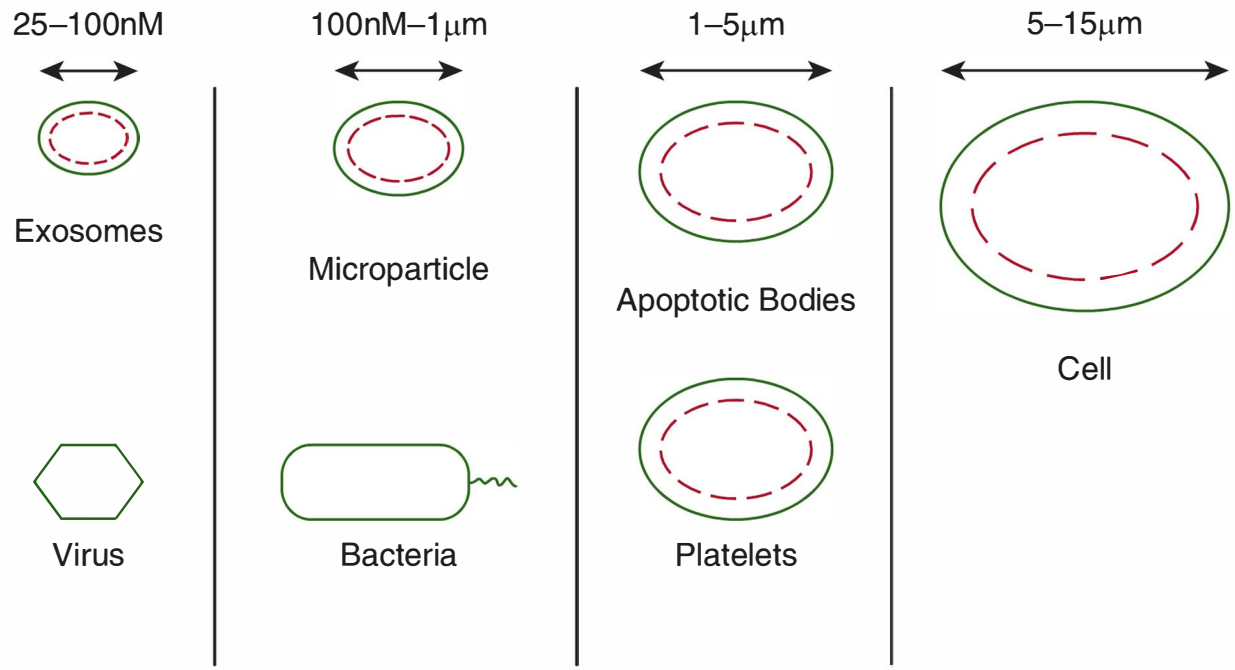

FIGURE 36.1 Depiction of the different membrane-bound vesicles based on size and comparison to other known biologic particles. As shown, apoptotic bodies, microparticles, and exosomes approximate the sizes of platelets, bacteria, and viruses, respectively.

in human blood: platelets, megakaryocytes, endothelial cells, erythrocytes, lymphocytes, monocytes, vascular smooth muscle cells, dendritic cells, and, often, tumor cells.

Platelet and Megakaryocyte Microparticles

The existence of platelet microparticles was first inferred by Chargaff and West[17] in 1946, when they noted that there was a precipitable factor in plasma capable of supporting thrombin generation. In 1967, Wolf[18] described the source of this procoagulant activity as being the platelet and also identified the microparticles morphologically. He termed them "platelet dust." Wolf also demonstrated that there was a linear relationship between the number of platelets and the quantity of microparticles in the blood, with thrombocytopenic patients having low numbers of microparticles compared to normals and those with polycythemia vera having elevated microparticle numbers.

Microparticles containing platelet markers account for 70% to 90% of circulating microparticles in healthy individuals.[19–21] Markers used to identify these microparticles by antibody staining and flow cytometry include CD41(the α subunit of the integrin $\alpha_{IIb}\beta_3$), CD42b (glycoprotein [GP] Ibα), CD42a (GPIX), CD61 (the β subunit of the integrin $\alpha_{IIb}\beta_3$), and CD62P (P-selectin).[13] Controversy exists over the extent to which platelet microparticles express PS, with some studies using the binding of annexin V as one of the defining characteristics of these microparticles[22] and one study finding that up to 80% of microparticles staining with platelet markers did not express PS.[23] This point is an important one, as some methods of microparticle quantification rely on annexin V capture of the microparticles[15] or base their definition of microparticles on the ability to bind annexin V. Despite these concerns, isolation by annexin V capture likely does give a reasonable estimate of the procoagulant capacity of the microparticles.[24]

Various stimuli are capable of eliciting microparticles from platelets, including thrombin, collagen, complement C5b-9,

Table 36.1
Cell-specific surface markers in microparticles

Cell of Origin	Surface Markers
Platelets	CD41 (αIIb) CD61 (beta3) Cd42b (GPIbα) CD62P (P-selectin)
Endothelium	CD31 (PECAM) CD54 (ICAM-1) CD62E (E-selectin) CD105 (endoglin) CD144 (VE-cadherin) CD146 (MUC18)
Red blood cell	CD235a (glycophorin A)
Monocytes	CD14
Lymphocytes	CD4 CD8 CD20 (B-lymphocyte antigen)
Neutrophils	CD66b (carcinoembryonic antigen-related cell-adhesion molecule 8)

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high shear stress, and calcium ionophore.[25–35] Microparticle generation is coupled to externalization of PS on the platelet membrane and is a key component of the ability of platelets to support the enzymatic reactions of the coagulation system that eventuate in thrombin production and formation of insoluble fibrin clots.

Platelets also generate microparticles as they undergo apoptosis, albeit of a different nature than the microparticles produced by agonist stimulation.[36,37–39]

Numerous studies have reported elevated platelet microparticle concentrations in association with a variety of pathologic conditions, including acute coronary syndromes, atherosclerosis, hypertension, diabetes, venous thromboembolism, heparin-induced thrombocytopenia, antiphospholipid syndrome, thrombotic thrombocytopenic purpura, sickle cell disease, human immunodeficiency virus (HIV) infection, sepsis, malignancies, and several other conditions (Table 36.2).

Although it was initially believed that microparticle staining with platelet markers arose entirely from platelets, recent evidence by Flaumenhaft et al.[40] call this interpretation into question. Using videomicroscopy, these investigators demonstrated the capacity of cultured mouse megakaryocytes to vesiculate, the vesicles arising along the lengths of micropodia. These microparticles expressed CD41, CD42b, and PS. However, they were distinct from platelet microparticles in that they failed to express CD62P and LAMP-1 and they contained uncleaved filamin A, whereas filamin A found in platelet microparticles was uniformly proteolyzed to smaller fragments. In the blood of mice and healthy human volunteers, most of the "platelet" microparticles detected by these investigators did not express CD62P and LAMP-1, leading them to conclude that the microparticles were derived from megakaryocytes. Because both CD62P and LAMP-1 are membrane proteins restricted to granule membranes in unactivated platelets, this finding could also indicate that, in addition to a megakaryocyte source for these particles, they might also arise normally *in vivo* from platelets that have not been stimulated to release granules.

Endothelium

A second major source of microparticles in the blood is the endothelium. These microparticles arise by blebbing of the endothelial plasma membrane,[41] and they carry a wide variety of endothelial membrane proteins, including CD54 (ICAM-1), CD62E (E-selectin), CD31 (PECAM), CD105 (endoglin), CD144 (VE-cadherin), and CD146 (MUC1).[6] Because platelet microparticles also express CD31, endothelial cell microparticles are distinguishable by the absence of CD41 staining. A wide variety of stimuli have been reported to induce microparticle production by endothelium, including tumor necrosis factor (TNF)-α, interleukin-1, uremic toxins, oxidized low-density lipoproteins, oxidants such as superoxide and hypochlorous acid, and agents that uncouple nitric oxide synthase.[42–46] Endothelial cells undergoing apoptosis also elaborate microparticles. Microparticles released by cells undergoing apoptosis express more CD31 and less CD62E than those elaborated by cells exposed to activation stimuli.[47]

Endothelial microparticles are elevated in a number of conditions, all of which involve endothelial pathology. These include acute coronary syndromes, hypertension, metabolic syndrome, diabetes, chronic renal failure, systemic lupus erythematosus, sickle cell disease, pulmonary artery hypertension, malaria, and others (Table 36.2).

Erythrocytes

Erythrocyte microparticles make up only a small fraction of microparticles in the blood of normal individuals,[19] but their quantity can be increased considerably in a number of disorders that primarily affect the erythrocyte and other systemic disorders. Hemolytic anemias, both hereditary and acquired, are associated with increased blood microparticle levels, including paroxysmal nocturnal hemoglobinuria,[48] sickle cell disease,[49–51] the thalassemias,[52–54] autoimmune hemolytic anemia, malaria,[55,56] and disorders of the erythrocyte membrane skeleton.[57,58] Erythrocyte microparticle levels are also often elevated in systemic disorders such as atherosclerosis and chronic renal failure.[59,60]

In addition to conditions that increase erythrocyte microparticles *in vivo*, microparticles are also produced during extended red cell storage and by conditions that can affect the stored red cells, such as ATP depletion or low pH.[58,61–64] Exposure to agents that elevate intracellular calcium concentrations, such as the calcium ionophore A23187, also induces erythrocytes to produce microparticles. Erythrocyte microparticles are usually identified by staining for glycophorin A (CD235a).[54,65]

Leukocyte Microparticles

Monocytes, neutrophils, and lymphocytes all produce microparticles.[65] Those derived from monocytes or tissue macrophages are identified by surface expression of CD14 and often are extremely procoagulant by virtue of carrying tissue factor (TF) in addition to expressing anionic phospholipids.[66] Neutrophils and lymphocytes also shed microparticles. Neutrophil microparticles are elevated in a number of inflammatory states known to be associated with neutrophil activation, including acute vasculitis, preeclampsia, rheumatoid arthritis, and meningitis.[67–72] Elevated neutrophil microparticle counts have also been reported in patients with extensive atherosclerosis and in those undergoing hemodialysis.[60,68]

Like monocytes, neutrophils have been reported to elaborate TF-bearing microparticles in response to activation with the anaphylatoxin C5a,[73–75] although this property might also be a consequence of fusion of TF-bearing microparticles with neutrophils, not from actual TF synthesis by the neutrophils themselves.[76]

MECHANISM OF MICROPARTICLE FORMATION

Several factors have led to platelets being the best studied cell for determining the mechanisms of microvesiculation. First, platelets were the first cell recognized to produce microparticles and for which microvesiculation was noted to be an important part of their physiology.[17,18,77] Second, microparticles containing platelet markers are the most prevalent type of microparticle in blood.[19–21,40] Finally, platelets are easy to isolate and produce microparticles rapidly when stimulated.[20,22]

Based on extensive research, it is clear that membrane remodeling and changes in cytoskeletal structure are both involved in the production of microparticles. A simplified illustration of the mechanism of microparticle formation resulting

(Text continued on page 480)

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Table 36.2

Circulating cell-derived microparticles in diseases

Clinical Condition	MP Origin	MP Level in Blood	References
Atherosclerosis	Platelet Endothelium Monocyte	Increased	van der Zee et al.[202] Bernal-Mizrachi et al.[203] Koga et al.[204] Bulut et al.[205] Werner et al.[206] Kockx[207] Leroyer et al.[60] Nomura et al.[208]
Hypertension	Platelet Endothelium	Increased	Preston et al.[209]
Hyperlipidemia	Endothelium Platelet	Increased	Nomura et al.[142] Boulanger et al.[210]
Acute coronary syndrome	Platelet Endothelium	Increased	Huisse et al.[211] Bernal-Mizrachi et al.[203] van der Zee et al.[202] Matsumoto et al.[212] Singh et al.[213] Mallat et al.[214] Werner et al.[206] Heloire et al.[215]
Diabetes	Platelet Endothelium Monocyte	Increased	Feng et al.[216] Bulut et al.[205] Nomura et al.[217] Nomura et al.[218] Tan et al.[219] Ogata et al.[220] Omoto et al.[221] Ogata et al.[222]
Deep venous thrombosis and pulmonary embolism	Platelet Endothelium	Increased	Ye and Wang. *Throm Res*. 2011.[286] Barnes et al.[223] Chirinos et al.[224] Wakefield et al.[225]
Heparin-induced thrombocytopenia	Platelet Monocyte	Increased	Kelton et al.[226] Warkentin et al.[227] Hughes et al.[148]
Antiphospholipid syndrome	Platelet Endothelium	Increased	Combes et al.[43] Dignat-George et al.[228] Morel et al.[89] Jy et al.[229] Ambrozic et al.[230]
Thrombotic thrombocytopenic purpura	Platelet Endothelium	Increased	Kelton et al.[231] Jimenez et al.[232] Jimenez et al.[233]
Paroxysmal nocturnal hemoglobinuria	Red blood cell Platelet	Increased	Hugel et al.[234] Kozuma et al.[48] Wiedmer et al.[235] Sims et al.[236]

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Table 36.2

Circulating cell-derived microparticles in diseases *(Continued)*

Clinical Condition	MP Origin	MP Level in Blood	References
Sickle cell disease	Platelet Endothelium Red blood cell Monocyte	Increased	Shet et al.[50] Wun et al.[237] Allan et al.[49] van Beers et al.[51]
Thalassemias	Platelet Red blood cell	Increased	Pattanapanyasat et al.[54] Pattanapanyasat et al.[238] Habib et al.[53] Freikman et al.[52]
Sepsis	Platelet Endothelium Neutrophil Monocyte Red blood cell	Increased	Meziani et al.[239] Nieuwland et al.[71] Itakura et al.[70] Soriano et al.[240] Fujimi et al.[69] Joop et al.[21] Ogura et al.[241] Walenta et al.[242]
HIV	Platelet Monocyte Lymphocyte	Increased	Mayne et al.[243] Corrales-Medina et al.[244] Gris et al.[245] Mack et al.[246] Aupeix et al.[129] Rozmyslowicz et al.[247]
Peripheral arterial disease	Platelet	Increased	Tan et al.[248] van der Zee et al.[202] Nomura et al.[208]
Strokes	Platelet Endothelium	Increased	Kuriyama et al.[249] Simak et al.[250] Jung et al.[251] Lee et al.[252]
Chronic renal failure	Platelet Endothelium Red blood cell	Increased	Faure et al.[253] Amabile et al.[59] Boulanger et al.[254] Ando et al.[255] Daniel et al.[256]
Immune thrombocytopenic purpura	Platelet	Increased	Nomura et al.[257] Jy et al.[258] Nomura et al.[259] Ahn and Horstman[260] Fontana et al.[261] Tantawy et al.[262]
Malaria	Endothelium Red blood cell Platelet	Increased	Combes et al.[263] Combes et al.[264] Coltel et al.[55] Faille et al.[265] Nantakomol et al.[56]

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Table 36.2

Circulating cell-derived microparticles in diseases *(Continued)*

Clinical Condition	MP Origin	MP Level in Blood	References
Systemic lupus erythematosus	Platelet Endothelium Leukocyte	Increased	Lazarus et al.[266] Dignat-George et al.[228] Nagahama et al.[267] Nagahama et al.[268] Pereira et al.[269] Combes et al.[43]
Rheumatoid arthritis	Platelet Leukocytes Lymphocyte	Increased	Knijff-Dutmer et al.[270] Berckmans et al.[67] Berckmans et al.[146] Umekita et al.[72]
Multiple sclerosis	Endothelium Oligodendrocyte (CSF)	Increased	Minagar et al.[271] Larkin[272] Jy et al.[273] Sheremata et al.[274] Scolding et al.[275]
Systemic sclerosis	Platelet Endothelium Leukocyte	Increased	Guiducci et al.[276] Nomura et al.[277]
Vasculitides	Platelet Endothelium Leukocyte	Increased	Brogan and Dillon[278] Daniel et al.[68] Erdbruegger et al.[279]
Pulmonary arterial hypertension	Endothelium Leukocyte	Increased	Amabile et al.[280] Bakouboula et al.[281]
Scott syndrome	Platelet	Decreased	Sims et al.[33] Zwaal et al.[77] Albrecht et al.[94]
Cancer	Platelet Monocytes Tumors Endothelium	Increased	Toth et al.[282] Tilley et al.[197] Campello et al.[198] Khorana et al.[200] Uno et al.[201] Amin et al.[283] Goon et al.[284] Kanazawa et al.[285] Owens and Mackman[66]

from cell activation or apoptosis is shown in FIGURES 36.2 and 36.3, respectively.

Membrane Remodeling and Loss of Phospholipid Asymmetry

The plasma membranes of unstimulated cells are asymmetric with respect to their phospholipid content,[78] the inner membrane leaflets being relatively enriched in PS and phosphatidylethanolamine (PE) and the external leaflets having a much higher content of phosphatidylcholine (PC) and sphingolipids.[79,80] Loss of membrane asymmetry has been assumed to be a *sine qua non* requirement for plasma membrane blebbing of microparticles. In favor of this notion is the observation that patients with Scott syndrome, an extremely rare bleeding disorder in which cells are unable to expose PS and PE on their external membrane leaflet, have very low levels of microparticles in the blood and their platelets display defective microvesiculation following agonist stimulation.[33,77] Recently, the requirement for initial PS exposure in microparticle formation has been called into question, based primarily on the fact that endothelial cells can release PS-negative microparticles when undergoing apoptosis[47] and that in the plasma of healthy individuals, 80% of microparticles carrying platelet markers did not bind annexin V.[23] These findings have several potential interpretations: first, they may truly indicate that PS exposure on the parent cell is

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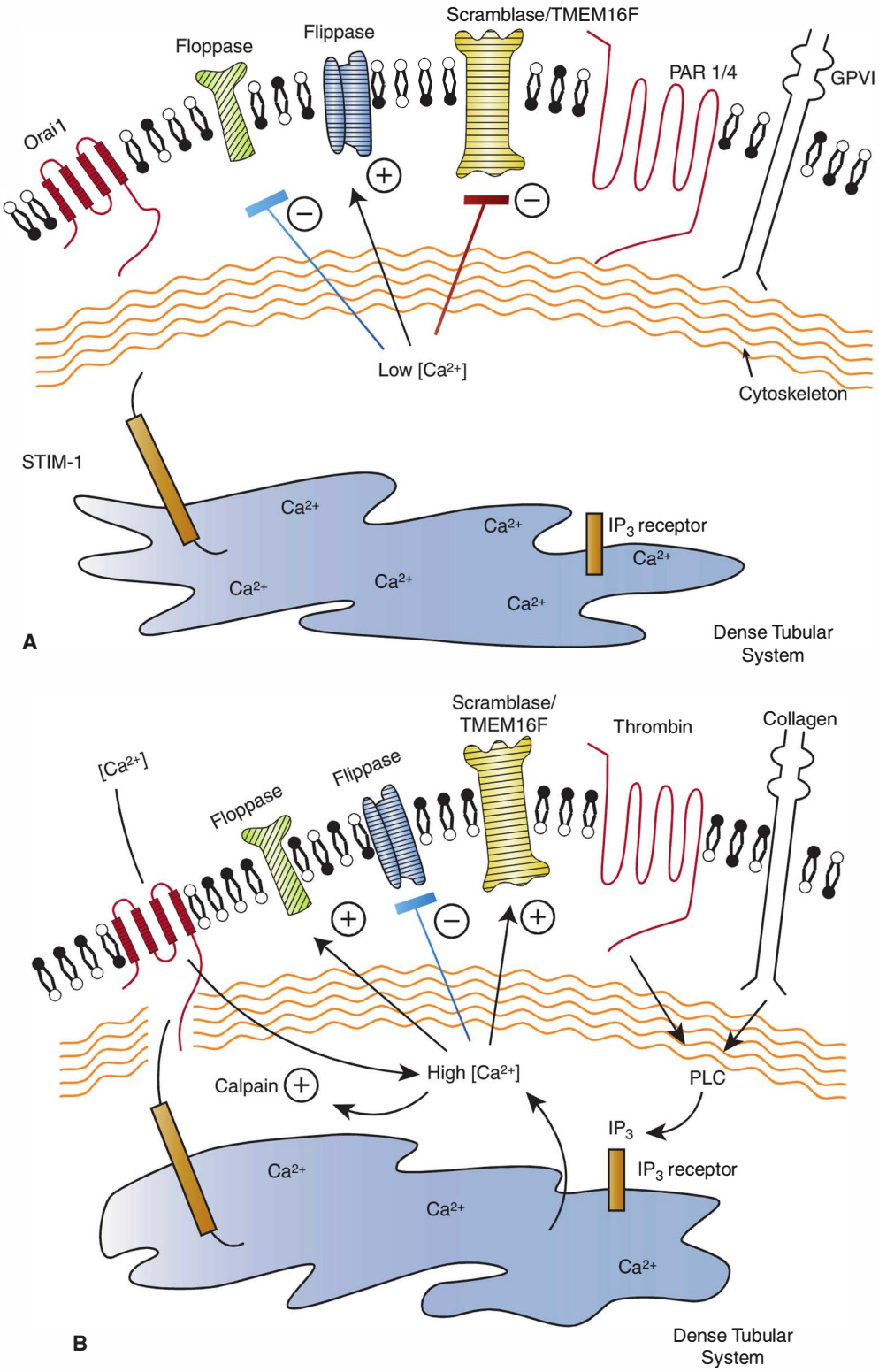

FIGURE 36.2 Proposed mechanism of cell activation–induced microparticle formation: **(A)** In the unactivated cell, the membrane asymmetry is maintained by the interplay between flippases and floppases, with flippases being more active in a low-calcium environment resulting in PS being sequestered in the inner leaflet of the plasma membrane. **B:** When the cell is activated, such as by thrombin or collagen in platelets, the intracellular calcium rises due to release of calcium from the intracellular stores. Increased calcium inhibits flippases and activates floppases and scramblases such as TMEM16F. These changes lead to externalization of PS. Emptying of the intracellular calcium stores also triggers Orai1/STIM1-mediated calcium influx.

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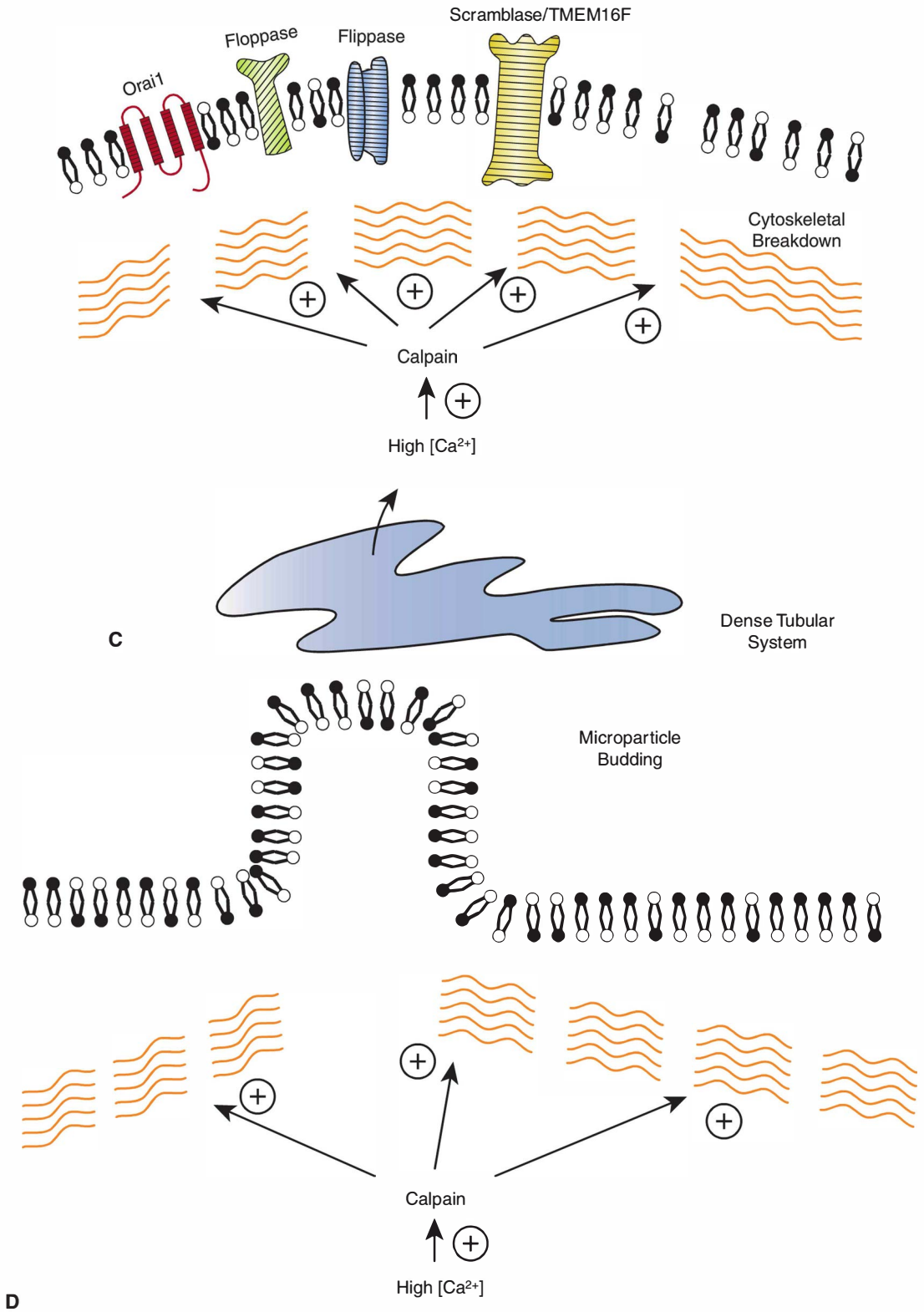

FIGURE 36.2 *(Continued)* **C:** Persistently high intracellular calcium levels also results in activation of calpain, which cleaves key cytoskeletal proteins. **D:** Calpain-mediated proteolysis of the cytoskeleton enables membrane budding and release of PS-exposing microparticles.

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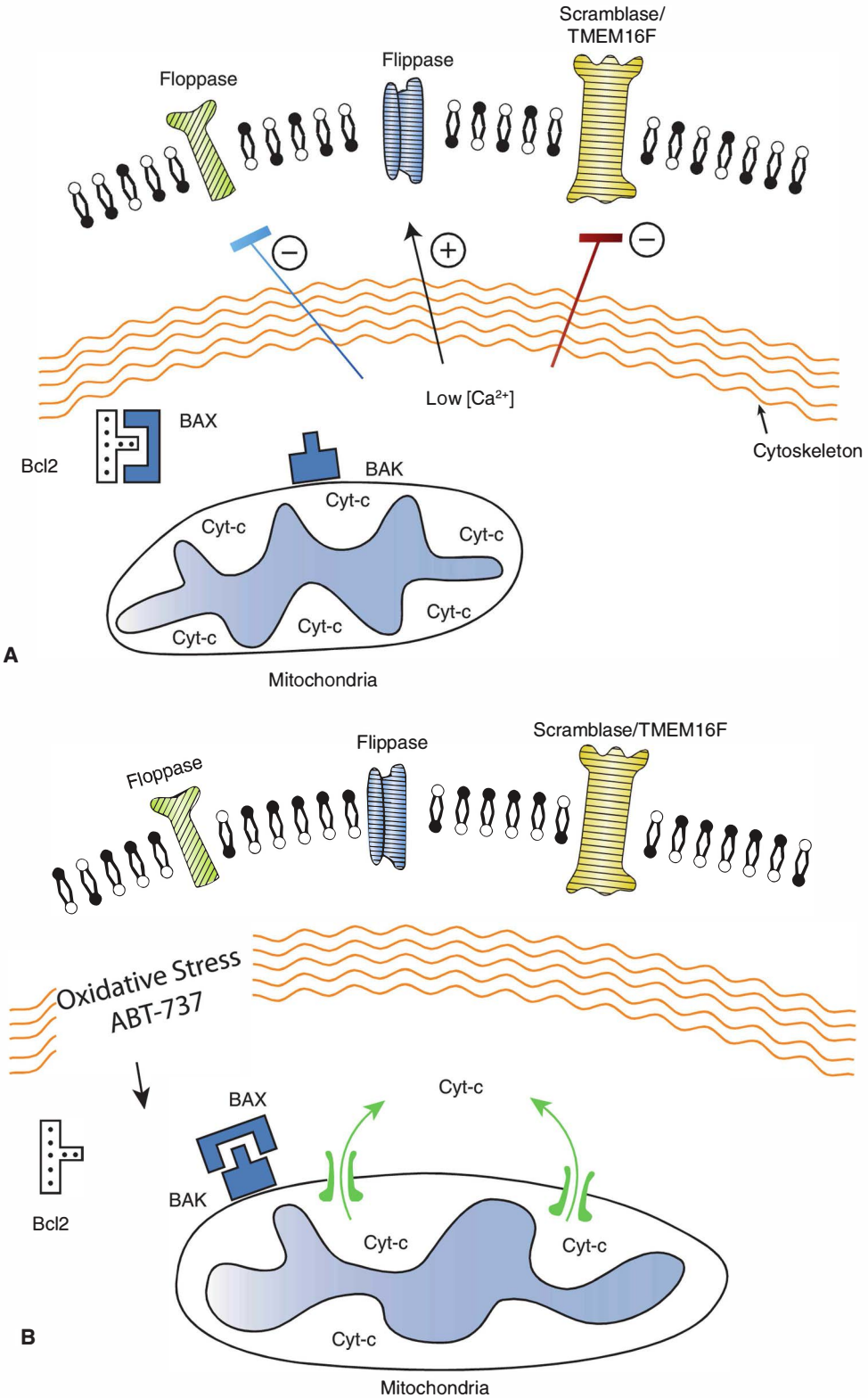

FIGURE 36.3 Proposed mechanism of apoptosis-induced microparticle formation: **(A)** In the unactivated cell, membrane asymmetry is maintained by the dominant action of flippases in a low-calcium state. Apoptosis is prevented by sequestration of Bax by Bcl-2 and Cyt c is sequestered in the intermembrane space of the mitochondria. **B:** When the cells experience an apoptotic trigger such as exposure to ABT737 or oxidative stress, Bax is released from Bcl-2 and is now free to bind to Bak present on the outer mitochondrial membrane. This binding leads to the formation of MOMP, which releases the Cyt c into the cytoplasm, where it contributes to the formation of the apoptosome.

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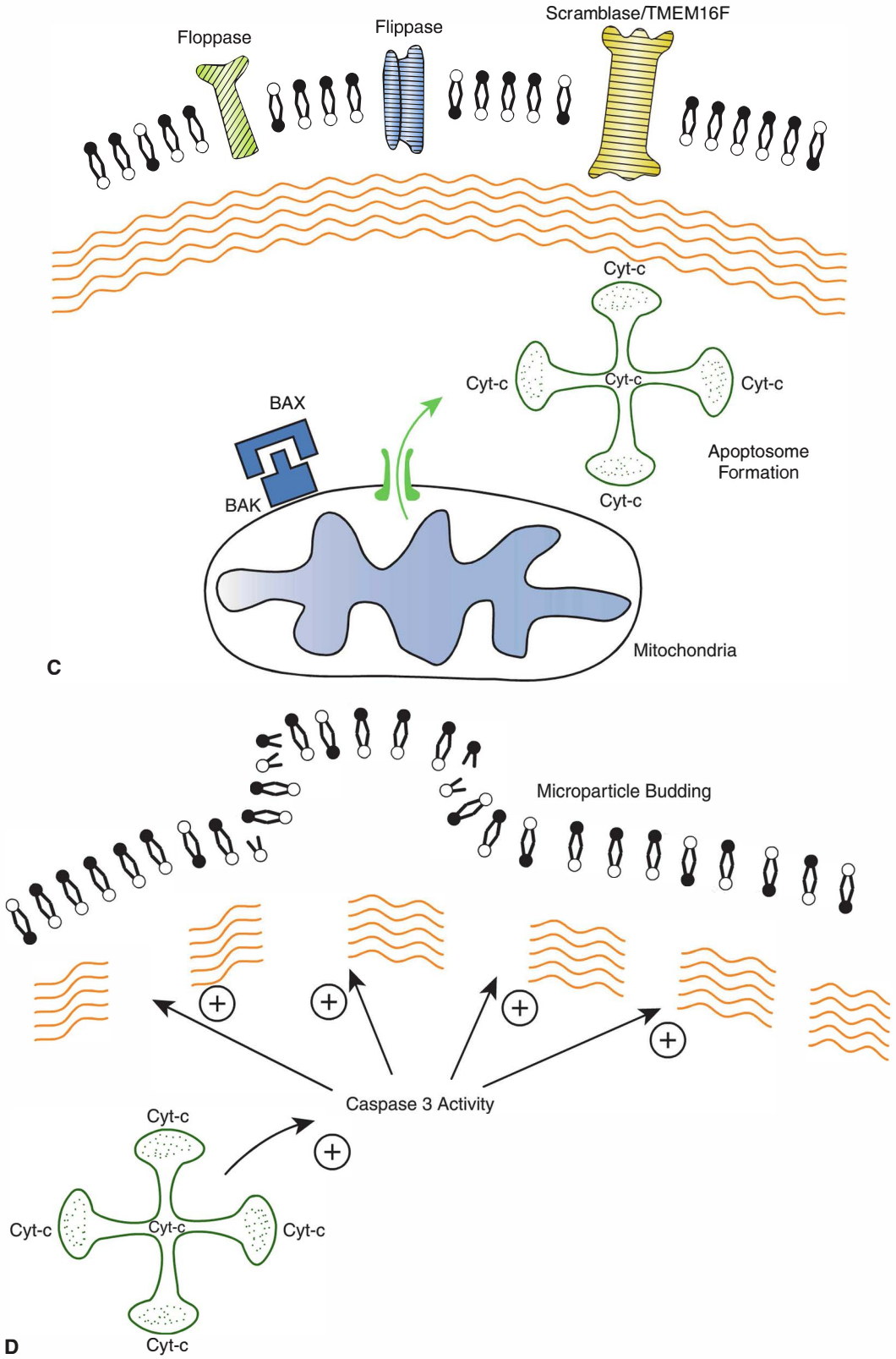

FIGURE 36.3 *(Continued)* **C:** Formation of the apoptosome results in the activation of executioner caspases such as caspase 3, which cleaves components of the cytoskeleton. **D:** Degradation of cytoskeletal elements aids in the budding of the membrane and subsequent release of microparticles. The exact mechanism by which PS is externalized in apoptotic cells is not known.

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not required for microparticle generation; second, the annexin V assay may be insufficiently sensitive to detect the quantity or arrangement of PS on the microparticle surface; or third, PS exposure on the parent cell is required for the formation of microparticles, but that this need not be reflected in enhanced PS on the microparticle.

Maintenance of plasma membrane phospholipid asymmetry requires energy expenditure, with ATP fueling the action of two phospholipid transporters, one that transports anionic phospholipids from the outer leaflet to the inner leaflet of the membrane, a so-called aminophospholipid translocase or flippase,[81–83] and one, a floppase, that transports PS to the outer leaflet from the inner leaflet, albeit much more slowly than the flippase.[83–86]

When cells are activated and the cytosolic Ca^{2+} concentration increases, the flippase is inhibited and another transporter is activated: a scramblase. Scramblase or scramblases transport aminophospholipids between the inner and outer membrane leaflets in both directions.[80,87] The unopposed activity of the floppase, coupled with the action of the scramblase, can rapidly increase the concentration of PS on the outer membrane leaflet.[80,83,87,88,89] At low intracellular Ca^{2+} concentrations, both the flippase and the floppase are active, with the scramblase being largely inactive. The threshold Ca^{2+} for activating the scramblase has not been determined.

Full phospholipid scrambling requires that the elevation in intracellular Ca^{2+} be sustained[90] as a rapid return of Ca^{2+} to baseline decreases scramblase activity, restoring phospholipid asymmetry.[91] In some situations, phospholipid asymmetry may be maintained even with a fairly rapid return of Ca^{2+} to baseline because the flippase may be irreversibly inactivated through proteolysis by calpains.[91]

The identity of the scramblase has been mysterious and highly sought after for many years and several candidates have been proposed. These include the ATP-binding cassette lipid transporter ABCA1[92] and phospholipid scramblase-1 PSCLR1, also called scramblase 1.[93] The case for ABCA1 seemed strong when a mutation in the transporter was discovered in a patient with Scott syndrome.[94] However, subsequent studies in deficient mice failed to demonstrate a defect in phospholipid scrambling.[87,95] PSCLR1 was also ruled out as the platelet scramblase when normal levels and functions of the transporter were detected in the lymphocytes of Scott syndrome patients.[96] In addition, PSCLR1-deficient mice do not manifest hemostatic defects.[97]

Another strong candidate was recently proposed. Suzuki et al.[98] identified a mutant mouse B-cell line that constitutively expressed PS on the surface, constructed a cDNA library from the cell line, and isolated a cDNA that could confer constitutive PS surface expression on other cell lines. The cDNA encoded a constitutively active mutant of a protein called TMEM16F, which has eight putative membrane-spanning helices. The case for its role as the scramblase defective in Scott syndrome was made when the investigators found a mutation in a splice-acceptor site of the gene encoding TMEM16F in a patient with Scott syndrome, leading to premature termination of translation of the encoded protein. Of interest, Scott syndrome lymphocytes undergoing apoptosis express PS normally, suggesting that the activation-depending increase in external leaflet PS occurs by a different mechanism than that occurring with apoptosis.

PS Exposure and Microparticle Release

The mechanism by which membrane phospholipid redistribution induces blebbing and microparticle release is unresolved. Changes in the shape of membranes (bending and puckering) can be induced by altering the composition or quantity of phospholipids; in isolated membranes, this is largely a consequence of energy minimization.[99] Unilamellar membranes with high local concentrations of PS form concave structures owing to the fact that PS assumes a cone-like shape by virtue of its small head group and relatively wider acyl chains. Such a configuration favors invagination rather than blebbing of membrane bilayers with increased external leaflet PS. However, it is also possible that the invagination can assume the form of a stalk at the base of the emerging microparticle.

Curvature of membranes can also be induced by an imbalance in the areas of the inner and outer membrane leaflets.[100] Thus, it is possible to envision that sudden local increases in the external membrane leaflet PS concentration could produce local areas of high convexity in the membrane, essentially producing a bleb. This mechanism may account for the observation that PS added to erythrocyte ghosts replete with ATP formed cytosolic inside-out vesicles resembling endocytic vesicles.[101] Presumably, the added PS was transported inward through the action of a flippase, producing a relative excess of PS in the internal leaflet. In intact cells, however, changes in membrane shape or composition can also trigger chemical changes in the cytoskeleton, for example, the polymerization of actin in platelets.[102]

Finally, proteins are likely to have large influences on the curvature of membranes, and on the formation of microvesicles. Negatively charged phospholipids, including PS and phosphatidylinositol 3,4 bisphosphate (PIP_2), interact with proteins containing clusters of basic residues (Lys and Arg), which in many cases facilitate membrane anchoring of the protein and also serve to fix the location of the acidic phospholipids on the inner leaflets. A subgroup of these proteins, known as BAR domain proteins (BAR for *B*in/*A*mphiphysin/*R*vs), are able to induce membrane curvature through their interactions with acidic phospholipids, in particular PS. Proteins with BAR domains are responsible for such membrane remodeling functions as formation of T-tubules in muscle and recycling of synaptic vesicles.[102,103] It remains possible that related proteins carry out similar functions in the generation of microparticles.

Role of the Cytoskeleton

The cytoskeleton has also been implicated in microparticle generation. Several reports indicate that cytoskeletal integrity is essential for maintaining the shape of cells and the asymmetry of their membranes.[78,104,105] This suggests that loss of the plasma membrane's cytoskeletal attachment could facilitate membrane blebbing.

Cytoskeletal disruption can be tied to increased Ca^{2+} through the activation of the calcium-dependent proteases known as calpains.[104,106–108] Activation of calpains requires high and sustained calcium levels, derived from influx and internal stores.[106] These thiol proteases are able to degrade several components of the cytoskeleton, including adducins, spectrin, actin, filamin A, talin, and α-actinin.[104,106–108] These changes are associated with increased microparticle release[109–111] but may not be a prerequisite for it, as calcium-driven PS exposure[112] and vesiculation[39] can occur even in the presence of calpain inhibitors, although

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one study did show a decrease in agonist-induced microparticle formation in platelets when calpain was inhibited.[113] Activation of calpain by itself is insufficient to induce microvesiculation because in Scott syndrome, calpain activation is normal even though PS externalization and microvesiculation are highly compromised.[114]

Proteolysis of cytoskeletal elements is also important for microparticle release associated with apoptosis. Effector caspases can cleave proteins such as filamin A, gelsolin, and talin in a manner similar to proteolysis by calpains.[105] This proteolysis precedes microparticle formation. However, the role of caspases in agonist-induced microparticle formation is controversial.[37,39,112,115]

Platelet apoptosis also yields microparticles and can be induced by the Bcl-2 inhibitor ABT737. Bcl-2 inhibition results in caspase-mediated proteolysis of filamin A, gelsolin, and talin and coincides with PS exposure and microparticle relsease.[39] Unlike the situations with calpains in agonist-induced microparticle formation, caspases are required for PS exposure and microparticle release in platelets treated with ABT737.

Caspases also play a role in microparticle release in T-lymphocyte cell lines undergoing apoptosis. In these cells, caspase-3 cleaves ROCK1, leading to phosphorylation of the myosin light chain, membrane contraction, and microparticle release.[116] A similar mechanism operates in microparticle release from endothelial cells during inflammation, which requires caspase-3 proteolysis of ROCKII.[44] Rho kinase has also been implicated in this pathway.[117]

Calcium Entry

Sustained elevations of intracellular Ca^{2+} are required for activation-mediated PS exposure and microparticle release.[110,111] Calcium activates both cytoskeleton-degrading enzymes such as calpains and scramblase activity.[90,106] Cytosolic calcium elevations originate from both intracellular and extracellular sources. The initial calcium wave comes from intracellular stores; when these are depleted, membrane channels are activated that allow calcium entry from external sources, a process called store-operated calcium entry (SOCE).[118,119] Activation of SOCE can induce PS externalization and microparticle release even in the absence of agonist stimulation, as evidenced by studies with the sarco/endoplasmic reticulum Ca^{2+}-ATPase ATPase inhibitor thapsigargin, which depletes intracellular calcium stores.[120]

SOCE is induced by the coordinated action of stromal-interacting molecule-1 (STIM1), which is present on membranes of the dense tubular system, and the Orai1 protein, a calcium channel present on the plasma membrane.[121] STIM1 senses low levels of dense tubular system calcium and induces the opening of Orai1, enabling the influx of calcium from outside the cell. The platelets of mice expressing a defective mutant Orai1 (Arg93Tyr) showed decreased SOCE upon agonist stimulation and decreased calcium transients.[122] However, their adhesion and aggregation on arterial collagen *in vivo* was normal, although the adherent platelets demonstrated decreased PS exposure.[122] In contrast, thrombin-mediated PS exposure was unaffected in the mutant platelets, suggesting that thrombin activates alternative calcium entry pathways.[123]

In addition to calcium, potassium and chloride have also been implicated in PS exposure and microparticle release. Elevated intracellular Ca^{2+} promotes the efflux of K^+ and Cl^-, leading to cell contraction, PS exposure, and membrane blebbing during apoptosis.[124] Blockade of the K^+ channel IKCa1 prevents these events. Ca^{2+} also activates Gardos potassium channels, another process implicated in PS exposure and microparticle release.[125] In addition, cellular sodium loading mediated through the Na/H exchanger has been proposed to be important for agonist-mediated PS exposure and microparticle generation.[126]

Role of Mitochondria in PS Exposure and Microparticle Release

Mitochondria are known to play a crucial role in apoptosis of nucleated cells and platelets.[38,127] Release of cytochrome c (Cyt c) from the mitochondria during apoptosis initiates the formation of an apoptosome and subsequent activation of executioner caspases.[128] The terminal phase of apoptosis is characterized by PS exposure, microparticle and apoptotic body formation, and cell shrinkage.[129,130]

Platelets contain much of the apoptotic machinery found in nucleated cells, including the Bcl-2 family of proteins and caspases.[131] Initiation of apoptosis-like changes by the Bcl-2 inhibitor ABT737 leads to Cyt c release from mitochondria and subsequent caspase-dependent microparticle formation and PS exposure.[132] It was initially assumed that the same pathway led to PS exposure and microparticle release in platelets undergoing apoptosis or activated by agonists because of the involvement of mitochondria in both processes.[36] This conjecture would indicate that agonist stimulation of platelets activated apoptotic pathways, but this has shown not to be the case in collagen/thrombin-stimulated platelets which externalize PS without undergoing apoptosis.[39,133]

Opening of the mitochondrial transition pore (MTP) induced by elevated Ca^{2+} is essential for collagen/thrombin-mediated PS exposure and microparticle formation.[115,134,135] However, inhibition of MTP opening does not completely block calcium ionophore–mediated PS exposure but does block microparticle shedding.[38] The MTP comprises many proteins including the adenine nucleotide transporter, cyclophilin D (CypD) and the voltage-dependent calcium channel (VDCC). The role of MTP was demonstrated both by pharmacologic blockade of the pore with cyclosporine, which inhibits the function of CypD,[136] and in mice deficient in CypD.[137]

The role of the apoptotic machinery in agonist-induced PS exposure and microparticle formation was also tested in mice that lack Bax/Bak, which is essential to form the mitochondrial outer membrane pore (MOMP).[39] These animals demonstrated normal PS exposure and microparticle formation in response to physiologic agonists.

Nevertheless, there is still debate regarding the exact contributions of MTP and MOMP in platelet PS exposure and microparticle formation mediated by activation or apoptosis. Some studies have shown that agonist activation of platelets can regulate Bcl-2 family proteins and activate caspases.[37,138] Yet other studies have shown that caspase inhibitors do not affect agonist-mediated procoagulant function.[37,138] The exact mechanism by which mitochondrial proteins participate in microparticle generation has not been fully delineated nor have the contributions of individual mitochondrial proteins and channels.

MICROPARTICLE COMPOSITION

Microparticles Originate from Rafts

Although they arise from cell plasma membranes, microparticles do not precisely reflect the composition of those membranes. This

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is because plasma membranes are not homogenous, but contain regions in which certain lipids or proteins are relatively enriched. Microparticles arise from areas of the membrane enriched in lipid termed rafts. Rafts represent regions of ordered lipid and protein floating within a sea of fluid, relatively disordered lipid.[139] Rafts comprise approximately 10% of the surface area of plasma membranes, the precise quantity depending on cell type, the state of cellular activation, and the cholesterol and sphingolipid content of the membrane.[140] Rafts are enriched in certain types of proteins: external membrane proteins attached to the membrane through glycosylphosphatidylinositol (GPI) anchors, cytosolic proteins posttranslationally modified through attachment of C16 (palmitate) and/or C14 (myristate) saturated fatty acids, and transmembrane proteins also largely modified by palmitate.[140] The precise relationship between microparticles and rafts is unknown, but it is clear that some proteins are overrepresented in microparticles (i.e., those that reside in detergent-resistant membranes in the parent cells) and some are underrepresented (i.e., those from the detergent-soluble portion of the plasma membrane).[141] Consistent with a raft origin of microparticles, treatment of parent cells with agents that deplete the membrane of cholesterol, a key raft-stabilizing lipid, decreases agonist-stimulated production of microparticles.[141] These findings have at least two important implications. First, they imply that microparticles are selectively enriched in signaling molecules, which they can transfer to other cells through membrane fusion. Second, the fact that microparticles arise from raft-rich regions implies that agents that lower membrane cholesterol, such as statins, may decrease production of microparticles under pathologic conditions. Consistent with this, statins have been shown to decrease the plasma concentration of platelet-derived microparticles in hyperlipidemic diabetic patients.[142]

In addition to differing considerably from the composition of the parent cells, the phospholipid and surface protein composition of microparticles differs between different parent cell types. Their composition also varies depending on the nature of the stimuli inducing them and on whether the cells produce them as a result of activation or apoptosis.

Membrane Lipids in Microparticles

Microparticle membranes isolated from healthy human subjects contain approximately 60% PC, 21% sphingomyelin, 9% PE, and 5% PS.[19,143] The lipid composition is reflective of the origins of microparticles from selected regions of the parent membrane. Platelet microparticles, for example, contain twice the PC and three- to sixfold more phosphatidylinositol than the platelet plasma membrane. In contrast, the contents of PS and PE were four- to fivefold and three- to fourfold less, respectively, in microparticles than in the platelet plasma membrane.[144]

Microparticles released from activated platelets are enriched in PS compared to those released from resting cells. The extent of microparticle PS exposure is determined by the nature of the agonist activating the platelets. For example, all of the microparticles released from calcium ionophore–activated platelets express PS on their surface. By contrast, only 50% of microparticles released from platelets activated with thrombin receptor activation peptide express PS.[145]

Similar differences in the phospholipid composition have been noted in microparticles released from endothelial cells. For example, IL-1-activated human umbilical vein endothelial cells (HUVECs) release microparticles enriched in PS and PE when compared to microparticles released from unactivated HUVECs. In addition, agonist-stimulated microvascular endothelial cells release more PS-containing microparticles than do those undergoing apoptosis.[47] Leukocyte-derived microparticles contain relatively more PE, sphingomyelin, and lysophospholipids when produced under inflammatory conditions.[27,146]

Microparticle Proteins

The protein profile of microparticles differs from that of the parent cell not only because they arise from selected regions of the plasma membrane but also because of the nature of the stimulus leading to their production. For example, platelets produce microparticles constitutively after stimulation or during apoptosis, but only microparticles produced as a consequence of activation express P-selectin (CD62P) and CD63.[32,33] These proteins are found in platelet α- and lysosomal granules, respectively, and only appear on the platelet surface when these granules fuse with the plasma membrane. Microparticles from activated platelets are also enriched in coagulation factor Va.[32] Similarly, microparticles from platelets activated with collagen express CD61, whereas those produced from complement-treated platelets do not.[33]

The situation is similar in other cells. For example, T-cell–derived microparticles express greater quantities of CD3ε and CD3ζ if produced in response to T-cell receptor activation rather than treatment with ionophore.[147] Likewise, microparticles from endothelial cells activated with TNF-α express more CD54 and CD62E and less PS than those released during apoptosis.[47]

Even a single stimulus can yield very heterogeneous populations of microparticles, a case in point being the vast heterogeneity in size and $\alpha_{IIb}\beta_3$ expression seen in microparticles from platelets exposed to sera of patients with heparin-induced thrombocytopenia.[148]

The protein composition of platelet microparticles has been examined by mass spectrometry–based proteomics. One study by Garcia et al.[149] identified 578 proteins in platelet microparticles. Many of these proteins were known to exist in platelets, but 380 had not been previously identified in platelets.

MICROPARTICLE QUANTIFICATION

To fully understand the role of microparticles in physiology and disease, it is imperative to be able to measure them accurately. Microparticle measurement is complicated by several factors, including the variety of cellular sources of microparticles found in blood, their heterogeneity in both size and composition even when derived from a single cell type, and the shortcomings inherent in virtually every technique used to measure them. These shortcomings, coupled with the inherent heterogeneity of microparticles from different cellular sources, render it almost impossible to compare different studies and call into question many of the conclusions derived from published studies.

Several methods are currently employed to measure microparticles and can be divided into optical and nonoptical methods. Optical methods include flow cytometry, nanoparticle tracking analysis, and fluorescence microscopy; nonoptical methods include scanning and TEM, atomic force microscopy (AFM), impedance-based flow cytometry, and functional and immunologic assays. None of these methods is considered standard for measuring and characterizing microparticles in

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biologic samples because each has unique advantages and liabilities. Flow cytometry has been the most common method used for detecting microparticles, but it has significant limitations. Perhaps the most prudent approach to microparticle measurement would be to use a combination of methods.

Light-Scattering Flow Cytometry

Flow cytometry is the most widely used method to detect and quantitate microparticles.[14] Most of the reported studies used this method to detect microparticles in biologic specimens because of the widespread availability of the equipment and several advantages inherent in its use. With this technique, microparticles can be analyzed singly and with high throughput, and a number of phenotypic parameters can be analyzed simultaneously.[24,65] The cytometer guides the microparticles in a focused fluid stream through a laser beam. The particle deflects light scattered forward and sideways, and the amount of light scattered in the two directions correlates with particle size and internal complexity, respectively. Conventional cytometers are unable to accurately resolve particles below a diameter of approximately 500 nm because this diameter is close to the wavelength of the light used to probe the sample (typically 488 nm).[12,145,150,151,152]

Efforts coordinated by the International Society on Thrombosis and Haemostasis (ISTH) have attempted to standardize microparticle analysis across laboratories by calibrating cytometers with polystyrene beads of uniform diameter (Megamix beads) across a range from 0.5 to 0.9 μm.[153,154] A bead of a particular diameter is meant to set the size of a microparticle with a similar forward scatter. However, because forward light scatter depends not only on particle diameter but also on its refractive index, a 0.5 μm bead displays the light-scatter characteristics of a microparticle of approximately 1 μm diameter.[16] This has led to significant underestimation of the number of microparticles in biologic samples. With this approach, conventional flow cytometry was able to detect close to 1,000 $CD41^+$ microparticles per 1 μL of plasma.[153,154] Other methods, such as AFM, detect close to 200,000 platelet microparticles per 1 μL.[12,155] Thus, enumeration of microparticles below 500 μm is currently not possible based on light-scattering characteristics alone. However, if microparticles can be labeled with a fluorescent marker of sufficient density, it is possible to identify those smaller than 500 μm. This advantage, however, is tempered by the fact that no universal marker of microparticle identity exists and because many phenotypic markers are present in insufficient quantity in smaller microparticles. Previously, microparticles were identified by their positivity for PS, but ample evidence now exists that not all microparticles express PS on their outer membranes and the percentage that do varies with the method used for PS detection.[23,47,145] Further, the extent of PS expression changes during sample preparation and storage.[11]

Perhaps, the biggest advantage of flow cytometry for microparticle measurement is the ability to identify the cellular source of the microparticles based on expression of unique protein markers detectable immunologically.[14] One caveat to this approach, however, is that a microparticle may express markers from more than one cell type because of fusion of microparticles from different sources, a possibility suggested by the fact that TF-bearing microparticles from monocytes are able to fuse with activated platelets in a process mediated by complementary counter-receptors on the two surfaces.[156]

Solid-Phase Capture Assays

Immunologic capture is not used as commonly in microparticle analysis as flow cytometry. The method is based on capture of microparticles with immobilized annexin V or cell-specific antibodies after appropriate centrifugation to remove cells.[24] Although this technique does not actually enumerate microparticles, it does give a very good indication of the number with certain characteristics. For example, although annexin V will only mark a subset of all microparticles, using it as the capture reagent could be a more accurate means of measuring the procoagulant potentials of plasma microparticles.[53,129]

This technique has several drawbacks. First, it depends on the ability of the immobilized reagent to recognize its target, the resistance of the interaction to washing, and the sensitivity of the detection.[15] Second, the technique is labor intensive and slow. Finally, antibody-based assays are subject to interference by soluble antigens.[157]

Functional Assays

These assays are primarily clot-based assays and may be the most clinically relevant if the intention is to assess the procoagulant activity of microparticles. Assays have been developed to detect both PS-expressing and TF-expressing microparticles.

PS-expressing microparticles can be evaluated with kits such as the Zymuphen MP activity assay.[129] This assay is similar to the solid-phase capture assay using immobilized annexin V except that coagulation factors V, X, and prothrombin are added to generate the prothrombinase complex and thrombin generated is detected using a chromogenic substrate. Another assay, called Proag PPL (Stago), assays the procoagulant activity of PS-expressing microparticles by the addition of phospholipid-free plasma to the test plasma and measurement of clotting time after the addition of factor Xa to the mixture.[158] A standard curve is used to extrapolate the quantity of phospholipid from the value obtained.

TF-bearing microparticles can be measured by capture using a TF antibody and then measuring the ability of the captured material to initiate coagulation by adding factors VIIa and X in the absence or presence of a TF antibody.[159] Another approach relies on isolation of the TF-bearing microparticles by ultracentrifugation and then testing their ability to initiate coagulation, as above.[160,161]

Atomic Force Microscopy

This infrequently used method was developed in 1986 by Binnig et al.[162] and is able to measure nanometer-sized particles, relying on the movement of a sharp-tipped cantilever over a biologic specimen affixed on a flat surface. Tip deflection and continuous scanning yield a three-dimensional representation of the surface, with a lateral resolution of 3 nm and a vertical resolution of 0.1 nm. Particles can be bound to the surface through antibodies, thus yielding information as to the quantity of particles of a certain phenotype.

Using this method, the quantity of platelet microparticles in normal plasma was estimated to be far greater than that estimated by conventional flow cytometry, approximately 200,000/μL.[12] The average diameter of the microparticles was 125 nm. However, AFM is unable to distinguish between microparticles and exosomes if

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the two share the membrane antigens used for capture, except by size since exosomes generally have diameters <100 nm.

AFM measurement of microparticles is limited by the complexity of the technique, the time required to run a single sample (i.e., hours), and the limited availability of the equipment.

Impedance-Based Flow Cytometry

This method relies on the Coulter principle that allows determination of the volume of a microparticle based on the change in impedance that occurs when a particle displaces electrolytes in a flow chamber. This technique is able to detect smaller particles than those detectable by conventional flow cytometry, with a lower limit of detection of 300 nm.[163] Given that AFM studies suggest that the majority of microparticles are below this size, impedance-based cytometry, while an improvement over conventional flow cytometry, may still be blind to the bulk of the microparticle population.

Most impedance-based flow cytometry only provides information about the size of particles. However, Zwicker et al.[164] were able to combine impedance with fluorescent labeling to quantify TF-bearing microparticles in a plasma sample.

Dynamic Light Scattering Spectroscopy

This method can detect particles ranging in size from 1 nm to 6 μm. Particle detection is based on the observation that their Brownian motion causes the intensity of scattered light to fluctuate.[165] The degree of fluctuation is inversely proportional to the size of the particle. This technique is useful for assessing particles of uniform size in a suspension but is not as useful when the particles vary substantially in size.[150]

Nanoparticle Tracking Analysis

This method also relies on the relationship between the size of a particle and its Brownian movement in a fluid medium. A laser beam is used to illuminate the particles and scattered light is detected with an optical microscope. Individual particles in the field of view are tracked by video to determine their velocity in the medium, and their size is calculated mathematically.[166] Recently, this technology was used to size and phenotypically characterize microparticles.[166,167]

Transmission Electron Microscopy

Because this technique uses electrons instead of photons to create an image, the shorter wavelength of electrons allows detection of smaller particles. The technique has a resolution of 1 nm, but is cumbersome because of the necessity to dehydrate and fix the samples. Further, these procedures can alter the size and morphology of the microparticles. TEM cannot be used to determine the microparticle concentration as it requires that the microparticles be concentrated by ultracentrifugation before analysis.

PREANALYTICAL CONSIDERATIONS IN THE ANALYSIS OF MICROPARTICLES IN BLOOD

Accurate quantification of microparticles is not only influenced by the technique used to measure them, but also by a number of important variables in how they are handled before that measurement. For the analysis of microparticles using flow cytometry, the ISTH workgroup has proposed a number of standardized methods for sample preparation, particle size calibration, and immunostaining, but did not specifically address the impact of preanalytical conditions on microparticle enumeration. Several of the conditions that can influence microparticle characterization are briefly discussed here.

Blood Collection

The use of smaller gauge needles increases the chance that blood cells will be damaged mechanically (or activated, in the case of platelets) due to the shear stress experienced in the needle lumen, thereby artifactually increasing the number of microparticles in the specimen to be analyzed.[51] This is particularly true when Vacutainer tubes are used to draw the blood because of the high velocity of the blood as it passes through the narrow tube. Ideally, needles of a larger gauge (19 gauge or larger) should be used, and the blood should be drawn without vacuum, preferably by gravity drainage. Artifactual elevations of microparticle counts due to endothelial damage can also be minimized by discarding the first 3 to 5 mL of blood drawn.[168,169]

Choice of Anticoagulant

It is important that the anticoagulant chosen not activate platelets. Commonly used anticoagulants include sodium citrate (3.2% or 3.8%), acid citrate dextrose (ACD), heparin, and ethylenediaminetetraacetic acid (EDTA). ACD significantly inhibits platelet function because of its low pH, minimizing platelet activation. In contrast, it was demonstrated that storage of platelet-rich plasma (PRP) obtained from sodium citrate–anticoagulated blood increased platelet activation and release of PS-expressing microparticles compared to PRP obtained from EDTA-anticoagulated blood.[170] It should be kept in mind that the binding of annexin V to PS requires a high concentration of calcium, and therefore blood collected into EDTA must be recalcified before evaluating the PS content of its microparticles.[23,171]

Another study demonstrated that the microparticle concentration of blood collected in heparin-containing tubes was much higher than that collected into sodium citrate anticoagulant.[172] Whether this represents an effect of the heparin on blood cells in the collection vessel or improved microparticle preservation in heparin is unknown.

Processing Time from Collection to Centrifugation

In general, as the interval between venipuncture and centrifugation increases, so does the concentration of PS-positive platelet microparticles in the blood due to activation of platelets in the tubes.[173] In one recent study, the concentration of microparticles almost doubled during the 2 hours between venipuncture and centrifugation.[174]

Centrifugation Speeds, Single versus Double Spins

As yet, a standardized protocol for blood centrifugation before microparticle analysis has not been established, with published studies using a wide variety of centrifugation speeds and times.[11]

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Protocols that include single centrifugation steps in general yield higher concentrations of PS-positive microparticles because they fail to remove all platelets from the suspension. The residual platelets continue to release microparticles when they are activated by agitation or centrifugation. A second centrifugation step was shown to decrease the quantity of residual platelets by approximately 65%.[24] High centrifugation speeds decreased the numbers of microparticles in plasma samples.[157,169]

Storage and Freezing of Samples

Platelet-poor plasma should ideally be prepared immediately following blood collection to avoid increasing the microparticle concentration in the sample.[173,174] If blood must be stored before centrifugation for logistical reasons, it should be kept at room temperature without agitation.[24] Storage of blood at cold temperatures can activate platelets and coagulation, increasing the production of microparticles.[11,175]

Although freezing and thawing of plasma samples is very common in medical investigations, standardized protocols have not been established for microparticle studies. One study on the effect of storage on microparticle number found that microparticle counts dropped dramatically in isolated microparticle samples even with only 4 hours of storage.[172] The decrease was not a result of protein proteolysis (marker removal). This finding contrasts to a study in which the number of platelet microparticles was found to be increased in frozen plasma samples prepared after a single centrifugation step.[176] In doubly centrifuged samples, the microparticle number was lower, probably reflective of more efficient platelet removal.[161]

Isolation of Microparticles

Although flow cytometry can be performed on plasma, methods such as TEM and AFM require concentrated microparticle preparations which are generally prepared by ultracentrifugation of plasma samples.[12] This step results in loss of microparticles and may prevent accurate enumeration of microparticles and comparison of their numbers between samples.[157,169]

MICROPARTICLES AND DISEASES

Microparticles have been implicated in the pathology of a number of pathologic states characterized by thrombosis, bleeding, inflammation, angiogenesis, or cancer. Because of the very large number of these studies, they are summarized in Table 36.2. Here, we focus on the procoagulant function of microparticles.

The procoagulant properties of microparticles relate primarily to the presence of PS and TF. Those containing both elements have higher procoagulant activity than those containing only one or the other.[66,177] PS on the external membrane leaflet of microparticles imparts on them the capacity to bind phospholipid-binding clotting factors, including those containing γ-carboxyglutamic acid (GLA) motifs (prothrombin and factors VII, IX, and X) and those with phospholipid-binding C domains (factors V and VIII).[29,32,178] In addition, PS-bearing microparticles are able to bind the GLA-containing anticoagulant proteins, protein C, and protein S.[21,179–181] This surface also allows formation of the enzyme complexes that catalyze activation of downstream serine protease zymogens in the amplification scheme of the coagulation cascade. One such complex is the tenase complex, composed of the activated forms of factors VIII and IX on a PS-rich surface in the presence of calcium. Tenase converts zymogen factor X to factor Xa, which itself participates in the prothrombinase complex that converts prothrombin to thrombin.

Before any of these reactions can take place in the blood, coagulation must be initiated by a complex of TF and factor VIIa, a small amount of which circulates in the blood as a result of autocatalytic activation of factor VII. The TF/VIIa complex activates the two zymogens, factors X and IX. The culmination of the coagulation cascade is the production of thrombin, which cleaves fibrinogen to fibrin, activates the transglutaminase factor XIII, and activates factors V, VIII, and XI to propagate its own generation. In addition, thrombin activates platelets, which can then aggregate, and endothelial cells, which can release large quantities of von Willebrand factor and display molecules on their surfaces that are adhesive for leukocytes.

Until relatively recently, TF was believed to be excluded from blood except under pathologic conditions.[182] In 1999, however, Giesen et al.[183] demonstrated the presence of low levels of TF in blood that were able to initiate coagulation. These investigators, and others,[184] found that most of the circulating TF was associated with microparticles,[185] although this group also described a circulating alternatively spliced isoform of TF that was incapable of activating coagulation pathways.[184]

During major blood vessel injury, coagulation is activated by TF in the vessel wall, of which the adventitia is a rich source.[186] In more circumscribed vessel wall lesions, the vessel wall TF may be quickly occluded by the platelet/fibrin thrombus, in which case the contribution of blood-borne TF may become more important. A similar situation exists in dealing with lesions that are more superficial and do not result in the exposure of TF. Such a mechanism of TF accumulation has been demonstrated in experimental models of arterial thrombosis.[156,187–189] Some investigators have argued that in healthy humans, the quantity of TF in the blood is insufficient to initiate coagulation[19,190,191]; the counter argument is that even if TF-bearing microparticles are present in low quantity, continual blood flow provides them in a virtually infinite and replenishing supply.

The association of TF-bearing microparticles with platelet-rich thrombi requires complementary membrane proteins on both the microparticles and platelet, which serve to dock the microparticles on the platelet surface. Activated platelets express P-selectin on their surfaces due to translocation of α-granule P-selectin to the plasma membrane. This protein serves to dock the microparticles through its counter-receptor P-selectin glycoprotein ligand-1.[192] Microparticle docking precedes fusion with the activated platelets, a process requiring expression of PS on both membranes and inhibited by annexin V.[193] Fusion of microparticles with activated platelets transfers to the platelets the contents of the microparticles, including TF, increasing the ability of the platelets to activate coagulation.

Elevated concentrations of microparticles, with potential procoagulant and proinflammatory roles, have been described in a large number of diseases associated with both arterial and venous thrombosis. Many of these studies are listed in Table 36.2. Among the diseases in which microparticles have been proposed as contributing to thrombosis, the most compelling evidence exists for cancer. Studies have demonstrated both increased microparticle-associated TF antigen[161,164,194] and TF activity[160,161,195–197] in cancers, even though the antigen and activity do not necessarily correlate. For example, increased

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concentrations of TF-bearing microparticles were found in patients with colorectal cancer, but there was no corresponding increase in the microparticle TF activity.[194] Elevated levels of TF-bearing microparticles in the blood of patients with cancers strongly correlate with occurrence of venous thromboembolism.[160,164,196,198,199] Some studies have also suggested a role for tumor-derived TF-bearing microparticles in venous thromboembolism in patients with ovarian and pancreatic cancers.[200,201] TF-bearing microparticles arising from the tumor have been demonstrated in a patient with florid Trousseau syndrome.[185]

CONCLUSIONS

There is substantial evidence that cell-derived microparticles circulating in blood play important roles in biologic phenomena and medical conditions, being both purveyors of information between cells and tissues and agents of disease. Much work has been done to unravel these roles, but much also remains to be done, particularly in the development of high-throughput, sensitive, and widely available means of measuring microparticles and in determining the appropriate parameters to measure.

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CHAPTER 37

Overview of Vascular Biology

WILLIAM C. AIRD

OVERVIEW OF VASCULAR BIOLOGY

The coagulation system has evolved to prevent excessive blood loss at sites of vascular injury. As much as the fluid phase of clotting is readily amenable to detailed molecular and biochemical dissection, thrombus formation is typically localized at the blood vessel wall. Indeed, the vascular wall plays a central role in orchestrating the hemostatic response. Thus, a full appreciation of the mechanisms of coagulation requires a sound understanding of vascular biology. In recognition of the central role of the blood vessel wall in hemostasis and owing to significant advances in this area over the past 6 years, we have expanded the section on Vascular Biology in this sixth edition of Hemostasis and Thrombosis. By way of an overview, we wish to emphasize several themes that are relevant to this section.

ANGIOGENESIS: MULTIPLE LINKS WITH HEMOSTASIS

The formation of new blood vessels is essential during embryonic development (ontogeny). Angiogenesis continues to play an important role in the early postnatal period in the lung, heart, brain, and retina[1] and resumes and/or continues into adulthood with the reproductive cycle, wound healing, and post-ischemic tissue repair. Qualitative and quantitative abnormalities in angiogenesis underlie many disease states. It has long been recognized that hemostasis must be highly coordinated with angiogenesis to prevent excessive blood loss or thrombosis prior to the stabilization of newly formed vessels. Fibrin provides a provisional matrix scaffold along which endothelial cells can migrate. Also, fibrin serves as a sustained release reservoir for endothelial growth factors and fibrinolytic enzymes. Platelets release vascular endothelial growth factor (VEGF), and VEGF signaling, in turn, alters the expression of procoagulant and anticoagulant proteins in endothelial cells.

Clotting factors not only cleave downstream substrates in the clotting cascade but they also cleave and activate receptors on the surfaces of cells, including endothelial cells (see Chapter 44). The importance of these cellular effects is evidenced by studies of knockout mice. For example, deletion of tissue factor, but not fibrinogen, is embryonic lethal and is associated with abnormal vascular development.[2,3] Mice that are null for the thrombin receptor, protease-activated receptor 1 (PAR-1) may survive, but they too develop abnormal vasculature.[4] The latter phenotype is rescued by expression of endothelial PAR-1.[5] These and other findings suggest that thrombin-PAR-1 signaling in the endothelium is important for vascular development, independent of its effect on clotting.

In addition to their role as signaling molecules, proteins of the coagulation system have been found to harbor cryptic antiangiogenic fragments. For example, Judah Folkman's group first reported that angiostatin corresponds to the first four kringle domains of plasminogen.[6]

Since the last edition of Hemostasis and Thrombosis, the field of angiogenesis has witnessed significant advances. For example, as outlined in Chapter 38, we now have novel insights into the mesodermal origin of endothelial cells from different sites in the vasculature, not just arteries and veins but also capillaries and the endocardium. There is new information about the interplay between tip and stalk cells and mechanisms of lumen formation. Finally, there is an evolving awareness of the importance of lymphatics and their endothelial lining as dynamic components of the cardiovascular system.

THE BLOOD VESSEL WALL: A STRUCTURAL AND FUNCTIONAL ENTITY

The anatomy and histology of the vascular wall have been accurately described for over two centuries (FIGURE 37.1). As originally portrayed, the vasculature comprises a system of blood vessels aligned in series, beginning with arteries, followed by arterioles, capillaries, venules, and veins. There are some interesting exceptions to this canonical arrangement. For example, the hepatic artery and portal vein both empty into the capillaries of the liver (hepatic sinusoids). The glomerular capillaries drain into arterioles, not venules. In general, arteries have thicker walls than veins and carry oxygenated blood. However, that is not always the case. For example, the oxygen content of the pulmonary artery is less than that of the pulmonary vein. The same is true for the umbilical artery compared with umbilical vein.

Light microscopy and special stains reveal site-specific differences not only in wall thickness between different segments of the vasculature but also in the size and composition of different layers in the wall, including the intima, media, and adventitia. Electron microscopic studies have demonstrated remarkable ultrastructural heterogeneity at the level of the endothelium. Histochemical analyses have provided important information about the molecular composition of the blood vessel wall. Endothelial cells and smooth muscle cells express distinct patterns of genes, the nature of which varies between different regions of the vasculature. The subendothelial surface is rich not only in collagen but also in heparan, von Willebrand factor, and tissue factor. Indeed, presence of tissue factor on the abluminal side of the endothelium (on smooth muscle cells and fibroblasts) has given rise to the concept of a "hemostatic envelope" surrounding the vasculature.

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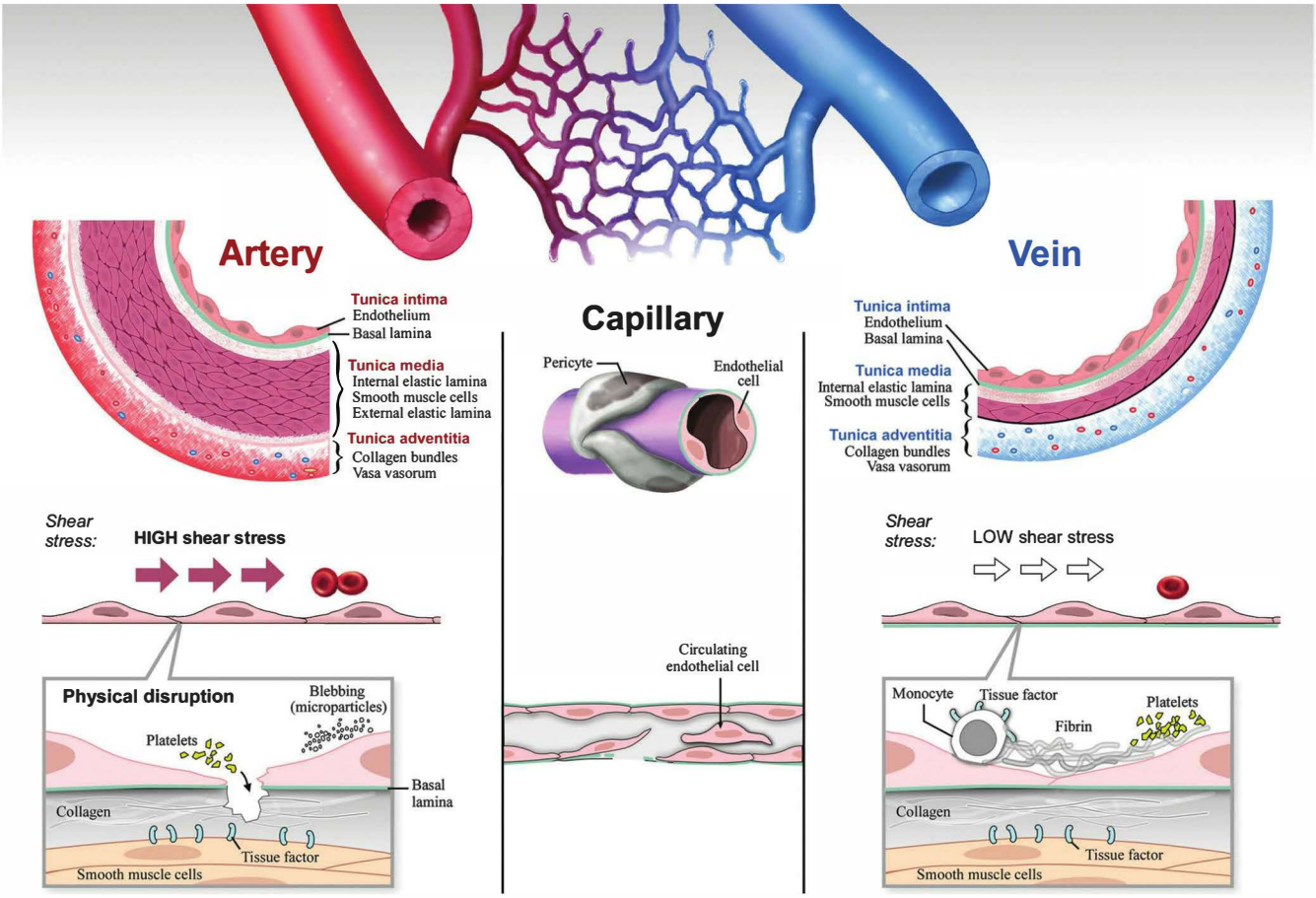

FIGURE 37.1 The blood vessel wall. Arteries and veins are surrounded by three distinct layers: the tunica intima, the tunica media, and the tunica adventitia. The tunica intima comprises endothelial cells and an underlying basement membrane or basal lamina, the media consists of an internal elastic lamina and one or more layers of smooth muscle cells, and the adventitia is composed of collagen bundles and vasa vasorum (with the interesting exception of intracranial arteries). Capillaries consist of a layer of endothelial cells (which may be continuous, fenestrated, or discontinuous), an underlying basal lamina (which may be incomplete, e.g., the case of liver sinusoids), and a variable number of pericytes (the degree of pericyte coverage varies between different vascular beds). All endothelial cells are derived from mesoderm, whereas other cellular components of the vascular wall may originate from mesoderm or neural crest. The vascular wall (especially the adventitia and the pericyte compartment) is a reservoir for different types of stem and progenitor cells. Flow patterns vary both between different blood vessel types (e.g., high flow in arteries and low flow in veins) and between different segments of the same blood vessel (e.g., at branch points in arteries and in valve pockets in veins). The pathophysiology of thrombosis differs between arteries and veins. In arteries, physical disruption of the endothelial lining leads to exposure of blood to underlying tissue factor (extravascular procoagulant activity). In veins, low shear stress and hypoxia lead to endothelial dysfunction and trapping of platelets and leukocytes, with secondary activation of the clotting cascade (intravascular procoagulant activity). Circulating endothelial cells and endothelial-derived microparticles are markers of endothelial cell dysfunction.

Like other components of the vessel wall, tissue factor levels vary between segments of the vasculature.[7] For example, expression is particularly high in the heart (tissue factor is expressed by cardiomyocytes), which may help explain why patients with hemophilia do not bleed into their hearts, despite the extreme forces associated with the cardiac cycle. Although tissue factor is readily expressed in cultured endothelial cells, there is little evidence that it is normally expressed in the intact endothelium.[7] Aberrant expression of tissue factor in the endothelium has been reported in certain diseases states, including atherosclerosis, tumors, and sickle cell disease, where it likely contributes to the underlying pathophysiology. Tissue factor is also expressed by circulating monoyctes. The relative role of vessel wall and hematopoietic tissue factor in mediating clot formation has been the subject of considerable debate and investigation.[8,9]

As discussed in Chapter 38, arteries and veins have different developmental origins. It is now widely accepted that all endothelial cells are derived from mesodermal progenitors. However, other components of the blood vessel wall may arise

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from other germ layers, namely, the neural crest. The importance of understanding the embryonic origins of the blood vessel wall lies in the fact that different origins may result in site-specific epigenetically programmed genes, including those that are involved in mediating hemostasis and thrombosis. The extent to which these differences are "locked in" will influence the modulability of the hemostatic balance from one location to the next. On a more general level, differences between arteries and veins have important implications for mechanisms of thrombosis. For example, it is widely believed that a breach in the vessel wall (and platelet activation) is a more common initiator of thrombosis on the arterial side of the circulation, whereas endothelial dysfunction and stasis of flow (and intravascular procoagulant activity) are more important in venous thrombosis.[10]

Whereas larger vessels (arteries, arterioles, veins, and venules) are invested with smooth muscle cells, capillaries are essentially three-dimensional tubes of endothelium surrounded to a variable extent by contractile cells termed pericytes. These cells are highly enigmatic. They express characteristic markers. However, none is specific to the pericyte lineage. Recent studies suggest that pericytes have properties of stem cells in both embryonic and adult tissues.[11] Stem and progenitor cells have also been identified in the adventitial layer of arteries. Interestingly, there is new evidence for the existence of hemogenic endothelium, which has the capacity to transdifferentiate into hematopoietic cells.[12]

Some but not all veins contain valves. These guide the direction of blood flow and prevent reflux. They are most numerous in veins of the lower extremity where the return of blood operates against gravity.[10] Venous thrombosis in the lower limbs typically occurs in the valve pockets, where endothelial cells are exposed to reduced flow velocities and shear stress during the valve cycle, where blood is relatively hypoxic, and where leukocytes and platelets tend to become trapped.

The walls of large arteries and veins contain a network of microvessels, termed vasa vasorum (see Chapter 41).[13] These tiny blood vessels provide oxygen and nutrients to the cells of the media and adventitia. There is increasing evidence for a role of the vasa vasorum in disease. For example, in atherosclerosis the vasa vasorum also extends into the intima and nourishes developing atherosclerotic plaques, and plaque growth is decreased through the inhibition of vasa vasorum angiogenesis and disruption of blood supply to the developing lesions.[13,14]

THE ENDOTHELIUM: ORCHESTRATOR OF LOCAL HEMOSTATIC BALANCE

The endothelium forms the inner cell lining of blood and lymphatic vessels. The endothelium was first recognized by Galen, who observed it grossly as a membrane on the inside surface of large arteries. In 1839, Theodor Schwann first noted the presence of cells lining capillaries using light microscopy of unstained samples.[15] In 1860, the use of silver nitrate staining provided definitive evidence for the existence of cells lining blood vessels. One year later, Wilhelm His named the endothelium, in order to distinguish it from epithelial surfaces that are directly or indirectly exposed to the outside world. The endothelium was once thought to serve as a physical barrier between flowing blood and underlying tissues. However, as discussed in Chapter 42, it is now appreciated that the endothelium participates in many physiologic functions. The endothelium plays several roles in hemostasis (see Chapter 43). First, it provides cell membrane surface and phospholipid for assembly of reactions. Second, it expresses and/or releases procoagulant and anticoagulant molecules. Third, it interacts with platelets and white blood cells, which in turn contribute to the hemostatic response. Fourth, it controls blood flow through the release of vasodilatory and vasoconstricting molecules. Endothelial cells display marked phenotypic heterogeneity. Thus, their contribution to hemostasis varies from one vascular bed to another. Site-specific impairment of endothelial function helps to explain the focal nature of many vasculopathic diseases, including thrombosis.

ENDOTHELIAL MICROPARTICLES: BLURRING THE LINE BETWEEN VESSEL WALL AND FLUID PHASES OF COAGULATION

There is now evidence that endothelial progenitor cells (EPCs), endothelial cells, and endothelial-derived microparticles circulate in the blood. There has been significant debate about how to define, enumerate, and phenotype these various elements (see Chapter 48). For example, in most studies, EPCs actually represent hematopoietic and not endothelial progenitors. True EPCs (also termed blood outgrowth endothelial cells or late outgrowth endothelial cells) are indeed present in blood but at extremely low numbers. Endothelial cells may detach from the vessel wall and circulate. These are termed circulating endothelial cells (CECs). They normally circulate in very low numbers (<10/mL). However, in certain disease states, such as sickle cell disease, their numbers may be increased. Endothelial plasma membrane blebbing leads to the formation of small (100 nm to 1 μm in diameter) anucleate microparticles. Endothelial microparticles (EMPs) can carry endothelial proteins such as vascular adhesion molecule 1, intercellular adhesion molecule 1, E-selectin, VEGF receptor 2, endoglin, and tissue factor. Thus, EMPs may contribute to the circulating pool of tissue factor. The protein composition of the EMP depends on the stimulus triggering their release. EMPs externalize phosphatidylserine and thus provide a surface for the assembly of activated coagulation factors. However, their procoagulant activity may be counter balanced to some extent by the expression of natural anticoagulants, such as thrombomodulin and endothelial protein C receptor. Interestingly, EMPs can result in the horizontal transfer of material from EMPs to other cells, including monocytes, platelets, and other endothelial cells. In this way, they are considered to be bioactive vectors that contribute in an endocrine manner both to physiology and pathophysiology. Their potential role as a diagnostic marker for vascular disease is discussed in Chapter 48. Importantly, the discovery of EMPs blurs the distinction between vessel wall and the fluid phase of coagulation.

VIRCHOW TRIAD: A USEFUL EXPLANATORY FRAMEWORK 150 YEARS LATER

Virchow triad represents a time-honored explanatory framework for understanding the mechanisms of thrombosis. A recent assessment of his work shows that Virchow proposed the triad (phenomena due to "irritation of the vessel," "blood coagulation," and "interruption of the bloodstream") in 1856 to describe conditions required for clot *propagation*.[16] At the

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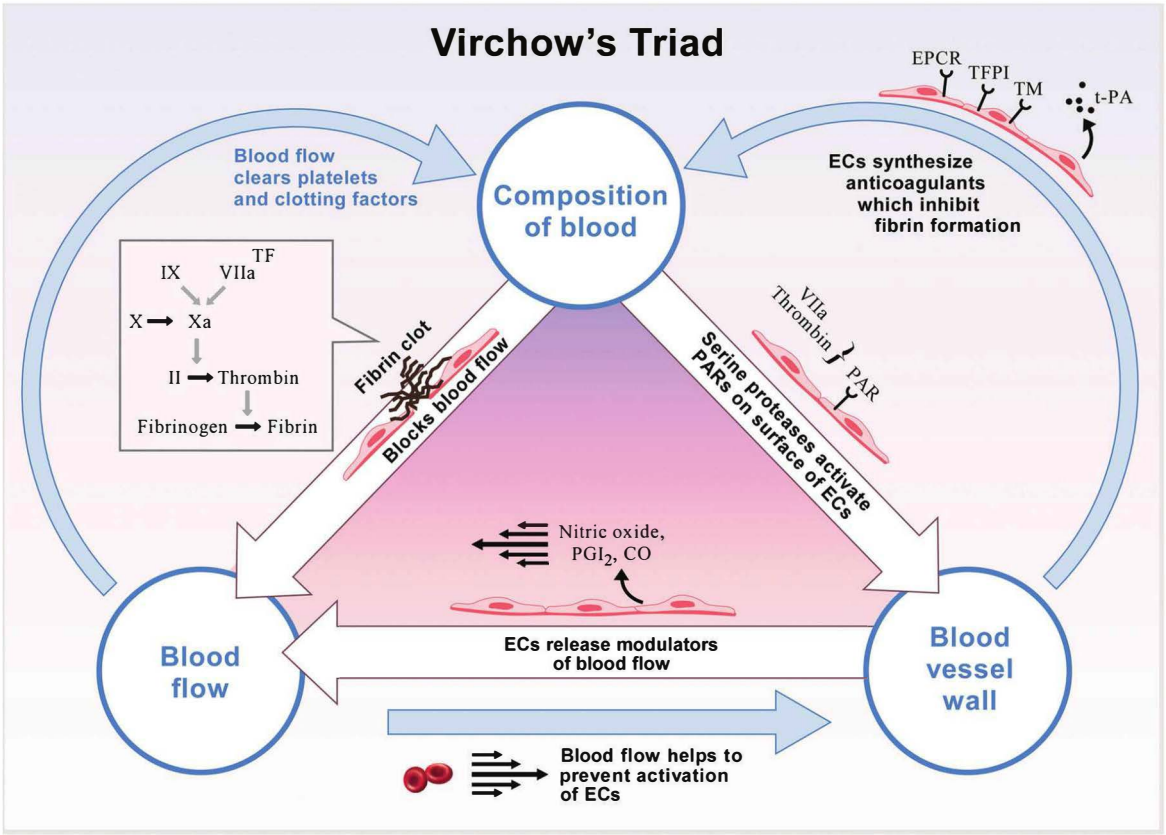

FIGURE 37.2 Virchow triad. Shown are the interconnections between the three components of the triad. ECs, endothelial cells; PGI2, prostacyclin; NO, nitric oxide; PAR, protease-activated receptor; TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; EPCR, endothelial protein C receptor.

time, there was no consensus as to the whether blood vessels had walls, let alone a cellular lining. Indeed, Virchow was unlikely to have been aware of the existence of endothelial cells when referring to the "vessel." Regardless of Virchow's original intentions, the triad has been adopted and refined over the past 150 years. Today, the triad may be classified as (a) the composition of blood, including circulating cells and plasma proteins; (b) the structural and functional integrity of the vessel wall; and (c) blood flow. The triad may be applied not only to the veins but also to the rest of the vasculature, including arteries and microvessels. It is now recognized that more than one abnormality in the triad is often required to provoke thromboembolism. Indeed, the three components of the triad are integrally linked (FIGURE 37.2). From the standpoint of the vessel wall, the following connections are worthy of consideration:

Vessel Wall–Flow Interactions

The endothelium contributes to and is in turn affected by blood flow. Endothelial cells are exposed to shear stress (a tangential force) and strain (a perpendicular force). These biomechanical forces alter signaling in endothelial cells and thus are important determinants of endothelial phenotypes (see Chapter 39). The architecture of the vasculature plays an important role in determining local flow regimes. For example, flow patterns differ between different regions of arteries (e.g., straight segments, branch points, curvatures, stenotic lesions) and veins (e.g., valve pockets). These different flow patterns translate into different susceptibilities to thrombosis. There are many reasons for this. For one, site-specific difference in shear stress can differentially influence the supply and clearance of clotting factors and cells at the blood vessel surface. Shear stress itself can activate platelets. Moreover, because shear stress is sensed and transduced by the endothelium, spatial differences in flow regimes contribute to phenotypic heterogeneity of the endothelium.

In addition to being affected by blood flow, endothelial cells contribute to vasomotor tone through the release of vasodilatory and vasoconstricting molecules, including nitric oxide (NO), carbon monoxide, endothelin-1, and epoxyeicosatrienoic acids. The discovery in the 1980s that NO is not simply a free radical but rather is a biologic signaling molecule in the cardiovascular system was recognized with the Nobel Prize in Physiology and Medicine in 1998. Since that time, there have been remarkable advances in the field (see Chapter 40). For example, there is new information on formation of NO adducts, the relative role of isoforms on homeostasis, and novel posttranslational modifications. There is compelling evidence that red blood cells carry an NO derivative. The precise nature of the interactions between NO and hemoglobin and their biologic significance have been hotly debated.[17,18] However, it is tempting to speculate that blood-transported NO functions in an endocrine manner to mediate distal vasodilation.

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Vessel Wall–Blood Components

As producers of procoagulant and anticoagulant molecules, endothelial cells contribute to the local balance of hemostatic factors. When activated, they provide a cell surface for the assembly of clotting factor complexes. Endothelial cells also release microparticles, which can promote clotting at distal sites. Several serine proteases, in turn, are capable of binding and activating PARs on the surface of endothelial cells, resulting in a proinflammatory and procoagulant state. In addition to the bidirectional communication between the endothelium and the various clotting factors, endothelial cells also engage in cross talk with circulating cells. The interaction between endothelial cells and platelets is an important theme throughout this volume. Chapters 46 and 47 detail the interactions between endothelium and circulating leukocytes, which also have important roles in prothrombotic states.

EVOLUTION

If we take a step back and ask why coagulation evolved in the first place, our answer focuses squarely on the vasculature. Clotting evolved in an ancestral vertebrate to prevent excessive blood loss and to maintain blood in its fluid state within blood vessels. Indeed, it is no coincidence that the clotting cascade arose during the same narrow window of time that saw the emergence of a highly pressurized closed circulation and that the clotting cascade acquired increasing complexity as blood pressures increased from fish to tetrapods. Thus, from an evolutionary perspective, it is the vasculature that drove the selection of coagulation.

In summary, it is not practical to approach blood coagulation without considering the role of the blood vessel wall. The field of vascular biology continues to make significant strides, and many recent advances have provided novel insights into mechanisms of hemostasis and thrombosis. This new and revised sixth edition section on Vascular Biology endeavors to capture many of the established and more recently recognized connections between the vascular and coagulation systems.

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Full Reference Lists can be found online

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CHAPTER

38

Development of the Vasculature, Angiogenesis, and Lymphangiogenesis

AERNOUT LUTTUN AND PETER CARMELIET

As species have become larger and more complex during evolution, nature has provided them with two tubular transport and communication systems, the blood vascular and the lymphatic system. While the former is responsible for oxygen and nutrient delivery to nearly all tissues, the latter mostly runs alongside the blood vascular system and serves to recirculate excess interstitial fluid (ISF) to take up lipids from the gastrointestinal tract and to regulate immune responses. The blood vascular system forms a closed loop with three main segments: the arteries carrying oxygen-rich blood from the heart, the capillaries that exchange oxygen with the tissues, and the veins that bring the deoxygenated blood back to the heart. In contrast, the lymphatic system is a blind-ended circuit beginning with the initial lymphatics (capillaries; specialized in fluid uptake) that connect to collector vessels (which pump the lymph fluid forward) that end up in larger ducts. These ducts (the thoracic duct and the right lymphatic duct) connect the lymphatic system with the venous arm of the blood vascular system. Given their ubiquitous presence in nearly all organs of the body, both vascular systems are frequent targets of disease. For instance, excessive blood vessel growth promotes cancer growth, while a shortage of blood vessels leads to ischemia and ultimately tissue death. Impaired lymph clearance results in lymphedema, characterized by disfiguring tissue swelling, while increased lymphatic growth can result in metastatic spreading of tumors. Hence, in order to remedy these diseases, a thorough understanding of the development of both vascular systems is paramount.

Both systems are lined on the inside ("endo" in Greek) by endothelial cells (ECs). While this monolayer of cells was long thought to be merely a passive barrier between tissues and blood or lymph, now we have come to understand that ECs are dynamic sensors that continuously adapt their behavior to their environment. This versatile behavior results in remarkable morphologic, functional, and molecular differences, a phenomenon known as "endothelial heterogeneity."[1,2,3] We distinguish different levels of heterogeneity. During vascular growth, the EC can adopt at least three distinct functional behaviors, that is, the tip, stalk, or phalanx cell. In addition, "macrovascular heterogeneity" refers to the differences between ECs from arteries, veins, and lymphatic vessels, while "microvascular heterogeneity" relates to the differences at capillary level in distinct organs. The existence of EC heterogeneity has vast consequences for disease development and hence should be taken into account when designing new therapies based on stimulating or inhibiting vessel growth. While proper blood/lymphatic vessel formation also involves non-EC types (e.g., smooth muscle cells or SMCs and hematopoietic cells), in this overview we describe the development of both vascular systems mainly from an endothelial perspective.

THE BLOOD VASCULAR SYSTEM

On the Origin of Blood Vascular Endothelium

Endothelia in the developing vascular system have a common origin, the mesoderm. The mesoderm is one of the three embryonic germ layers that segregate during gastrulation, starting around embryonic day (E) 6.25 in the mouse embryo (reviewed in Refs.[4,5]). At this time, cells from the epiblast converge toward the posterior proximal pole of the embryo to form the primitive streak (PS), which subsequently extends in the anterior direction to the distal tip of the embryo (reviewed in Ref.[6]). Mesoderm is formed when epiblast cells entering the streak undergo epithelial-to-mesenchymal transition (EMT). Distinct mesodermal zones become patterned according to the time and site of passage through the PS (FIGURE 38.1; reviewed in Refs.[4,7]): the earliest, most posterior cells become extraembryonic mesoderm (the progenitor of yolk sac blood islands), the intermediate somewhat later invading cells become lateral plate (giving rise to the intraembryonic axial vessels), paraxial (giving rise to the somites), and cardiac mesoderm and the epiblast cells that enter through the anterior tip of the streak become axial mesoderm (comprising the notochord, the prechordal plate, and the node). Interestingly, each of these mesoderm layers has the intrinsic capacity to form ECs, with the exception of the axial mesoderm.[8]

Mesoderm induction, EMT, and mesoderm patterning involve a complex interplay and gradient of signals (including bone morphogenetic proteins [BMPs], Wnt, fibroblast growth factors [FGFs], nodal, and Hox proteins).[4–6,9,10] As epiblast cells have to move extensively during PS formation and mesoderm patterning, they do so by following certain physical (e.g., a basal lamina underneath the moving cells) and chemical guidance cues. In the avian embryo, this involves attractive and repulsive signals (e.g., SDF-1/CXCR4, cAMP, FGFs/FGFR; Refs.[4,6]). While moving, epiblast cells extend lamellipodia/filopodia in the direction of migration.[6] The idea that this process may resemble the movement of angiogenic sprouts or neural growth cones, which involves certain classes of guidance molecules (see below), is intriguing. For instance, the EphB4 receptor (expressed together with its ligand ephrinB2 in embryoid bodies) was recently shown to be involved in modulation of mesoderm induction, which may imply a role in migration/patterning as well.[11] Regarding the location of the emergence of ECs, two main regions can be distinguished, corresponding to two temporal waves of vascular development, extraembryonic

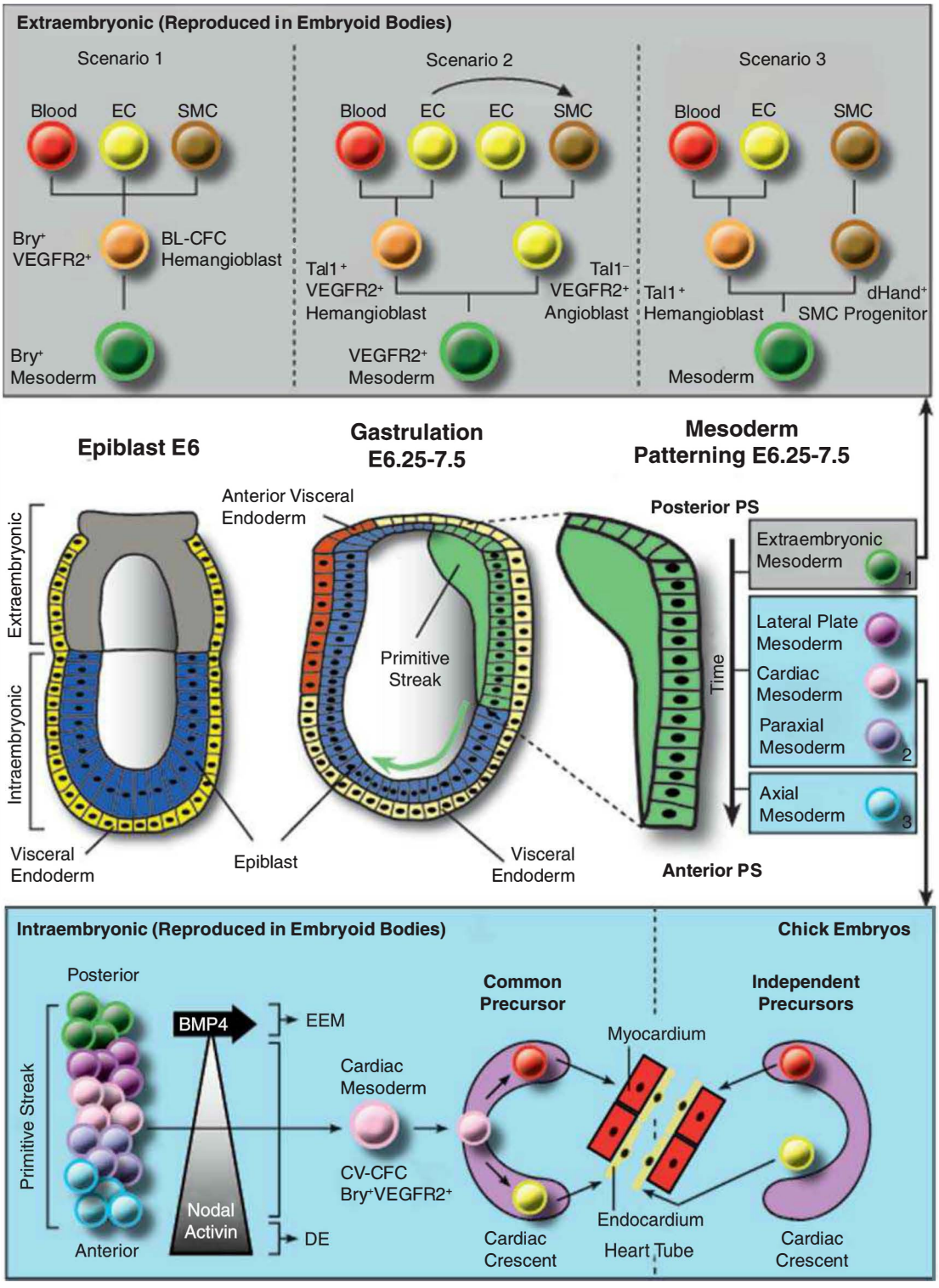

FIGURE 38.1 On the origin of blood vascular ECs. All embryonic tissues derive from the epiblast (*blue* in **left middle panel**). During gastrulation (**central middle panel**), a subset of epiblast cells converges toward the posterior proximal pole to generate the primitive streak (PS; *green structure* in **right** and **central middle panel**) that expands toward the anterior pole *(green arrow)*. Depending on timing (indicated by a time axis and numbering in boxes on the right) and location (across the anterior–posterior PS axis) of passage through the PS, epiblast cells give rise to distinct mesodermal derivatives. Extraembryonic hematopoiesis and vasculogenesis can be reproduced in a culture system based on ESCs, that is, embryoid bodies. At least three scenarios (*gray box* at the **top**) have been described for the cellular hierarchy that gives rise to the first hematopoietic and vascular precursors. In scenario 1, hematopoietic cells, endothelial cells (ECs), and smooth muscle cells (SMCs) arise from a common precursor, "the hemangioblast" (its corresponding ESC-derived culture equivalent is called "blast colony-forming

and intraembryonic, the former starting with the migration of EC precursors into the yolk sac (around E6.5 to 7.0 in the mouse[12]) and the latter starting slightly later around E7.3 with the appearance of EC precursors in the cranial region.[13]

Emergence of Extraembryonic EC Precursors

The extraembryonic mesoderm in birds and mammals is organized in two layers separated by the coelom, the somatopleural mesoderm (adjacent to ectoderm), and the splanchnopleural mesoderm (aligned with endoderm). EC emergence in the splanchnopleural mesoderm of the yolk sac occurs simultaneously with the appearance of primitive erythrocytes. This has fueled the idea of the existence of a common precursor, "the hemangioblast," a term that was already coined in 1932 by Murray.[14] *In vitro* evidence for the hemangioblast was given by the discovery of a bipotential precursor, called the "blast colony-forming cell" (BL-CFC) during mouse and human embryonic stem cell (ESC) differentiation, a system that faithfully recapitulates the early stages of vasculogenesis and (primitive) hematopoiesis (reviewed in Refs.[15,16]). *In vivo* evidence for the hemangioblast was shown in reporter mice expressing GFP under the *brachyury* (Bry) promoter[12] and in zebrafish.[17] Interestingly, the hemangioblast concept is evolutionary conserved since a similar vascular/hematopoietic precursor has been described in *Drosophila melanogaster*.[18] Around E6.5 to 7.0 in the mouse, the Bry-GFP$^+$ precursors, coexpressing vascular endothelial growth factor receptor-2 (VEGFR2), emerge in the posterior region of the PS and move into the yolk sac. There, they form polarized aggregates in which the outer cells give rise to flat ECs and the inner cells become primitive erythrocytes. Several of these structures subsequently fuse to form a rudimentary capillary network.[7,19]

Not all yolk sac blood vessels arise from hemangioblasts. Initial static observations of stained mouse embryos suggesting that some unipotential "angioblasts" are present in the yolk sac[13] were later confirmed by kinetic analysis of mouse embryonic/stromal cell cocultures (a system equivalent to the early gastrulating embryo), where two waves of EC precursors were found, one consisting of angioblasts and a second composed of hemangioblasts.[20] The relative importance of angioblast versus hemangioblast contribution to ECs is still a matter of debate since clonal analysis revealed that only a minor portion of ECs derives from hemangioblasts.[21] In addition to ECs and blood cells, the yolk sac mesoderm also harbors SMCs. Several scenarios exist concerning their origin (FIGURE 38.1). One theory claims that a common Bry$^+$VEGFR2$^+$ precursor (the hemangioblast) exists for the three lineages (blood, EC, and SMC).[12,15,22] According to another study, a VEGFR2$^+$ mesodermal precursor first segregates into a VEGFR2$^+$Tal1$^+$ hemangioblast (which can give rise to hematopoietic stem cells or HSCs and ECs) and a VEGFR2$^+$Tal1$^-$ angioblast (which can give rise to ECs and SMCs). A similar VEGFR2$^+$ progenitor with EC and SMC potential was described earlier by Yamashita et al.[23] This second model also considers the possibility that SMCs can arise by transdifferentiation from ECs, which would mean the hemangioblast can also be a common source for the three lineages.[24] This transdifferentiation may however be a culture-restricted phenomenon that does not occur *in vivo*.[25] A third possibility was recently proposed in the avian embryo, where the extraembryonic mesoderm would give rise to a Tal1$^+$ bipotential endothelial/hematopoietic precursor (the hemangioblast) and a separate dHand$^+$ SMC precursor, the segregation of which would be positively regulated by Notch signaling in cooperation with Wnt signaling.[26] A stimulatory effect of Notch on SMC differentiation at the expense of hematopoietic/endothelial fate was also shown during mouse ESC differentiation.[27] Finally, the transient nature of the hemangioblast has made it difficult to prove its existence during development; however there is now also evidence to suggest that such a bipotential progenitor may also persist during adulthood.[28,29]

Emergence of Intraembryonic EC Precursors

The first intraembryonic VEGFR2$^+$ EC precursors emerge in the cranial region of the mouse embryo around E7.3 where they contribute to the endocardium, the EC monolayer lining the primitive heart tube. A little thereafter (at E7.8), the VEGFR2$^+$ primordia of the bilateral dorsal aorta (DA) arise, followed by the lateral vascular networks (between E8 and E8.5) (see below). It has been suggested that the vast majority of ECs within the embryo arises from angioblasts with limited contribution of hemangioblasts.[30] In zebrafish, hemangioblast-derived ECs all clustered in a specific posterior region of the axial vessels[17] but were later also found more anteriorly.[31,32] Hemangioblast contribution to ECs has also been suggested in other regions, for example, the heart (see below). In agreement with hemangioblast activity around the axial vessels in zebrafish, in the mammalian embryo, the floor of the DA in the aorta-gonad-mesonephros region was shown to contain HSCs in intimate contact with the endothelial wall.

Two hypotheses exist concerning the relationship of the ECs and HSCs at this location.[16,33,34] According to one view, HSCs

← **FIGURE 38.1** *(Continued)* cell" or BL-CFC). According to scenario 2, SMCs and blood cells derive from separate precursors; however, hemangioblast-derived ECs may transdifferentiate to form SMCs (indicated by the *curved arrow*). In scenario 3, SMCs arise from a dHand$^+$ unipotent precursor. In the lower blue box, one example is shown of how the embryoid body system has been used to further reveal cell hierarchies during the formation of intraembryonic mesoderm derivatives. Passage through the PS can be mimicked by exposing the cultures to different levels of activin/nodal and BMP-4 expression. Intermediate activin/nodal concentrations result in the formation of a common cardiogenic mesoderm precursor, the cardiovascular colony-forming unit or "CV-CFC," giving rise in the cardiac crescent to (endocardial) ECs, cardiomyocytes (together forming the primitive heart tube), and SMCs (not shown). Studies in chick embryos have suggested an alternative scenario where endocardial and myocardial lineages derive from independent precursors in/outside the cardiac crescent. Bry, brachyury; VEGFR2, vascular endothelial growth factor receptor-2. (Adapted from Kattman SJ, Adler ED, Keller GM. Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. *Trends Cardiovasc Med* 2007;17(7):240–246; Harris IS, Black BL. Development of the endocardium. *Pediatr Cardiol* 2010;31(3):391–399.)

may arise independent of the ECs from a mesodermal precursor in a region underneath the aortic endothelium, called "the subaortic patches" (only seen in mammals, not in lower vertebrates), and then subsequently move across the DA floor into the lumen. However, recent studies have favored another scenario that HSCs arise within the DA lumen as descendants of cells with endothelial characteristics, called "the hemogenic endothelium." Recent *in vivo* tracing experiments as well as ESC cultures have strongly supported such a process.[35–38] Interestingly, in birds, the aorta has been shown to be a chimeric structure, with ECs of the floor originating from the splanchnopleural mesoderm (also called the lateral plate mesoderm or LPM) and the roof deriving from the paraxial/somitic mesoderm (see below). In a later stage, the floor ECs are replaced by ECs coming from the somites, and this transition coincides with cessation of hematopoietic activity.[16,25,33] Since the paraxial mesoderm and LPM derive from distinct regions and at distinct time points during mesodermal patterning in the PS (see above), it is possible that the different hematopoietic capacities are already determined at that level.

While the ESC system was originally thought to represent the early, mostly extraembryonic stages of vasculogenesis/(primitive) hematopoiesis, recent advances have made it possible to adapt the system to also study several aspects of intraembryonic mesodermal fate mapping.[15,16,22] Based on the expression of an anterior PS marker, Foxa2, anterior (Bry^+Foxa2^{high}; induced by activin/nodal and giving rise to cardiogenic mesoderm) and posterior ($Bry^+Foxa2^{low/-}$; induced by Wnt signaling and giving rise to extraembryonic mesoderm) PS could be segregated.[39] Another group also demonstrated that different mesoderm types can be segregated in ESC cultures using specific tracers and culture conditions and that the common or single potential to make ECs and blood was different among these types.[40] Using their murine and human ESC differentiation system, Keller's group showed that in addition to the $Bry^+VEGFR2^+$ or BL-CFC population that is the *in vitro* equivalent of the hemangioblasts colonizing the yolk sac and originating from the posterior PS, a second $Bry^+VEGFR2^+(Islet1^+)$ population, the "cardiovascular colony-forming cell" (CV-CFC; FIGURE 38.1), can be obtained that is representative of the cardiogenic mesoderm precursor that originates from the anterior PS and gives rise to (endocardial) ECs, cardiomyocytes, and SMCs *in vitro*.[22] Others have confirmed that in embryoid bodies, these three lineages can arise from a common $VEGFR2^+(Islet1^+)$ precursor.[41] While these ESC-based studies have thus suggested a common origin for endocardial and myocardial lineages, another model of endocardial specification based on lineage tracing by cell labeling in chick and zebrafish embryos suggests that the endocardial lineage develops from an independent precursor in the cardiac crescent—the structure that gives rise to the primitive heart tube consisting of an endocard and myocard layer (both conceptual models have been reviewed in Ref.[42]; FIGURE 38.1). A recent study using fluorescent mapping/time-lapse imaging and quail tissue/mouse EC implantation in chick embryos supports the view that endocardium at least in part derives from endothelial-restricted precursors devoid of myocardial differentiation potential and which may reside outside the cardiac crescent (FIGURE 38.1).[43]

Vasculogenesis

Once EC precursors have emerged from their mesodermal origin, they need to acquire a more differentiated EC signature and be assembled and shaped into a primitive and disperse tubular network to lay down the basic architecture of the embryonic vasculature. This process is collectively called "vasculogenesis." Much of this happens before the first heartbeat, while further reshaping occurs after the onset of blood flow. The molecules that orchestrate this complex process are only partially known. Nevertheless, VEGF-A most likely is the conductor of this orchestra since it is expressed in spatial and temporal association with most—if not all—events during vascular formation. Indeed, lack of a single *VEGF* allele disrupts embryonic vascular patterning.[44,45] Complementary to this, the VEGFR2 receptor is the earliest marker to define the endothelial lineage, and cells subsequently express additional markers such as junctional molecules (VE-cadherin and CD31), CD34, and later Tie2.[13] This temporal sequence of expression is more or less recapitulated during EC differentiation of mouse ESCs.[46] In the mouse, the first $VEGFR2^+$ EC precursors are spotted at E7.3 in the cranial region corresponding to the endocardial primordia, while precursors that will give rise to the bilateral DA are first detectable around E7.6 flanking the notochord.[13] The region around the notochord remains avascular at this stage due to notochord-derived negative signals, that is, BMP antagonists chordin and noggin.[47] Subsequently, these DA EC precursors gradually merge into a tubular structure in a bidirectional fashion along the craniocaudal axis. Additionally, between E8 and E8.5, primary vascular networks arise laterally from this axis, called the "lateral vascular networks," that connect to the extraembryonic vasculature.[13] The first axial vein (the cardinal vein) emerges slightly later than the DA. In the following paragraphs, we give an overview of the different steps in vasculogenesis during development, thereby using specific examples and highlighting some recently identified novel concepts.

Although the extraembryonic tissues also go through a vasculogenic phase slightly earlier than within the embryo,[7] here we describe the different steps of the process using the sculpture of the first intraembryonic main axial vessels, the DA, and the posterior cardinal vein (PCV), as an example (FIGURE 38.2). Axial vessel formation has been studied in various species, including quail embryos, chick embryos, (transgenic) mouse fetuses, zebrafish embryos, and *Xenopus* tadpoles. There are some important differences between these species. In birds and mammals, the DA is initially paired and needs to merge into a single tube. In amphibians and fish, DA patterning is in part driven by the hypochord, a temporal tubular structure that does not exist in birds and mammals and lies just ventral from the notochord.[48] As such, there are slight variations in the mechanisms by which the axial vessels are formed (FIGURE 38.2). A first critical step in DA and PCV formation is migration of the EC precursors to the correct location, that is, the midline of the embryo ventral from the notochord. The process involves a combination of soluble factors, cell–cell interactions and cell–extracellular matrix interactions.[49] In zebrafish and *Xenopus*, axial vessel EC precursors originate and migrate mainly—if not exclusively—from the LPM.[50,51] The underlying endoderm and the overlying midline structures (notochord/hypochord) seem to direct this migration. In *Xenopus*, expression of the diffusible $VEGF_{122}$ isoform in the hypochord attracts precursors to the midline.[50] In contrast, in zebrafish, while somite-derived VEGF-A secretion (induced by Shh in the notochord) is important for arterial specification of these angioblasts (reviewed in Ref.[52]), VEGF-A does not seem to be absolutely required for their migration from

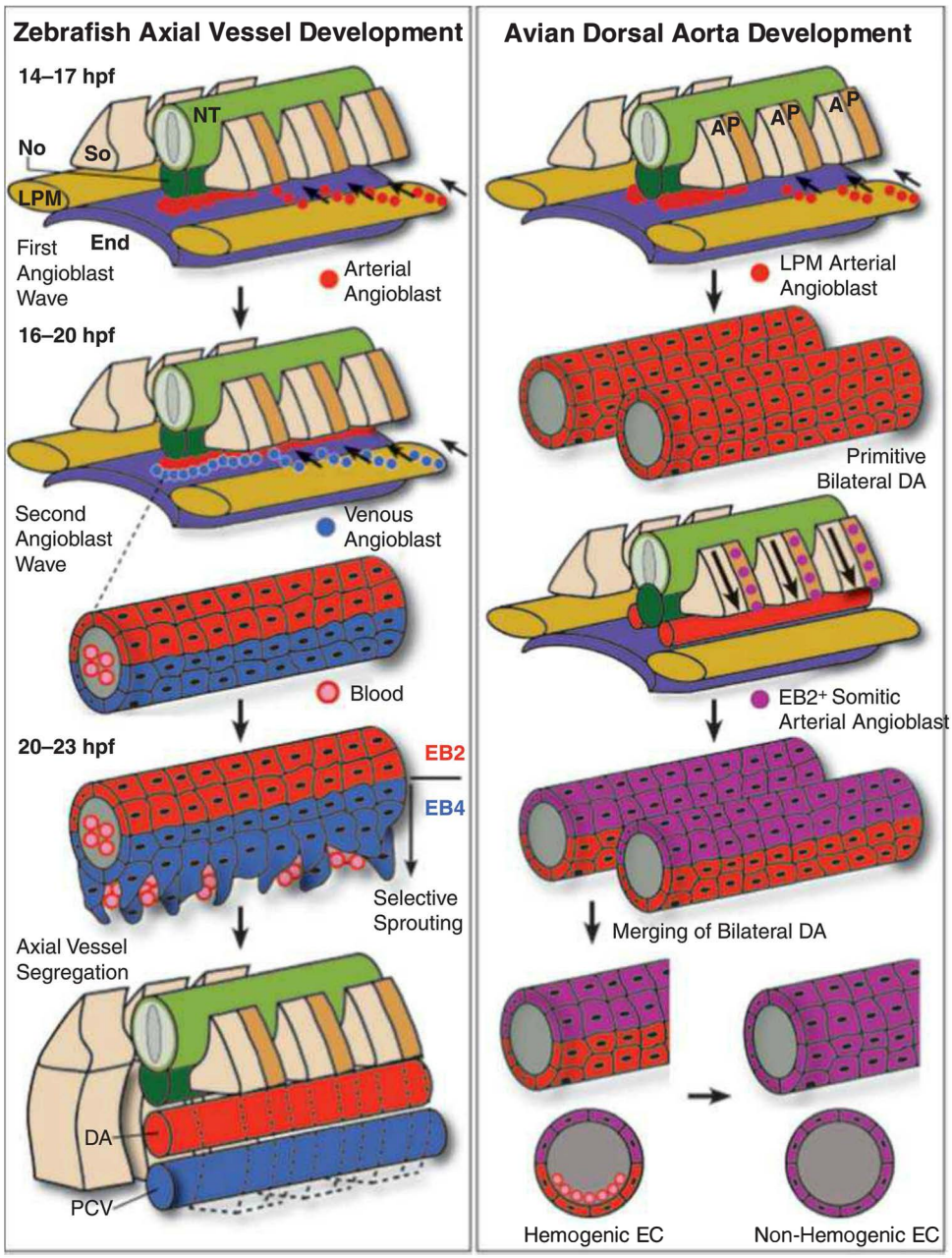

FIGURE 38.2 Vasculogenesis: the making of the main axial vessels. **Left:** *Axial vessel formation in zebrafish.* During early somitogenesis (14 to 17 hours postfertilization or hpf; somites indicated by "So"), the first wave of angioblasts (*red circles*) moves over the endoderm (End) from the lateral plate mesoderm (LPM) to the midline, just underneath the notochord (No). At some point during/after this journey, these angioblasts start to express ephrinB2a or "EB2" (under the influence of an arterial EC-inductive Shh-VEGF-Notch cascade) and give rise to a primitive vascular cord. Later (16 to 20 hpf), a second wave of LPM-derived angioblasts (*blue circles*, not expressing EB2) moves to the midline to join the cord. Subsequently (20 to 23 hpf), this common, mixed vascular cord gives rise to two axial vessels by selective sprouting, regulated by differential expression of EB2 and EphB4 ("EB4") where EB4-expressing cells sprout ventrally to form the PCV, while the EB2-expressing cells stay put and form the dorsal aorta (DA). Red blood cells (with *pink* nucleus) invading the nascent PCV are associated with lumen expansion of the PCV. **Right:** *Avian dorsal aorta development.* In contrast to zebrafish, the DA is initially paired in avian embryos. The primitive paired DA forms by lateral movement and merging of LPM-derived angioblasts (*red circles*). Later, the roof of the DA is replaced with angioblasts (*purple circles*) coming from the posterior ("P") part of the somites. These angioblasts express EB2 already at the onset of their journey. The paired chimeric DA then merges to form a single axial vessel. Around that time in the LMP-derived ventral part of the DA, "hemogenic" ECs give rise to hematopoietic cells (with *pink* nucleus), an activity that is terminated when the ventral part of the aorta is replaced by somite-derived ECs. NT, neural tube. (Adapted from Coultas L, Chawengsaksophak K, Rossant J. Endothelial cells and VEGF in vascular development. *Nature* 2005;438(7070):937–945.)

the LPM to the midline.[51] In addition, contact with endoderm causes the EC precursors to migrate as individual cells in zebrafish.[51] Additional signals may assist in angioblast navigation to the midline (reviewed in Ref.[49]): semaphorins (e.g., semaphorin 3a1, a repulsive signal expressed in the somites[53]), CXCR4/SDF-1, and sucrose nonfermenting-related kinase (Snrk)-1.[54] We recently identified the PDZ domain-containing scaffold protein synectin as a regulator of angioblast migration. Its absence in zebrafish resulted in angioblast stalling at the LPM or in between the LPM and the midline.[55] In zebrafish, there are two waves of migrating angioblasts, one starting around 14 hours postfertilization (hpf), another around 16 hpf. While the first wave will give rise to the DA, the second wave will contribute to the PCV (see below). In avian—and most likely mouse but not zebrafish—embryos, in addition to the laterally migrating angioblasts, there is a second source of angioblasts, migrating ventrally from the posterior half of the somites to contribute to the DA in response to a yet unidentified signal[56] (FIGURE 38.2). While the LPM angioblasts contribute to the floor of the primitive DA, this somitic angioblast population first invests the roof of the primitive DA and then spreads over the entire aorta to form the definitive DA. This coincides with the conversion of ECs in the floor of the aorta from hemogenic to nonhemogenic endothelium (see above)[56] (FIGURE 38.2).

The next step following migration is the establishment of a tube/cord-like structure. In *Xenopus*, avian, and mouse embryos, endoderm-derived hedgehog signals (Shh in chicks; an unidentified hedgehog signal in mice) provide necessary cues for tube formation.[57,58] Unlike its indirect effect (through stimulation of VEGF-A production) during arteriovenous specification and postnatal angiogenesis,[59] Shh most likely directly interacts with EC precursors during tubulogenesis.[58] Interestingly, however, regional differences seem to exist along the anterior–posterior axis of the DA of zebrafish. While in the posterior part of the DA, tube formation can occur in the absence of endoderm,[51] a recent study showed that the formation of the bilateral DA (the anterior portion of the DA) is critically dependent on the endoderm-derived cxcl12b (a zebrafish orthologue for SDF-1).[60] Analogous to what is known from epithelial tube formation,[61] the establishment of vascular tubes involves at least three coordinated activities,[62,63] that is, the increased cellular contact (mediated by junctional molecules, e.g., vascular tubulogenesis is severely affected in VE-cadherin knockout mice[64]), the establishment of apical–basal polarity (involving translocation of junctions to the lateral side, exocytosis to the apical surface of negatively charged antiadhesive proteins, such as CD34-sialomucins that form a cell–cell repulsive glycocalix, and rearrangement of the cytoskeleton), and interaction with the surrounding matrix (e.g., fibronectin[51,65]) through integrins (e.g., β_1 integrins[65,66]). In the zebrafish, angioblasts from the first migration wave initially aggregate without the expression of adhesion molecules, then a fibronectin matrix is deposited between the cells, and zona occludens-1 (ZO-1) only marks cell–cell junctions 2 hours later at 17 hpf.[51] Lumenization starts at 18 hpf according to a "hollow out" mode to form the future DA. Recently, it was shown in mice that lumenization of the DA is driven by VEGF-A that activates Rho-dependent kinases leading to cytoskeletal changes that open up a patent extracellular lumen between two apposing ECs.[67] This extracellular mode of tube formation thus requires junctional rearrangement between ECs as opposed to another model based on fusion of intracellular vesicles within single ECs described for lumen formation during intersomitic vessel (ISV) formation in the zebrafish.[68] The latter intracellular model has recently been complemented by another study showing that the lumens in the ISV of zebrafish are formed by rearrangement of ECs around an intercellular lumen.[69] The mechanisms of vascular lumen formation thus seem context dependent.

The first signs of arteriovenous differentiation appear in the zebrafish around 17 hpf, upon expression of the arterial marker *ephrinb2a* in the cell aggregate that will form the DA.[51] At this time, some cells in the ventral part of the cluster as well as those that constitute the second migratory wave do not express *ephrinb2a* and are therefore likely venous angioblasts. This is before the onset of circulation, meaning that this cell fate decision is independent of flow and hence genetically predetermined. When exactly arteriovenous fate decisions are made is not entirely clear. Similarly, in mice, polarized expression of ephrinB2 in the arterial cells and EphB4 in venous cells becomes apparent before the onset of blood circulation.[70,71] While ephrinB2 expression studies suggest that LPM angioblasts only acquire arterial specific markers after migration, some molecules, such as synectin, seem to specifically affect arterial angioblast behavior during (or perhaps before) migration.[55] Interestingly, the somitic angioblasts identified in birds and mice acquire ephrinB2 expression before/during their migration to the DA.[56] By 24 hpf, both axial vessels (ephrinB2a$^+$ DA and flt4$^+$ PCV) are formed in the zebrafish trunk.[51]

The molecular cascades responsible for the induction of arteriovenous fate have been mostly determined in zebrafish and mouse embryos (including a Shh-VEGF-Notch cascade, TGFβ, Sox, Fox, adrenomedullin, Spreds, phosphatidylinositol-3, and COUP-TFII or chicken ovalbumin upstream promoter-transcription factor II). This process has been elaborately described in many comprehensive reviews.[72–75] Due to space restrictions, here we only highlight the recent discovery that the Notch and Wnt/β-catenin pathways synergize to induce an arterial fate in vascular progenitors *in vitro* and *in vivo*.[76,77]

In mice, the size and segregation of the axial vessels seems to be critically determined by Notch and ephrinB2/EphB4 signaling, the former regulating the balance of arterial versus venous ECs and the latter "sorting" arterial and venous ECs into their respective vessels.[78] A recent time-lapse analysis of main axial vessel formation in zebrafish confirmed such a sorting process whereby arterial and venous angioblasts first reside in a common vessel primordium at the midline. Subsequently, ephrinB2a/EphB4 reciprocal signaling regulates the selective ventral sprouting of venous ECs (which then form the PCV) and the restricted dorsal sprouting of arterial ECs (which then generate the ISVs)[79] (FIGURE 38.2). Thus, according to this model, the PCV is not formed by vasculogenesis but rather by sprouting angiogenesis. Whether this is a characteristic of the zebrafish model or also applicable to other species remains to be determined.

Angiogenesis

Angiogenesis, or the generation of new endothelial-lined vessels from preexisting ones, expands and remodels the rudimentary vascular primordium resulting from vasculogenesis during development and most likely also is a ("the") main mechanism by which new vessels arise after birth. Angiogenic mechanisms have been mostly unraveled by studying a few stereotyped forms of developmental angiogenesis, that is, the formation of the ISVs

in the zebrafish and the formation of the retinal vasculature in the mouse, the latter occurring after birth. Many of the events during development are recapitulated in adult angiogenesis. Furthermore, different modes of vascular expansion/remodeling can be distinguished: sprouting angiogenesis (or the formation of vascular branches mostly triggered by hypoxia or other stimuli), intussusception (or transluminal pillar formation; reviewed in Ref.[80]), and circumferential growth (which for instance occurs during collateral enlargement driven by shear forces, also known as "adaptive arteriogenesis."[81]) Here, we review the process of angiogenic sprouting since our understanding of this process has significantly enhanced in the last decade.

Angiogenic sprouting is a dynamic and highly coordinated process during which ECs must perform distinct tasks and adopt specific functional identities[81,82,83,84,85] (FIGURE 38.3): tip cell selection/sprout initiation, tip cell navigation/sprout guidance, stalk cell proliferation/sprout extension, tip–tip cell anastomosis/sprout fusion, pericyte recruitment/vessel stabilization, and "phalanx" cell conversion/return to quiescence. In addition, during angiogenic remodeling, initial arteriovenous fate decisions can be "reconsidered." Again, VEGF-A takes a center stage position in this coordinated effort, with the help of another signaling pathway, the Notch pathway, which modulates VEGF signaling.[86,87]

Tip Cell Selection/Sprout Initiation

Expansion of the developing vasculature is largely driven by the need for oxygen in growing organs. Hypoxia, or the lack of oxygen, can trigger the local production of VEGF-A, which results in the creation of a VEGF-A gradient. The extracellular distribution of VEGF-A is additionally shaped by the existence of different splice isoforms with distinct matrix binding or diffusible properties, the bivalent $VEGF_{165}$ isoform being the most crucial for correct angiogenic sprouting.[88] In the retina, VEGF-A is secreted by astrocytes that move a little bit ahead of the expanding blood vascular network and are therefore indispensable for normal vascular expansion in the retina.[89] Upon contact with higher VEGF-A concentrations, certain individual ECs expressing VEGFR2 nearest by the source of VEGF-A in the avascular area, called "tip cells," flip their apical–basal polarity, send out long VEGFR2-expressing protrusions or "filopodia," and break out of the frontline.

At the same time, VEGF signaling upregulates the Notch ligand delta-like 4 (Dll4). Dll4 subsequently activates Notch signaling in the neighboring cell, which results in downregulation of VEGFR2 and upregulation of soluble VEGFR1, a decoy receptor. As such, this neighboring cell becomes less sensitive to VEGF-A presented at the vascular front and becomes unable to upregulate Dll4. This lateral inhibition and self-reinforcing negative feedback loop thus induces a "stalk cell" phenotype in cells immediately adjacent to the tip cell and makes sure that tip cells retain their leading position. Detailed time-lapse analysis has however shown that tip/stalk cell identity is very dynamic and transient and that cells in a given sprout constantly compete, by differential expression of VEGFR1 and VEGFR2, for the tip position which involves repeated tip-stalk cell identity switches.[90]

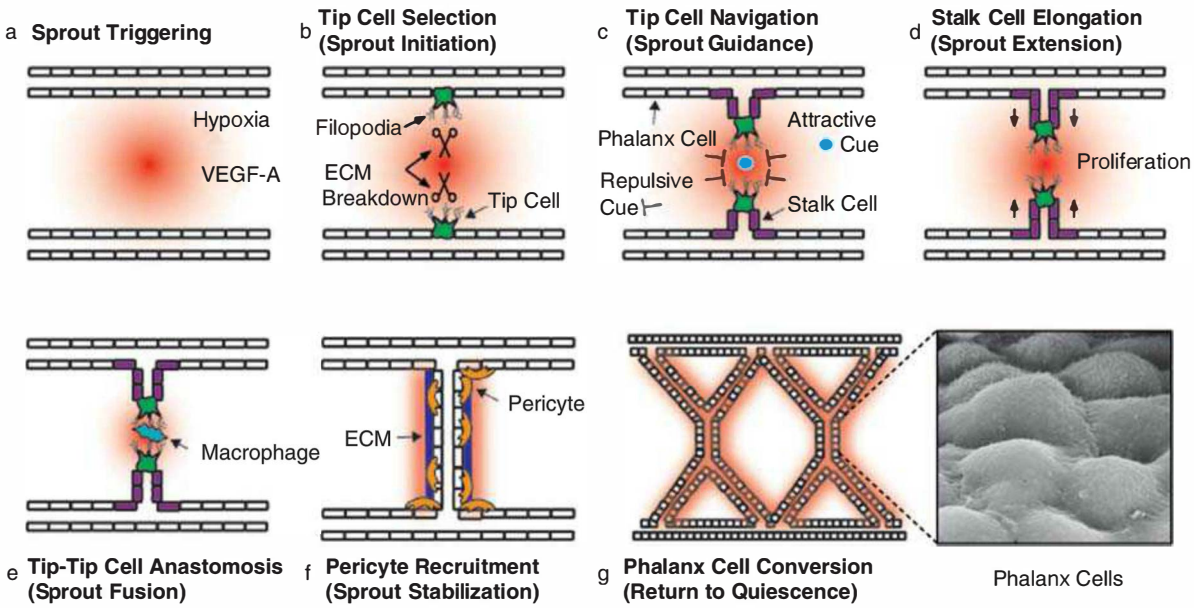

FIGURE 38.3 Angiogenesis: the different steps of the angiogenic ladder. Following a hypoxic trigger, a VEGF-A gradient arises (*red*; **panel a**). Next, cells encountering high VEGF-A concentrations change their polarity, break out from the vessel by breaking down the surrounding matrix, and develop filopodia to sense their immediate environment (**panel b**). These cells, "tip cells," are guided on their way to another tip cell by attractive and repulsive cues (**panel c**). Cells following the tip cell ("stalk cells") proliferate to elongate the sprout (**panel d**). Macrophages make both sprouting ends meet by fine-tuning tip cell guidance (**panel e**). After blood flow is restored, the new sprout is stabilized by deposition of extracellular matrix (ECM) and coverage with pericytes (**panel f**). The new vessel delivers oxygen to the tissue in need, thereby resolving hypoxia. As a result, the ECs revert to a quiescent phenotype, called "phalanx cells," as they arrange themselves in a tight formation resembling the ancient Greek soldiers (**panel g**). (Adapted from Carmeliet P, De Smet F, Loges S, et al. Branching morphogenesis and antiangiogenesis candidates: tip cells lead the way. *Nat Rev Clin Oncol* 2009;6(6):315–326.)

In contrast to tip cells, stalk cells form fewer filopodia, but instead trail behind the leading tip cell to elongate the sprout (see below). Interestingly, these two distinct functional behaviors are reflected in a different expression profile: tip cells express high levels of VEGFR2, VEGFR3, Nrp1, UNC5b, Dll4, CXCR4, Ang2, ESM-1, SLP-76, and PDGF-BB, while stalk cells express more Jagged-1 (a Notch ligand), VEGFR1, and Robo4.[87,91] Jagged-1 expression in stalk cells was recently shown to antagonize Dll4, thereby preventing Notch activation (and thus stalk cell induction) in neighboring cells. When modified by fringe glycosyl transferases, Notch1 is indeed activated more by Dll4 than by Jagged-1. Given that some Dll4 protein is detectable in stalk cells, Jagged-1 also helps to maintain differential Notch activity by antagonizing Dll4 that signals back to tip cells.[92] In addition to Notch signaling, VEGF-A/VEGFR2 signaling in tip cells is further modulated by other mechanisms (reviewed in Ref.[84]) including cooperation with other VEGF receptors and a newly identified transcriptional repressor complex composed of translocation ets leukemia gene (Tel) and carboxy-terminal–binding protein (CtBP). Proximity ligation assays revealed that VEGFR2 can heterodimerize with VEGFR3 in tip cell filopodia upon VEGF-A or -C binding.[93] The VEGFR2 coreceptor Nrp1, on the other hand, was shown to be involved in lateral filopodia extension, which is critical for tip cell turning and fusion (see below).[94] Alternatively, Nrp1 alone may induce vessel branching by direct signaling in ECs through synectin.[55] While VEGF coreceptors spatially modulate VEGF branching activity, the Tel–CtBP complex was shown to temporally restrict VEGF-A–stimulated Dll4 expression in sprouting ECs.[95] Furthermore, this complex also regulated the expression of antagonistic sprouting cues (e.g., VE-cadherin and Sprouty4). In addition to their sensing behavior through filopodia, tip cells also need machinery to invade the surrounding basement membrane to be able to break out and advance. Matrix metalloproteinases (MMPs), such as MT1-MMP, may be one of the molecular tools to dissolve the matrix (FIGURE 38.3), at least during postnatal angiogenesis where the "mother vessels" from which the new sprouts are formed are embedded in a thicker layer of basement membrane.[84]

Tip Cell Navigation/Sprout Guidance

The tip cell filopodia function as sensors that constantly probe their environment. In this respect, tip cells resemble the growth cone of navigating axons.[96] Therefore, certain guidance signals used by axons seem to have been co-opted by endothelial tip cells on their way to meet another tip cell[82,97] (FIGURE 38.3). Tip cell movement needs to be correctly monitored by attractive and repulsive guidance posts. There are at least four ligand/receptor classes of guidance molecules with a documented role in vascular navigation: Slits/roundabout4, netrins/Unc5B, Semaphorin 3E/Plexin-D1, and ephrinB2/EphB4. Due to space restrictions, we only briefly illustrate some examples as prototypic examples. One roundabout (Robo) family member (Robo4) is expressed in growing vessels. Robo4 deficiency in mice leads to hypervascularization during oxygen-induced retinopathy, and its high expression in stalk cells may prevent these cells from being activated by VEGF-A.[98] Mechanistically, Robo4 counteracts the leakage-promoting activity of VEGF-A by impairing VEGFR2-mediated activation of the kinase Src.[98] The nature of the Robo4 ligand is debated, since Robo4 lacks Slit-binding domains, and Robo4 also binds to Unc5B, another guidance receptor, suggesting that Robo4/Unc5B maintains vessel integrity via Unc5B activation.[99]

Unc5B is highly expressed in tip cells, and binding of netrin-1 to Unc5B causes filopodia collapse in the retina while Unc5B knockdown in zebrafish induces ectopic ISV sprouting.[100] Repulsion by netrin-1–Unc5B interaction requires the cytoplasmic domain of the receptor.[101] However, netrin-1–deficient mice do not have a vascular phenotype, suggesting that netrin-1 signaling might involve other yet unidentified receptors or that other molecules may take over its role in mice.[97] Alternatively, Unc5B could act as a dependence receptor that, in the absence of ligand, causes EC death.[102]

While secreted semaphorins usually bind to neuropilin receptors (functioning as a coreceptor for Plexins) and often inhibit vessel sprouting, Semaphorin 3E directly binds Plexin-D1 also causing vessel repulsion. Absence of either binding partner results in excessive branching in the trunk vessels of the mouse.[103] Within the developing mouse retina, neuron-derived Semaphorin 3E binding to Plexin-D1 activates RhoJ in ECs, thereby restricting and orienting VEGF-induced filopodia projection.[104] In addition, this Semaphorin 3E/Plexin-D1 interaction fine-tunes the balance between tip and stalk cells by coordinating VEGF's activity in a negative feedback.[105] Similarly, the zebrafish mutant *out of bounds* maps to a mutation in the *plexin-D1* gene which results in excessive and aberrant ISV branching into the somites; however, unlike in mice, the action of plexin-D1 in zebrafish is antagonized by Semaphorin 3E/plexin b2 signaling.[106]

Eph receptors and their ephrin ligands regulate cell contact-dependent signaling through bidirectional signaling in cells expressing the receptor (forward signaling) or ligand (reverse signaling), in most cases generating repulsive signals.[82,97] Notably, ephrinB2 was shown to regulate VEGFR2 endocytosis upon VEGF-A ligation during sprouting angiogenesis[107,108] (reviewed in Ref.[109]). EphrinB2/EphB4 have also been shown to be involved in directional sprouting of ECs during the formation of the PCV in zebrafish (see above).[79]

Stalk Cell Proliferation/Sprout Extension

While tip cells lead the way, stalk cells follow the tip cell and are responsible for sprout elongation and lumen formation. Unlike tip cells that seldom proliferate, stalk cells do proliferate as a means to make the sprout longer (FIGURE 38.3). As mentioned, stalk cells acquire their phenotype by activation of Notch signaling. Since Notch signaling is generally known to inhibit EC proliferation,[87] there must be another mechanism at play that overrules this inhibition. One of the downstream targets of Notch signaling in stalk cells is Nrarp which functions as a negative regulator of Notch through degradation of the Notch intracellular domain (NICD),[110] thereby creating a permissive environment for proliferation. At the same time, Nrarp also activates Wnt signaling in stalk cells which stabilizes the stalk by inducing firm intercellular junctions.[110] Instead of just passively trailing behind the tip cell, stalk cells may also actively push the sprout forward by secreting certain matrix components, for example Egfl7.[111] As a result of Notch signaling, stalk cells have lower expression of VEGFR2 while they preferentially express VEGFR1.[90] Notch activation also lowers endothelial expression of placental growth factor (PlGF), a VEGFR1-specific ligand.[112] Gamma-secretase (which generates NICD by cleaving Notch) was shown to cleave VEGFR1, leading to release of its soluble form which can scavenge VEGF-A in the vicinity of the stalk cells, further dampening its effect.[113] At the same time, the intracellular VEGFR1 fragment

may block VEGFR2 signaling. Collectively, these effects reduce and spatially restrict the overall response of ECs to VEGF-A and may fine-tune stalk cell morphogenesis. A recent paper revealed an additional fine-tuning mechanism of stalk cell behavior, whereby the response of an EC to Notch activation is negatively modulated through deacetylation—and hence destabilization—of NICD by SIRT1, an NAD^+-dependent deacetylase.[114]

Another task of cells in the stalk is the formation of a lumen, so blood flow can be initiated in the new sprout. Lumen formation in angiogenic sprouts may occur either before or after connection of the sprout to another tip cell[83]; it remains unknown how this occurs precisely. Possibly, lumen formation in anastomosing vessels mechanistically resembles that of vessels formed by coalescing angioblasts during vasculogenesis (see above).[62]

Tip–Tip Cell Anastomosis/Sprout Fusion

To make new connections, tip cells move toward each other. As they make contact, they need to convert their behavior of a migratory, navigating cell into a stalling connective cell. Recently, VE-cadherin, a junctional molecule, was shown to be expressed in filopodial protrusions of advancing tip cells, suggesting that interactions between cadherins of contacting tip cells may stabilize the connection.[115] Formation of these junctions may then induce the phenotypic change from an actively navigating tip cell to a more quiescent cell. Failure to make such a stabilized contact, for example by repulsive forces between approaching tip cells, may on the other hand lead to sprout retraction, leaving behind an empty matrix sleeve. ECs may get assistance from helper "bridge" cells to acquire the precision needed to find another tip cell, as recently demonstrated in zebrafish where proangiogenic macrophages serve as guiding posts for tip cells and promote tip cell fusion[116] (FIGURE 38.3). Recently, it was shown that VEGFR3 signaling in tip cells triggered by VEGF-C derived from macrophages positioned around sprout fusion sites results in fusion stabilization and phenotypic conversion of the fused tip cells to a stalk-like cell by reinforcing Notch signaling.[117]

Pericyte Recruitment/Vessel Stabilization

Maturation and stabilization of the nascent tube requires the deposition of extracellular matrix as well as the recruitment of pericytes (FIGURE 38.3). Pericytes, expressing PDGFRβ, are attracted to the endothelial tubes by PDGF-BB signals derived from the ECs. These EC–pericyte interactions additionally involve angiopoietin/Tie receptor signaling, S1P/sphingosine-1 phosphate receptor (S1PR) signaling, and Notch signaling (for comprehensive reviews[118,119]). Mural cell-secreted angiopoietin-1 activates its Tie2 receptor on ECs, thereby promoting pericyte adhesion to and junctional tightening between ECs. However, a recent study revealed that angiopoietin-1 seems less important for pericyte recruitment than originally thought and rather determines vessel diameter and number during vascular development.[120] S1P1/S1PR coupling strengthens EC/pericyte contacts by relocating *N*-cadherin to the abluminal side of ECs.[121] *NOTCH3* mutations in CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) patients lead to vascular SMC degeneration and results in arteriopathy, which was recently phenocopied in mice.[122]

Stabilization further prevents the newly formed sprout from pruning. While all previous steps of angiogenic sprouting can be studied in zebrafish, it has not been clear whether the zebrafish vasculature during early development becomes coated with a pericyte or SMC layer.[123] A recent paper demonstrated that only the proximal part of the DA is invested with a limited number of periendothelial ("mural") cells during zebrafish development.[124]

Phalanx Cell Conversion/Return to Quiescence

The flow of oxygenated blood in the lumen will further cause the hypoxia signal that initiated the sprout to subside, leading to a decrease in VEGF-A levels. This restores the balance between pro- and antiangiogenic factors and leads to quiescence. Quiescent ECs have yet another behavior than tip or stalk cells, since they do not (or to a much lesser extent) migrate nor proliferate in response to VEGF-A.[125] Indeed, VEGF-A mostly elicits a survival response in quiescent ECs, since the absence of VEGF-A in ECs causes widespread EC dysfunction and disintegration.[126] These cells have tight connections and are aligned in a smooth monolayer, resembling the "phalanx formation" of ancient Greek soldiers, hence their name "phalanx cells" (FIGURE 38.3). Recently, some of the molecules that determine this cell behavior were identified. Quiescent ECs in a normal vessel can be compared with sensors that constantly probe their environment for oxygen, thereby estimating the need for vascular expansion. Prolyl-hydroxylase domain-2 (PHD2) belongs to the family of oxygen sensors that regulate the stability of hypoxia-inducible factor (HIF), a major stimulator of VEGF-A production. When oxygen supply is normal, PHDs use oxygen to hydroxylate specific proline residues in HIFs, thereby targeting them for proteasomal degradation.[127] During hypoxia, the oxygen-dependent activity of PHDs is attenuated allowing HIFs to mount an adaptive response. In a recent study, it was shown that lowering the activity of the PHD2 sensor (by heterozygosity) in tumor ECs causes the normally hyperactivated tumor endothelium to become quiescent and adopt the phalanx phenotype, a process called "vessel normalization"[125] (reviewed in Refs.[85,128,129]). $PHD2^{+/-}$ tumor ECs had elevated levels of VE-cadherin and soluble VEGFR1, the latter which acts as a VEGF-A trap. Increased VE-cadherin expression is known to shift the EC response to VEGF-A from migration/proliferation to survival/quiescence.[130] This resulted in a better perfusion and oxygenation of the tumors which hence became less invasive and less metastatic.[125] Thus, by lowering the activity of an oxygen sensor, ECs can readjust their shape and behavior to improve oxygen delivery when needed. In addition to oxygen sensors, additional molecular pathways have been shown to regulate quiescence and survival, for example BMP-9/Alk1, thrombospondin, FGFs, HoxC9,[131] miR-132,[132] angiopoietin-2/FOXO1,[133] and angiopoietin-1/Tie2. Mural cell-derived angiopoietin-1 induces Tie2 clustering at EC–EC junctions in *trans* to promote EC survival and quiescence.[84,134,135]

Arteriovenous (Re)specification

Even though arterial/venous cell fates are acquired early in development during the vasculogenic phase independent of blood flow, these fate decisions are not completely irreversible and are sometimes reshaped during the angiogenic remodeling phase (coinciding with the onset of blood flow). The plasticity of ECs was demonstrated in the chick yolk sac where experimentally induced flow changes could transform arteries into veins and *vice versa*.[136] Also during development of the zebrafish trunk vasculature, such fate switches occur.[137] For the generation of the ISVs, primary bilateral sprouts emerge around 20 hpf from the DA which give rise to the primary ISVs. Despite

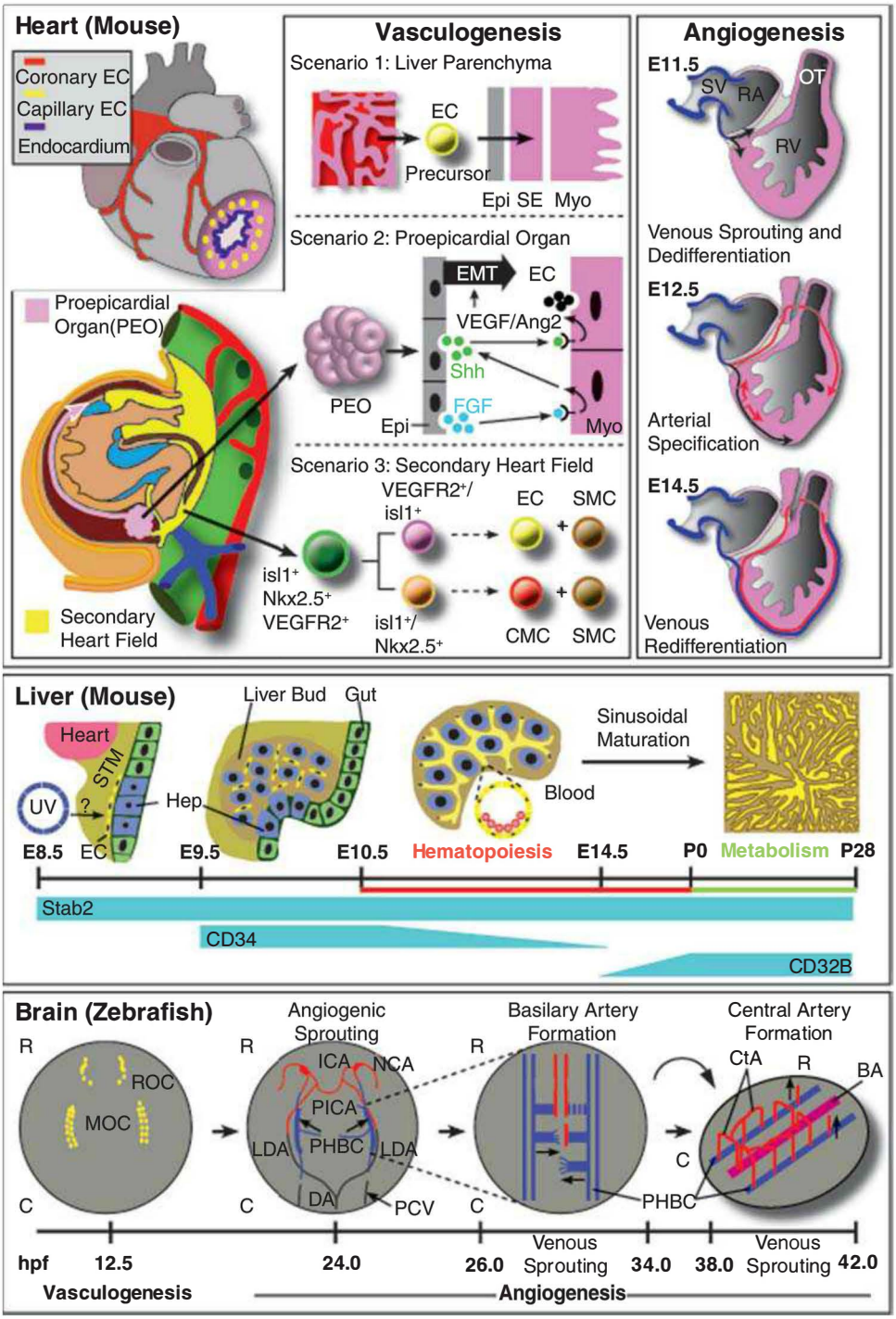

FIGURE 38.4 Generation of organ-specific vascular beds. **Upper panel:** *mouse heart*. There are three endothelial beds in the heart (the coronary, capillary, and endocardial bed; upper left pictogram). At least four different scenarios were proposed to explain the origin of the coronary vascular bed(s), three claiming a vasculogenic initial step (*left box*), challenged by a recently published fourth theory proposing an angiogenic mechanism (*right box*). The first vasculogenic theory claims that endothelial cell (EC) precursors move from the liver parenchyma to the subepicardium (SE). Alternatively, the PEO (*pink* structure in lower left pictogram), a grape-like protrusion of the sinus venosus (SV), is the common precursor of ECs, fibroblasts, SMCs (and part of the cardiomyocytes). This PEO first gives rise to the outer heart layer, the epicardium (Epi), by moving across the myocardium (*pink arrow*). Through an FGF-Shh-VEGF/angiopoietin-2 molecular cascade, the ECs arise from the epicardium by EMT. The third scenario proposes a common cardiomyocyte (CMC), EC, and SMC islet(isl)1^+Nkx2.5^+VEGFR2^+ precursor originating from the secondary heart field (*yellow* structure in lower left pictogram). According to the angiogenic scenario, the coronary vasculature derives by angiogenic sprouting from the SV, during which venous ECs (in *blue*) first dedifferentiate (in *black*), then transdifferentiate to arterial ECs (to give rise to the arterial

their aortic origin, the sprouting vessels, including their tip cells, express flt4, which is a venous marker in zebrafish. Only later, the expression of flt4 becomes restricted to the PCV.[138–140] Around 35 hpf, a secondary round of sprouting occurs, this time emanating from the PCV. About half of the secondary sprouts make connections with a primary ISV (originating from the DA) which converts them to venous ISVs, while the other half becomes part of the lymphatic system.[137,141,142] Therefore, the arteriovenous identity of trunk vessel ISVs is not fixed until after secondary sprout connection.

It is likely that flow dynamics determine the final differentiation to venous or arterial phenotypes.[137] For instance, in the developing mouse retina, Unc5B seems to be expressed in tip cells at the forefront of both arterial and venous sprouts, while Unc5B expression is otherwise mostly restricted to arterial vessels.[100] This may suggest that tip cells may lose their arteriovenous identity during sprouting, but may regain arteriovenous polarity once incorporated in an established vessel. Also adult ECs retain some arteriovenous plasticity since ECs of vein grafts, implanted into an arterial segment, acquire arterial characteristics (reviewed in Ref.[72]). Thus, refinement by hemodynamics (or other environmental signals) adds another layer of regulation on top of a genetically predetermined decision program.

Establishment of Organ-Specific Vascular Beds

Different organs fulfill distinct tasks that require a customized vascular supply. This is not only reflected in the existence of highly specialized angioarchitectures but even more so in the unique properties of the microvasculature within every organ. Indeed, the microvessels or capillaries are the exchange vessels that are in immediate contact with the cells that perform each organ's specific functions. This intimate relationship gives rise to remarkable functional, morphologic, and molecular differences among ECs from different organs.[1] While these differences have been recognized for decades, there is yet much to learn about how they were created and when exactly they appeared. Many of these differences already appear early in development. As an example, we discuss the heart, the liver, and the brain, since these organs are frequent targets of life-threatening vascular diseases. Studying the mechanisms underlying the ontogeny of specific capillary beds may give important clues for the design of life-saving "tailor-made vascular bed-specific" therapies.[143]

The Heart

Of all tissues, the heart is a frequently studied organ when it comes to how its vasculature is established during development. However, most studies have focused on the coronary arteries—a hot spot for atherosclerosis—while the cardiac microvasculature has received much less attention. Within the heart, there are at least three major types of blood vascular endothelium[1] (FIGURE 38.4): the endocardium (which lines the entire interior surface of the heart muscle, including the valves), the coronary macrovascular endothelium (consisting of arterial and venous ECs), and the myocardial capillaries that connect the coronary arteries to the veins. The endocardium is the first EC type appearing around E8 in the mouse,[13] when the heart is only a tube consisting of a few layers of cardiomyocytes lined on the inside by an EC monolayer that subsequently becomes trabeculated to allow maximal oxygen diffusion to the heart muscle.[144] The heart valves develop by endothelial-to-mesenchymal transformation from the endocardium.[1,145]

While the origin of the endocardium is debated (see above), there is also discussion concerning the origin of the ECs covering the coronary arteries, veins, and capillaries. Until recently,

FIGURE 38.4 *(Continued)* coronary tree in *red*), and subsequently redifferentiate to venous ECs (to form the venous coronary tree in *blue*). **Middle panel:** *mouse liver*. The first VEGFR2$^+$ EC (precursor)s (in *yellow*) appear in the septum transversum mesenchyme (STM) near the liver primordium (*blue* hepatoblasts or "Hep") around embryonic day (E)8.5. Another theory hypothesizes venous transdifferentiation from the umbilical vein (UV) as origin for liver EC (precursor)s. At E9.5, triggered by the EC (precursor)s, hepatoblasts invade the STM to form the liver bud, intermixed with EC (precursor)s that do not yet form lumenized structures. From E10.5 on, lumenized structures appear and the liver starts its activity as hematopoietic organ. During late development and several weeks after birth, the liver microvasculature gradually acquires its typical sinusoidal organization (*right* pictogram) which coincides with conversion to a metabolic function. During maturation, LSECs acquire expression of early (Stabilin2 or Stab2; CD34) and late markers (Stab2 and CD32B). **Lower panel:** *zebrafish brain*. The formation of the brain vasculature involves a vasculogenic phase, followed by an angiogenic and maturation phase (the latter not shown). Around 12.5 hours postfertilization (hpf), two angioblast centers arise, one in the rostral (R) part (the rostral organizing center or ROC) and one more caudally (C) in the midbrain (the midbrain organizing center or MOC). These angioblasts merge to form a primitive plexus that expands by angiogenic sprouting, giving rise to the first venous (the primordial hindbrain channels or PHBC) and arterial vessels (the nasal ciliary artery or NCA; the primitive internal carotid artery or PICA; the internal carotid artery or ICA). The extracranial arteries, the LDA or lateral dorsal aorta and dorsal aorta or DA, and extracranial veins (the posterior cardinal vein or PCV) that connect to the trunk vasculature are indicated in *black*. Starting at 26 hpf, the basilary artery (BA) arises by sprouting (*black arrows*) from the venous PHBC. Subsequently, the sprouts disconnect from the vein (indicated by *dashed blue lines*) and the disconnected ECs merge to form a lumenized BA. Similarly, the central arteries (CtA) arise by sprouting (*black arrows*) from the PHBC somewhat later in development. (Adapted from Proulx K, Lu A, Sumanas S. Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. *Dev Biol* 2010;348(1):34–46; Lie-Venema H, van den Akker NM, Bax NA, et al. Origin, fate, and function of epicardium-derived cells (EPDCs) in normal and abnormal cardiac development. *ScientificWorldJournal* 2007;7:1777–1798; Red-Horse K, Ueno H, Weissman IL, et al. Coronary arteries form by developmental reprogramming of venous cells. *Nature* 2010;464(7288):549–553; Zaret KS. Regulatory phases of early liver development: paradigms of organogenesis. *Nat Rev Genet* 2002;3(7):499–512.)

there were three (not necessarily mutually exclusive) hypotheses (FIGURE 38.4). One claims that the ECs lining the coronary vasculature are derived from angioblasts of the liver region, while a second theory argues that the ECs, together with the SMCs and fibroblasts and perhaps some cardiomyocytes,[146] have a common origin, that is, the proepicardial organ (PEO)—a transient grape-shaped protrusion that attaches to the looped heart tube around E9.5 in the mouse and subsequently spreads over its entire outer surface to form the epicardial layer by E11.[145,147–150] According to the "PEO theory," endothelial precursors are generated within the subepicardial layer by EMT (FIGURE 38.4). The inductive signals responsible for EC precursor generation are the result of cross-communication between the epicardium and the myocardium where an FGF-Shh-VEGF/Ang2 pathway has been described.[151] A third source of coronary ECs could be a population of Islet1$^+$VEGFR2$^+$Nkx2.5$^+$ multipotent progenitor cells residing within the secondary heart field, giving rise to the proximal part of the coronary endothelium and vascular smooth muscle (along with part of the cardiomyocytes).[152] According to each of these theories, the coronary vasculature initially arises by vasculogenesis, that is, the formation of a primitive network of vessels by EC precursor fusion. Some studies have suggested the presence of hemangioblasts and blood islands during cardiac development.[151,153,154,155,156] The rudimentary network then is remodeled into a hierarchical tree of arteries, veins, and capillaries. Very recently, these vasculogenesis theories have been challenged by a mouse study in which clonal analysis revealed that the majority of the endothelium of coronary vessels (including arteries, veins, and capillaries) originates from the sinus venosus by angiogenic sprouting[155] (FIGURE 38.4). This new theory also challenges the classical view that arteries and veins derive from distinct EC precursors and again underscores the plasticity of arteriovenous fate.

Due to the different hypotheses on the origin of coronary ECs, it is not really known when the capillaries in the heart acquire their specific characteristics. While these characteristics have been mainly defined by comparing the capillary ECs with the endocardial ECs,[1,157] few—if any—studies have compared the heart capillaries with those in other organs. The late connection of the coronary tree to the aorta implies that capillary formation occurs largely independent of blood flow and that there is a time window during which capillary ECs are only exposed to contractile forces coming from the contracting myocardium.[156] Therefore, these forces may codetermine the unique characteristics of capillary heart ECs. In addition, the very close apposition of these ECs to cardiomyocytes allows for intense reciprocal communication through secretion of (growth) factors, some of which may be specific for the cardiac vascular bed.[151,158] Transplantation and EC/cardiomyocyte coculture studies have revealed the importance of such signals by showing that von Willebrand factor expression in ECs was specifically upregulated by cardiomyocytes but not by hepatocytes.[159] Cardiomyocyte-specific deletion of VEGF-A resulted in an underdeveloped microvasculature but did not affect coronary artery structure revealing the specific sensitivity of the capillary endothelium to VEGF-A.[160] Specific loss of the heparin-binding isoforms of VEGF-A ($VEGF_{188}$ and $VEGF_{165}$) however did not affect microvascular development but disrupted arteriovenous differentiation, suggesting that different VEGF-A isoforms may target different steps in heart vascular development.[161] On the other hand, several recent studies revealed that VEGF-B, which is highly expressed in the heart, may affect the growth of large coronary vessels, while its effects on vessels outside the heart may be more limited.[154,162–164] Another cardiac-specific growth factor is brain-derived neurotrophic factor, since its general absence caused specific defects in myocardial arteries and capillaries. Accordingly, its receptor trkB is selectively expressed by neonatal and adult heart vessels.[165]

The Liver

The vascular architecture of the liver is particular since the liver capillaries, also called the sinusoids, are fed by two afferent vessels: the hepatic artery for delivery of oxygen (comprising 20% of normal hepatic flow) and the portal vein for providing nutrients from the intestine (constituting 80% of the normal hepatic flow). The sinusoids on their turn empty into the hepatic venules, the hepatic vein, the inferior vena cava, and ultimately the right atrium of the heart. The ECs in the hepatic sinusoids (called liver sinusoidal ECs or LSECs) have acquired unique anatomical/molecular features that allow them to function as selective "sieves" (by the presence of large nondiaphragmed cytoplasmic holes, called "fenestrae," and the absence of a basal lamina), scavengers for soluble waste molecules (by the expression of scavenger receptors), and inducers of immune tolerance (by the expression of MHC class I, II and costimulatory molecules).[1] The development or regeneration of the liver parenchyma is intimately related to its investiture with blood vessels—in part because ECs from these vessels produce specific inductive "angiocrine" signals[166]—and the same scenario is true for other gut tissues[167,168] (FIGURE 38.4). At approximately E8 in the mouse, the liver primordium (the liver "bud") arises from the ventral foregut endoderm. As early as E8.5, VEGFR2$^+$ angioblasts (the origin of which is not known) are found between the nascent hepatic epithelium and the septum transversum mesenchyme.[167,169] The essential role of these cells for liver development is shown by the complete failure of hepatoblast budding in VEGFR2$^{-/-}$ mice.[170] At E9.5, these cells begin to merge into vascular structures and hematopoietic cells appear in their lumens, indicating the onset of liver hematopoiesis.[167] These VEGFR2$^+$ cells start to express Stabilin-2 (Stab2), a scavenger receptor specifically expressed in sinusoidal ECs (including LSECs, bone marrow (BM), and spleen ECs), before they are organized in a lumen.[169] Another hyaluronan receptor, LYVE-1, becomes expressed 1 day later, and both remain expressed until adulthood. CD34 was expressed in fetal LSECs (from E9.5 to E14.5) but was downregulated later on. In contrast, the expression of CD32b, a receptor for the Fc fragment of immunoglobulins, started later in development (around E15.5).[169] This stepwise phenotypic transition mirrors the functional conversion of the liver as a hematopoietic organ during development to a metabolic organ after birth and also runs in parallel with morphologic changes of the sinusoids.[171,172]

After birth, the sinusoids further adopt regional changes resulting in "zonation" across the portal tract (see below).[173] LSECs share various molecular and structural features with lymphatic ECs. The latter emerge by transdifferentiation and budding from veins (see below). Intriguingly, since Stab2 expression was both observed in ECs of the vitelline vein as well as in VEGFR2$^+$ cells that give rise to LSECs during liver development, the hypothesis that LSECs may also arise by transdifferentiation from venous ECs is certainly worth pursuing[169,172] (FIGURE 38.4).

While zebrafish have been mainly used to study axial vessel formation since their introduction in the vascular community,

a new trend has emerged in which this animal model is also becoming applicable to study organ development (and its dependency on contemporary vascular development).[174,175] Liver vasculogenesis starts around 50 to 55 hpf in the zebrafish.[175,176] Unlike in mice, liver development was initially independent of the vasculature[177]; however the presence of sinusoids (starting at 68 hpf), and later on (at 72 hpf), blood flow in these sinusoids, was essential for further liver growth.[176]

The signals that control the acquisition of an LSEC-specific gene signature, function, and morphology remain largely unknown. Likely, the zebrafish model along with studies in mice will boost the discovery of these factors. By sequestering VEGF-A specifically in the mouse liver, upon overexpression of signaling-incompetent VEGFR2 in hepatocytes, this angiogenic growth factor was shown to be essential for maintaining fenestrae in LSECs throughout development.[178] Angiopoietin-like 3 (ANGPTL3), an integrin-binding member of the angiopoietin family that is specifically expressed in the liver during development and in adults, was shown to induce blood vessel formation in a rat corneal assay, but a role in LSEC behavior has not been documented.[179] In a recent differential transcriptome analysis, Wnt2 was found to be highly enriched and Frizzled4 was identified as a novel Wnt2 receptor in LSECs. VEGFR2 was a target of Wnt2 signaling, suggesting that autocrine Wnt2 and VEGF signaling cooperate to determine LSEC behavior.[180] In addition to this autocrine effect, LSEC-derived Wnt2 (triggered by VEGF-A signaling) also induced an "angiocrine" effect on hepatocytes by promoting their proliferation during liver regeneration after partial hepatectomy in adult mice.[166] Importantly, LSECs and hepatocytes were shown to have different characteristics depending on their location in the liver lobules. This so-called zonation reflects the performance of different metabolic tasks in different areas of the lobules and is in part determined by Wnt/β-catenin signaling.[181]

The Brain

In order to maintain a stable environment for neurons, the central nervous system has developed specialized epithelial and endothelial barriers for regulation of ionic balance and nutrient transport and for blocking transit of potentially toxic molecules.[182] The endothelial barrier that protects the brain is called "the blood–brain barrier" (BBB). This barrier is established by tight junctions that seal the intercellular clefts and hence restrict paracellular diffusion of water-soluble molecules. ZO-1, junctional adhesion molecule, occludins, and claudins are the most important building blocks of tight junctions.[183] Caveolae, present in small numbers at the luminal surface, regulate transcellular transport.[182] In addition, brain capillary ECs express transporter molecules (e.g., P-glycoprotein or multidrug resistance 1) that function as efflux pumps for excitotoxic compounds or to deliver vital molecules to the neurons (e.g., glucose transporter [GLUT] 1). In addition to brain capillary ECs, the BBB has two additional cellular components that share their basement membrane with the ECs, astrocytes (which make contact with the ECs through their end feet), and pericytes.[182] Although astrocytes and ECs have been thought to be more important in barrier formation, the crucial role of pericytes in BBB integrity has recently been highlighted by the observation that pericyte loss in brain vessels may progressively disrupt the BBB and result in neurodegeneration.[184]

The establishment of the brain vasculature can be divided into three phases: a vasculogenic phase, an angiogenic phase, and the maturation phase during which the specific BBB characteristics arise. The central nervous system originates from the surface ectoderm. Shortly thereafter, angioblasts originating from the somites and the LPM migrate to the cranial region where they merge into a primitive tubular network called "the perineural vascular plexus" that envelopes the neural tube.[185] The brain tissue becomes vascularized by angiogenic sprouts from the perineural plexus. While the early fetal brain vasculature is quite permeable (frequently showing fenestrations), the barrier gradually tightens during late development, although this may happen at a different rate in different species.[186] The acquisition of BBB characteristics is in large part not predetermined, but is induced by the environment, as evidenced by transplantation studies of nonbrain vessels into a brain environment.[187] In humans, acquisition of BBB features, such as the expression of P-glycoprotein, occurs quite early in development, and this rapid change has been suggested to constitute a "second line of defense" for the fetus in addition to the placental barrier.[188]

As for the liver, the zebrafish has been recently introduced as a model system to study brain vasculogenesis/angiogenesis and barrier formation.[31,189] Similarly as for mammals, brain vessels in zebrafish develop by a combination of vasculogenesis and angiogenesis. First, two bilateral angioblast clusters emerge at 12.5 hpf, one rostrally giving rise to the rostral portion of the brain vasculature and one more caudally being the common source of the posterior brain vessels and the endocardium.[31] Angiogenic sprouting from these clusters begins around 16 hpf. In a recent study, the formation of the basilar artery around 36 hpf was shown to arise by a novel mechanism, that is, by sprouting from the primordial hindbrain channels (PHBCs), a bilateral venous structure which itself arises by vasculogenesis from the caudal angioblast cluster[190] (FIGURE 38.4). Moreover, the central arteries subsequently were generated by a second wave of sprouting from this primitive venous vessel, another illustration that arteriovenous fate decisions are versatile (see above). Interestingly, the emerging sprouts subsequently disconnected from the PHBC whereby the resulting isolated ECs merged *in situ* into the basilary artery. Thus, the basilary artery forms by a combination of angiogenesis and vasculogenesis. These processes were guided by cxcr4a (expressed on PHBC ECs) and cxcl12b (expressed in the neural hindbrain keel immediately adjacent to the basilary artery).

An accompanying study revealed that the same chemokine signals in coordination with hemodynamics regulates proper arteriovenous connectivity in the hindbrain.[191] By using a transgenic zebrafish expressing an endogenous tracer to monitor BBB integrity, Xie et al.[189] found that the BBB starts to develop at 72 hpf, preceded by the expression of claudin5a in the central brain arteries at 48 hpf. Claudin5 was also identified in mice as being determinant for BBB permeability since its absence results in a loss of barrier function specifically against small molecules.[192] Neurothelin, an extracellular MMP inducer, is upregulated during BBB maturation while plasmalemma vesicle associated protein (Plvap, also known as Meca-32) expression inversely correlates with BBB differentiation.[193–196]

The molecular mechanisms that instruct BBB maturation are only beginning to be unraveled. Since VEGF-A is known to induce/maintain fenestrations in ECs[178] and induce vascular

permeability (hence its original name "vascular permeability factor"[197]), it may have a less significant role during BBB maturation. It does however play a role during the vasculogenic phase of brain vessel development by functioning as an attractant signal for angioblasts to migrate in the cranial direction and position themselves around the neural tube.[185,198] In support of the rather negative role of VEGF-A during BBB establishment, the Src-suppressed C kinase substrate (SSeCKS or gravin) is induced by hypoxia in astrocytes and reduces VEGF-A expression while upregulating angiopoietin-1 (a vascular maturation factor[199]). This event may mark the transition of the angiogenic phase to the BBB maturation phase in brain vascular development.[200] Recently, Wnt signaling was shown to be involved in BBB formation by upregulating claudin3. Wnt signaling was active as early as E9.5, when the immature perineural vascular plexus is completed, was maintained throughout development, but then subsided in the adult stage, suggesting that Wnt signaling is a requisite for BBB ontogeny but less important for long-term BBB maintenance.[196] Wnt3a, but not Wnt5a, induced claudin3 expression, suggesting a differential effect of Wnt ligands. In addition to Wnt3a, Wnt7a and 9a also seem specifically important for BBB development, since their absence in mice leads to a selective defect in the vasculature of the central nervous system.[201,202] Given the effect of Wnt2 on LSECs (see above), one may hypothesize that Wnt ligands may have tissue-specific functions.[203] One of the powerful advantages of the zebrafish system is the opportunity to perform forward genetic screens by chemical induction of genome-wide random mutations. Such screens have identified mutations that compromise vascular integrity, some specifically in the brain (e.g., *bubblehead* and *redhead* corresponding to *βPix* and *Pak2a* [Ref.[123] and references therein]). Recently, the orphan receptor GPR124 was shown to be highly expressed on brain ECs and to cell-autonomously regulate expression of Glut1 and induction of other BBB properties.[204,205]

THE LYMPH VASCULAR SYSTEM

On the Origin of Lymph Vascular Endothelium

Like the blood vascular system, the lymphatic system is present in most organs, except for the central nervous system, BM, the uterine mucosa, cartilage, the epidermis, hair, nails, and cornea. New lymphatic vessels form in certain physiologic situations after birth (e.g., wound healing and pregnancy[206]) and under pathologic circumstances (e.g., cancer, inflammation). In addition to the lymphatic vessels, the lymphatic system in higher organisms also comprises lymphoid organs (which include the lymph nodes, tonsils, Peyer's patches, the spleen, and the thymus).[207] Like blood vessels, lymphatic vessels are of mesodermal origin. Since the beginning of the last century, there has been a debate concerning the precise nature of this mesodermal origin.[208,209] While the original descriptive studies based on ink injections and analysis of serial histologic sections did not decisively determine the way the lymphatic vessels arise, more sophisticated technology based on interspecies chimerism, the use of specific lymphatic endothelial cell (LEC) markers, transgenic mice, and genetic lineage tracing has presented more conclusive evidence for two different scenarios.

According to the first theory, LECs arise by transdifferentiation from venous ECs and then, after moving away from the vein, arrange themselves into the first lymphatic structures, the lymph sacs ("the centrifugal theory" proposed by Florence Sabin in 1909; Ref.[210]). Another scenario states that LECs arise as descendants from separate scattered mesenchymal precursors ("lymphangioblasts") which move toward the venous system and make contact with it later on ("the centripetal theory" proposed by Huntington and McClure in 1910; Ref.[211]). While both theories are not mutually exclusive, the relative importance of both mechanisms may however be different between species. Indeed, the contribution of lymphangioblasts to the lymphatic system in higher organisms (such as the mouse) may be much less important than in birds or frogs.[207,208,212,213] The existence of lymphangioblasts has been most elaborately shown in avian embryos, with several embryonic tissues harboring these cells, including the cardiac mesenchyme, the dermatomes, and the somites.[214–217] Interestingly, it was recently shown that the initial development of lymph nodes does not require the presence of lymph sacs; hence the origin of these lymphoid organs remains elusive.[218]

While nothing is known concerning the precise origin of and the molecular mechanisms underlying the differentiation of lymphangioblasts from their mesodermal precursors, recent studies have yielded insight into the molecular cascade that gives rise to the first LECs by transdifferentiation from venous ECs.[213,219,220] The earliest marker, LYVE-1, expressed by a subset of venous ECs in the anterior cardinal vein is already apparent around E8.5 in the mouse. Yet, the functional significance of this is unclear since mice lacking LYVE-1, alone or in conjunction with its homolog CD44, do not have a lymphatic defect.[221] Subsequently, these cells start to express Sox18, which supposedly makes them "competent" to respond to a lymphatic inductive signal. Sox18 can directly induce the expression of another transcription factor, Prox1, around E9.75, by binding to its promoter[222] (FIGURE 38.5). The signal that is responsible for the asymmetric expression of LYVE-1 and Sox18 remains to be determined. A number of recent studies in mice and small animal models have shown that COUP-TFII also induces and temporarily maintains Prox1 expression in LECs and therefore is absolutely required for lymphatic development.[223–225] COUP-TFII, by forming heterodimers with Prox1, can then codetermine the LEC-specific gene signature.[226,227] The ubiquitous expression of COUP-TFII in the entire cardinal vein makes it unlikely that this factor is involved in asymmetric LYVE-1/Sox18 expression. Recently, synectin was shown to regulate Sox18-driven lymphatic reprogramming *in vitro*, suggesting that it may have a role in the commitment phase, in addition to its involvement in lymphangiogenic sprouting later on (see below).[228] Finally, microRNAs (miR-181 and miR-31) may be involved in the regulation of Prox1 protein expression.[229,230]

Lymphangiogenesis

While the onset of Prox1 expression defines the commitment of the first LECs during development, many additional steps and accompanying molecular mediators are required to establish the first rudimentary lymphatic vascular network, which initially consists of a number of lymph sacs spread across the anterior–posterior axis[207,213,220,231,232] (FIGURE 38.5). Subsequently, lymphatic vessels arise from these structures and are further remodeled into a hierarchical network of initial lymphatics (capillaries), collectors, and larger ducts (the thoracic duct and the right lymphatic duct) that connect to the blood vascular

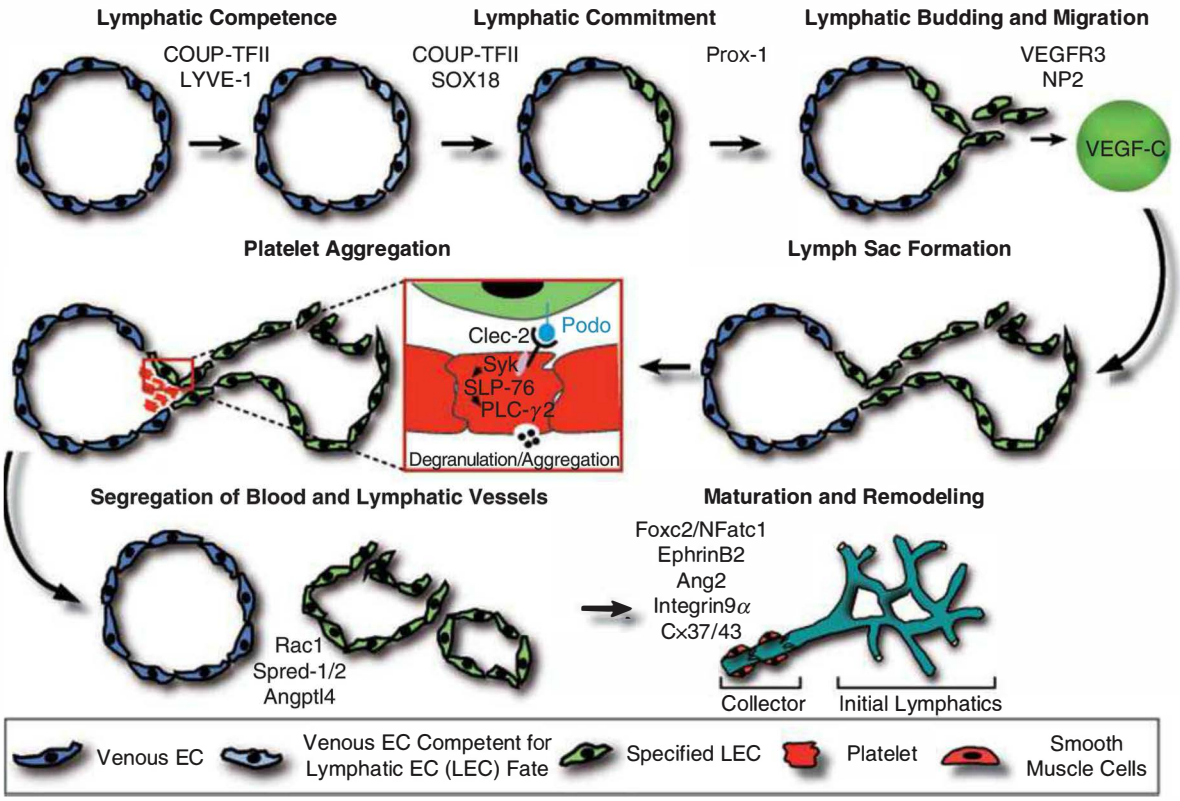

FIGURE 38.5 Lymphangiogenesis: stepwise toward a lymphatic tree. The most common route through which LECs arise in mammals is by transdifferentiation from venous ECs. First, a subset of ECs in the anterior cardinal vein become competent to respond to a lymphatic inductive signal. LYVE-1, as the earliest detectable LEC marker, was thought to be the "competence" factor; however, since mice deficient for LYVE-1 do not feature a lymphatic defect, other factors, perhaps Sox18 in cooperation with COUP-TFII, are involved in this step. Subsequently, Sox18 together with COUP-TFII induces the expression of Prox1 by which the cells become committed to an LEC fate. In response to lymphangiogenic signals (mainly VEGF-C), Prox1$^+$VEGFR3$^+$ LECs bud off from the vein and migrate outward. Thereafter, cells merge into primitive lymphatic structures, called "lymph sacs." To separate these newly formed lymphatic structures from the vein, platelets localized at the interface communicate through their Clec-2 receptor with podoplanin-expressing LECs. As a consequence, a molecular cascade is triggered resulting in platelet degranulation and aggregation. The platelet clot physically separates the two vessels, after which they segregate. In addition, other molecules have been shown to regulate lymphatic venous segregation, such as Rac1, Spreds, and Angptl4. Finally, the rudimentary lymphatic network is remodeled into a hierarchical system composed of bare initial lymphatics and SMC-coated collector vessels, the latter which have valves to prevent backflow of lymph. (Adapted from Tammela T, Alitalo K. Lymphangiogenesis: molecular mechanisms and future promise. *Cell* 2010;140(4):460–476; Schulte-Merker S, Sabine A, Petrova TV. Lymphatic vascular morphogenesis in development, physiology, and disease. *J Cell Biol* 2011;193(4):607–618.)

system at the level of the subclavian vein. A first step following LEC commitment is the acquisition of additional LEC markers, for example, VEGFR3 and neuropilin-2, and budding of Prox1$^+$ LECs from their venous mother vessel. This process, as well as the sprouting of LECs during the formation of lymph vessels from the lymph sacs, may be similar to angiogenic sprouting and likely requires destruction of the basement membrane, removal of SMC coating, and the induction of a tip cell phenotype. Such lymphangiogenic tip cells were already described at the beginning of the previous century.[208] A recent study has shown that, in analogy to angiogenic sprouting, lymphangiogenic sprouting (and hence the tip cell vs. stalk cell phenotype) is regulated by Notch signaling, since Notch induced a stalk cell phenotype in LECs.[233]

It is not clear whether the merging of two approaching lymphangiogenic tip cells is guided by macrophages (as is the case in angiogenic sprouting), since increased accumulation of macrophages seems to rather induce aberrant fusion of lymphatic and venous vessels.[234] Instead, macrophages may have a role in determining lymphatic vessel size by limiting LEC proliferation.[235] The "centrifugal" movement away from the vein occurs in response to VEGF-C. However, given the ubiquitous expression of this lymphangiogenic growth factor in the surrounding mesenchyme, additional guidance cues are likely required to guide the LECs to their precise locations.

Intriguingly, in zebrafish, the first functional lymphatic trunk vessels, that is, the thoracic duct and the dorsal longitudinal lymph vessel, are formed when the PCV-derived parachordal

lymphangioblasts move ventrally and dorsally, respectively. These lymphangioblasts arise by secondary sprouting from the PCV, a process that was specifically affected in the absence of synectin.[228] The ventral or dorsal lymphangioblast movement seems to require signals from the arterial vessels, since the lymphangioblasts systematically follow the arterial ISVs to reach their final destination.[142] As for sprouting, this guidance also required Notch signaling.[236] In addition, environmental signals, such as Ccbe1, a secreted collagen and calcium-binding EGF-domain-1 protein, were also necessary for correct lymphatic sprouting.[141] "Classical" guidance signals for blood vessels and nerves may also be used by LECs for path finding and sprouting. Recent studies in mice have revealed a role for ephrinB2 in LEC sprouting by regulating VEGFR3 internalization and signaling.[108] Furthermore, Robo1 is expressed by LECs and was found to mediate LEC migration and tube formation upon Slit2 stimulation.[237] Finally, netrin4 stimulated LEC proliferation, migration, adhesion, and tube formation, through a yet unidentified receptor.[238]

A next crucial step is the separation of the nascent lymphatic structures from the vein (FIGURE 38.5). While the involvement of hematopoietic cells was already demonstrated 8 years ago, it was only last year that the identity of the responsible hematopoietic cells was revealed.[239,240] LECs, which by now have acquired podoplanin expression, interact with the corresponding Clec-2 receptor on platelets located at the border of the budding lymphatic vessel. This interaction results in the activation of a signaling cascade involving Syk, Slp-76, and Plcγ-2 leading to platelet aggregation. The aggregate seals off the vein from the lymphatic vessel causing them to separate. Meis-1 that controls thrombopoiesis also is required for venolymphatic separation.[241] In certain organs, appropriate blood vascular-lymphatic segregation requires specific signals, such as fasting-induced adipose factor (fiaf or Angptl4); however, the underlying signaling mechanism remains to be determined.[242]

Further maturation of the lymphatic network involves the specification of the proximal blind-ending initial lymphatic vessels (capillaries) from the distal collector vessels (FIGURE 38.5). While the former lack a continuous basement membrane, pericyte/SMC coating, and intraluminal valves, collector vessels have all these three features. In addition, the junctional organization between LECs of these two different segments of the lymphatic vasculature is distinct: initial lymphatic vessel LECs feature button-like junctions optimal for fluid absorption, while the collector vessel LECs are joined by zipper-like junctions that support better their transport function.[243,244] The functional and morphologic differences between LECs in collectors versus capillaries are reflected by a differential expression profile (with collector LECs expressing more VEGFR2, ephrinB2 and capillary LECs expressing more LYVE-1, VEGFR3, neuropilin-2, Cox1/2, eNOS, and iNOS).[209,245] The molecular mechanisms underlying this functional, molecular, and morphologic heterogeneity have only recently been revealed (reviewed in Refs.[213,220,240]). Here, we only highlight a number of them showing that many are common to blood vascular development.

In Foxc2$^{-/-}$ mice, collecting lymphatics lack valves and the capillaries wrongfully acquire an SMC coat and basement membrane.[246] Upon VEGF-C stimulation, the nuclear factor of activated T-cell c1 transcription factor synergizes with Foxc2 for regulating lymphatic maturation.[247] The PDZ domain of ephrinB2 is also crucial for lymphatic maturation since deletion of this domain aborts valve formation and induces collector hyperplasia.[248] Angiopoietin-2 is important for SMC recruitment[249] while in the absence or upon lower expression of Tie1, an angiopoietin-2 receptor, there is an abnormal remodeling/patterning of the lymph sacs and a breach in lymphatic vessel integrity due to LEC apoptosis.[250,251] Furthermore, lymphatic remodeling defects have also been observed in Akt1$^{-/-}$ mice and in PI3 kinase signaling–deficient mice, perhaps because these pathways are acting downstream of angiopoietin-2 receptor Tie2 signaling.[252–254] Lymphatic valve formation is also regulated by integrin9α and its ligand fibronectin containing the EIIIA domain.[255] In the absence of integrin9α, the valve leaflets cannot be elongated, leading to the formation of ring-like constrictions that do not prevent lymph backflow. Finally, connexin-37 and -43 are potential targets of Foxc2 and are highly expressed on valvular LECs, and their single or combined absence leads to lymphatic valve disruption in lymphatic collectors.[256]

Establishment of Organ-Specific Lymphvascular Beds

While remarkable differences concerning the blood microvasculature in different organs have been elaborately documented, and in contrast to the rapidly emerging notion that there is clearly morphologic, functional, and molecular differences among ECs in collector versus initial vessels, few studies have considered the possibility that the same heterogeneity may also exist among lymphatic microvessels in different organs.[257–259] Vascular bed–restricted lymphatic phenotypes occurring in knockout mice lends further support for such heterogeneity.[260] Even less is known about how these lymphatic vessel networks develop in order to adopt their final organ-specific architecture. Since the lymphatic system needs to balance the amount of ISF that leaks out from the blood vessels, the absence of lymphatic vessels within certain vascularized tissues, such as the brain and the BM, is unexpected. Like we did for the blood vasculature, here we discuss the heart, liver, and brain as examples to document interorgan differences in the lymphatic system.

The Heart

In the adult heart, there are three major lymphatic plexi, a subendocardial, a myocardial, and a subepicardial plexus.[261] The two main lymphatic collectors run closely alongside the subepicardial branches of the coronary arteries.[262] A recent study in the mouse reveals that the first LYVE-1$^+$ cells appear around E11 scattered in the subepicardium and the trabecular myocardium with a positive gradient from the base to the apex of the heart.[263] In addition, LYVE-1 expression was also found at the venous pole of the heart in the proximal part of several early systemic veins (e.g., the left and right cranial caval vein and the pulmonary vein). From E14.5 on, these scattered cells started to merge into lumenizing tubules extending in the apical direction, and the first collecting lymphatic vessel could be observed running cranially on the surface of the coronary sinus. In many cases, scattered LECs had an "arborized" appearance reminiscent of nerve outgrowths and vessel sprouts coming of the primitive tubular plexus were frequently observed. Around birth, the heart contained two main drainage areas, one collecting lymph from the left, another from the right ventricle, and two major collectors were apparent. Interestingly, some endocardial cells also stained for LYVE-1. Partial SMC coverage was seen around E17.5 in LYVE-1$^+$ vessels in the aortopulmonary sulcus and, in

accordance with the human heart,[264] maturation and expansion of the lymphatic vasculature continued at least until 4 weeks after birth at which time scattered LYVE-1$^+$ cells were no longer present.[263] While the static LYVE-1$^+$ stainings do not deliver direct mechanistic evidence, they suggest that the heart lymphatic system arises by a combination of venous transdifferentiation and "lymphvasculogenesis," that is, the merging of lymphatic precursors scattered within and originating from the heart mesenchyme. Another study confirmed the presence of scattered LYVE1$^+$CD45$^+$ lymphangioblasts in the avian embryonic heart supporting the "lymphvasculogenesis" theory.[214] While according to one scenario, blood vascular EC precursors originate from the PEO (see above), this temporary structure does not deliver lymphangioblasts to the heart.[214] Given the significant overlap between LSECs and LECs, and in analogy to the presumed liver origin of the heart coronary ECs,[145] it has been hypothesized that lymphatic vessels of the heart may have a hepatic origin.[263] In the avian heart, ECs on the inner side of the semilunar heart valves express Prox1.[214] Since ECs from the endocardium give rise to valvular ECs (see above), it is tempting to speculate that the LYVE-1$^+$ cells observed in mouse endocardium are precursors of valvular ECs. Finally, while the lymphatic and blood vascular systems in general stay separate, except for a connection at the level of the thoracic duct and right lymphatic duct to the venous system, some lymphovenous anastomoses have been reported in human, porcine, and avian hearts.[214,265]

The Liver

For the liver, studies on the lymphatic network have been merely descriptive, and not much is known about when and how the lymph vessels arise in this organ.[266] Nevertheless, the liver produces a large amount of lymph, estimated to constitute as much as 25% to 50% of the total lymph that is drained by the thoracic duct.[267] Like in the heart, there are three major lymphatic plexi, a portal, a sublobular (absent in the rat[268]), and a superficial (or capsular) plexus, the portal plexus being the most significant lymph drain. Lymph is primarily produced in the sinusoids, from where it enters the space of Disse underneath the sinusoidal endothelium and flows through the channels traversing the limiting plate either independently of blood vessels or alongside the inlet venules to end up in the interstitial space of the portal tract.[266] As mentioned earlier, the ECs lining the blood vascular and the lymphatic microvessels in the liver, LSECs, and LECs, respectively, are very similar in their molecular characteristics.[169] As for the developmental origin of the latter, it has been suggested by quail/mouse chimera experiments that liver tissue has its own intrinsic capacity to give rise to VEGFR3$^+$ lymphatic vessels, suggesting a "lymphvasculogenic" scenario.[269]

The Brain

Since the brain (as well as the rest of the central nervous system) does not feature conventional lymphatic vessels, drainage of extracellular fluid requires alternative mechanisms. Interestingly, despite the absence of lymphatic vessels, VEGF-C, the main lymphangiogenic signal, is amply produced in the brain where it serves as a trophic factor for VEGFR3-expressing neural progenitors during development.[270,271] Unlike in other organs, two sources of extracellular fluid exist in the brain: the cerebrospinal fluid (CSF; produced by the choroidal plexus and serving as a liquid shield to protect the brain from traumatic damage) and the ISF (produced by brain capillaries). For drainage of CSF, two routes have been described: the arachnoid villi and granulations (projections of the arachnoid mater into the cranial venous system) or the extracranial lymphatic vessels (those in the vicinity of the olfactory bulb in the nasal submucosa, those in the dura mater, or those around cranial nerves); however, the relative importance of each of these routes is species dependent and has been debated.[272,273,274] Drainage of ISF follows a perivascular route which is largely separate from the routes described above for CSF drainage.[274] Tracer studies in animals have revealed this route that runs along the basement membrane of capillaries and along the basement membranes and between the SMCs of arteries to the extracranial cervical lymph nodes. Efficient drainage is dependent on an active propulsive force, most likely, the contraction of the arteries. Most intriguingly, such a route has also been revealed in the human brain by looking at the clearance of the "natural tracer" amyloid-β (Aβ) peptides (derived from the amyloid precursor protein) that accumulate in the brain tissue of Alzheimer disease patients. This may suggest that the acquired arterial stiffness in older individuals (which may reduce active ISF drainage) is responsible for the accumulation of Aβ peptides in Alzheimer patients.[274] Interestingly, impaired perivascular drainage of protein may not be limited to the brain and may occur at other sites leading to protein elimination failure arteriopathy. For instance, periarterial protein accumulation has been observed in patients with CADASIL in the skin and kidney in addition to the brain.[275]

CONCLUSIONS AND FUTURE PERSPECTIVES

The existence of the blood vascular and lymphatic systems has been recognized since ancient times; however, the first studies were mainly descriptive, thereby providing little information about cellular and molecular mechanisms that underlie their ontogeny and final appearance. Understanding these mechanisms is however an important mission since this may teach us how to design new rationalized therapies for the growing list of diseases that find their origin in defects in the blood or lymph vascular systems.[213,220,231,276] With the advent of increasingly more sophisticated technology, substantial progress is being made in identifying key molecules and mechanisms that can be therapeutically exploited. The first drugs to treat vascular-related diseases have already reached the clinic in the form of strategies to combat cancer by inhibiting blood (and lymphatic) vessel growth. Perhaps not surprisingly, these drugs are targeted against VEGF-A, a molecule that has an orchestrating role in almost every step during the formation of blood vessels. Nevertheless, another recurrent theme related to the availability of more advanced technologies is that they have often forced us to revisit and reconsider certain concepts that became dogmatic over the years. For instance, since Judah Folkman launched the concept in the 1970s that cancer cells need blood vessels for their growth, antitumor strategies have been focusing on how to reduce vessel growth in cancer. Now, it has become clear that it may be also relevant to "normalize" the tumor blood vasculature, that is, to change the phenotype of tumor blood vessels from a hyperactive to a quiescent one, thereby limiting tumor invasiveness/metastasis and making them more accessible for chemotherapeutic drug delivery.[129,277] Another important example is the realization that ECs are not all the same in every vascular bed and that more specific "tailored" therapies may be required. In addition, this EC heterogeneity can be exploited for vascular bed–specific drug delivery.[278]

At the same time, technologic refinement also has surfaced new concepts that likely will gain more attention in the near and distant future. Here we only highlight one example. A cross-control link between angiogenesis and metabolism has been recently demonstrated.[279,280,281] Similar to the previously described neurovascular link,[96] these two areas of research seem more closely related than previously anticipated. When there is sufficient oxygen, most cells use this for generating energy, as its reduction provides the largest free energy release per electron transfer. When there is shortage of oxygen, cells switch from aerobic to anaerobic metabolism (known as "the Pasteur effect"), which is a less efficient means of generating energy. The link with angiogenesis (which improves oxygen supply) ensures that oxygen consumption by oxidative metabolism remains in close balance with vascular oxygen supply. Certain metabolic regulators, such as PGC-1α, function by mounting an angiogenic response to improve oxygen reserves.[282] Other metabolic sensors, like PHD1, do not induce an angiogenic response but instead make cells tolerant against hypoxia by reprogramming oxygen consumption.[283] In ischemic (oxygen-deprived) limbs, the absence of PHD1 results in reduced oxygen consumption in muscle fibers, which protects them from oxidative stress. This may open perspectives for the use of PHD inhibitors in ischemic diseases.

In conclusion, here we summarized our current knowledge about the mechanisms behind blood/lymphatic vessel development which are quickly being unveiled thanks to sophisticated technology allowing research at incremental resolution. Obviously, this has been realized by many researchers and we apologize to those whose work we have not discussed here, but was nevertheless of central importance. By using technology as an "engine of progress," we will soon arrive at the next generation of (lymph)angiogenic therapies that will help millions of patients suffering from vascular disease.

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CHAPTER

39 Rheology and Vessel Wall Stress

LARRY V. MCINTIRE, JUAN M. JIMÉNEZ, SUZANNE G. ESKIN, AND PETER F. DAVIES

The classical conditions considered favorable for thrombosis and attributed to the triad of observations assembled by Virchow[1] in 1856 remain central to the modern interpretation of thrombotic mechanisms. They are (a) alterations in normal blood flow, particularly low flow or its cessation; (b) vessel wall injury, particularly of the protective anticoagulant endothelium; and (c) blood hypercoagulability. To promote most forms of pathological coagulation, blood-borne elements are delivered to a relatively fixed interface of the vessel wall surface. This places rheology (study of the flow of materials) and its principal components—molecular transport, shear stresses, and strain deformation of cells—as important physical properties in the regulation of coagulation. In this chapter, we first define and outline in context the fluid mechanical aspects of rheology and the experimental *in vitro* models that address mechanisms of interactions between blood and surfaces and vascular cells. We then consider how different natural vascular geometries *in vivo* promote spatial variations of rheology that can influence the phenotype of the endothelium with implications for its anticoagulation properties. Finally, we consider how similar rheologic principles apply at the microscale to flow around intracoronary stents to promote a local environment conducive to stent-related thrombosis.

Blood flow–induced shear stresses and pulsatile-induced vessel wall strain can considerably modulate blood kinetics and vascular cell function. Local mechanical forces can directly alter the metabolism and gene expression of vascular cells.[2,3,4] While the kinetics of thrombus formation depends on local flow conditions, flow-responsive cell receptors and ligands support cell–cell and cell–blood protein adhesion under arterial, venous, and pathologic shear conditions. Additionally, mass transport of cells and proteins to and from reactive surfaces, such as a damaged vessel wall, varies in different flow regimes. Understanding hemodynamics requires the application of the fundamentals of classical fluid mechanics and rheology coupled with deformable vessel mechanics. Theoretical models of the vascular system have been developed by applying the governing principles of fluid flow and mass transport. On the basis of these relatively simple models, dynamic experimental systems have been designed that simulate vascular shear conditions. These systems are valuable tools for investigating the molecular mechanisms supporting vascular processes important to thrombosis under flow, as seen *in vivo*.

BLOOD FLUID DYNAMICS

Principles of Rheology

Physical blood flow characteristics have long been considered to be as important to vascular function as are the blood vessel itself and the circulating components. A simple, but relevant, model of the vascular system may be developed by considering the steady, pressure-driven laminar flow of an incompressible newtonian fluid through a long cylindrical tube with a constant cross-sectional area.[5–8] These conditions yield an axisymmetric flow field that can be described by the radial position (r), the mean fluid velocity (v), and the vessel radius (R):

$$v_z = 2(v)\left[1-\left(\frac{r}{R}\right)^2\right] \tag{39.1}$$

As suggested by Equation 39.1, the axial velocity, v_z, reaches a maximum at the center of the tube where r is equal to zero (see FIGURE 39.1). Moving away from this center position, velocity decreases as the wall of the tube is approached. The fluid is assumed not to slip along the wall; that is, the fluid velocity at the wall is assumed to be zero.[6] The presence of maximum velocity at the center and zero at the wall yields a velocity gradient called *Shear*, which refers to the relative motion of the different fluid planes in the velocity profile. The shear rate (γ) is a measure of the velocity gradient and indicates the rate at which fluid layers are sliding past one another.

$$\gamma = \frac{dv_z}{dr} = -\frac{4(v)r}{R^2} \tag{39.2}$$

The units of the shear rate are inverse time, commonly reported as inverse seconds. Although the fluid velocity is greatest at the center of the tube, the velocity gradient, and therefore the shear rate, is zero there. Conversely, at the wall of the tube, the velocity gradient and therefore the shear rate reach a maximum (FIGURE 39.1), but the actual velocity is zero. Important to the rheology of the vascular system is the shear rate at the wall of the tube ($r = R$), which corresponds to the shear rate at the vessel surface (γ_w):

$$\gamma_w = -4v/R \tag{39.3}$$

The mean fluid velocity, (v_z), may be expressed in terms of physical quantities as in Equation 39.4:

$$(v) = \frac{Q}{\pi R^2} \tag{39.4}$$

where Q is the volumetric flow rate. Substituting Equation 39.4 into the wall shear rate equation (Equation 39.3) leads to a more practical expression of γ_w:

$$\gamma_w = -\frac{4Q}{\pi R^3} \tag{39.5}$$

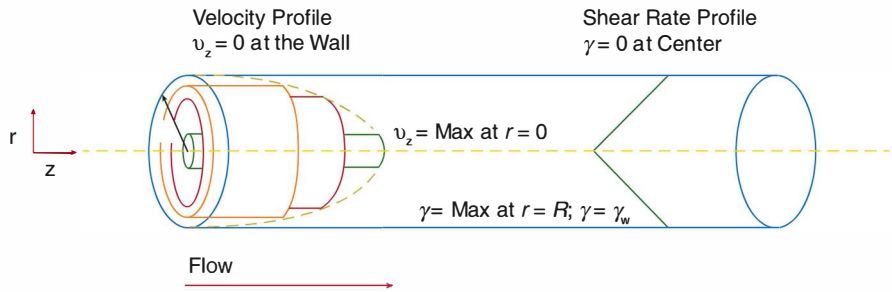


FIGURE 39.1 Velocity (v_z) and shear rate (γ) profiles produced by steady, laminar flow of an incompressible, newtonian fluid through a long cylindrical tube with a constant cross-sectional area. Shear refers to the relative motion of the different fluid planes in the velocity profile. The shear rate indicates how fast the fluid layers are sliding past one another. The fluid velocity is greatest at the center of the tube while the shear rate is zero. Conversely, at the wall of the tube, the shear rate (γ_w) reaches a maximum and the fluid velocity is zero. r, radial position; R, vessel radius. (Modified from Alevriadou BR, McIntire LV. Rheology. In: Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage*. Boston, MA: Blackwell, 1994:369–381; Goldsmith HL, Turitto VT. Rheological aspects of thrombosis and haemostasis: basic principles and applications. ICTH9 Report—Subcommittee on Rheology of the International Committee on Thrombosis and Haemostasis. *Thromb Haemost* 1986;55:415–435.)

As predicted from the range of vessel sizes and volumetric blood flow rates, the wall shear rates corresponding to the different regions of the vascular system can vary greatly (see Table 39.1).

In addition to shear rate, the fluid *shear stress* is commonly used to describe rheologic conditions in the vascular system. Shear stress is a measure of the force per unit area exerted by the flowing blood as different fluid layers slide past one another. Shear stress is typically expressed in dynes per square centimeter (dyn per cm^2) or Pascals (1 Pa = 10 dyn per cm^2). For newtonian fluids, shear stress (τ_z) is directly proportional to both the viscosity of the fluid (μ) and the shear rate[7]:

$$\tau_z = -\mu \frac{dv_z}{dr} = \mu\left(\frac{4(v)r}{R^2}\right) \tag{39.6}$$

At the vessel wall, $r = R$, and Equation 39.6 becomes the equation for the wall shear stress (τ_w):

$$\tau_w = \mu\gamma_w = \mu\left(\frac{4Q}{\pi R^3}\right) \tag{39.7}$$

Table 39.1

Shear rates in the vascular system

Vessel	Shear Rate (Per Second)
Large arteries	300–800
Veins	20–200
Arterioles	500–1,600
Stenotic vessels, stents	800–10,000

(Modified from Alevriadou BR, McIntire LV. Rheology. In: Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage*. Boston, MA: Blackwell, 1994:369–381.)

Fluids resist shear, and so pressure gradients along the tube length are necessary to produce the work required to overcome the frictional resistance to flow. In fully developed laminar flow, a balance exists between the force generated by the pressure gradient (acting across the luminal surface) and the force of shear resistance generated by viscous drag (acting on the vessel wall)[5]:

$$\frac{\Delta P}{\Delta z}\pi R^2 = 2\pi R\tau(R) \tag{39.8}$$

where ΔP is the pressure difference across a given tube length in the axial direction, Δz. Substituting for $\tau(R)$, which equals τ_w in Equation 39.7, yields the Hagen-Poiseuille equation that relates the pressure drop along a vessel to the volumetric flow rate[7]:

$$\frac{\Delta P}{\Delta z} = \frac{8\mu Q}{\pi R^4} \tag{39.9}$$

This model of the vascular system describes flow regimes where viscous forces rather than inertial forces dominate, a type of flow known as *Poiseuille flow*.[5–7] The Reynolds number (N_{Re}) is a dimensionless parameter that describes the relative importance of the inertial and viscous forces present under flow[6]:

$$N_{Re} = \frac{\text{inertial force}}{\text{viscous force}} = \frac{\rho(v)^2}{\left(\mu\frac{(v)}{2R}\right)} = \frac{2R(v)\rho}{\mu} \tag{39.10}$$

where ρ is the fluid density. When the viscous forces are dominant relative to the inertial forces, specifically when N_{Re} is less than approximately 2,100, the fluid flow is laminar and Equations 39.1 through 39.9 are valid descriptions of the velocity and shear profiles.[5,7] This is nearly always the case in the straight portions of blood vessels, with the possible exception of the largest vessels with high blood flow rates, such as the ascending aorta, and in severely stenotic atherosclerotic vessels.

When N_{Re} is >2,100, the steady, Poiseuille flow becomes unstable and is replaced by complex, turbulent flow regimes.[5–7] Under these conditions, the inertial forces required to accelerate and decelerate the fluid along its flow path dominate. The pulsatile motion of blood flow contributes to the inertial forces present, and a specific modified Reynolds number has been developed to determine when inertial forces resulting from the pulsations in the cardiac cycle must be considered. This dimensionless parameter is called the *Womersley alpha parameter*[5]:

$$\alpha = R\left(\frac{\omega\rho}{\mu}\right)^{1/2} \qquad (39.11)$$

where ω is the frequency of the oscillation in radians per second. Pulsation effects predominantly impact the flow fields in major arteries that have large diameters and are located proximal to the heart where oscillations have not been significantly dampened by vascular compliance.[5]

Adding to the complexity of flow in the vascular system is the elasticity of the blood vessels. As indicated by Equations 39.5 and 39.9, the shear profiles and the pressure gradients required to produce flow are highly sensitive to changes in the vessel radius. Although most vessels do not substantially dilate under the oscillatory conditions of normal blood flow, very large vessels (e.g., the aortic and the pulmonary arteries) experience radial changes during the different phases of the cardiac cycle.[5] Additionally, the velocity and shear profiles in a broad distribution of vessels are affected by events such as vasoconstriction and vessel stenosis that also lead to changes in vessel radius.

Applicability to Blood Vessels

The model of the blood vessel as a long cylindrical tube is applicable to much of the vascular system, and in the straight portions of vessels, turbulence due to a Reynolds number higher than 2,100 is rarely observed. Even in the presence of physiologic shear rates, however, complex vessel geometries may change the flow from simple Poiseuille to a more complex form while remaining laminar. This complexity can induce flow separation from the vessel wall, stagnant regions, transient flow reversal, and eddy formation.[7,8] In the vascular system, recirculating flow conditions increase the residence time between the vessel wall and circulating cells and proteins, leading to a potential increase in the local deposition of the components necessary for plaque and thrombi formation. FIGURE 39.2 outlines some common sites of vascular flow complexity, which include bifurcations, T-junctions, highly curved vessels (e.g., aortic arch), regions near valves, and vessels with abruptly changing diameters such as a stenosis.[5,9–12] These sites correspond with areas of increased thrombi and plaque formation.[13,14,15–19]

Nonnewtonian Characteristics

In addition to geometric and flow-rate influences, blood flow regimes vary because the viscosity of blood depends on the shear rate applied; that is, blood is not strictly a newtonian fluid.[20] At very low-shear rates, blood does not flow in response to the subthreshold levels of shear stress applied.[20–22] This property of having a yield stress is characteristic of elastic materials rather than newtonian fluids. After the yield stress is applied, blood flows and, as the shear rate is increased further, the effective viscosity of blood decreases (see FIGURE 39.3).[20,23,24,25] In contrast to whole blood, *plasma* is a newtonian fluid.[21] Therefore, the nonnewtonian properties of blood can be attributed to the presence of cells and cell crowding. All cells in the bloodstream experience shear from the flowing blood. If the cells were simply hard spheres, the shear would cause simple rotation and

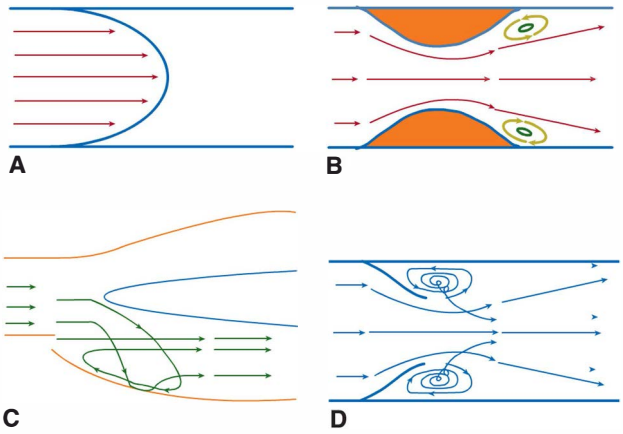


FIGURE 39.2 Streamlines in normal Poiseuille flow **(A)** and flow at sites of complex laminar flows, including vessels with abruptly changing diameters **(B)**, bifurcations **(C)**, and regions near valves **(D)**. (Modified from Karino T, Goldsmith HL, Motomiya M, et al. Flow patterns in vessels of simple and complex geometries. *Ann N Y Acad Sci* 1987;516:422–441.)

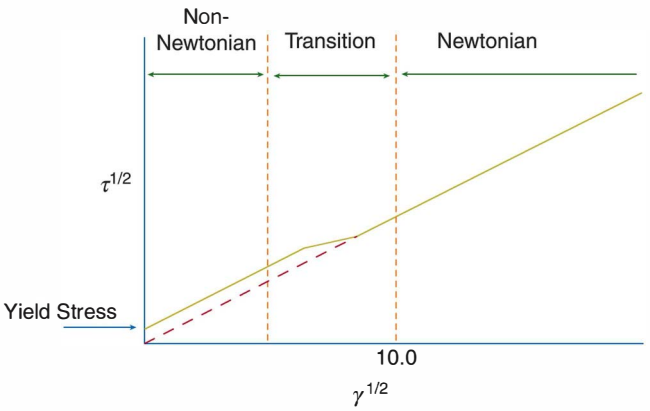


FIGURE 39.3 The nonnewtonian nature of blood. At very low shear rates (γ), blood will not flow in response to the subthreshold levels of shear stress (τ) applied. After the yield stress is reached, blood will flow, and as the shear rate is increased further, the viscosity of blood will decrease, as indicated by the decrease in slope with increasing shear rate. For newtonian fluids, the shear stress is directly proportional to the shear rate over the entire range of shear rates and no yield stress is present. Blood behaves as a newtonian fluid at shear rates >100 to 200/s. (Modified from Merrill EW. Rheology of blood. *Physiol Rev* 1969;49:863–888.)

translation.[26,27] Owing to the viscoelastic nature of cells, however, shear can twist, stretch, or compress the cells causing deformations.[26,28] Red blood cells, constituting 99% of the blood cellular volume, contribute significantly to the nonnewtonian nature of blood. At low shear rates (<50/s), fibrinogen mediates red cell aggregation into chains called *rouleaux*.[5,20,29] As the shear rate is increased, the rouleaux breaks down and the effective blood viscosity decreases.[25,30,31] At shear rates >20/s, red cells deform and align in the direction of flow.[32,33] Deformations on single cells in dilute suspensions lead to cell migration toward the center of the flow stream[32,33] due to the Magnus effect. However, in concentrated suspensions, crowding restricts the rotation and translational movements of the red cells and increases the number of red blood cell collisions. These crowding effects drive the cells toward the wall and offset the migration of deformable particles to the vessel center.[32] In vessels with diameters larger than 100 μm, the flow of a suspension with a physiologic concentration of red cells will result in a cell-free layer of approximately 4 μm at the wall.[5,32] Red cell crowding and collisions also complicate the flow paths of circulating white blood cells, platelets, and large plasma proteins.[32,33] Additionally, under physiologic red cell concentrations, the velocity profile is no longer parabolic as in Poiseuille flow, but becomes blunted at the leading edge.[5,32] Despite all these limitations, blood can normally be considered a homogeneous, newtonian fluid for shear rates >100 to 200/s.[5,20] At these shear rates, the viscosity of blood remains relatively constant and is determined primarily by the hematocrit, temperature, and the concentration of fibrinogen and other proteins.[20]

Blood Cell Rolling and Adhesion

The shear forces due to flowing blood also influence the interactions between the vascular wall and adherent cells. The shear force and torque that a cell experiences when it is tethered to a surface depend on the cell size, the fluid viscosity, and the shear rate.[34–36] At higher shear forces, stronger cell–surface bonds are required to support firm cell adhesion. If the energy from shear in the bloodstream is high enough to deform the cell membrane and overcome the adhesive bond strength, cells will not adhere firmly to the wall.[36] Under these conditions, rapid cell–surface bond formation may support transient adhesion, or rolling, on the vascular surface (see FIGURE 39.4A). Cells that constitute the vascular wall itself, such as endothelial cells and smooth muscle cells, also experience tangential shear stress when exposed to flowing blood. Additionally, these cells may experience circumferential stress resulting from deformations caused by expansion and contraction of the blood vessel as well as compressive stress resulting from hydrodynamic pressure (FIGURE 39.4B).[3,4]

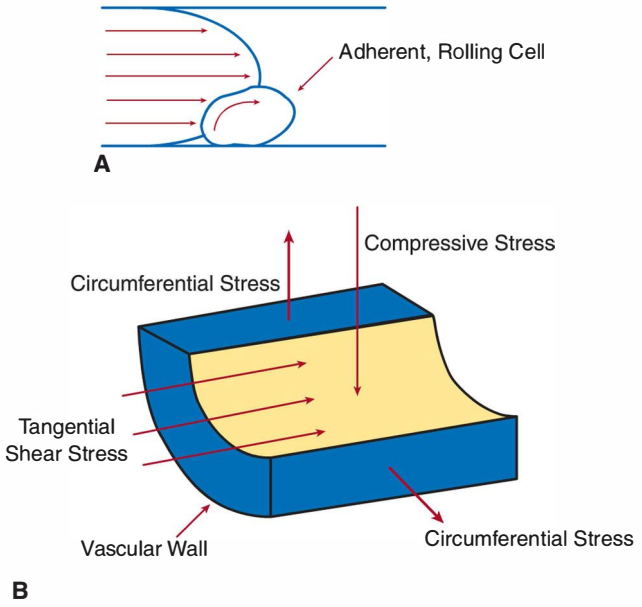


FIGURE 39.4 **A**: Shear forces acting on an adherent, rolling cell in a flow stream. **B**: Stresses acting on cells that constitute the vascular wall, such as endothelial cells and smooth muscle cells. Tangential shear stress results from the flow of blood over the vascular wall. Circumferential stress results from deformations caused by the expansion and contraction of the blood vessel, whereas compressive stress results from hydrodynamic pressure. (Modified from Papadaki M, Eskin SG. Effects of fluid shear stress on gene regulation of vascular cells. *Biotechnol Prog* 1997;13:209–221.)

BLOOD TRANSPORT PROCESSES

Diffusion and Convection

The primary function of blood is to transport materials to and from the organs, thereby sustaining life. Although vital, this transport may also augment pathological states such as thrombosis and atherosclerosis. The transport of materials occurs by two processes: diffusion and convection. *Diffusion* is the movement of material relative to the average fluid motion, irrespective of whether the fluid is flowing or stationary. Diffusion is random and results from Brownian motion of the particles. *Convection* is the movement of materials with the fluid and results from flow. Diffusion of materials is a very slow process in liquids, whereas convection can be very rapid. If flow is present, convection forces will dominate the mass transfer in blood, and diffusion effects will be negligible in the directions of flow. The *Peclet number* (N_{Pe}) is a dimensionless parameter that describes the relative importance of convection and diffusion in a given fluid stream[7,37]:

$$N_{Pe} = \frac{\text{convection effects}}{\text{diffusion effects}} = \frac{(v)2R}{D} \qquad (39.12)$$

where D is the molecular diffusion coefficient. Large Peclet numbers correspond to fluid flow where convection dominates, whereas for small Peclet numbers, diffusion is the dominant method of mass transport.

Considering again the model of the vascular system using Poiseuille flow of a newtonian fluid through a cylindrical tube of constant cross-sectional area, convection moves fluid along streamlines parallel to the wall and, therefore, the effects of diffusion are negligible in the axial direction. In the radial direction, convection does not occur and mass transfer is due solely to diffusion. The rate of mass transfer is proportional to the product of the molecular diffusivity and the local concentration gradient. Under these conditions, radial diffusion is required to transport material to the wall, and the steady state mass balance

at any point in the tube for a single species reduces to (neglecting homogeneous reactions):

$$v_z \frac{\partial C}{\partial z} = \frac{1\partial}{r\partial r}\left(rD\frac{\partial C}{\partial r}\right) \tag{39.13}$$

where C is the concentration of the species in the bulk fluid and D is the effective diffusion coefficient.[8,38] When turbulence, which generates an effective radial convection that directly pushes cells and soluble proteins to the wall (*cf* FIGURE 39.2), is present, Equation 39.13 does not apply. Turbulence and complex laminar flows in the vascular system most commonly result from complex vessel geometries or high flow rates, although blood pulsations and vessel elasticity may also contribute to the development of secondary flows. Independent of its origin, any radial transport present greatly enhances the transfer of material from the bloodstream to the wall of the vessel.

Cell and Protein Transport

Mass transfer processes are also significantly affected by the presence of red blood cells, even in steady, laminar flow regimes where blood can be considered newtonian. Because red cells deform in response to shear, the cells align in the direction of flow and migrate to the center of the flow stream. This effect is less pronounced in blood with a normal hematocrit flowing at high shear rates, but even under these physiologic conditions, a thin red cell–free layer exists at the wall. Radial mixing of circulating platelets, white blood cells, and large protein components occurs as these components are pushed to the vessel surface by red cell crowding in the vessel center. Increasing hematocrit increases the frequency of platelet, white cell, and protein collisions with the vessel wall.[5] Relative to Brownian motion alone, movements by red cells can increase the diffusivity of platelets by two to three orders of magnitude.[39] The diffusion of platelets in response to red cells has been quantified and is a function of the shear rate[37–39]:

$$D = b\gamma^n \tag{39.14}$$

where b is a proportionality constant and n is a power law constant.

On a reactive surface, such as a damaged vessel wall, both fluid dynamics and reaction kinetics influence mass transport. To solve the mass transfer equations, the mass and the momentum boundary layers must first be evaluated.[5,7] These boundary layers indicate the distance from the wall at which the concentration profile (mass boundary layer) or the velocity profile (momentum boundary layer) reaches maximum values. When the Peclet numbers are large, indicating convection forces are dominant, the height of the mass boundary layer is much smaller than the height of the momentum boundary layer. Under these conditions, it can be assumed that within the mass boundary layer the diffusion coefficient is essentially the diffusion coefficient at the wall and the velocity profile is a linear function of the distance from the wall. Applying these two assumptions, Equation 39.13, developed for steady state, laminar, Poiseuille flow through a cylindrical tube with constant cross-sectional area, can be solved using the following boundary conditions[5,7]:

$$C = C_\infty \text{ at } r \le \delta \tag{39.15}$$

$$-D\frac{\partial C}{\partial r} = kC \text{ at } r = R \tag{39.16}$$

where δ is the thickness of the boundary layer, C_∞ is the concentration of the homogeneous reactant outside the boundary layer, and k is the reaction rate constant. The boundary conditions also assume the reaction is first order and the surface does not become saturated or exhibit decreasing reactivity. Evaluation and substitution of these boundary conditions leads to the general solution for the steady state flux (j) of platelets at any point on the surface[4,37]:

$$j = \left[-D\frac{\partial C}{\partial r}\right]_{r=R} = \frac{C_\infty}{\underbrace{\frac{1}{k}}_{\text{reaction control}} + \frac{1.48}{\left(\frac{D^2\gamma_w}{2z}\right)^{1/3}}} = \frac{C_\infty}{\frac{1}{k} + \frac{1.48}{\left(\frac{D^2}{2z}\left(\frac{4(v)}{R}\right)\right)^{1/3}}} \tag{39.17}$$

As shown in Equation 39.17, the flux of platelets at any point on the vessel surface is determined by the wall shear rate of the fluid (γ_w), the axial position (z), the reactant concentration in the homogeneous phase outside the boundary layer (C_∞), the reaction rate constant (k), and the effective diffusion coefficient (D).

Platelet Transport

To investigate the thrombotic process, it is useful to determine the parameters controlling platelet transport to the surface under three different vascular conditions. The first condition occurs when platelets flow across an intact monolayer of endothelial cells. In this case, the surface is nonthrombogenic and $k \ll D$. Therefore, the flux of platelets at the vessel wall is limited by the reaction kinetics and no longer depends on the axial position or the shear rate:

$$j_z = kC_\infty \tag{39.18}$$

The second condition occurs when platelets flow across a mildly reactive surface, $k \simeq D$, and Equation 39.17 cannot be simplified. In this circumstance, in order to determine the flux of platelets at the wall, the equations of motion must be solved first. For simple cases, such as steady Poiseuille flow, these solutions can be found analytically. However, when more complex flow regimes are present, the equations of mass transfer must be determined simultaneously with the equations of motion, and an analytical solution is difficult.[6,8]

The third condition occurs when platelets flow across a very reactive surface (e.g., exposed subendothelium), $D \ll k$, and the flux is limited by the diffusion of platelets to the surface[7,38]:

$$j_z = 0.67C_\infty\left(\frac{D^2\gamma_w}{2z}\right)^{1/3} = 0.67C_\infty\left(\frac{D^2}{2z}\left(\frac{4(v)}{R}\right)\right)^{1/3} \tag{39.19}$$

In this case, flux depends on the shear rate, the axial position, and the effective diffusion coefficient. As shown in Equation 39.14, the effective diffusion coefficient of platelets in flowing blood is a function of the shear rate. This indicates that for a given increase in shear rate, platelet flux at the vessel wall will increase by more than $\gamma_w^{1/3}$. The inverse relation between the axial position and the platelet flux is compounded by the depletion of platelets in the mass boundary layer as a result of upstream thrombi growth.[40] In addition to platelets, circulating proteins and other soluble compounds that support platelet adhesion and growth of thrombi may become depleted in the mass boundary layer. Platelets partially offset the decrease in the delivery of soluble blood components by secreting activating factors and other necessary compounds that then accumulate downstream of the growing thrombi.[41] Despite the presence of these secreted components, the rate of thrombus growth on the thrombogenic components of the subendothelial matrix is decreased in the axial direction.[40]

MODELS OF THROMBOSIS UNDER FLOW

Thrombosis in the vascular system may occur in the bulk fluid phase leading to circulating platelet aggregates or on the vessel wall leading to mural thrombus growth. To identify the molecular mechanisms supporting each type of thrombosis, the dynamic nature of the vascular system must be incorporated into experimental models. This requires the application of engineering principles to develop systems that are physiologically relevant to a number of conditions present in the vascular system.

Aggregometry

The platelet aggregometer was one of the first experimental systems developed to study thrombosis occurring in the bulk fluid phase.[42] In this system, aggregation in platelet-rich plasma can be monitored over time by measuring light scattering or particle counts. The observed platelet aggregation depends on the addition of chemical agonists to the platelet-rich plasma that induce platelet binding to fibrinogen or von Willebrand factor (vWF).[43–46] Agonists include substances present in the blood, such as adenosine diphosphate (ADP),[42] but nonphysiologic modulators, such as the antibiotic ristocetin[47] or the snake venom botrocetin,[44] also induce aggregation and have been used extensively to study thrombosis. Agitation is required for aggregation to occur, and, therefore, the system can be described as shear dependent. However, the shear produced by the aggregometer is low and cannot be quantified with certainty owing to the variable flow patterns produced in response to agitation.

Viscometry

In contrast to the aggregometer, rotational viscometers can produce constant and uniform shear stress on all cells and proteins in liquid suspensions, independent of the individual particle location.[6] The shear profiles in viscometer systems are well characterized and can be varied widely to match a number of physiologic and pathologic vascular conditions. Shear stresses high enough to directly induce platelet aggregation can be developed in viscometer systems, so that the addition of chemical agonists is not necessary.[48–52] In concentric, or Couette, viscometers, the sample to be investigated is placed between a stationary bob and a rotating outer cylindrical shell (see FIGURE 39.5A). In cone-and-plate viscometers, the sample of interest is placed between a rotating cone and a stationary platen (FIGURE 39.5B). To study arterial thrombosis, the rotating cone and stationary platen are separated by approximately 25 μm, and the cone angle ranges from 0.3 to 1.0 degree. Over the range of shear stresses used to investigate arterial thrombosis, blood viscosity is constant and

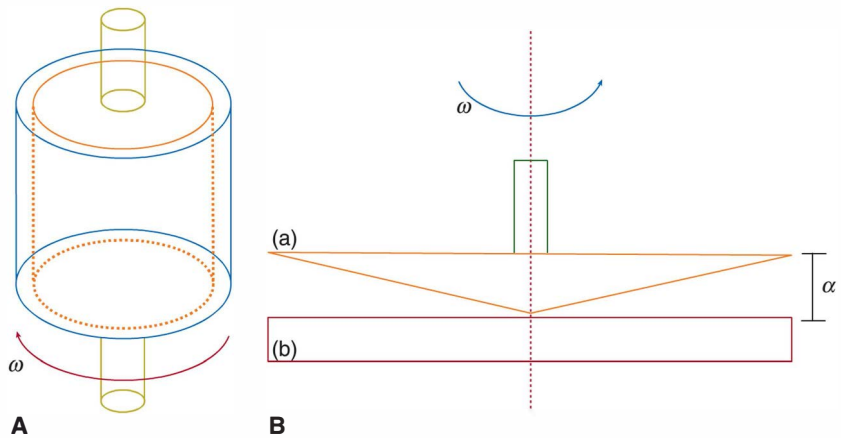


FIGURE 39.5 Schematic of a concentric, or Couette, viscometer **(A)** and cone-and-plate viscometer **(B)**. These rotational viscometers can produce a constant and uniform shear stress on cells and proteins in liquid suspensions, independent of the individual particle location. In a Couette viscometer, samples are placed between a stationary bob and a rotating outer cylindrical shell. In a cone-and-plate viscometer, samples are placed between a rotating cone (*a*) and a stationary plate (*b*). The cone angle (α) typically ranges from 0.3 to 1.0 degree. The shear stress that particles experience in a cone-and-plate viscometer depends on the cone angle, the rotation rate (ω), and the fluid viscosity (Equation 39.20). (Modified from Alevriadou BR, McIntire LV. Rheology. In: Loscalzo J, Schafer AI, eds. *Thrombosis and hemorrhage*. Boston, MA: Blackwell, 1994:369–381; Wang LN, Keller KH. Augmented transport of extracellular solutes in concentrated erythrocyte suspensions in Couette flow. *J Colloid Interface Sci* 1985;103:210–225.)

the shear stress is varied by changing the shear rate. The shear rate in a cone-and-plate viscometer depends on the cone angle (α) and the rotation rate of the cone (ω):

$$\tau = \mu\gamma = \frac{\mu\omega}{\alpha} \qquad (39.20)$$

The extent of shear-induced platelet aggregation can be determined by directly comparing the number of single platelets in samples before and after exposure to shear.[52,53,54] Additionally, the size of the platelet aggregates formed in response to shear can be quantified.[55,56]

Platelet aggregation in the bulk fluid phase can be investigated in viscometer systems using whole blood, platelet-rich plasma, or washed platelet suspensions. The presence of other cellular and protein components will affect platelet function and, therefore, whole blood is particularly useful for studying samples from patients receiving therapeutics,[57] recovering from a thrombotic event,[58] or both. However, the use of washed platelets is extremely valuable to isolate and investigate distinct molecular mechanisms supporting the thrombotic process. For example, by using washed platelet suspensions in physiologic concentrations, it has been shown that shear stress induces the binding of vWF to the platelet glycoprotein (GP) Ib and GP IIb/IIIa receptors.[59] More recently, using dilute suspensions of washed platelets, it has been determined that vWF binding to platelet GP Ib precedes binding to platelet GP IIb/IIIa.[60] The cone-and-plate viscometer system can also be used to study cell–surface interactions by immobilizing cells or proteins on the stationary plate. This method has been used to study the responses of smooth muscle cells and endothelial cells to shear including quantifying the released compounds and changes in gene expression.[61–66,67,152]

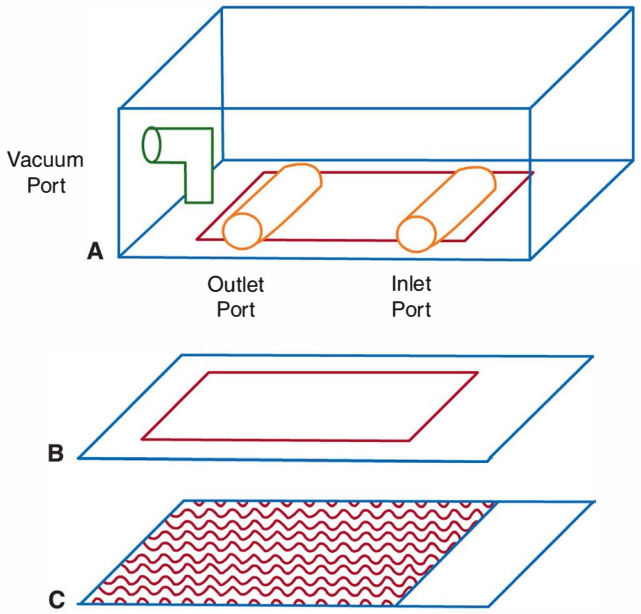


FIGURE 39.6 Schematic of a parallel plate flow chamber. The chamber consists of a polycarbonate slab **(A)**, a silicon gasket **(B)**, and a glass coverslip **(C)** held together by vacuum. The thickness of the silicon gasket determines the height of the gap between the coverslip and the polycarbonate slab. A syringe pump connected to the outlet port draws fluid across this gap through the chamber. The wall shear stress depends on the height of the gap, the width of the chamber, the fluid viscosity, and the flow rate through the chamber (Equation 39.21).

Flow Chambers

Cell–surface interactions have also been investigated through the use of flow chambers. In these systems, whole blood or a cell suspension is perfused through the chamber across a surface of immobilized cells or proteins. Parallel plate,[68–71] annular,[72–76] and tubular[65,77–80] flow chamber geometries have been developed. The parallel plate flow chamber, consisting of a polycarbonate slab, a silicon gasket, and a glass coverslip, can be used to illustrate the fundamental principles of these systems (see FIGURE 39.6). The glass coverslip forms the bottom side of the parallel plate flow chamber, and cells or proteins may be immobilized on this surface. The thickness of the silicon gasket determines the height of the gap between the coverslip and the polycarbonate slab. Fluid is pumped across this gap through the chamber. The wall shear stress in the system depends on the height of the gap (G), the width of the chamber (W), the fluid viscosity (μ), and the volumetric flow rate through the chamber (Q)[6,80]:

$$\tau_\omega = \frac{6Q\mu}{G^2W} \qquad (39.21)$$

In the range of shear stresses applied to investigate arterial thrombosis, plane Poiseuille flow is developed and the velocity and shear profiles are similar to those shown in FIGURE 39.1. The use of washed platelets or platelet-rich plasma in flow chambers is limited because the absence of red blood cells considerably changes the mass transfer of platelets to the surface. Therefore, to study platelet-mediated events in arterial thrombosis, whole blood is generally perfused through the chambers across glass coverslips coated with thrombogenic components of the subendothelial matrix, such as collagen or vWF. Significant insight into the molecular mechanisms supporting mural thrombosis, including the roles of fibrinogen and vWF and the platelet receptors GP Ib and GP IIb/IIIa, has been gained through the use of whole blood in flow chambers.[74–76,81–86] Distinct pathways in the thrombotic process are commonly investigated by the addition of blocking antibodies or by the use of patient blood that is deficient in an individual component. Specific interactions may also be studied by using mammalian cells transfected with only the receptor or ligand of interest.[87–89] The transfected cells may be in the fluid suspension, immobilized on the surface, or both. In addition to basic mechanism studies, flow chambers have been used to evaluate the effects of potential or current therapeutic compounds on mural thrombosis from patient samples under arterial shear conditions.[90,91]

Both arterial and venous shear conditions can be produced using flow chambers of the same geometry, with the only modification required being a change in the volumetric flow rate (Equation 39.21). A wide range of laminar shear conditions have been used to study the interactions of platelets and leukocytes with immobilized proteins, endothelial cells, or transfected

mammalian cells. Turbulent or complex laminar flow regimes, such as those in regions of stenoses or valves, may be produced by changing the geometry of the chamber or tube to resemble the complex geometry of interest. Under these conditions, the equations of Poiseuille flow (Equations 39.1 through 39.10, 39.21) no longer apply.

Measurement of Platelet Deposition by Imaging

Endpoint measurements of platelet deposition after perfusion of whole blood through a flow chamber may be measured either by fixing and staining the mural thrombi formed[81,92–95] or by using radiolabeled platelets reconstituted in blood.[15,92,94,96,97] Using these methods, the total number of platelets deposited may be evaluated by observing thrombi morphology with light microscopy or by radioactive measurements and imaging. Although these methods provide a snapshot of thrombi growth at a specific point, direct visualization of platelet deposition over time may be determined by combining epifluorescent microscopy and digital image processing.[68,71,86,98–100] In this method, platelets are fluorescently labeled before perfusion through the flow chamber. Platelets can be labeled by adding the fluorescent dye mepacrine[101] or fluorescent platelet monoclonal antibodies[102] directly to whole blood. During experiments, the flow chamber is mounted on an inverted-stage microscope equipped with an epifluorescent illumination attachment and a CCD camera connected to computers for image acquisition and processing. Whole blood is pumped through the chamber, and quantitative values for the number of platelets deposited and the total surface coverage over time are measured to calculate the real-time growth of thrombi.[68,98] The imaging can be extended to evaluate the surface interactions with larger cells such as leukocytes and transfected mammalian cells. These larger cells are phase bright, and fluorescent illumination is not necessary. Leukocytes and transfected mammalian cells in suspensions can be clearly distinguished by phase-contrast microscopy even if the suspensions are perfused over a cell monolayer immobilized on the surface. The mean velocity of rolling platelets or larger cells may be calculated by overlapping sequential digital images obtained and determining the distance the cells rolled during the period of the image acquisition.[103]

FLOW EFFECTS ON COAGULATION

The coagulation cascade is a complex series of linked enzymatic reactions that support the formation of mural thrombi by producing thrombin and fibrin at sites of vascular injury.[104,105] The reactions are catalyzed by complexes composed of circulating proteases and surface-associated protein cofactors. The key regulating enzyme complexes and a number of the reaction products form on phospholipid surfaces.[104,105,106] This surface requirement localizes reactions to certain elements in the subendothelial matrix, as well as membranes of endothelial cells (intact or damaged) and peripheral blood components such as platelets.[106,107] The formation of enzyme complexes depends on the delivery of the required circulating protease to the vessel wall where the protein cofactor is immobilized. Under noncomplex flow conditions, the protease may only reach the wall by radial diffusion. Increasing velocity, and therefore increasing shear, enhances the mass transfer of the protease to the surface.[79,106,108] Thrombin production measured in a phospholipid/cofactor-coated capillary tube clearly demonstrates the effects of mass transfer on the formation of surface-bound enzyme complexes in the coagulation cascade.[108] The production of thrombin is accelerated by the phospholipid-bound enzyme complex, prothrombinase, formed by circulating factor Xa associating with surface-bound factor Va. In the capillary tube system, the steady-state production level of thrombin can be limited by a low concentration of factor Va bound to the phospholipid surface. When factor Va is limiting, increasing the concentration of circulating factor Xa or increasing the shear does not change the final, steady-state thrombin production values. However, under these conditions, the time to reach the steady-state production values is reduced by increasing the concentration of circulating factor Xa or by increasing the shear rate. These results indicate that shear increases the formation of the prothrombinase complex by increasing the delivery of factor Xa to the surface. When the concentration of the circulating component rather than the surface component is limiting, increasing shear will lead to an increase in steady-state production values. In addition to increasing the supply of circulating components to the vessel surface, increasing shear will increase the removal of species produced at the wall but not bound to the vessel. This may explain the decrease in fibrin deposition on exposed subendothelium observed in response to increasing shear.[109] Alternatively, the decrease in fibrin deposition may be attributed to shear-induced secretion of tissue-type plasminogen activator (tPA) by endothelial cells upstream of the wound.[110]

MOLECULAR MECHANISMS OF CELL–SURFACE AND CELL–CELL ADHESION UNDER FLOW

Endothelial cells lining the blood vessels constitute a nonthrombogenic surface over which platelets flow during normal homeostasis. When the endothelium is compromised after angioplasty, atherosclerotic plaque rupture, or other vascular injury, mural thrombi form on the thrombogenic components of the exposed subendothelial matrix. Platelets are transported to the injury site by convection. If platelet transport were the only determinant of thrombi growth, increasing shear would always increase the deposition of platelets. The growth of thrombi on some surface components isolated from the subendothelium is decreased by increasing shear, however, suggesting that the bonds between platelets and these components either are not strong enough to withstand the increasing drag force on the platelet or do not have a fast enough on rate to fully form during the decreased contact time between the cell and the surface. Increasing shear also decreases the local concentration of activating factors produced at the vascular wall, potentially decreasing the total number of platelets deposited. A final explanation for observed inverse relation between shear and growth of thrombi on these surfaces is that shear may change the affinity of platelet–surface interactions by directly affecting the structure of the ligand or the platelet receptor. Dynamic experimental models have been used successfully to determine the molecular mechanisms supporting thrombus formation under the different shear conditions present in the vascular system.

Under arterial shear conditions, vWF is the principal mediator of both initial platelet adhesion to exposed subendothelium and subsequent platelet aggregation on the vessel wall, although fibrinogen and other proteins may also participate.[74–76,81,86,93,111–114]

vWF is well suited to support platelet adhesion because a single platelet may form multiple bonds to the repeating subunits within the vWF molecule.[115] Multiple bonds increase the strength of interaction, potentially allowing the platelet to remain attached to the vessel surface in the presence of high shear forces. Soluble vWF, released into the blood from the α-granules of platelets and from the Weibel-Palade bodies of endothelial cells, is rapidly insolubilized on exposed subendothelial matrix.[116] Endothelial cells may also secrete vWF directly into the subendothelium.[116] Unusually large forms of vWF, not found in plasma but present in platelets and endothelial cells, are most effective in promoting thrombus formation.[52] Further research is necessary to fully determine the primary components supporting the binding of soluble vWF to exposed subendothelial matrix, but it has been shown that collagen type I plays a role in the process.[117]

The platelet GP Ib/IX/V complex binds immobilized vWF under high shear conditions, mediating the initial step of platelet adhesion to the subendothelium of a damaged vessel.[86,93,111] This result has been demonstrated in flow chambers using patient blood deficient in GP Ib/IX/V (Bernard-Soulier syndrome)[93,111] and normal blood with added monoclonal GP Ib/IX/V antibodies.[86] Aggregation to the initial and subsequent layers of platelets on a damaged arterial wall is mediated by soluble vWF and possibly other proteins such as fibrinogen binding to GP IIb/IIIa complexes on adjacent platelets. This has been demonstrated in flow chambers using patient blood deficient in GP IIb/IIIa (Glanzmann thrombasthenia)[93,111] and normal blood with added monoclonal GP IIb/IIIa antibodies.[86,90,91] Adhesion of platelets to the subendothelial matrix under high shear conditions may also involve platelet GP IIb/IIIa.[93,118]

In contrast to thrombosis occurring in high shear conditions, mural thrombus formation under low shear conditions is supported mainly by fibrinogen–platelet rather than by vWF–platelet interactions.[119,120] Collagen VI[121] and fibrillin[122] have also been shown to support mural thrombosis under low shear conditions only. Adhesion on these surfaces is supported by the platelet GP IIb/IIIa complex, although other platelet receptors may play a role.[123] As with high shear conditions, the platelet GP IIb/IIIa complex is required for subsequent platelet aggregation.

Circulating platelet aggregates form in the presence of elevated shear conditions, such as are found in stenotic vessels, as a direct result of shear-induced platelet activation.[48–52] This high shear–induced platelet aggregation depends on vWF binding to GP Ib and GP IIb/IIIa receptors.[52,53,86,124] It has been established that calcium influx, platelet activation, and platelet GP Ib–vWF binding precede GP IIb/IIIa–vWF binding.[59,60,125,126] Platelet–vWF binding is minimal in the absence of shear. Currently it is unknown whether a change in vWF itself or a change in the platelet complexes enhances vWF–platelet binding under high shear conditions, although evidence suggests the change occurs on the platelet receptors.[52] Under low shear conditions (<12 dyn/cm^2), platelet–vWF binding becomes less important and platelet–fibrinogen binding predominately supports the observed shear-induced platelet aggregation.[127]

Leukocytes, which have been observed to accumulate in atherosclerotic plaques[128,129] and thrombotic clots[130,131] *in vivo*, may amplify the thrombotic process through the release of secretory products that activate platelets, promote vasoconstriction, aggravate endothelial damage, and increase local generation of thrombin and fibrin.[132–135] Additionally, the physical presence of leukocytes on mural thrombi may augment the coagulation cascade by providing an assembly site for the prothrombinase complex.[136,137] Monocytes may contribute further to the coagulation cascade because these cells express tissue factor (TF) after certain types of stimulation.[138] The effect of shear on leukocyte adhesion to the vascular surface has been characterized using parallel plate flow chambers.[103,139–147] Leukocyte rolling on stimulated, intact endothelium is mediated primarily by selectins, and rolling is optimal at wall shear stresses of 1 to 2 dyn per cm^2.[103,142] Under these conditions, firm adhesion (no rolling) of leukocytes to the stimulated endothelial cells requires the formation of integrin bonds.[141,142] At higher shear stresses, the number of rolling leukocytes decreases rapidly and at 10 dyn per cm^2 it is nearly zero.[103,142] In contrast, P-selectin expressed on activated platelet monolayers supports leukocyte rolling at higher levels of shear characteristic of the arterial system.[146] Circulating leukocyte–platelet aggregates may also contribute to the vascular response to injury and have been observed to increase in number after percutaneous transluminal coronary angioplasty[134] or cardiopulmonary bypass.[148,149] Shear enhances the formation of these circulating aggregates by activating platelets to express P-selectin.

It is of interest that unactivated platelets appear to be able to bind to activated endothelium, perhaps through an interaction of GP Ib and endothelial cell P-selectin.[88] In addition, GP Ib can utilize the I-domain of the leukocyte Mac-1 integrin as a ligand, providing a mechanism for platelet accumulation at the site of activated leukocyte adhesion (see[150] and FIGURE 39.7).

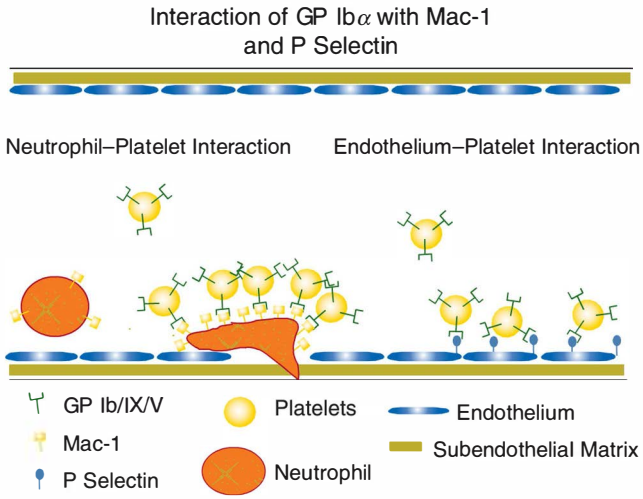


FIGURE 39.7 Additional ligands for platelet receptor GP Ib. Unactivated platelets can interact with activated endothelial cells through GP Ib/P-selectin interactions or with activated leukocytes at sites of inflammation via GP Ib/Mac-1 binding through the I-domain of the α chain (CD 11b) of Mac-1. This provides another possible direct strong connection between thrombosis and inflammation.

RHEOLOGIC MECHANOTRANSDUCTION MECHANISMS INFLUENCING VASCULAR WALL FUNCTION

The role of endothelial cells in the vascular system extends beyond providing a simple cover for the thrombogenic components of the subendothelial matrix. Instead, endothelial cells,

responding to both chemical and mechanical stimuli, express and secrete a wide variety of molecules that regulate thrombosis, fibrinolysis, coagulation reactions, vasoconstriction, leukocyte adhesion, and recruitment.[151] The mechanical stimuli acting on endothelial cells include tangential, circumferential, and compressive stresses (FIGURE 39.4B).[6,7] Circumferential stress is generated primarily by cyclic strain in compliant arterial vessels. Mechanotransduction in response to these stresses on and within endothelial cells are considered to respond both by activating signal transducers and by deforming the cytoskeletal structure to transmit forces to other locations throughout the cell.[152,153,154] Since intracellular mechano*transmission* is very fast[155,156] and focuses the strain to adhesion sites, junctional complexes and the nucleus, mechanotransduction can occur simultaneously at multiple cellular locations, and it is probable that they are interconnected and integrated within the cell, a decentralization model.[152] Mechanical stimuli, propagated through several cascade systems distributed at multiple subcellular sites, ultimately leads to altered gene and protein expression resulting in sustained functional responses (see FIGURE 39.8). There are many possible mechanisms for the initiation of mechanotransduction (conversion of deformation to a biochemical response), including force-induced conformational changes in membrane proteins, physical effects upon the lateral mobility of membrane molecules, direct force effects on ion channels, separation of assembled cell–cell oligomeric proteins at junctions, deformation of caveolar structures, and physical inhibition of integrin dynamics and clustering especially at the basal sites of cell adhesion to extracellular matrices.[152,154] Physical deformation may also displace local cofactors engaged in homeostatic regulation, including soluble and bound forms. Experimentally, parallel plate flow chambers, cone-and-plate viscometers, and other dynamic models have been used to determine the molecular mechanisms that translate mechanical stresses into cellular

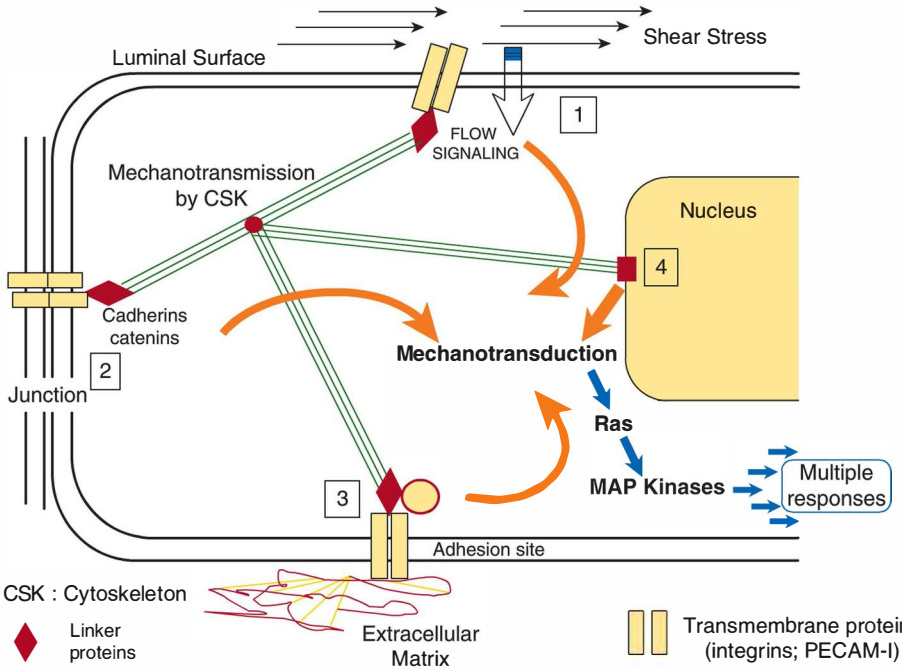


FIGURE 39.8 Decentralized model of endothelial mechanotransduction by shear stress. The cytoskeleton plays a central role in mechanotransmission of tension changes throughout the cell. Examples of direct signaling at the luminal surface (*1*), junctional signaling (*2*), and adhesion site signaling (*3*) are shown. Nuclear deformation also likely results in mechanosignaling (*4*). The locations are based on direct or indirect experimental evidence, are not mutually exclusive, and are likely interconnected.

At *1*, deformation of the luminal cell surface, possibly via the glycocalyx. Examples include localized activation of K^+, Na^+, and Cl^- ion channels, phospholipase activity leading to calcium signaling, G-protein activation, and caveolar signaling. At *2*, transmission of forces to the intercellular junction protein complexes via the cortical and/or filamentous cytoskeleton. Vascular Endothelial Growth Factor Receptor 2 (EGFR2) located at the luminal surface (site 1) or near the junction (site 2) may associate with VE-cadherin, b-catenin, and phosphatidylinositol 3-kinase to phosphorylate Akt and the primary transmembrane protein at this location, PECAM-1. At *3*, cytoskeletal force is transmitted to adhesion sites. Transmembrane integrins bound to extracellular matrix serve as a focus for deformation that results in autophosphorylation of focal adhesion kinase (FAK). FAK binds the SH2 domain of c-Src, a kinase family that phosphorylates paxillin and p130 Cas leading to integrin-dependent activation of MAPKs via Ras G-proteins. A second integrin-mediated pathway is via RhoA activation that profoundly influences actin assembly. At *4*, nuclear deformation/displacement may result in mechanotransduction possibly via lamins in the nuclear membrane. Other possible direct effects are on macromolecular conformation relevant to gene regulation. (Reprinted From Davies PF, Helmke BP. Endothelial mechanotransduction. In: Mofrad MRK, Kamm RD, eds. *Cellular mechanotransduction: diverse perspectives from molecules to tissue*. New York: Cambridge University Press, 2010:20–60, with permission.)

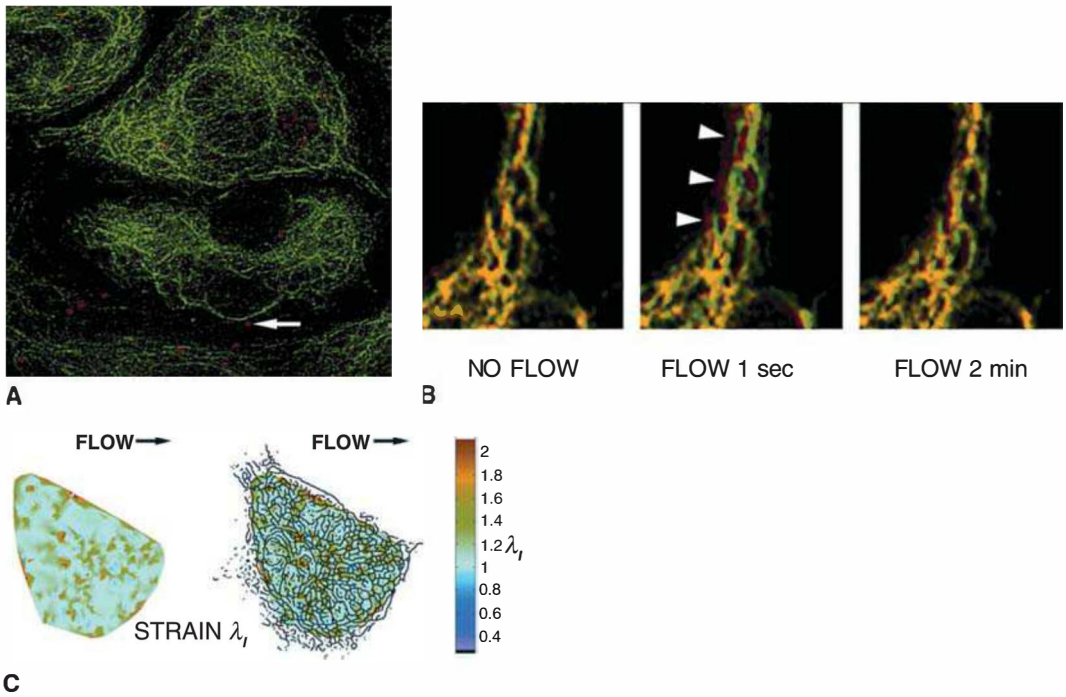


FIGURE 39.9 The cytoskeleton is instantaneously deformed by flow. **A**: Stable transfection of living confluent endothelial cells with green fluorescence protein (vimentin) permits the dynamics of intermediate filaments of the cytoskeleton to be measured. **B**: Intermediate filaments deform (red-to-green shift) within 1 second after imposition of flow over the monolayer. **C**: Calculation (*left image*) of the strain concentrations (yellow/red) in the basal plane of a confluent endothelial cell following exposure to flow. The distribution of the cytoskeleton in the same plane is also shown (*right image*). (Reprinted from Helmke BP, Rosen AB, Davies PF. Mapping mechanical strain of an endogenous cytoskeletal network in living endothelial cells. *Biophys J* 2003;84:2691–2699, with permission from Elsevier.)

responses. Both venous and arterial levels of shear stress and strain have been investigated, and there is now appropriate consideration of the complex flow waveforms encountered *in vivo* when designing *in vitro* experiments.[157]

The Central Role of the Cytoskeleton in Force Transmission

Flow deformation of a connected system under tension—adherent endothelial cells in a confluent monolayer—results in instantaneous transmission of the forces throughout the connected elements. The transmission conduit is principally the cytoskeletal fibers distributed throughout the cell body and the submembranous spectrin-like cortical cytoskeleton around the cell. Both systems provide rigidity, shape, and structure to the cell and are under tension. Demonstrations of flow-initiated intermediate filament displacement,[158] actin filament deformation,[159] and directed motion of mitochondria attached to microtubules[160] support the view that apical forces are transmitted to "remote" sites via cytoskeletal displacements. Interference with cytoskeletal structure inhibits flow responses as defined for various experimental systems.[161] Thus, as proposed in the decentralization model, the actual mechanotransduction event may occur not only at the site of fluid shear interaction with the cell but also at considerable subcellular distances (microns) from the apical surface. Furthermore, the deformation effects may extend to adjacent cells, communicated through cell junctional structures[158] as shown by live cell imaging of green fluorescent protein (GFP)-cytoskeletal dynamics under flow and calculation of strain concentrations at a limited number of subcellular sites[156] (FIGURE 39.9). Such studies also draw attention to the dynamic motion of the cytoskeleton consistent with continuous remodeling of adhesion sites[162] and junctions.[156]

Responses at the Cell Surface

Ion channels, G-protein–linked receptors, tyrosine kinase receptors, and integrins have been identified as mechanoreceptors, although it has not yet been determined if all of these receptors initiate the primary response, or whether some act downstream of additional mechanoreceptors. Mechanoresponsive ion channels include a nonselective stretch-responsive ion channel that depolarizes the cell on activation.[163] Additionally, at least three shear-responsive ion channels have been identified. Two of the shear-responsive ion channels, an HCO_3^-/Cl^- exchanger and an Na^+/H^+ exchanger, acidify the intracellular cytosol within minutes of endothelial cell exposure to shear.[164] The third shear-responsive ion channel, a potassium channel, becomes activated within 10 seconds of exposing endothelial cells to shear.[165,166] Activation of this potassium channel leads to hyperpolarization of the cell in contrast to the cell depolarization observed in response to cyclic strain. There is some evidence that this potassium channel is linked to a G-protein that elevates intracellular guanosine 3′,5′-cyclic monophosphate levels in response to shear.[167]

Functional expression studies suggest that Kir2.2 and Kir2.1 are primary determinants of endogenous K^+ conductance in HAECs under resting conditions and that Kir2.2 provides the dominant conductance by these channels.[168] They are also sensitive to the concentration of plasma membrane cholesterol, higher levels of which inhibit their function.[169] The presence of other mechanoresponsive G-protein–linked receptors is suggested by the shear-induced elevation of the levels of other signal transducers, such as inositol 1,4,5-trisphosphate (IP_3)[170,171] and cyclic adenosine 3′,5′-monophosphate (cAMP),[172] which are generated by the activation of G-proteins. Cyclic strain also elevates the level of the G-protein activation–dependent signal transducers, IP_3, and diacylglycerol.[173,174] Direct shear-induced G-protein activation has been observed within 1 second of endothelial cell exposure to shear.[175] This fast activation process requires no mechanoreceptor and may be due to shear effects on membrane fluidity.[176] The presence of tyrosine kinase mechanoreceptors is suggested by the shear-induced elevation of multiple protein kinases. The major protein kinases activated by shear stress are extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase (MAPK)/ERK. Protein kinase C is also responsive to both cyclic strain[177] and shear stress.[178] Integrins, transmembrane GPs that link the cytoskeleton and extracellular adhesion proteins, may also serve as mechanoreceptors[179] and transmit decentralized signals to the cell through the cytoskeleton as outlined below. Shear has been shown to enhance the integrin-mediated activation of the MAPK and ERK 1/2[179,181] and also enhances the integrin-mediated phosphorylation of the focal adhesion kinase (FAK), which may alter the cytoskeleton and lead to rearrangements of focal adhesion sites, eventually producing changes in the cell shape.[179,180,181] Cytoskeletal changes also regulate the gene expression of a number of proteins, including the urokinase-type plasminogen activator, which modulates the generation of plasmin and, therefore, affects blood clot dissolution.[182]

Transcription Factors

Changes in cellular pH, production of G-protein activation-dependent signal transducers, and stimulation of protein kinases may impact cellular responses by regulating transcription factors that activate or inhibit gene expression by binding to promoter regions within DNA. Two transcription factor families have been shown to be shear responsive: nuclear factor activator protein-1 (AP-1) and *Rel*-related nuclear factor κB (NFκB).[183] AP-1 can bind cAMP response element and tumor-promoting agent response element promoter regions of DNA. In response to shear, AP-1–binding activity increases fourfold in the first 20 minutes, then falls back to basal levels.[183] The shear-induced response of AP-1 is biphasic; however, and after 2 hours of shear exposure, the binding activity of AP-1 is increased substantially relative to both the basal level and the initial burst of activity. AP-1 has binding sites on the promoters of many proteins important to thrombosis and vascular wall function, including tPA, plasminogen activator inhibitor 1 (PAI-1), endothelin-1 (ET-1), monocyte chemotactic protein-1 (MCP-1), collagenase, intercellular adhesion molecule-1 (ICAM-1), transforming growth factor-β_1 (TGF-β_1), platelet-derived growth factor (PDGF)-A, and PDGF-B.[64,184,185] However, only MCP-1 has been shown to depend on shear-induced AP-1/DNA binding.[185] Binding of NFκB to DNA increases within 30 minutes of cell exposure to shear and reaches maximum levels of activity in 1 hour. Binding sites of NFκB have been identified on vascular cell adhesion molecule-1 (VCAM-1), E-selectin, interleukin (IL)-1, IL-6, IL-8, *c*-myc, and TF.[186] NFκB has also been shown to interact with a shear stress–responsive element (SSRE) in the promoter region in PDGF-B.[187] This SSRE is distinct from other DNA-binding sites of NFκB and is required for shear-induced elevation of PDGF-B messenger RNA levels in endothelial cells. Promoter regions with identical sequence homology to this SSRE are present on tPA, ICAM-1, and TGF-β_1, all of which are increased in response to shear.[61,187]

Of particular importance to thrombus formation is the increase in tPA messenger RNA level in response to arterial levels of shear.[110] Endothelial secretion of tPA increases by a factor of three after 4 to 6 hours of exposure to arterial, but not venous, levels of shear. In contrast, neither arterial nor venous shear levels increase the secretion of the prothrombotic compound, PAI-1. Further illustrating the selective response of endothelial cells to mechanical stimuli are the mechanisms that may support shear-induced vasodilation. Only minutes of arterial shear exposure are required to increase the production of prostacyclin (PGI_2), a potent platelet aggregation inhibitor and vasodilator.[66,188,189] Within 4 hours, the production of the vasoconstrictor and smooth muscle mitogen, ET-1, decreases.[190] Shear-induced vasodilation may also depend on nitric oxide (NO) production that increases with increasing shear.[64,191,192,193,194] The synthesis of several other biomolecules is regulated by shear, including prostaglandin $F_2\alpha$[195] and fibronectin.[196] The response of endothelial cells to cyclic strain differs from their response to shear. Physiologic levels of cyclic strain increase the secretion of PAI-1, whereas the secretion of tPA is unchanged.[197,198] Cyclic strain has also been shown to increase the secretion of both ET-1 and PGI_2 from endothelial cells.[197–199]

Integrin-Associated Mechanisms

Transmembrane integrins are well represented in regions of subcellular concentration of strain in endothelial cells subjected to flow change, and there is a burgeoning literature associated with this mechanism. Shyy and Chien[200] investigated the role of adhesion site integrins in mechanotransduction. They combined the results of endothelial flow studies with the data obtained from platelet activation and growth factor signaling (both being integrin mediated). In their proposed mechanism, flow activates integrins, increasing their avidity for the extracellular matrix (ECM). Integrin activation by many different stimuli is influenced by extracellular or intracellular factors, particularly small GTPases. If activation is blocked with RGD peptides or by monoclonal antibodies against integrin b subunits, typical downstream responses to flow/shear stress are inhibited, for example, MAPK phosphorylation and NFκB activation. One mechanism of involvement of integrins in flow mechanotransduction is proposed to be through the assembly of signaling complexes on the cytosolic side of the membrane where integrin-dependent autophosphorylation of FAK leads to its association with the SH2 domain of c-Src that in turn phosphorylates paxillin and p130 Cas.[201] These act as a scaffold to assemble C3G, a guanine nucleotide exchange factor for Ras G-protein family member Rap1, and adapter protein Crk that leads to the activation of MAPKs. There is spatial evidence (using GFP as a reporter) for the recruitment of activated FAK to new focal adhesion sites when endothelial cells (ECs) are subjected to shear stress, suggesting that the scaffold assembly is likely to be highly compartmentalized to portions of the plasma membrane.

A similar spatial relationship may occur in a second mechanism of integrin activation by shear stress in which the protein Shc is activated and binds Src family tyrosine kinases through SH2 binding.[202] Fyn is one such kinase which, together with its associated caveolar protein Cav-1, binds to Shc, phosphorylates it, and recruits an adaptor protein growth factor receptor–binding protein 2. This leads to activation of the Ras-MAPK pathway prominent in endothelial flow responses.

RhoA is also activated by shear stress through mechanisms that require integrin–ECM binding. Given the association of Rho with regulation of the cytoskeleton through Rho-associated kinase ROCK, and LIM kinase via cofilin,[203] it is likely that the flow-responsive adhesion site complexes involving integrins are intimately related to the binding of cytoskeletal elements at adhesion sites and cell junctions. Integrins are not restricted to the abluminal surface; flow activation via integrins may occur without the engagement of the ECM. Furthermore, it has also been proposed that nonintegrin mechanisms of activation of the Ras pathway occur by direct G-protein activation in the plasma membrane as a direct result of deformation of membrane lipids without cytoskeletal or integrin involvement.[204]

Transduction through Junctional Mechanosensors

Fujiwara et al.[205] first demonstrated the phosphorylation of the endothelial junction adhesion protein platelet EC adhesion molecule-1 (PECAM-1), its binding to SH2 tyrosine phosphatase, and the subsequent accumulation of signaling molecules at the junction in response to shear stress; they proposed PECAM-1 to be a mechanosensor. Shay-Salit et al.[206] demonstrated flow activation of vascular endothelial growth factor receptor 2 (VEGFR2) and its assembly with phosphorylated vascular EC cadherin (VE-cadherin), b-catenin, and PI3-K, leading to the phosphorylation of Akt-1. Building on these findings, Tzima et al.[202] reported that a mechanosensory complex involving junctional proteins regulates a subset of mechanotransduction pathways through integrins. In transfection studies of non-ECs, they showed that PECAM-1, VE-cadherin, and VEGFR2 are sufficient to transduce shear stress in the presence of an intact cytoskeleton and its associated proteins. Conversely, their selective inhibition or deletion in ECs inhibited important flow-related transduction; flow-induced activation of VEGFR2 did not occur in PECAM-1$^{-/-}$ and VE-cadherin$^{-/-}$ ECs nor did pathways that are dependent upon integrin activation. *In vivo* evidence in support of the mechanism was provided by local regions of endothelial activation (NFκB nuclear translocation) that correlated with disturbed hemodynamic sites in wildtype mice but were not activated in PECAM-1$^{-/-}$ mice.

The cell must either filter or integrate the spatially and temporally variable signaling events of mechanotransduction.[152] These mechanisms are not likely to be exclusive to endothelial cells.

Smooth Muscle

Vascular smooth muscle cells also produce and secrete biomolecules in response to mechanical stimuli. Like endothelial cells, vascular smooth muscle cells are continually exposed to circumferential and compressive stresses. Direct smooth muscle cell exposure to tangential shear stress results from deep vessel injuries produced by angioplasty or atherosclerotic plaque rupture. Even in the presence of an intact endothelium, transmural pressure gradients lead to interstitial fluid flow that continuously produces a shear stress on smooth muscle cells under normal physiologic conditions.[207] It has been found that either with[208,209] or without[210,211] an intact endothelium, increasing tangential shear stress reduces the proliferation of smooth muscle cells. Increasing shear also increases smooth muscle cell secretion of tPA, NO, prostaglandin E_2, PGI_2, PDGF, and basic fibroblast growth factor.[210–213] The shear-induced release of mitogens may partially inhibit the observed shear induced decrease in smooth muscle cell growth. Under dynamic conditions, however, it is more likely that mitogens released in high shear regions will accumulate in low shear regions downstream and potentiate the higher smooth muscle cell proliferation rates observed in regions of low shear. In addition to the secreted products discussed previously, in response to either shear or cyclic strain, smooth muscle cells release a factor, potentially carbon monoxide, which inhibits the ADP-induced aggregation of platelets.[64] Activated smooth muscle cells may also express VCAM-1 and ICAM-1 that support leukocyte–smooth muscle cell binding.[214–217] After angioplasty or atherosclerotic plaque rupture, expression of these surface molecules may recruit leukocytes to sites of vascular injury.[218] The shear-induced mechanisms that lead to smooth muscle cell expression and secretion of biomolecules remain largely undetermined, but cellular pH may play a role. It has been shown that shear induces alkalinization of smooth muscle cells mediated by an Na^+/H^+ exchanger.[219]

HEMOSTASIS-RELEVANT RHEOLOGIC EFFECTS UPON ENDOTHELIAL PHENOTYPE *IN VIVO*

The endothelium modulates coagulation and is normally antithrombotic through its heparin-like surface properties and its synthesis and release of both NO to inhibit platelet adhesion and of prostacyclin which inhibits platelet aggregation. Its surface ectonucleotidase enzymes also rapidly deplete ADP released by activated platelets in the early stages of aggregation, thereby reducing the recruitment and degranulation of other platelets. The disruption of endothelium is associated with many acute thrombotic events in arteries. Exposed subcellular matrix, particularly collagen, interacts with platelet GpVI, vWF (factor VIII; vWF), and Gp1b-V-IX complex, leading to platelet adhesion and activation. Platelet activation also occurs through ADP binding to P2Y receptors, leading to aggregation. Rheology contributes to platelet activation via two significant mechanisms. First, platelets passing through high shear regions in blood flow (and high gradients of shear) may be exposed to levels that physically promote activation,[220] and second, activated platelets captured within a vortex of flow separation have relatively long residence times that favor sustained interactions with vascular wall matrix and procoagulant bulk phase molecules also trapped there.[220,221]

Platelet Activation in Branches, Stenoses, and Devices

As platelets pass through complex shear fields associated with normal vessel geometries such as branching and curves, or traverse pathological stenoses,[220] or flow around intravascular

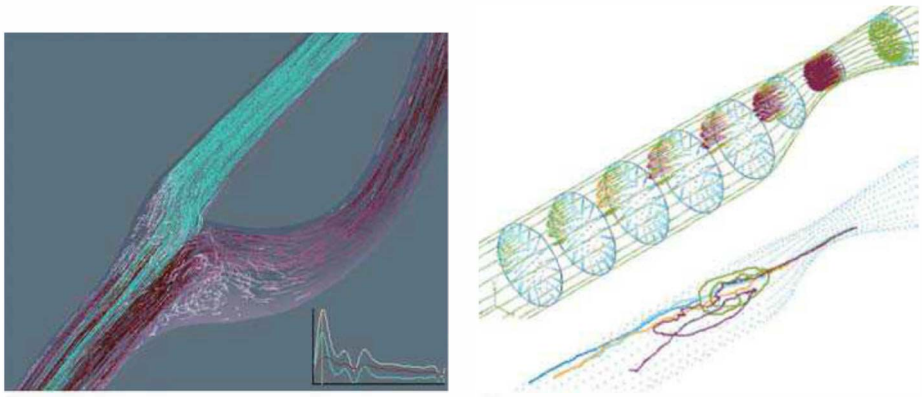


FIGURE 39.10 **A**: Flow characteristics in the normal male human carotid artery at the bifurcation into internal and external branches near peak systole showing spatial characteristics of the flow. Note the transition from unidirectional laminar flow (and shear stress) in the carotid artery sinus, a site of high susceptibility for atherosclerosis, where complex disturbed flow develops with oscillating flow reversal and the formation of transient vortices. Flow varies in velocity but is laminar and unidirectional elsewhere. This frame captures an instantaneous event at peak systole during unsteady arterial blood flow. As diastole develops, the flow velocities (indicated by bar length) fall sharply in the flow separation region as predicted from the steady flow model of panel A. Mean common carotid artery diameter in males is approximately 6.3 mm. Frame from a combined medical imaging and CFD sequence of human blood flow by Professor David A. Steinman of the Department of Mechanical and Industrial Engineering, University of Toronto (Image gallery URL: http://www.mie.utoronto.ca/labs/bsl/). (Kindly provided by Dr. Steinman as a high resolution image.) **B**: Velocity vectors and platelet trajectories through an 84% eccentric model stenosis. (From Einav S, Bluestein D. Dynamics of blood flow and platelet transport in pathological vessels. *Ann N Y Acad Sci* 2004;1015:351–366.)

devices,[222,223] they frequently experience shear rates that exceed their activation threshold and predispose them to aggregation. Such regions typically contain vortices created by flow separation, which capture a fraction of activated platelets into a zone of low flow velocity with oscillatory characteristics and within which they are retained for a longer period than in fast-flowing arterial bulk flow. Soluble procoagulant factors released from the vessel wall (e.g., free radicals and other reactive oxygen species, ROS) and from platelets themselves (e.g., ADP) are also retained within the recirculation zone. When this occurs adjacent to a stenosis or an intravascular device deployed during angioplasty where endothelium is lost, conditions are highly favorable for thrombosis. A potent mixture of slow-moving activated platelets, the absence of a functional antiplatelet endothelium, and localized prothrombotic chemical gradients tip the equilibria toward coagulation. Thus local shear rates and fluid dynamics patterns together with increased plasma procoagulation factors and diminished anticoagulation factors promote the activated state of platelets, leading to higher probabilities of thrombosis. The generation and characteristics of flow separation in such geometries are predictable by computational fluid dynamics (CFD).[220] *In vitro* experimental models of micro- and macrospatial relationships of particles, platelets, and other blood cells and proteins encompassing periods of extensive flow separation throughout the cardiac cycle have validated CFD predictions. FIGURE 39.10A demonstrates the complex flow separation in the sinus of the human internal carotid artery and FIGURE 39.10B illustrates the trajectory of platelet particles downstream of a simulated eccentric coronary region with >80% stenosis; a similar process on a microscale in the peristrut region of a commercial coronary stent is shown and discussed in the next major section. Measurements of thrombus formation itself are spatially consistent with predicted and measured platelet activation in a number of *in vitro* systems.[220,224] Flow separations close to significant stenoses shed smaller vortices of greater dynamic complexity from the primary separation region.[225] These regions will further trap procoagulant platelets, cells, and proteins. At the microscale around stent struts, it is less likely that vortex shedding occurs, but strong evidence for retention of the primary vortex region throughout the entire cardiac cycle is predicted by CFD with only minor oscillations of the separation boundaries. The contribution of such regions to late stent thrombosis (LST) is discussed in more detail below.

Natural Endothelial Heterogeneity Influenced by Arterial Geometry

The endothelium is highly sensitive to the local geometry of arteries via variability in hemodynamic force distribution and cell surface transport characteristics.[152] Throughout the majority of the arterial circulation, the flow is pulsatile but always moving forward. However, regions of flow separation such as arterial branches, bifurcations, and curvatures experience low average shear stress, steep temporal and spatial shear stress gradients, and oscillatory flow.[221] Since temporal multidirectional forces act on the cells in disturbed flow regions, the endothelium, unlike elsewhere in the arterial circulation, lacks preferential cell alignment and the cells frequently adopt a polygonal morphology; transition of cell shape from aligned to polygonal at the boundary of flow separation is clearly shown in FIGURE 39.11. Atherosusceptibility and disturbed flow are closely correlated *in vivo*. There is significant differential endothelial gene

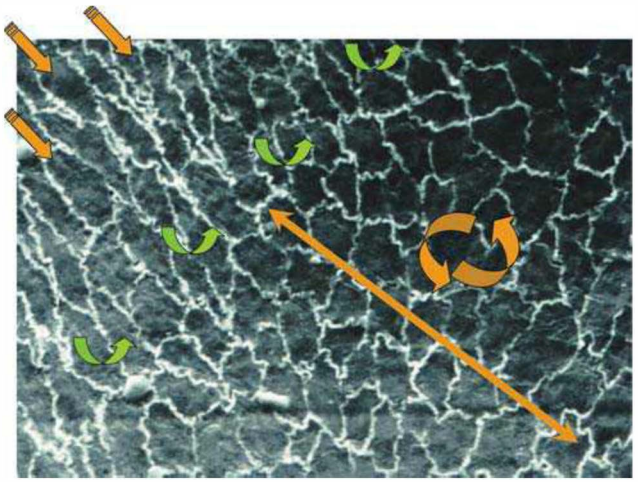


FIGURE 39.11 Transition of endothelial cell morphology in a region of flow disturbance near an intercostal branch from the aorta. When undisturbed flow (*top left*) separates to disturbed flow (boundary at *curved arrows*) cell shape changes from aligned to polygonal (no preferred orientation) in the region to the right of the separation line.

expression between susceptible and protected (undisturbed flow) regions.[226,227] Thus, a site-specific important rheological influence upon susceptibility to, or protection from, pathological change mediated by the endothelium is proposed.[228,229]

Flow–phenotype relationships were initially addressed by gene expression studies using cultured endothelium.[230–232] Candidate genes were identified *in vitro* and were subsequently demonstrated to have *in vivo* relevance.[233–236] However, high-throughput methodologies applied to cells isolated from small regions of the endothelium *in situ*[226] can directly address endothelial phenotype heterogeneity *in vivo*.

Endothelial phenotypes in atherosusceptible sites can be considered to reflect the kinetic status of multiple interacting cellular pathways in cells that are adapted to locally complex flow characteristics. Since the interpretation of gene expression networks *in vivo* is informative for optimal design of *in vitro* experiments that address the underlying mechanisms, a combined *in vivo/in vitro* approach greatly expands the relevance of *in vitro* investigations alone.

Function to Dysfunction

The subtle and complex pathways that determine functional phenotype *in vivo* are reflected in multiple levels of regulation of gene and protein expression. There is a range of endothelial phenotypes in the arterial circulation—and significant variability within the same artery—each arising from the complex interplay of cellular pathways that regulate function and are subjected to different physical environments including local rheology differences. Unbiased genomic analyses have shown that atherosusceptible and atheroprotective regions express significant differences of endothelial phenotypes before any pathology develops.[226,237–242,243,244] The transition from normal phenotype to overt dysfunction at these sites is not defined; it is generally assumed to be a threshold event but may represent subtle and gradual chronic changes of function—subtle adjustments in multiple subcellular equilibria. Within this heterogeneity lie differences in the endothelial expression of molecules that regulate or participate in thrombogenicity and that are associated with the rheology because of their location.

Passerini et al.[226] identified the coexistence of differential pro-and anti-inflammatory gene expression in endothelium at disturbed flow sites in normal swine. This was accompanied by differential expression of coagulation molecules. vWF and factor XIII, both procoagulant, were significantly elevated in regions of flow disturbance. Expression of plasminogen activator and its substrate plasminogen, molecules that promote the degradation of formed clots (fibrinolysis) by plasmin cleavage of fibrin, were also elevated while expression of PAI-1 was decreased. Thus, endothelial phenotype in disturbed flow appears to be sensitized toward platelet proaggregation offset by enhanced degradation of formed/forming clots. *In vitro* experiments that applied disturbed atheroprone disturbed flow waveforms to cultured human umbilical vein endothelial cells demonstrated decreased expression of thrombomodulin (TM).[245] TM forms a complex with thrombin that is responsible for the conversion of protein C to activated protein C. This cleaves factors VA and VIIIA (activated cofactors of the coagulation mechanism) and thereby reduces the amount of thrombin generated. The lower expression in disturbed flow therefore suggests a procoagulation balance consistent with the *in vivo* phenotype.

ER Stress, UPR, and Reactive Oxygen Species

A consistent finding in endothelial genomics analyses of disturbed flow locations *in vivo* is enhanced endoplasmic reticulum stress (ER stress).[243,244] ER stress is an adaptive protective mechanism that arises because of excessive protein biosynthesis or interference with normal protein folding mechanisms in the ER lumen in response to multiple kinds of cellular stress. Excessive newly synthesized and/or misfolded polypeptides in the ER lumen exceed its protein-folding capacity. It results in the activation of the unfolded protein response (UPR), a ubiquitous adaptive cell response that assists cell survival in an adverse environment by activating a set of intracellular signaling pathways.[246] The UPR signals a coordinated transcriptional upregulation of ER chaperones and folding enzymes to promote the correct assembly of unfolded polypeptides and prevent incompletely folded proteins from aggregating. The products of the activated UPR transducers converge as transcriptional regulators in the nucleus to upregulate ER chaperones and UPR transducer synthesis and to ubiquitinate unfolded proteins for degradation through the proteosome; both processes relieve ER stress accumulation of unfolded proteins and restore homeostasis. Failure to restore ER protein equilibrium to a normal range leads to apoptosis through transcriptional induction of the transcription factor C/ERB homologous protein, inflammation through activation of NFκB, and generation of ROS through excessive protein oxidation in the ER.

In a multisite study in 45 normal adult swine, endothelium in atherosusceptible regions of the aortic arch, proximal brachiocephalic artery, aortorenal branch region, and abdominal aorta was analyzed relative to protected sites of the common carotid artery, descending thoracic aorta, and the distal renal artery. All atherosusceptible regions are associated with complex disturbed blood flow. From this multisite study, the most

abundant common feature of the endothelium of all disturbed flow regions was the upregulation of genes associated with ER processing of proteins, ER stress, and the UPR.[243,244]

ER stress/UPR are protective mechanisms in response to a variety of stresses that include free radicals, physical perturbation, osmotic, radiation, metabolic stress, and inflammation. Their elevations in regions of flow separation reflect a low-level chronic stress associated with these locations. This may arise by adverse localized chemical concentrations resulting from suboptimal transport at the endothelial surface or from physical force differences resulting from oscillatory flow in regions of disturbed flow. *In vitro* analyses of ER stress/UPR in cultured endothelium under different flow conditions are consistent with a hemodynamic contribution to ER stress *in vivo*.[247,248] ER stress is a signature of multiple sites in the swine arterial circulation where proinflammatory NFκB pathway molecules are activated,[226,249] and where regulatory transcription factors[236,250] and microRNAs[249] are differentially expressed. ER stress pathways are both stimulated by ROS and can generate ROS. Direct visualization of ROS by the dihydroethidium in swine has shown a markedly increased ROS load in the inner curvature of the aortic arch (flow separation) when compared with the descending thoracic aorta[244] (FIGURE 39.12A). In swine coronary arteries, ROS is distributed throughout the right coronary, circumflex, and left anterior descending coronary arteries (FIGURE 39.12B), demonstrating a heavier overall ROS load in the coronary circulation than noncoronary arteries that may be linked to enhanced coronary sensitivity to cardiovascular disease.[244] The mechanisms of ROS load and Redox signaling for thrombosis/hemostasis may be via effects upon integrin signaling, particularly platelet integrins.[251] Ligation of integrins in endothelial cells and platelets induces activation of the Ras/MAPK, NFκB, and phosphatidylinositol 3-kinase and Rho-GTPases pathways. Following vessel wall injury and associated with activation and recruitment of platelets, there is a production of ROS concomitant with the stimulation of the blood coagulation.[252] Moreover, ROS are capable of inducing

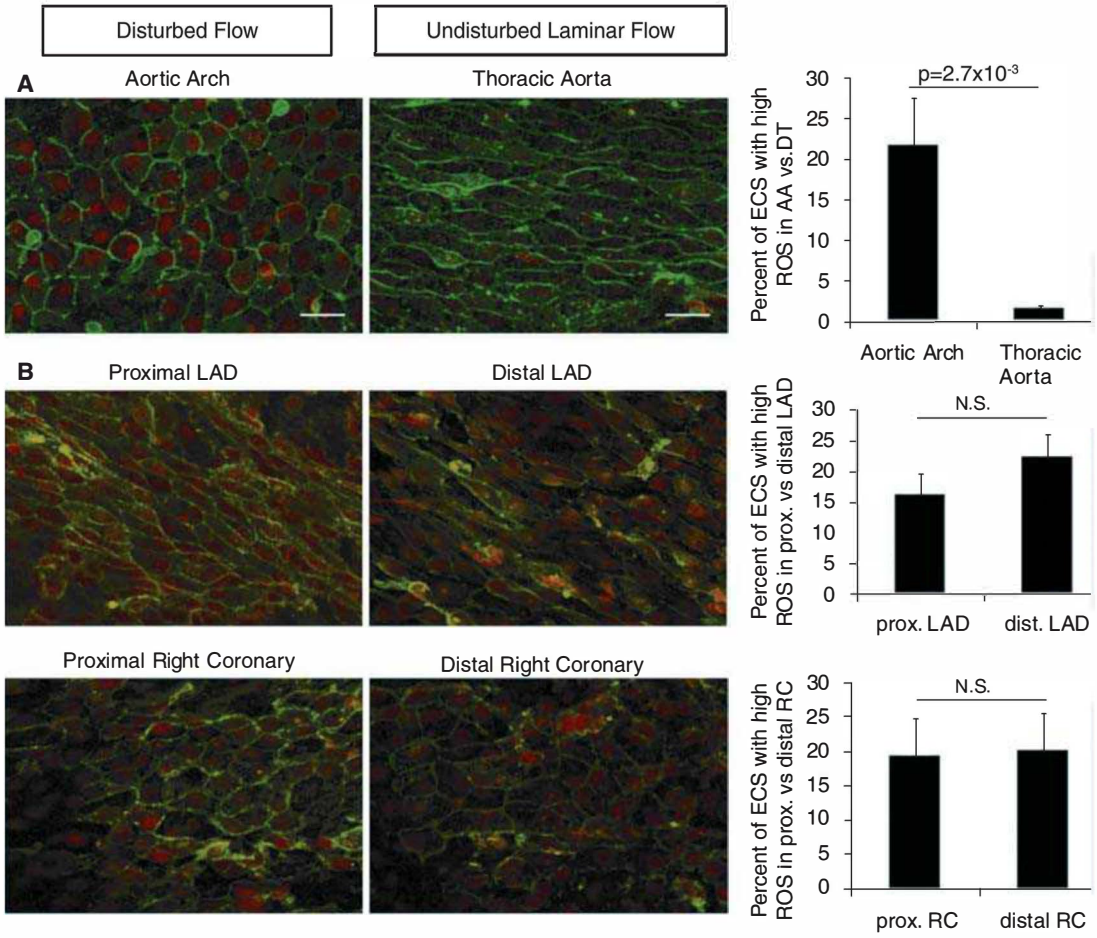


FIGURE 39.12 Endothelial ROS detection in freshly harvested normal swine. **A**: aorta. **B**: coronary arteries incubated with dihydroethidium (oxidized product red) and isolectin B_4 (endothelial identity; green) and imaged *en face* using confocal microscopy under identical conditions. Percentages of endothelial cells with high ROS were determined by counting the red nuclei. This percentage was used to calculate a ratio for each pair of atherosusceptible and atheroprotected samples. Data represent mean ± SEM. *P*-values were calculated by one-sample, one-sided, paired Wilcoxon test. Scale bar 25 μm. $^{**}P < 0.01$, NS, not significant. (From Civelek M, Manduchi E, Riley R, et al. Coronary artery endothelial transcriptome *in vivo*: identification of endoplasmic reticulum stress and enhanced ROS by gene connectivity network analysis. *Circ Cardiovasc Genet*. 2011;4:243–252.)

conformational changes in integrins to change their binding affinity and function. ROS is therefore a likely participant in the interplay of differentially expressed pathways in endothelial cells from regions where flow separation is a prominent characteristic influencing the force and transport environment in a proinflammatory, prothrombotic direction.

MICROSCALE RHEOLOGY AND STENT-ASSOCIATED THROMBOSIS

Rheological factors play an important role in the potential for thrombus generation around intravascular devices. Although anticoagulant surface materials are readily available, arterial devices promote flow separations that contribute to platelet activation, aggregation, and thrombus formation. Coronary artery stents are an important example at the microscale.

Stent Rheology May Influence Late Stent Thrombosis (LST)

The standard of care for coronary artery occlusion commonly includes the deployment of drug-eluting stents (DESs) into the lumen of the affected vessel. The stents provide immediate relief by widening the lumen, and the eluted drugs are effective in inhibiting restenosis. However, episodes of LST that are frequently fatal occur in 1% to 2% of patients up to 4 years after stent insertion. Typically, DES struts remain at, or close to, the vessel/blood interface and therefore represent an obstacle to the flow field for extended periods. Reendothelialization of DESs is typically slow and often incomplete, contributing further to a procoagulant environment.[253] Although <150 microns in height, DES struts provide a physical perturbation in the flow field that yields local pockets of disturbed flow,[222,223] which are associated with low endothelial cell migration rates. Simon et al.[254] demonstrated that endothelialization of the strut surface was inversely proportional to the thickness of the strut, and areas devoid of cells were largest on the downstream side, which is the side with the largest flow separation zone. The ISAR-STERE0 and ISAR-STERE0 2 clinical trials reported a proportional relationship between strut thickness and angiographic restenosis.[255,256] CFD demonstrated that as the stent strut thickness increases, the flow recirculation zones in the vicinity of the struts also increase in magnitude.[222] Lack of endothelialization, endothelial dysfunction, and hypersensitivity to the stent coating have been implied as possible factors in LST. However, an additional feature of current commercial DESs (FIGURE 39.13A) is significant peristrut flow separation.[223] As the stent strut thickness decreases, there is a nonlinear decrease in the proximal and distal flow recirculation zones around the struts[223] as shown in FIGURE 39.13B(I–III). The rate of endothelialization is proportional to the local flow rate (and shear stress), with slower moving flow in a recirculation zone yielding lower shear stress levels and retarding endothelialization.[257,258]

Flow Separation Creates Shear Values Sufficient for Platelet Activation

Within the flow separation zones of current rectangular commercial stent struts, the low flow velocities yield low wall shear stresses. However, they generate peaks of shear at the rectangular corners that approach 10,000/s at the upstream edge and >3,000/s at the downstream edge of the strut as shown computationally in FIGURE 39.14A. These levels exceed the shear required to activate platelets.[220,222]

A Procoagulation Reaction Zone in Regions of Flow Separation

In the undamaged coronary artery, a noncoagulation state is maintained where the secreted and surface-presented anticoagulant molecules of the endothelium help maintain hemostatic balance (FIGURE 39.15B(I)). These molecules include ADP, TF, vWF, PGI_2, NO, tissue factor pathway inhibitor, tPA, and TM. FIGURE 39.15B(II) shows how the deployment of current commercial (nonstreamlined) stents promotes flow separation and consequent recirculation zones in the proximal and distal regions of the strut. Thrombosis is promoted by disturbed flow, lack of endothelial cells, and increased residence time in a "microreaction chamber" created by flow separation.

Streamlined Geometry Mitigates Coagulation

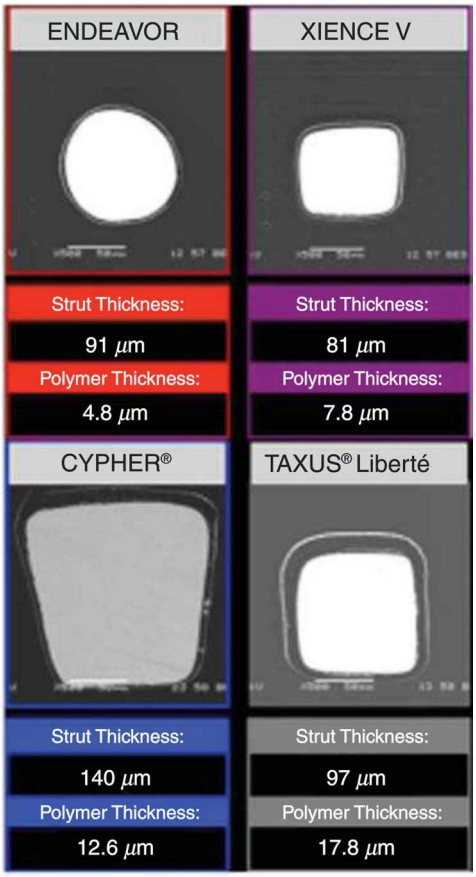
A modest streamlining of stent struts has therefore been proposed to mitigate flow separation.[222,223] Streamlining the stent contours significantly reduces flow separation as shown experimentally by particle streamlines (FIGURE 39.15A) and by numerical analysis (FIGURE 39.13(IV–VI)). Furthermore, sharp peaks of high shear are attenuated by a streamlined geometry of a 100 μm height circular arc (FIGURE 39.14B). Fully streamlined stents would minimize or eliminate flow disturbances in the vicinity of the struts to restore flow conditions that allow the natural washout of the vessel wall. Short residence times of platelets and rapid dilution of procoagulation factors and intermediates (FIGURE 39.15III) should promote healing (FIGURE 39.15IV). The goal of modifying the stent strut cross-sectional geometry is to decrease the morbidity and mortality associated with LST via rheological mechanisms.

Transport in the Flow Separation Regions

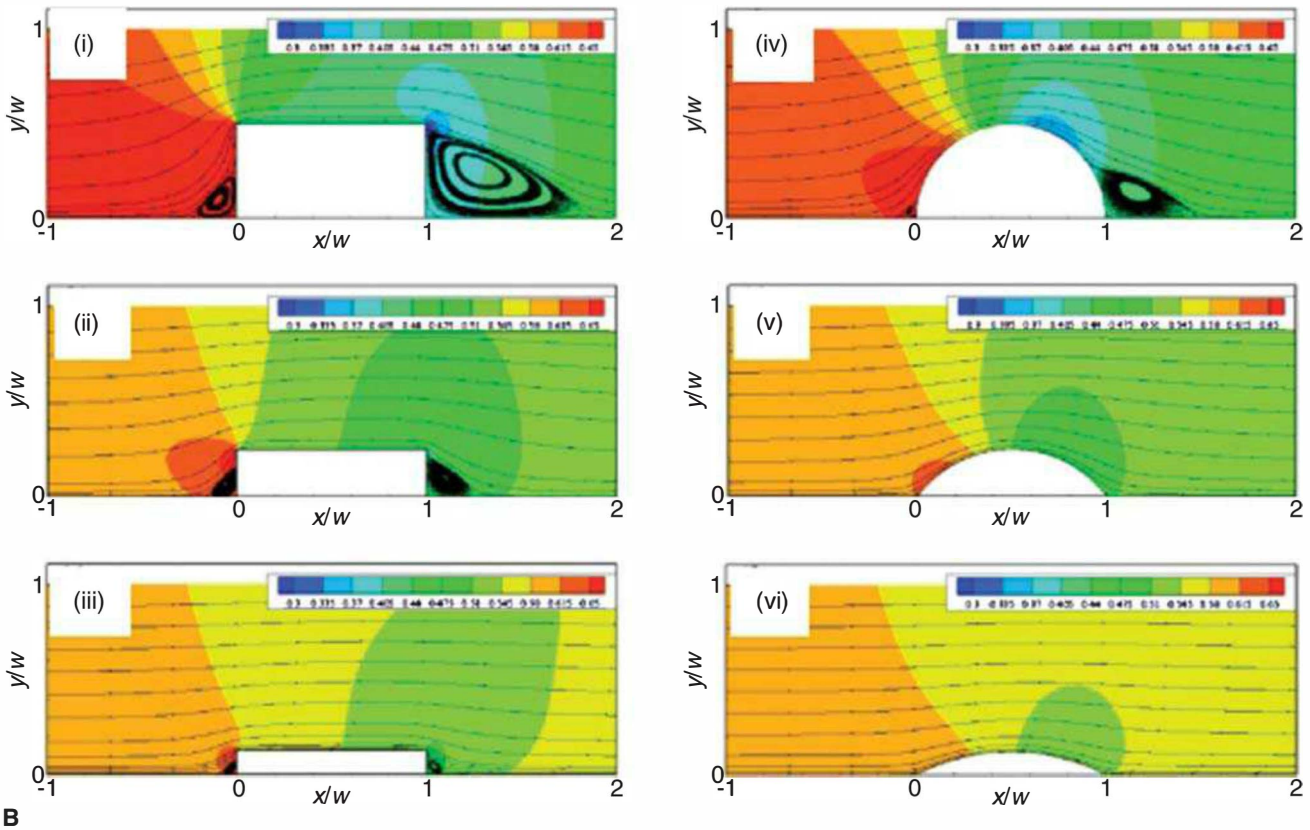
A consequence of flow separation around nonstreamlined stent strut geometries is an impact on surface enzyme reactions. A number of critical enzymatic reactions in the coagulation cascade, such as the generation of active factor X (Xa) by both extrinsic and intrinsic tenase complexes and the generation of thrombin by prothrombinase, are heterogeneous reactions that require a lipid surface for proper enzyme–cofactor assembly and proteolytic activity. Unlike platelets, which due to their size are minimally affected by Brownian diffusion, soluble proteins readily diffuse into the recirculation zones created by nonstreamlined stent struts, whereas platelets have to follow pathlines to be transported to a recirculation zone. The effects that recirculation zones have on the generation of high local concentrations of reactive enzymes, specifically thrombin, surrounding stent struts could be crucial to understanding LST events.

Numerical Prediction of Strut Geometry on Heterogeneous Surface-Catalyzed Reactions

A diffusion-convection-reaction numerical model for a stented vessel flow field was developed to investigate the role of stent strut cross-sectional geometry in the increase of the local



A



B

FIGURE 39.13 **A**: Cross-sectional geometry of four commercial DESs in common use. **B**: Streamlines in the foreground of a nondimensional pressure, p^*, flow field in the vicinity of (*i*)–(*iii*) rectangular stent strut and (*iv*)–(*vi*) circular arc stent geometry for strut thicknesses of 100, 50, and 25 μm. (From Jiménez JM, Davies PF. Hemodynamically driven stent strut design. *Ann Biomed Eng* 2009;37:1483–1494.)

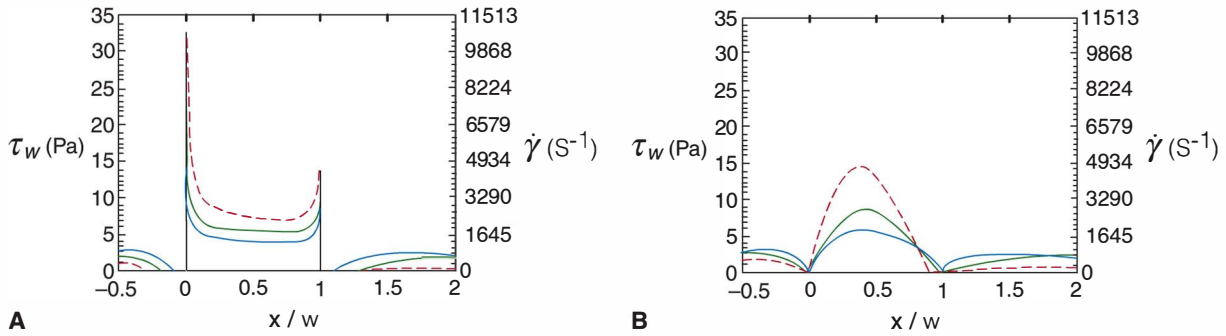


FIGURE 39.14 Wall shear stress (τ_w) and shear rate ($\dot{\gamma}$) distributions corresponding to **(A)** rectangular and **(B)** circular arc stent struts for strut thicknesses of 100, - - -, 50, —, and 25 μm, •••. (From Jiménez JM, Davies PF. Hemodynamically driven stent strut design. *Ann Biomed Eng* 2009;37:1483–1494.)

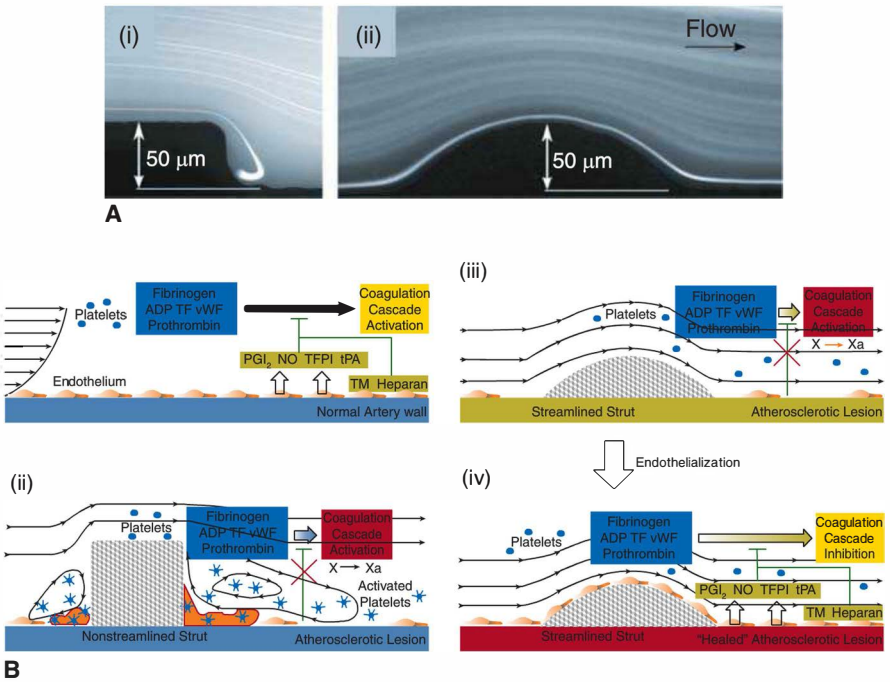


FIGURE 39.15 Flow separation adjacent to commercial stent geometry. **A**: Flow visualization around a (*i*) rectangular and (*ii*) streamlined stent strut. **B**: Blood contains multiple procoagulant proteins as well as natural anticoagulants that together with the endothelium normally maintain a noncoagulant state. (*i*) In the normal artery wall the secreted and surface anticoagulant molecules of the endothelium help maintain hemostatic balance. ADP, adenosine diphosphate; TF, tissue factor; vWF, von Willebrand factor; PGI2, prostacyclin; NO, nitric oxide; TFPI, tissue factor pathway inhibitor; tPA, tissue plasminogen activator; TM, thrombomodulin. (*ii*) The deployment of current commercial rectangular or circular nonstreamlined stents promotes flow separation in the proximal and distal regions of the strut. Procoagulant conditions are greatly increased around the stent strut by the following: (*1*) accelerated flow over the strut edges yields shear rate magnitudes that can activate platelets some of which will enter the distal flow separation zone, (*2*) recirculation zones retain activated platelets and procoagulant factors that can reach critical concentrations for activation of the coagulation cascade, (*3*) the removal of endothelium during angioplasty and stenting eliminates anticoagulant mechanisms and exposes the highly thrombogenic extracellular matrix surface. Furthermore, (*4*) low flow velocity (shear stress) inhibits endothelialization of the vessel and strut surface. (*iii*) A streamlined stent strut inhibits fluid flow separation and high shear rate peaks that can activate platelets. Furthermore, a streamlined geometry will be less likely to generate recirculation zones, thereby decreasing the residence time of procoagulant molecules and the probability of thrombogenesis despite the absence of endothelium. Undisturbed flow proximal and distal to the streamlined strut favors endothelialization of the vessel and strut surface. (*iv*) An endothelialized vessel and strut surface provide an anticoagulant environment that helps maintain hemostatic balance and protect against stent-related thrombosis. (Adapted from Jiménez JM, Davies, PF. Hemodynamically driven stent strut design. *Ann Biomed Eng* 2009;37:1483–1494.)

concentration of a soluble product from a reactive surface. At a lumen diameter of 3 microns, Re of 100, and strut height 50 microns, the maximum concentration regions coincide with the proximal and distal recirculation zones for a rectangular stent, but after streamlining (circular arc; peak 50 microns) only a very small concentration zone was predicted to remain on the proximal side of the strut (Jimenez; manuscript in preparation). The combination of a high concentration zone of reactive enzymes in the flow separation zone generated by a nonstreamlined strut in a blood vessel denuded of endothelial cells provides an optimal environment for thrombogenesis when combined with the transport of platelets from the mainstream to the recirculation zone by flow pathlines (*cf.* FIGURE 39.15).

Experimental support for the numerical predictions has been obtained in a *macro*fluidics *in vitro* coagulation model operated at coronary artery flow velocities using polymer casts of rectangular and streamlined stent struts. In this model, thrombin was first deposited in peristrut regions of the stent after which a fibrinogen solution was perfused into the model coronary system. The transport of soluble fibrinogen to the regions where thrombin was deposited was dictated by the local flow field generated by the different stent strut geometries. In the case of the rectangular (nonstreamlined) stent struts, the flow patterns carried fibrinogen and entrapped it in the long residence time recirculation zone, enabling the fibrinogen/thrombin interaction and yielding local formation and deposition of fibrin. Greater fibrin accumulation was measured in the vicinity of rectangular strut flow separation regions than when streamlined geometries were tested.[259]

SUMMARY

The process of thrombosis depends on complex interactions between fluid dynamics, convective mass transfer, reaction kinetics, and cell adhesion. Dynamic experimental models that mimic the local environment *in vivo* have been used successfully to determine the molecular mechanisms supporting thrombus formation under different shear conditions present in the vascular system. Under arterial shear conditions, it has been shown that mural thrombosis requires the binding of vWF to platelet GP Ib and GP IIb/IIIa receptors. Inhibiting these interactions during the onset of thrombosis not only decreases the formation of thrombi but also helps to prevent long-term restenosis of the vessel.[200,201,202] The use of these dynamic models has been extended to investigate how shear modulates endothelial and smooth muscle cell metabolism, smooth muscle cell proliferation, the coagulation cascade, the inflammatory process, and tumor cell metastasis. *In vivo* site-specific analyses of endothelial phenotypes have identified the steady-state expressions of many endothelial pathway molecules and prioritized their importance for *in vitro* studies. All of these studies lead to the conclusion that the local rheologic environment can have a profound effect on the molecular mechanisms of thrombosis. With the advent and accessibility of massive parallel (next-generation) sequencing facilities, substantial progress is to be expected in understanding the complexity of thrombotic mechanisms in an appropriate *in vivo* site-specific context in blood vessels that should accelerate the development of novel, more potent therapeutic agents perhaps tailored to the individual patient.

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CHAPTER

40

Nitric Oxide: The Vascular Biology of Nitric Oxide and Nitric Oxide Synthases

BRADLEY A. MARON AND THOMAS MICHEL

Nitric oxide (NO) is a ubiquitous, biologically versatile, and structurally unique molecule that is central to a diverse range of cell signaling processes that influence vascular function. The chemical properties of NO are ideally suited for participation in the inter- and intracellular reactions that modulate blood vessel relaxation.[1,2] NO is distinctive among other endothelium-derived relaxing factors in several ways. NO is a small (30 Da) lipophilic gaseous molecule. These properties allow NO to easily traverse vascular endothelial and smooth muscle cell membranes and participate in intercellular signaling.[3,4,5] Moreover, NO is a labile free radical that may react with a diverse range of molecules including thiols, heavy metals, and other biologically active free radicals such as superoxide anion. The relatively short half-life (5 to 10 seconds)[6] of NO ensures a rapid and localized signaling effect,[4,7] permitting NO to exert a tightly regulated, juxtacrine effect on the function of neighboring cells while also modulating intracellular signaling pathways. Importantly, the endothelial isoform of nitric oxide synthase (eNOS), which synthesizes NO, plays an important role in maintaining vascular homeostasis by virtue of the heterogeneous array of extracellular signals that modulate NO levels through influencing the *eNOS* gene, transcript, and protein. Posttranslational modifications to eNOS regulate its enzyme activity, subcellular targeting, and, importantly, the role of eNOS in vascular homeostasis; alterations in eNOS are implicated in the pathogenesis of several vascular disease states.

NITRIC OXIDE BIOCHEMISTRY

Targets of NO

NO produced by eNOS in vascular endothelial cells rapidly diffuses into adjacent vascular smooth muscle cells (VSMCs) and into the blood vessel lumen. The chemistry of NO allows for numerous reactions, including those with (a) proteins that contain metallic centers and/or sulfhydryl groups; (b) low molecular weight thiol compounds; (c) hemoproteins; or (d) other radicals.

Soluble Guanylyl Cyclase

In VSMCs and circulating platelets, the heterodimeric (α_1/β_1 or α_1/β_2) enzyme soluble guanylyl cyclase (sGC) is a critical NO target. NO-sGC signaling is essential for normal vascular reactivity and for suppression of abnormal platelet aggregation. NO binding to the heme prosthetic group of sGC disrupts a nitrogen–iron interaction, thereby freeing an axial histidine residue on the catalytically active β_1 subunit of sGC. Nanomolar concentrations of NO may induce a 100-fold increase in the sGC-dependent conversion of cytosolic GTP to cGMP, which in VSMCs activates cyclic GMP-dependent protein kinase (also known as protein kinase G [PKG]) to promote vascular relaxation (discussed below). Diminished eNOS abundance and decreased enzyme activity can lead to reduced NO synthesis, leading to the attenuated activation of sGC and causing increased vascular tone and blood vessel injury. These alterations in eNOS bioactivity are implicated in the pathophysiology of important vascular diseases including stroke and myocardial infarction.[8,9] Moreover, the potent vasodilatory effects of NO may be limited significantly owing to pathologic posttranslational modifications of sGC or other downstream signaling intermediaries. For example, oxidation of the heme moiety or key cysteinyl thiol(s) located near the NO-sensing pocket of sGC converts sGC to an NO-insensitive state and attenuates NO-dependent vasodilation.[8,10] This model illustrates a key distinction in NO biology: NO *bioavailability*, which suggests the presence of NO levels that are sufficient to induce a biologic response, is not synonymous with NO *bioactivity*. The latter implies an appropriate interaction between NO and NO targets that results in a downstream functional effect on cellular physiology.

Formation of NO Adducts: Nitrosothiols

The relatively short half-life of NO has generated speculation regarding potential storage forms of NO that may serve to sustain adequate basal levels of NO and permit actions of NO to occur at targets distant from its sites of synthesis.[11] The redox reactivity of nitrogen oxides with sulfhydryl groups is well characterized, and various nitrosothiols, including proteins covalently modified by a NO adduct, may represent a molecular "depot" for storage of NO *in vivo*.[12] Both protein and nonprotein nitrosothiols have been found in cells and biologic fluids *in vivo*. Compared with a free plasma NO concentration of approximately 3 nM, the concentration of plasma nitrosothiol is approximately 1 μM, of which 85% is nitrosoalbumin.[13–15] *S*-nitrosothiols are endogenously formed in both the cardiovascular and pulmonary systems,[13,14] and micromolar concentrations of *S*-nitrosoglutathione have been isolated from the lung.

Nitrosothiols share a qualitatively similar pharmacodynamic profile with NO, such as stimulating sGC,[11] inducing vascular relaxation and hypotension, and exerting antithrombogenic effects via inhibition of platelet aggregation.[16] The molecular mechanism(s) by which endogenous nitrosothiols induce biologic effects remains speculative: nitrosothiols may undergo spontaneous nonenzymatic decomposition to release NO, induce transnitrosylation of other proteins, or directly exert nitrergic effects.[17]

Hemoglobin is proposed to be a nitrosoprotein with potential biologic relevance. While it is clearly established that hemoglobin has the ability to bind NO at its heme moieties, hemoglobin

may also undergo nitrosylation at Cys93 in the β globin protein to form a protein nitrosothiol. Although there is evidence to suggest that nitrosohemoglobin may release NO, the utility of exploiting NO binding by hemoglobin as a pharmacotherapeutic system for NO delivery to distal points in the vascular system remains under investigation.[18] However, other mechanisms that utilize *S*-nitrosothiols for NO delivery have been tested *in vivo*. Through *S*-nitrosothiol linking, numerous NO molecules may be covalently bound to polyethylene glycol-conjugated bovine serum albumin, a macromolecule that is resistant to intermolecular disulfide bond formation. It has been demonstrated that intravenous injection of this compound in mice is associated with an increase in whole blood levels of the stable NO metabolite, nitrite, and a reduction in mean arterial blood pressure.[19] The translation of this technology into an effective therapy remains the focus of ongoing investigations.[20,21]

In addition to functioning as a storage reservoir of NO, NO-protein adducts may serve as an important posttranslational modification of biologically active proteins.[22] For example, a novel molecular mechanism by which *S*-nitrosylation influences vascular tone has recently been reported. Zhou et al. demonstrated that activation of angiotensinogen, a key vasoactive octapeptide that regulates blood pressure in mammals, is dependent, in part, on oxidation-induced disulfide bond formation between Cys18 and Cys138. Under reduced conditions in which this disulfide bond is broken, however, *S*-nitrosylation at these critical cysteines may occur. In turn, this induces a stereometric change to angiotensinogen that prevents Cys18-Cys138 disulfide bond formation, and thereby attenuates the angiotensinogen-mediated hypertensive response *in vivo*.[23]

Nitrosylation pathways may also regulate proteins associated with hemostasis. For example, *S*-nitrosoglutathione may interact with fibrinogen and impair fibrin cross-linking, without chemically reacting with fibrinogen but possibly by inducing a conformational change in fibrinogen.[24] Nitrosylation of factor XIII may be an antihemostatic mechanism that could attenuate fibrin cross-linking and render a nascent clot more susceptible to fibrinolysis.[25] While nitrosylation of tissue-type plasminogen activator (S-NO-t-PA) has no effect on its interaction with fibrin or the catalytic activation of plasmin, S-NO-t-PA may act as a nitrosothiol carrier of NO to inhibit platelets and cause vasodilatation at sites of thrombus where the local endothelium may be dysfunctional and producing insufficient quantities of NO.[26]

NO Adducts and Reactive Oxygen Species

Formation of NO adducts may be affected by the cellular redox potential, and in turn may influence the nature of redox-active species (e.g., reactive oxygen species) present in the vascular wall and influence NO bioavailability. The most prominent example of this phenomenon is the formation of peroxynitrite through the reaction of NO and superoxide. Although this process may be a mechanism for detoxifying superoxide, it also leads to decreased levels of bioavailable NO through several mechanisms. First, generation of peroxynitrite results in depletion of the NO substrate. Second, peroxynitrite may react with nitrite to form peroxynitrate, thereby decreasing levels of nitrite, which is a key substrate for NOS-independent NO synthesis.[10] Finally, peroxynitrite itself is a chemically reactive species that may adversely modify cellular proteins involved in NO-dependent cell signaling pathways.

SOURCES OF NITRIC OXIDE

Nitric Oxide Synthases

NO is synthesized endogenously in mammalian tissues by a family of three nitric oxide synthase (NOS) isoforms, each the product of a distinct gene. The mammalian NOSs are homodimeric enzymes containing an *N*-terminal heme-binding domain and a *C*-terminal reductase domain, separated by a calmodulin-binding domain. The *C*-terminal domains of the three proteins in the mammalian NOS enzyme family are structurally similar to cytochrome P450 reductases.[27–29] The *N*-terminal domain of NOS, also known as the oxygenase domain, contains binding sites for heme (protoporphyrin IX), tetrahydrobiopterin, and the substrate L-arginine.[28] The *C*-terminal flavoprotein oxidoreductase domain binds flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and NADPH.[30] As these two functions are often the products of separate polypeptides, the combination of the oxygenase and reductase functions of NOS suggests that the prototypical NOS protein evolved by gene fusion, with a binding site for the Ca^{2+}-binding protein calmodulin separating the *N*- and *C*-terminal domains.

eNOS Catalysis

Under basal conditions, electron transfer between NOS domains is suppressed; however, it appears that calmodulin promotes the flow of electrons from the reductase to the oxygenase domains.[31] The fact that NOS binds five redox-active cofactors—more than any other enzyme known—suggests an intricate catalytic mechanism. NOS catalyzes the five-electron oxidation of the guanidine nitrogen of L-arginine, although reactions of NADPH and flavoproteins typically involve an even number of electrons. NOS catalysis proceeds in two distinct monooxygenation steps, each involving electron transfer to molecular oxygen: in the first step, one mole of NADPH transfers, via FAD and FMN,[32] two moles of electrons to a heme-bound oxygen, which reacts with L-arginine to form one mole each of omega-*N*-hydroxy-L-arginine and water.[33] In the second step, one half mole of NADPH transfers electrons to a second heme-bound oxygen, which reacts with omega-*N*-hydroxy-L-arginine to release one mole each of NO and L-citrulline.

NOS Isoforms

Of the three distinct NOS isoforms that have been identified, two are constitutively expressed in their archetypal cells of origin: eNOS in vascular endothelial cells and neuronal NOS (nNOS) in neurons. As the name implies, inducible NOS (iNOS) is an inducible isoform found in a broad range of immunoactivated cells (see Table 40.1). Such terminology is somewhat misleading, however, because NOSs are expressed in a diverse range of tissues other than endothelium, neurons, and immunoactivated cells. Furthermore, the designation of constitutive and iNOS expression patterns are not strictly maintained, as nNOS and *eNOS* genes can undergo tissue-specific transcriptional regulation. Moreover, while eNOS is constitutively expressed, it may be induced by extracellular mediators. The overall catalytic scheme of the three mammalian NOS isoforms is conserved, and the members of this gene family share similar gene structure and exhibit 50% to 60% mammalian amino acid sequence homology,[34–36] as well as nearly identical locations of cofactor binding sites. There is considerable heterogeneity among the isoforms at the *N*-termini. For example, nNOS contains an additional sequence of approximately 290 amino acids not found in either eNOS or iNOS. Moreover, the *N*-terminus of eNOS is distinguished from nNOS and iNOS by

Table 40.1

Cellular and tissue localization of NOS expression

eNOS (NOS 3)	iNOS (NOS 2)	nNOS (NOS 1)
• Vascular endothelial cells, including arteries and veins	Immunoactivated cells, including: • Macrophages • Neutrophils • Vascular smooth muscle • Cardiomyocytes • Small vessel endothelial cells	• "Nitrergic" nerves innervating large vessels in diverse vascular beds
• Blood platelets		• Central nervous system
• Cardiomyocytes		• Skeletal muscle
• Brain (hippocampus)		• Gastrointestinal system
• Mast cells		• Cardiomyocytes

the presence of consensus sites for acylation, which are thought to determine eNOS subcellular localization.[37]

All three NOS isoforms catalyze the same chemical reaction and have similar redox cofactor requirements. A major difference lies in the isoforms' differential affinity for calmodulin: although all three NOS isoforms are dependent on calmodulin binding for activity, there are important differences in the calcium dependence for calmodulin binding. Whereas eNOS and nNOS require elevated intracellular calcium levels in order to bind calmodulin and thereby become activated, the affinity of iNOS for calmodulin is sufficiently high that iNOS fully binds calmodulin at the ambient intracellular calcium concentrations present in resting cells, and typically iNOS is persistently activated once the protein is synthesized.[38]

VASCULAR FUNCTIONS OF NOS-DERIVED NITRIC OXIDE

NOS, NO, and the Blood Vessel Wall

NO is an essential endogenous vasodilator, platelet inhibitor, and natural antithrombotic agent. Numerous experiments in both animal models and humans indicate that the physiologic role of eNOS is to produce NO in response to diverse extracellular stimuli and thereby maintain both a baseline level of systemic vasodilatation and establish vasomotor tone. In addition to its anticoagulant activity, endothelium-derived NO also attenuates endothelial proliferation and leukocyte adhesion. In turn, endothelial dysfunction that occurs as a consequence of decreased eNOS-derived NO may be a central mechanism involved in the pathobiology of vascular diseases characterized by vasoconstriction, hypertension, and a prothrombotic state.

The functional consequences of abnormal eNOS activity have been extensively studied. Both knockout and overexpression experiments suggest a role of eNOS and NO in maintaining basal vascular homeostasis. eNOS^{null} mice are hypertensive, demonstrate increased leukocyte-endothelial adherence, and diminished bleeding time.[39–41] These eNOS^{null} mice also develop pulmonary hypertension, hypoxia, and right ventricular hypertrophy.[42] Conversely, transgenic mice that overexpress eNOS show increased basal levels of NO and cGMP, associated with decreased blood pressure.[43]

Compensatory Bioactivity of NOS Isoforms

Animals genetically engineered to express various combinations of NOS isoforms (including the absence of all three isoforms), provide insight into the compensatory bioactivity of these enzymes *in vivo*. For example, pyramidal tissue-initiated long-term potentiation (i.e., sustained signal transmission) of hippocampal neurons is abolished in doubly mutant n/eNOS^{null} mice, but preserved in mice singly mutant for nNOS or eNOS.[44] Although eNOS protein expression levels are elevated in pyramidal tissue relative to central nervous tissue overall, the ratio of eNOS: nNOS protein expression in pyramidal tissue is quite low.[45] Thus, the observation that eNOS-derived NO levels are sufficient to sustain long-term potentiation suggests that functional efficiency of NOS varies between enzyme isoform and/or with respect to tissue type.

Studies performed in mice lacking all three isoforms (e/i/n NOS^{null} "triple knockout" mice) provide insight into the importance of NOS-independent mechanisms of NO synthesis *in vivo*. This "triple knockout" model also provides an informative model for understanding compensatory actions in the absence of NOS isoforms. Although the e/i/n NOS^{null} mice develop normally, they demonstrate significantly decreased rates of survival compared to wild type counterparts.[46] Interestingly, basal levels of NO are detectable in vascular tissue harvested from the triple-null animals, which probably reflects the conversion of dietary or bacteria-derived nitrite to NO.[47] In fact, there is accumulating evidence to suggest that nitrite (NO_2^-) and nitrate (NO_3^-), present in plasma of mammals via metabolism of endogenous NO, or, in the case of NO_3^-, from dietary intake of foods rich in inorganic nitrate, are biologically important through their recycling to NO.[48] A typical Western diet, for example, is rich in NO_3^-, which is readily absorbed in the gastrointestinal tract. Although mainly excreted in urine, the balance (as much as 40%) of circulating NO_3^- is ultimately excreted into the saliva.[49] There, NO_3^- may be converted to NO_2^- by endogenous oral flora in a reaction that requires nitrate reductase. In the circulation, NO_2^- may be reduced by deoxyhemoglobin and/or deoxymyoglobin to form bioavailable NO.[50] Cycling of NO, NO_2^-, and NO_3^- may be facilitated in the presence of xanthine oxidoreductase, hypoxia, or, even by NOS.[48]

In addition to the implications of NOS-independent NO synthesis on vascular function that are gathered from NOS knockout rodent models, it has been demonstrated previously that

dietary NO_3^- loading or the administration of inorganic NO_2^- via nebulizer or intravenous access influences vascular tone to decrease blood pressure. This has been demonstrated in the peripheral, pulmonary, and cerebrovascular circulations.[51–54,48]

As expected, the triple null mice exhibit many of the features associated with the absence of single NOS isoforms. For example, they have equivalent rates of hypertension and bradycardia as compared to eNOS-deficient mice. However, the loss of more than one isoform results in pathophysiologic changes not observed in singly NOS^{null} mice. For example, the number of disrupted *NOS* genes appears to positively correlate with the development of hypotonic polyuria, polydipsia, and renal responsiveness to vasopressin (i.e., nephrogenic diabetes insipidus).[46] The development of nephrogenic diabetes insipidus in triply NOS^{null} mice may be linked with decreased NO-dependent cGMP formation and membrane insertion of aquaporin-2 in renal collecting ducts, two biomolecular processes that regulate urine tonicity.[46]

Despite the inhibitory effects of eNOS-derived NO on pathobiologic processes linked to the development of coronary artery disease, such as promoting vasodilation and inhibiting platelet aggregation and leukocyte adhesion, eNOS-deficient mice do not spontaneously develop arteriosclerosis. In contrast to $eNOS^{null}$ mice, nNOS-deficient mice demonstrate elevated rates of metabolic syndrome, vascular lesion formation, and spontaneous myocardial infarction while iNOS deficiency is linked to atherogenesis.[47] These findings suggest that nNOS and/or iNOS may play a vascular-protective, compensatory role under conditions in which eNOS levels are diminished.

eNOS Uncoupling

Synthesis of NO by eNOS is dependent upon the transfer of electrons from NADPH to FAD and then to FMN in a reaction that occurs in the reductase domain of the eNOS dimer complex. Tetrahydrobiopterin (BH_4) is an eNOS cofactor required for electron donation from FMN to heme on the oxygenase monomer of eNOS that catalyzes the conversion of L-arginine + O_2 → L-citrulline + NO.[55] However, under conditions in which levels of BH_4, L-arginine, or NADPH are depleted, the eNOS dimer "uncouples" and oxygen is converted to superoxide in preference to NO (FIGURE 40.1).

Decreased levels of bioavailable NO that occur as a consequence of eNOS uncoupling is a critical downstream effect of increased vascular oxidant stress, and is linked to the pathobiology of vascular dysfunction in hypertension, diabetes mellitus, hypercholesterolemia, and atherosclerosis.[56] For example, eNOS uncoupling is postulated to be a mechanistic link between hyperaldosteronism and systemic hypertension. In peripheral blood vessels, elevated levels of aldosterone are associated with a vasculopathy characterized by activation of NADPH oxidase(s) that generates vascular endothelial superoxide formation. In turn, superoxide may oxidize BH_4 to BH_3 that results in eNOS uncoupling, decreased eNOS-dependent NO synthesis, and impaired endothelium-dependent vascular relaxation.[57]

In mice treated with streptozotocin to induce diabetes via pancreatic β-cell destruction, mouse aortas demonstrate increased total eNOS expression levels that are associated with diminished BH_4 and low levels of eNOS dimer formation.[58] Vascular dysfunction despite eNOS protein overexpression has been observed under a variety of experimental models of increased vascular oxidant stress. This paradox appears to reflect the effects of accelerated BH_4 consumption by increased eNOS activity,

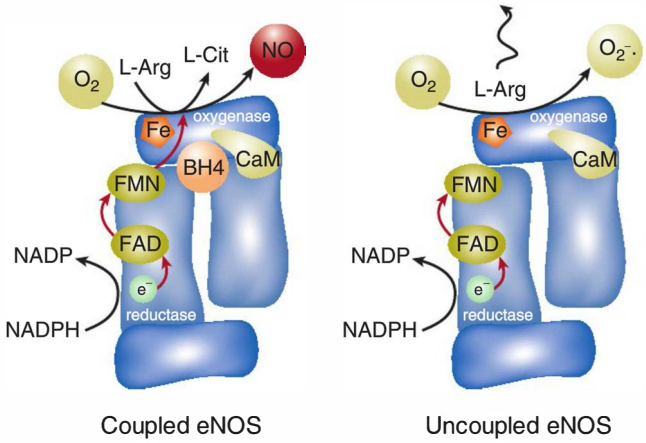

FIGURE 40.1 eNOS catalysis under normal conditions and in the uncoupled state. The synthesis of NO and L-citrulline (L-Cit) from molecular oxygen (O_2) L-arginine (L-Arg) by eNOS requires five cofactors that permit the flow of electrons from the reductase domain to the oxygenase domain. NADPH donates an electron to the FAD-FMN complex. FAD/FMN facilitates the transfer of an electron to the iron of heme on the oxygenase domain, while calmodulin is believed to promote the flow of electrons from the reductase to the oxygenase domains. This, and the donation of an electron and proton from tetrahydrobiopterin (BH_4) to O_2/L-Arg are necessary for the synthesis of NO/L-Cit. In turn, if levels of the eNOS cofactors BH_4 or NADPH, or the substrate L-Arg are insufficient, eNOS may become "uncoupled." Under these conditions, O_2 is converted to the reactive oxygen species superoxide ($\cdot O_2^-$) in preference to NO. CaM, calmodulin; Fe, iron; e^-, electron. (From Schulz E, Jansen T, Wenzel P, et al. Nitric oxide, tetrahydrobiopterin, oxidative stress, and endothelial dysfunction in hypertension. *Antioxid Redox Signal* 2008;10:1115–1126.)

which overall contributes to further superoxide formation by uncoupled eNOS, diminished levels of bioavailable NO, and worsening endothelium-dependent vascular relaxation.[56,59]

Other important mechanisms in addition to BH_4 depletion are implicated in the uncoupling of eNOS. For example, the endogenous NOS inhibitor asymmetric dimethylarginine (ADMA) is linked to eNOS-derived superoxide formation in pulmonary artery endothelial cells,[60] possibly by competitively inhibiting the binding of L-arginine. Circulating ADMA levels positively correlate with cardiovascular mortality.[61] Interestingly, the ADMA-degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) is redox sensitive: under conditions of increased vascular oxidant stress, inhibition of ADMA by DDAH is decreased resulting in elevated plasma ADMA levels.[61,62,63]

Genetic Etiologies of eNOS Dysfunction

A number of human studies have reported increased prevalence of specific eNOS polymorphisms in hypertensive cohorts.[64] For example, one major eNOS polymorphism is Glu298Asp, which has been associated with decreased NOS activity in the renal artery,[65] reduced basal production of NO,[66] coronary vasospasm,[67] essential hypertension,[68,69] hypertension in pregnancy,[70,71] high-altitude pulmonary edema,[72] as well as elevated risk of atherosclerosis[73] and possible myocardial infarction.[74,75]

However, the mechanism by which this genetic polymorphism alters eNOS function remains to be determined.

NO and Vascular Smooth Muscle Relaxation

NO produced in the endothelium stimulates sGC in VSMCs to produce cGMP. Increase in cGMP functions as a potent second messenger to promote vascular smooth muscle relaxation by activating cGMP-dependent PKG-myosin light chain phosphatase (MLCP) signaling, as well as by regulating intracellular calcium levels (FIGURE 40.2). The latter response may be mediated by the effects of cGMP on voltage-gated calcium channels, sodium/calcium exchangers, calcium-ATPase, phospholamban (PH), the IP3 receptor, and G proteins.[76] Alternatively, cGMP may independently regulate monovalent cation channels to affect intracellular calcium levels.

NO may also influence vasomotor tone through cGMP-independent pathways, including the activation of calcium-dependent potassium channels, which serve to hyperpolarize vascular smooth muscle.[77] NO can also rapidly and reversibly inhibit cytochrome c oxidase (complex IV). Additional evidence suggests that prolonged exposure to NO may persistently inhibit other heme or iron-sulfur cluster enzymes of the respiratory chain including aconitase, NADH-ubiquinone oxidoreductase (complex I), and succinate-ubiquinone oxidoreductase (complex II).[78] The rapid, reversible inhibition of cytochrome c oxidase is believed to provide a mechanism whereby NO may limit cell respiration and available cellular ATP, thereby attenuating smooth muscle contraction and causing passive vasodilatation.[78,79] Longer-term inhibition of other enzymes in the respiratory chain may lead to the suppression of key metabolic pathways and to pathologic changes in VSMCs.[78]

NO and Vascular Smooth Muscle Proliferation

NO inhibits the migration and proliferation of VSMCs.[80] Pharmacologic NO donors exhibit antiproliferative effects *in vitro*,[81] possibly by attenuating activity through activation of the cGMP-dependent protein tyrosine phosphatase, or "PTP-PEST."[82] Elevated levels of cGMP also activates cAMP-dependent protein kinases, which may account, in part, for the antiproliferative effect of NO (see below for additional details on NO and cAMP-dependent protein kinase).[83]

Antiproliferative characteristics of NO-donating drugs may also have an antiatherogenic effect in healthy endothelium vis-à-vis preventing neointimal formation in vascular smooth muscle.

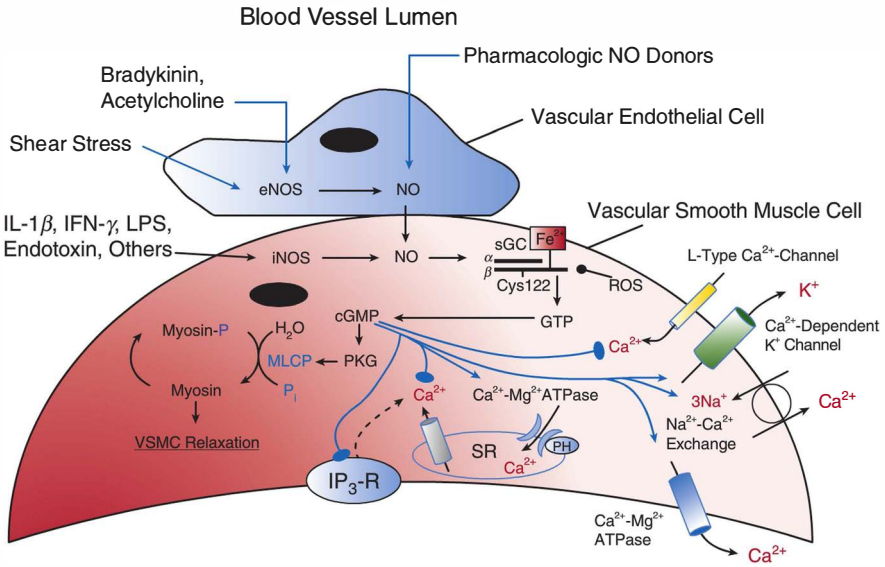

FIGURE 40.2 NO-induced cGMP formation modulates VSMC relaxation. In vascular endothelial cells, shear stress and/or agonist-mediated activation of a broad range of cell surface receptors increases eNOS activity and results in NO synthesis. NO derived from eNOS and/or pharmacologic donors may traverse the vascular endothelial cell membrane into the vascular smooth muscle cell (VSMC) layer. Alternately, induction of iNOS by various cytokines and/or endotoxin increases levels of bioavailable NO in VSMCs. The interaction of NO with soluble guanylyl cyclase (sGC) results in the conversion of cytosolic GTP to cGMP. Importantly, oxidative modification of sGC at the heme site or at key cysteinyl thiols (e.g., Cys122) on the catalytically active β_1-subunit may impair NO-sensing by sGC and decrease conversion of GTP to cGMP. Protein kinase G (PKG)-dependent activation of myosin light chain phosphatase (MLCP) by cGMP induces VSMC relaxation. Additionally, cGMP modulates VSMC relaxation by decreasing intracellular levels of Ca^{2+}. This occurs via cGMP-induced inhibition of Ca^{2+} mobilization through L-type Ca^{2+} channels or the inositol triphosphate-3 receptor (IP_3-R) located on either the plasma membrane or in the sarcoplasmic reticulum (SR). In turn, cGMP also decreases intracellular Ca^{2+} levels by stimulating Ca^{2+}-dependent K^+ exchange, Na^{2+}-Ca^{2+} exchange, Ca^{2+}-Mg^{2+} ATPase(s), and phospholamban (PH) in the SR. *Arrows* and *circular* heads indicate agonist and inhibitory effects, respectively. eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; ROS, reactive oxygen species; LPS, lipopolysaccharide. (Adapted from Lucas KA, Pitari GM, Kazerounian S, et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* 2000;52(3):375–414. Ref. 238.)

However, endogenous NO signaling pathways are also linked to migration[84] and proliferation[85] of VSMCs, as well as vascular endothelial growth factor (VEGF)-NO signaling–associated angiogenesis. Despite this and other similar observations, the preponderance of data currently available suggests that the attributable contribution of NO to the development of atherosclerosis is small, and is offset significantly by the proven beneficial effects of NO on maintaining normal endothelial function.

NO and Vascular Apoptosis

NO is a key mediator of apoptosis in various cell types including vascular endothelial and VSMCs.[86] The effect of NO on apoptosis is concentration-dependent: low levels of NO are protective against apoptosis, whereas elevated levels of NO promote apoptosis and are cytotoxic.[87] This provides a framework for interpreting the variable effects of each NOS isoform on the cell cycle. For example, iNOS induction in VSMCs or macrophages, which is associated with apoptosis, tends to generate significantly higher levels of NO compared with eNOS. In turn, eNOS exerts a predominantly antiapoptotic effect in vascular endothelial cells.

The mechanisms whereby NO inhibits apoptosis in the endothelium appear to be independent of cGMP and may involve the increased expression of heat shock proteins,[88] inhibition of apoptotic kinase cascades, as well as inhibition of a family of proapoptotic cysteine proteases (termed caspases), probably via cysteine sulfhydryl nitrosylation and/or tyrosine nitrosation.[89,90] Shear stress over the endothelial layer, which is an important determinant of basal NO synthesis by eNOS, may be an important antiapoptotic factor that serves to protect the endothelium against apoptosis in the initial stages of atherogenesis.[91]

The ability of iNOS-derived NO to exert proapoptotic and antiproliferative effects on vascular smooth muscle may contribute to the putative role of iNOS in vascular wall remodeling.[92] NO-induced apoptosis of VSMCs may also be important in atherogenesis and plaque instability.[93,94] The proapoptotic functions of iNOS-derived NO also appear to be cGMP-independent, and may relate to NO-induced oxidative damage of cytosine residues, which results in DNA fragmentation.[95] Other mechanisms that have been proposed include: increased formation of peroxynitrite; activation of caspases; activation of poly(ADP ribose) polymerase; and increased mitochondrial permeability. Some evidence indicates an upregulation of p53 and/or p73 protein in apoptotic cells, and experiments demonstrate vascular smooth muscle from $p53^{null}$ mice are specifically more susceptible to NO-induced apoptosis as compared to wild type.[96,97]

Endothelial expression of iNOS may also induce apoptosis in endothelium in diabetic vasculopathy, where circulating glycosylated albumin derivatives have been reported to upregulate iNOS activity in small vessels.[98] Additionally, expression of iNOS has been suggested as a mechanism of neuronal apoptosis that contributes to cardiovascular autonomic depression in septic shock.[99]

eNOS and Leukocyte–Endothelial Interactions

eNOS-derived NO exerts broad anti-inflammatory and antithrombogenic effects that arise in large part by the inhibition by NO of leukocyte–endothelial interactions. NO downregulates expression of P-selectin on endothelial cells.[100] NO-mediated inhibition of P-selectin expression prevents the entire process of leukocyte recruitment. NO may also limit the expression of leukocyte adhesion molecules such as L-selectin or CD11/CD18.[101] Since superoxide in the endothelium and leukocytes can promote oxidant-dependent adhesion, the inhibitory effects of NO on cell adhesion may be mediated in part by detoxification of superoxide.[102] Inhibition of eNOS enzyme activity may result in the enhanced adherence of leukocytes to the vascular endothelium.[102] Since monocyte adhesion may be one of the initial events in atherogenesis, eNOS may function as an antiatherogenic agent in part by inhibiting recruitment of leukocytes to endothelium. eNOS enzyme inhibition by arginine analogs also leads to increases in vascular permeability, extravasation of plasma proteins, and microvascular edema, involving both cGMP-dependent[103] and cGMP-independent pathways of vascular permeability.[104] These increased vascular permeability responses may be mediated in part by the increased adherence of leukocytes to endothelium in the absence of NO.[105]

eNOS and Blood Platelets

The same eNOS isoform originally described in vascular endothelium is also expressed in blood platelets, but the vascular endothelium is more significant quantitatively as a source of NO.[106,107] Endothelial cell–derived NO is important to the regulation of platelet function, and is a key determinant of the balance between physiologically appropriate hemostasis and pathologic thrombosis. Nevertheless, platelet-derived NO from endogenous platelet eNOS may also play an important role in inhibiting platelet adhesion,[39,80,108] aggregation,[80] and activation. NO may affect platelets through several interrelated signaling pathways that variously involve cGMP, calcium, eicosanoids, and phosphoinositides as key effectors of the NO response.

Platelet adhesion to the endothelium depends on a number of adhesion molecules expressed on both cells, including intercellular adhesion molecule (ICAM)-1 and α/β 3 integrin on the endothelium, and β_1 integrin, P-selectin, glycoprotein Ib, and glycoprotein IIb/IIIa on platelets.[109] NO released by nitrovasodilator drugs inhibits expression of P-selectin on platelets.[110] In addition to antagonizing platelet–endothelial interactions, the downregulation of P-selectin also inhibits platelet–leukocyte interactions. A PKG-modulated pathway also disrupts activation of GP IIb/IIIa, one of the more critical mechanisms of platelet adhesion to the endothelium. In this pathway, cGMP-activated PKG phosphorylates both phospholipase C and vasodilator-simulated phosphoprotein,[111] which results in a decline in intraplatelet calcium. cGMP can also lower intraplatelet calcium by inhibiting release of intracellular calcium stores and shuttling more calcium out of the cell while limiting extracellular calcium reabsorption.[108] Low calcium hinders protein kinase C signaling, hindering the activation of GP IIb/IIIa.[110] The GP IIb/IIIa receptor is also necessary for aggregation of platelets in nascent thrombi by cross-linking platelets and fibrin. The potent platelet aggregating effects of thrombin are inhibited by NO[112] from eNOS, which may also be activated by insulin.[113] In platelets, insulin stimulates the Akt-mediated phosphorylation of eNOS Ser1177 via phosphoinositide-3-kinase (PI3K) and AMP-activated protein kinase (AMPK) pathways.[113] In addition to insulin's direct effects on eNOS in endothelial cells, insulin may also induce vasodilatation by activating platelet eNOS and

PKG,[114] resulting in release of vesicles containing the vasoactive substances ATP, adenosine, and serotonin, which may in turn activate eNOS.[115] The lipid mediator sphingosine-1-phosphate (S1P) is involved in a signaling between platelets and the endothelium: activated platelets secrete S1P, which stimulates activation of eNOS, thus releasing NO into the vascular lumen, which serves to attenuate platelet activity.

NO inhibits a number of arachidonic acid pathways that normally function to activate platelets. NO inhibits platelet release of ADP[16] as well 12-lipoxygenase, thus preventing formation of leukotrienes and hydroxyeicosatetraenoic acids. NO attenuates the activity of phospholipase A2, the rate-determining enzyme in the arachidonate pathway by which platelets ultimately produce thromboxane. PKG in platelets also impairs thromboxane-mediated signaling by directly phosphorylating the G-protein–coupled TXA_2 receptors, further inhibiting thromboxane-mediated platelet activation via the downstream phospholipase C cascade.[116] These mechanisms of thromboxane antagonism coordinately attenuate a potent stimulus of platelet activation and recruitment.

NO also positively affects the *in vivo* synthesis of prostacyclin, the physiologic antagonist of thromboxane.[117] Synergy in the antiplatelet effects of prostacyclin and NO may result from cGMP-mediated inhibition of cAMP-specific phosphodiesterase III, which elevates intraplatelet cAMP and attenuates platelet activation. NO-mediated inhibition of platelet function may also occur via cGMP-independent mechanisms, as suggested for the NO-dependent suppression of thromboxane synthesis.[118,119]

Platelets derived from $eNOS^{null}$ mice show several abnormalities in agonist-induced platelet aggregation,[39] confirming the role of platelet eNOS–derived NO in normal hemostasis. The loss of NO-cGMP signaling in these $eNOS^{null}$ mice shifts the balance of platelet function toward platelet adhesion and aggregation. In mice with normal eNOS but with eNOS-deficient platelets, bleeding time is decreased owing to increased platelet-endothelial adhesion.[39] In humans, NO-mediated inhibition of platelet function results in decreased bleeding time[120] and it has been suggested that nitrovasodilator-mediated inhibition of platelet aggregation may affect the progression of coronary thrombosis in patients with unstable angina pectoris.[121]

NO is antithrombotic through mechanisms that are independent of its inhibitory effects on platelet aggregation and NO-dependent activation of ICAMs. For example, increased eNOS-derived NO synthesis is a well-established countermeasure to the prothrombotic effects of shear stress on vascular endothelial structure and function.[122] NO synthesized under these conditions is protective against the development of prothrombotic molecular and phenotypic changes in the blood vessel wall such as lipid oxidation, smooth muscle cell proliferation, and apoptosis.[123] Protecting the integrity of the endothelial cell layer is associated with a decrease in the thrombogenicity of the blood vessel lumen. In turn, eNOS deficiency is linked to increased thrombosis following endothelial injury induced by mechanical force or by activation of the procoagulant protein von Willebrand factor.[124] Other factors that suppress eNOS activity, such as the endogenous NOS inhibitor known as ADMA, are found in thromboembolic diseases.[125] For example, elevated ADMA levels are present in chronic thromboembolic pulmonary hypertension, which is a disease characterized by pathologic changes to the normal pulmonary vascular architecture and *in situ* thrombosis.[125]

Regulation of eNOS in Endothelial Cells

eNOS is expressed in endothelial cells, and to a lesser extent in platelets,[106] mast cells, and neutrophils.[126] The tissue-restricted expression of eNOS has lead to analyses of the eNOS promoter. The promoter for the *eNOS* gene lacks a prototypical TATA promoter, and instead contains promoter sites for the binding of SP1 and GATA transcription factors, two mechanisms that are utilized by other constitutively expressed genes.[127,128] Despite constitutive expression, the precise levels of eNOS protein are further controlled by specific transcriptional stimuli and by posttranscriptional modulation of eNOS mRNA stability.[129]

Several extracellular factors influence eNOS mRNA levels. For example, shear stress is a key determinant of basal eNOS transcription. This effect may be mediated, in part, by nine consensus shear stress–response elements in the eNOS promoter.[76] The transcription factor KLF2 is specifically upregulated by shear stress in the endothelium,[130] and KLF2 itself serves as an important regulator of basal eNOS transcription in response to laminar shear stress.[131] Shear stress also induces eNOS transcription via a c-Src/NF-κB dependent pathway.[132,133] Hypoxia has been shown to induce *eNOS* gene transcription. This effect is mediated by a hypoxia-responsive element 5.3 kb upstream of the transcription start site.[134] However, the role of hypoxia in regulating eNOS is not entirely clear, as some evidence suggests that hypoxia may actually inhibit eNOS expression via the Rho kinase pathway.[135] Cytokines, including tumor necrosis factor (TNF)-α, downregulate eNOS transcription in part by inhibition of SP1.[136] High levels of oxidized low density lipoprotein (LDL) have been shown to impair eNOS mRNA stability.[137] Statins, which may exert an indirect effect on eNOS mRNA via LDL-lowering properties, furthermore have an independent effect in increasing eNOS mRNA,[137,138] which may be mediated by limiting the cytokine-mediated downregulation of eNOS transcription,[139] or by statin-mediated inhibition of RhoA GTPase.[140] Many other mediators exert effects at the level of eNOS transcription, including thrombin and Rho kinase, both of which attenuate the half-life of eNOS mRNA.[141,142]

In endothelial cells, the eNOS enzyme is subject to an intricate scheme of modifications and controls that result in a dynamic pattern of stimulation and inhibition in response to a number of physiologic stimuli. Five themes have emerged from a wide variety of experiments that elucidate some of the biochemical systems controlling eNOS in vascular endothelial cells: (a) eNOS is targeted to plasmalemmal caveolae, (b) in the caveolae, eNOS is tonically inhibited by its interaction with the scaffolding-regulatory protein caveolin, (c) certain extracellular signals, including shear stress and specific agonists, establish a basal level of eNOS activity for NO signaling in the endothelium, (d) a diverse family of eNOS agonists—generally involving calcium- or phosphorylation-mediated pathways—dynamically regulates eNOS activity by promoting a rapidly reversible activation of eNOS followed by the return of eNOS activity to basal levels, and (e) part of this inhibitory mechanism and return to baseline may involve subcellular translocation of eNOS from caveolae in a reversible agonist-mediated process.

eNOS and Caveolae

Most eNOS in a resting endothelial cell is targeted to specialized plasmalemmal domains called caveolae. Caveolae are small invaginations of the plasma membrane that are relatively

enriched with cholesterol and sphingolipids, and are further distinguished by the presence of the transmembrane scaffolding protein caveolin-1. The unique biochemical composition of caveolae generates an "ordered liquid phase" that may facilitate various protein–protein and protein–lipid interactions.[143] Caveolae are now recognized as important domains for the sequestration of diverse receptors and signaling molecules, including G-protein–coupled receptors and growth factor receptors and their downstream activators, as well as calcium regulatory proteins, and sphingolipids. The targeting of eNOS to caveolae is dependent on dual acylations of the protein by myristoylation and palmitoylation. Myristoylation of eNOS is irreversible and cotranslational, and occurs on the amino group of glycine-2 after removal of the *N*-terminal methionine; this modification is absolutely essential to caveolar targeting.[144] Palmitoylation of eNOS is posttranslational and occurs at two cysteine residues, Cys15 and Cys26, near the *N*-terminus. The thiopalmitoyl bond is relatively labile, and palmitoylation is reversible in the presence of the enzyme acyl protein thioesterase-1 (APT-1).[145]

When anchored in caveolae, eNOS is tightly associated with caveolin-1 via protein–protein interaction; eNOS can be coimmunoprecipitated with anticaveolin-1 antibodies. The association with caveolin maintains eNOS in an inactive state in resting endothelial cells. Dynamic regulation of eNOS activity is achieved through various calcium- and phosphorylation/dephosphorylation-mediated pathways that appear to operate in tandem with cycles of enzyme acylation/deacylation. Regulation of intracellular calcium levels appears to provide the most rapid increase in eNOS activity through the binding of calcium/calmodulin to the enzyme. eNOS agonists, including acetylcholine (acting *via* M2 muscarinic receptors), bradykinin, and estradiol,[146] are known to initiate receptor-specific intracellular signaling pathways in endothelial cells. For example, bradykinin and acetylcholine activate a Gq pathway that increases phospholipase C activity, leading to inositol triphosphate generation and the release of calcium from intracellular stores.[76] These agonist-induced transient increases in intracellular calcium lead to the activation of the ubiquitous calcium regulatory protein calmodulin. Binding by eNOS of the calcium–calmodulin complex disrupts the caveolin-eNOS inhibitory protein–protein interaction, and increases the catalytic activity of eNOS, apparently by facilitating the transfer of electrons from the NADPH/FAD/FMN cofactors in the reductase domain to the oxygenase domain in eNOS. Displacement of caveolin from eNOS occurs in concert with the binding of calcium–calmodulin to the enzyme.[147] In turn, calcium–calmodulin binding to eNOS facilitates the depalmitoylation of eNOS catalyzed by the enzyme APT-1.[145] In the cytosol, eNOS activity is downregulated further, possibly via reversible phosphorylation at Ser 116.[148] Recent evidence indicates that calmodulin itself is under control of kinase CK2; phosphorylated calmodulin is less efficiently bound, and thus the response of eNOS to intracellular calcium is abbreviated and eNOS activity is attenuated. Since calmodulin levels in the cell are low relative to total capacity of calmodulin binding proteins, this CK2 phosphorylation mechanism may serve to selectively displace calmodulin from eNOS without decreasing total cellular calmodulin levels or disturbing other signaling pathways.[149]

Caveolin-1 may have important roles in the pathobiology of cardiovascular diseases as a consequence of the inhibitory effect of caveolin-1 on eNOS activity (see a detailed review of caveolae and caveolins by Patel et al.[150]). Blood vessels isolated from caveolin-1–deficient (Cav-$1^{-/-}$) mice show an exaggerated response to agonists that promote eNOS activation, yet systemic blood pressure in these animals is not substantively changed. Indeed, there are mixed results regarding the functional effects of caveolin-1 inhibition on key hemodynamic indices. For example, Wunderlich et al.[151] demonstrated that in Cav-$1^{-/-}$ mice, increased levels of eNOS activity, but not BH_4 are observed, resulting in eNOS uncoupling and an increase in pulmonary artery pressure compared to wild type mice. Others have observed no significant change in central aortic blood pressure in Cav-$1^{-/-}$ mice despite biochemical evidence to suggest increased levels of NO.[152] Understanding further the complexities of caveolin-1–eNOS signaling *in vivo* to develop this as a therapeutic target is a goal of ongoing translational investigations.[153]

eNOS Phosphorylation

In addition to these mechanisms involving acylation and calcium, the complete picture of eNOS regulation includes extensive pathways of phosphorylation and dephosphorylation at multiple sites on the enzyme (FIGURE 40.3). Phosphorylation at Ser1179 in bovine eNOS (Ser1177 in human eNOS) leads to enzyme activation, whereas phosphorylation at Thr 497 and Ser 116 leads to attenuation of eNOS activity. Recent studies suggest that Ser 635 is a stimulatory phosphorylation site, possibly responsive to shear stress,[154] while phosphorylation of Ser 617 may sensitize eNOS to calmodulin binding.[155] Src-dependent phosphorylation at Tyr81 in the oxygenase domain of eNOS appears to be one mechanism by which stimulation of endothelial cells by bradykinin, ATP, and acetylcholine, among others, promotes NO generation.[156,157] Indeed, compared to wild type eNOS, site-directed mutagenesis in which phenylalanine, which is resistant to phosphorylation, is substituted for Tyr81 (Y81F) is unresponsive to stimulation with bradykinin or VEGF. Proposed but as yet unconfirmed mechanism(s) by which eNOS phosphorylation at Tyr81 increases eNOS-derived NO production include changes to eNOS activity levels, alterations in Ca^{2+}-sensing by eNOS, and direct effects on eNOS stability.[156,157]

In contrast to Tyr81, phosphorylation at Tyr657 results in a complete loss of eNOS-dependent NO, citrulline, and superoxide production.[158] The inhibitory effect of Tyr657 phosphorylation on eNOS activation may be linked to the location of this tyrosine residue, which lies within the FMN domain on the reductase eNOS monomer. It has been hypothesized that limitations to FMN mobility imposed by phosphorylation restricts FMN-mediated delivery of electrons to the heme moiety of the oxygenase monomer, which is necessary to facilitate the conversion oxygen/L-arginine to NO/L-citrulline.[159] Phosphorylation at Tyr657 is proline-rich tyrosine kinase 2 (PYK2) dependent; in mice aortas, angiotensin II infusion, which is associated with increased vascular hydrogen peroxide formation and subsequent activation of PYK2, attenuates NO synthesis and endothelium-dependent vascular relaxation.[160]

Phosphorylation of eNOS Ser1179 is mediated directly by kinase Akt, also known as protein kinase B,[156] and numerous other protein kinases have been implicated in phosphorylation of this site. Akt is itself activated by phosphorylation pathways downstream of PI3K. Although Akt is predominantly found in the cytosol, membrane targeting of both eNOS and Akt is

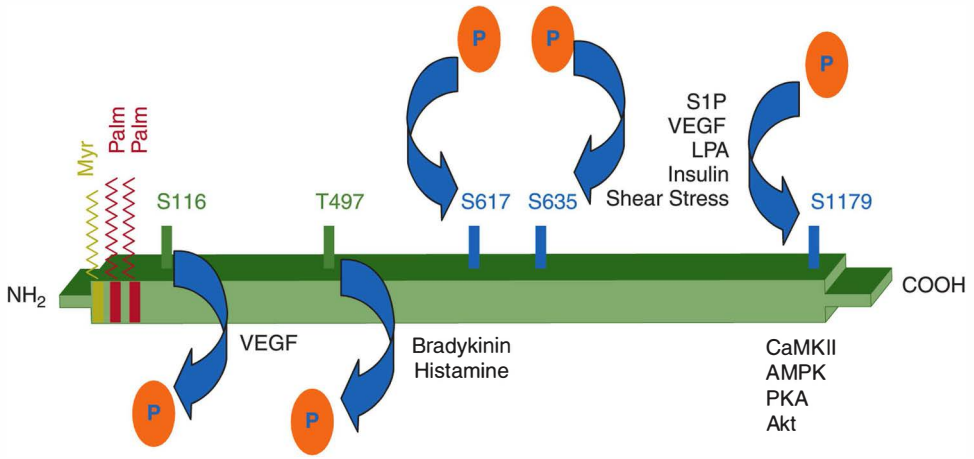

FIGURE 40.3 Regulation of eNOS activity. eNOS activity is regulated by agonist-mediated phosphorylation and dephosphorylation at a number of eNOS serine and threonine sites (amino acid sequence numbers refer to bovine eNOS). Phosphorylation at Ser1179 and Ser 635 are believed to stimulate eNOS activity, while phosphorylation at Ser 617 may promote calmodulin binding and thus increase eNOS activity. Many eNOS agonists, including shear stress, VEGF, S1P, estrogen, and leptin, lead to phosphorylation at Ser1179. Phosphorylation at Ser 116 or Thr 497 attenuates eNOS catalytic activity. VEGF induced dephosphorylation at Ser 116 increases eNOS activity; similarly, protein phosphatase 2A may dephosphorylate Thr 497 and Ser1179.

necessary for eNOS to undergo phosphorylation at Ser1179.[144] Upon activation, Akt is known to translocate to the membrane, as its upstream activators are membrane associated. Several molecules superimpose another level of control on eNOS by interacting with kinase Akt in the vascular endothelium. Thrombin, acting via the Rho/Rho kinase pathway, inhibits Akt-mediated phosphorylation and activation of eNOS.[141] The heat shock protein hsp90 appears to bind to eNOS and/or kinase Akt to promote eNOS activation.[161] Adipocyte-released hormones appear to cause vasodilatation: leptin may activate endothelial Akt in a tyrosine kinase–dependent mechanism,[162] while adiponectin, an insulin mimetic, activates insulin receptor tyrosine kinases leading to eNOS activation via a PI3K/AMPK pathway.[163]

Part of the overall endothelial response to shear stress is mediated by phosphorylation of eNOS at Ser1179. Shear stress is likely a key determinant of the tonic stimulation of NO production and the maintenance of basal vascular tone.[164] Shear stress is transduced via a mechanochemical coupling that increases both calcium and potassium currents and leads to activation of the PI3K/Akt kinase and PKA pathways.

VEGF is a potent stimulus of eNOS activity via kinase Akt–mediated phosphorylation at eNOS Ser1179.[148] Activation of the VEGF receptor KDR results in PI3K α and PI3K β activation, and leads to increased activity of Akt. In addition to its effects at Ser1179, VEGF induces dephosphorylation at eNOS Ser116 in a pathway that involves the activation of the phosphatase calcineurin.[148] Since phosphorylation of eNOS at Ser116 appears to inhibit enzyme activity, the involvement of calcineurin in eNOS dephosphorylation may provide a clue to the hypertensive effects of the calcineurin inhibitor cyclosporine.

Other agonists activate eNOS by a PI3K/Akt-dependent mechanism, including estrogen,[165,166] and a class of platelet-derived lipid mediators that includes S1P and lysophosphatidic acid (LPA).[167] In addition to S1P release from activated platelets and endothelial cells, S1P release is induced by platelet-derived growth factor and released by platelets into the extracellular space, where it activates endothelial cells through its interaction with G-protein–coupled S1P receptors (formerly known as EDG, or endothelial differentiation gene receptors). Moreover, S1P has many functions in endothelial cells including angiogenesis, vasorelaxation, and proliferation. S1P induces translocation of S1P receptors to caveolae.[168] This translocation may facilitate coupling of the S1P receptor to downstream effectors that lead to eNOS Ser1179 phosphorylation *via* a PI3K β/Akt pathway.[169]

LPA is released by platelets activated by agonists, including thrombin. Although LPA exerts actions similar to S1P, including phosphorylation of kinase Akt and eNOS, LPA acts independently of S1P receptors,[170] and appears to bind the related family of LPA receptors.[171] S1P and LPA signaling provides a paradigmatic example of cross-talk between platelets and endothelial cells in the regulation of eNOS.

VEGF is a critical regulator of eNOS in that it directly exerts effects via PI3K/Akt but also sensitizes the endothelium to the effects of lipid mediators.[172] VEGF induces S1P receptor mRNA and receptor levels, and thereby augments eNOS activity and endothelial responses to S1P. S1P and VEGF may synergize and coordinately promote angiogenesis by stimulating NO-dependent endothelial cell migration.

PI3K/Akt pathways are but one of a number of phosphorylation cascades that modulate the activity of eNOS. VEGF, S1P, and bradykinin all stimulate various mediators in the MAP kinase pathway, especially ERK1 and ERK2.[148,173–175] The pleiotropic effects of various agonists evince a possible developing paradigm of complex temporal regulation of eNOS. A wide variety of protein kinases have been implicated in phosphorylation of eNOS. In addition to the role clearly established for kinase Akt, other reports have implicated PKA, PKC, PKG, MAP kinases, and the AMPK in eNOS phosphorylation. The relative roles of different kinase pathways represent an active area of investigation, and it is safe to say the pattern of eNOS regulation by

protein kinases and phosphoprotein phosphatases is complex. The phosphorylation of eNOS at different sites has profound effects on stimulating and inhibiting eNOS activity, and it is clear that various phosphatases, as well as kinases, contribute to these processes. Part of the eNOS-activating effect of bradykinin may derive from calcineurin-mediated dephosphorylation of Thr 497.[176] In addition to the phosphatase calcineurin, protein phosphatase 2A appears to be a key regulator with an overall inhibitor effect on eNOS activity that dephosphorylates eNOS on Thr 497 and Ser1179.[177] Reciprocal control of phosphorylation at Thr 497 may contribute to the balance of production of NO and superoxide, respectively, by eNOS.[149]

eNOS (De)acetylation

There is growing evidence to support a role for (de)acetylation in modulating eNOS bioactivity. For example, Mattagajasingh et al.[178] demonstrated that by deacytylating lysine 496 and 506 in the calmodulin-binding domain of eNOS, silent mating type information regulation 2 homolog (SIRT1), a lysine deacetylase histone deacetylase-3 (HDAC3), increases levels of the stable NO byproducts nitrite and nitrate (NOx) and promotes endothelium-dependent vasodilation. Laminar flow, which is associated with shear stress and eNOS activation, increases SIRT1-mediated deacetylation activity in cultured endothelial cells. In contrast, oscillatory flow, in which flow is disturbed and without uniform direction, has no effect on SIRT1 activity.[179] Overall, these findings are consistent with observations made by others that aortic endothelial cell exposure to the SIRT1 stimulator resveratrol increases eNOS activity.[180] Furthermore, calorie restriction–induced eNOS deacetylation may provide one potential mechanism to explain the beneficial effects of calorie restriction as an established strategy to improve blood pressure and vascular reactivity in both obese and nonobese mammals.[181,182]

Similar to the divergent site-dependent effects of serine phosphorylation on eNOS activation, it has been demonstrated that acetylation of lysine 607 (Lys609 in mammals), which is located in the autoinhibitory domain of calmodulin (i.e., activation of this domain interferes with eNOS binding to calmodulin to decrease eNOS activity), is a critical protein posttranslational modification that increases eNOS-dependent NO synthesis.[183] Jung et al. investigated the possibility that Lys609 acetylation is one mechanism by which aspirin increases vascular NO levels to induce a decrease in systemic blood pressure. Indeed, their findings suggest that by inducing Lys609 acetylation, low-dose aspirin promotes eNOS–calmodulin binding to increase eNOS-dependent (but cyclooxygenase-independent) NO generation *in vitro* and in mice aortas *in vivo*. Conversely, these authors demonstrate by inducing deacetylation of Lys609, SIRT1 inhibits aspirin-mediated eNOS activation by preventing lysine acetylation.

AMP-Activated Protein Kinase

AMPK is a heterodimeric enzyme that is stimulated by an increase in cellular levels of AMP:ATP and is also a target of several protein kinases.[184] Within many cell types, the role of AMPK may be most closely related to the effects of this kinase on cellular energetics. Studies of AMPK in vascular endothelial cells have focused on signaling pathways that regulate cellular metabolism and vasodilatory function. Chen et al.[185] demonstrated that AMPK coimmunoprecipitates with eNOS in rat cardiac myocytes and promotes phosphorylation of eNOS at Ser1177 in the presence of Ca^{2+}-calmodulin to increase eNOS activity. Moreover, under conditions of myocardial ischemia, which are associated with decreased levels of bioavailable ATP, a twofold increase in AMPK levels was associated with a significant increase in P-eNOS (Ser1177) protein expression levels and Ca^{2+}-calmodulin–dependent eNOS activity.

In aortic endothelial cells, it has been discovered that factors conventionally associated with eNOS activation—shear stress, statins, and adiponectin—induce phosphorylation of AMPK at Thr172, which, in turn, is linked to phosphorylation of eNOS at Ser633 and increased levels of eNOS-derived NO.[186] Increases in statin-induced eNOS phosphorylation at Ser633 are not maintained in aortas harvested from AMPK α_2 null mice *in vivo*.[179] Moreover, it appears that Ser633 may compete with Ser1177 for phosphorylation by AMPK. Overall, these findings suggest that AMPK functions in an important role to regulate NO bioavailability in vascular endothelial cells.

The framework of AMPK-eNOS signaling has been expanded recently. For example, VEGF-stimulated AMPK results in eNOS phosphorylation at Ser1177, and provides one potential mechanism to account for the observation that inhibition of VEGF-induced activation of PI3K does not fully attenuate eNOS activation.[187] Furthermore, it has recently been reported that AMPK is a regulatory target of eNOS. In cultured aortic endothelial cells, molecular or pharmacologic inhibition of eNOS is associated with increased intracellular levels of H_2O_2, which, in turn, is a potent agonist for Ca^{2+}/calmodulin-dependent protein kinases II β (CaMKII β)-dependent phosphorylation and activation of AMPK.[188] As stated above, AMPK is an established agonist for eNOS activation. Thus, collectively these findings raise the possibility that oxidant stress-mediated AMPK activation, either from eNOS-dependent (i.e., NOS uncoupling) or eNOS-independent reactive oxygen species generating sources (i.e., NADPH oxidase, mitochondrial sources, others), is a key counter regulatory mechanism to maintain cellular levels of NO under conditions in which eNOS may be dysfunctional or present at low levels.

INDUCIBLE NOS-DERIVED NITRIC OXIDE AND VASCULAR PHYSIOLOGY

iNOS has been isolated from macrophages, endothelial cells, hepatocytes, neutrophils, airway epithelium, fibroblasts, bone, microglia, retina, and mast cells; its prototypical function is as a component of the innate immune response to pathogens and turnover cells.[189,190] iNOS is generally absent in quiescent cells, but iNOS mRNA levels rise rapidly within hours following exposure to proinflammatory stimuli, including bacterial lipopolysaccharide (LPS), lipoteichoic acid, and some exotoxins like TSST-1; heat stress and hypoxia may induce similar transcriptional responses. Bacteria-generated proinflammatory cytokines including IFNγ, TNF-α, TNF-β, interleukin (IL) 1, IL 12, and platelet activating factor[191] promote iNOS transcription via NF-κB–dependent signaling pathways.[192,193] Induction of iNOS leads to persistent NO production, as the tight binding of calmodulin to iNOS at low levels of intracellular calcium leads to "constitutive activation" of this inducible enzyme once it is synthesized in response to proinflammatory stimuli. The relatively high concentrations of iNOS-derived NO destroy cellular structures and inhibit iron-containing enzymes. Intricate

molecular cross-talk between the cyclooxygenase and NOS pathways interconnects NO with inflammation. Specifically, iNOS-derived NO may stimulate cyclooxygenase-2 directly or via cGMP that is generated by NO-sGC signaling. Activation of cycloloxygenase-2 results in the subsequent production of prostaglandin E_2/I_2, each of which, in turn, may induce iNOS.[194]

iNOS and Infection

Widespread induction of iNOS in macrophages, VSMCs, hepatocytes, and cardiomyocytes is a central feature in the pathophysiology of endotoxemia, sepsis, and circulatory shock. Supraphysiologic induction of iNOS in macrophages and VSMCs significantly raises NO production in the vasculature, such that blood levels of NO and its metabolites can reach micromolar levels.[195] iNOS-dependent increases in NO has been proposed as the major impetus for systemic hypotension that is observed clinically in patients with septic shock. In fact, the role of iNOS in modulating abnormal hemodynamics in sepsis is undoubtedly more complex. For example, endotoxemia-associated iNOS induction is associated with disrupted vascular endothelial metabolic signaling pathways, skeletal muscle insulin resistance, and abnormal hypothalamic arginine vasopressin synthesis, all of which may contribute to hypotension in sepsis.[196,197] In addition to the influence of iNOS on vascular function in shock, iNOS induction in the heart is a negative inotrope, and thus by decreasing cardiac output exacerbates hypotension directly.[198] Therefore, through this pathway, iNOS is a likely stimulator of worsening local metabolic derangement (e.g., lactic acidosis) in vascular and skeletal tissue that contributes to end-organ injury via hypoperfusion-induced mitochondrial dysfunction.

iNOS and Vascular Diseases

In addition to its roles in inflammation and infection, iNOS has important roles in vascular biology, and iNOS-derived NO has been associated with several vascular disease states. In iNOS-deficient mice, cholesterol levels and the incidence of atherosclerotic plaques were found to be significantly increased.[199] Several studies have reported that iNOS is induced in vascular smooth muscle within human atherosclerotic lesions, and the expression of iNOS in macrophages colocalizes with peroxidized lipids and necrotic cores in advanced atheromas.[200,201] Activation of iNOS by the inflammation-regulating nuclear receptor of peroxisome proliferator-activated receptor gamma results in VSMC proliferation and neointimal formation in carotid arteries following vascular injury.[202]

The precise roles of iNOS in atherogenesis are still being defined, and it is not yet clear whether iNOS reflects a pathologic or protective response, or both. For example, iNOS-derived NO may possibly function as an antioxidant in atherosclerotic plaques.[203] Additionally, by combating certain pathogens, iNOS may prevent the initiation or exacerbation of the inflammatory and immunologic reactions of atherosclerotic responses in the vascular wall.[204] Statins have been demonstrated to upregulate iNOS mRNA levels in vascular smooth muscle and may contribute to the atheroprotective effect of this class of therapeutics.[205] In turn, iNOS expression has been correlated with decreased extracellular collagen content and atherosclerotic plaque instability. Moreover, aortas from rats fed a high fructose diet to induce the metabolic syndrome are characterized by elevated levels of ADMA, an endogenous eNOS inhibitor, but also have increased protein expression levels of iNOS and nitrotyrosine (i.e., consistent with peroxynitrite formation).[206] These findings reflect the unresolved role of iNOS in atherosclerotic disease: increased iNOS expression could be a compensatory measure to offset ADMA-induced degradation of eNOS, or, conversely may be induced by elevated oxidant stress levels to function as a promoter of vascular injury.[206,207]

iNOS may also play a role in the pathogenesis of hypertensive states, including preeclampsia and some forms of pulmonary hypertension. In preeclampsia, vascular reactivity of peripheral blood vessels is impaired as early as the first trimester when assessed by flow-mediated dilation, and is associated with increased serum levels of iNOS and NO metabolite formation, but also increased placental levels of protein tyrosine nitrosation.[208] In a rabbit ischemia–reperfusion lung injury model, iNOS inhibition was associated with decreased pulmonary vasoconstriction and platelet–arteriolar wall interactions.[209] Following pulmonary embolism, pathophysiologic levels of iNOS-derived NO and subsequent increases in vascular reactive oxygen species formation are implicated in the development of lung injury and abnormal pulmonary vascular reactivity.[210] Although iNOS inhibition appears to reduce the levels of pulmonary vascular oxidant stress in animal models of pulmonary embolism, this therapeutic strategy has not been proven effective for mitigating pulmonary embolism-induced pulmonary hypertension *in vivo*. Thus, the potential of iNOS inhibition as a treatment target to modify systemic or pulmonary hypertension remains unresolved and is a topic of ongoing investigation.[211]

NEURONAL NITRIC OXIDE AND VASOREGULATION

nNOS is found in relatively high concentrations in specific regions of the mammalian central nervous system.[212] However, nNOS is also expressed in myocardium, skeletal muscle and in parasympathetic nervous systems of the gastrointestinal tract, bladder, ureter, prostate, and in neurons that innervate the walls of corpus cavernosa large blood vessels.[213] nNOS transcription is complexly regulated in pathways involving the p38 MAPK[214] and OCT2[215] transcription factors, while differential splicing of nNOS results in at least three tissue-specific variants.[216,217]

Neurogenic Vasodilatation Mediated by NO

Experiments demonstrating a vasodilatory response mediated by nonadrenergic, noncholinergic nerves that was not mediated by other well known neurotransmitters, was independent of denuded endothelium, and yet was susceptible to NOS inhibitors led to the hypothesis that nitrergic (also termed nitroxidergic) nerves modulate local NO release to mediate vasodilation.[218] This phenomenon of nitrergic innervation has been revealed in the cerebral, mesenteric, temporal, and ophthalmic arteries and arterioles of dogs, cows, and monkeys, as well as the human uterine artery.[219] Some evidence from experiments in humans suggests that NO functions in vasoneuronal coupling of cerebral blood flow to neuronal activity, which may be relevant to maintenance of cerebral blood flow and the pathophysiology of migraine.[220] Nitrergic nerves, like noradrenergic nerves, directly innervate vascular smooth muscle. nNOS is localized

to postsynaptic terminals of perivascular nerves, and there is immunohistochemical evidence for nNOS in the adventitia and outer media of nitrergic innervated vessel.[219] Dual innervation of VSMCs by noradrenergic and nitrergic nerves provides a mechanism for reciprocal neurogenic control of vasoconstriction and vasodilatation.[218]

nNOS is expressed in cardiomyocytes and localized in the sarcoplasmic reticulum (SR).[221] Owing to the close association between nNOS and ryanodine receptor Ca^{2+} release channel (RyR2) and SERCA2, both key intermediaries necessary for myocyte r(d)epolarization, it has been hypothesized that nNOS is important for maintaining normal ventricular function. In fact, $nNOS^{null}$ mice demonstrate pathologic left ventricular remodeling[222] and an increased predilection to L-type Ca^{2+} channel–dependent ventricular arrhythmias and death following myocardial infarction. NO donor therapy, in turn, attenuated this effect by maintaining intracellular calcium levels. nNOS-derived NO may accomplish this by inducing *S*-nitrosylation of the L-type Ca^{2+} or SERCA2, and/or inhibition of L-type Ca^{2+} channel-mediated intramyocyte Ca^{2+} overloading, mechanisms linked to the prevention of electromechanical disassociation.[223,224–228]

EFFECT OF PHARMACOLOGIC SOURCES OF NITRIC OXIDE ON VASCULAR SIGNALING

Exogenous Sources: Nitrovasodilator Drugs and NO-Donating Molecules

Nitrovasodilator drugs encompass a chemically heterogeneous group of molecules that have the ability to generate NO *in vivo*. As such, NO has been termed the "endogenous nitrovasodilator" and nitrovasodilator drugs have been dubbed "endothelial cell replacement therapy."[229] However, it is important to note that exogenous and endogenous sources of NO can differ importantly in their kinetics and reactivity. Of the various organic nitrovasodilators in clinical use, the NO donating group is either a nitrate group, as in nitroglycerin, isosorbide dinitrate, and isosorbide mononitrate, or a nitrite group as in amyl nitrite. The pathways whereby organic nitrovasodilators release NO are incompletely understood, but several mechanisms for the enzymatic transformation to NO have been proposed.[230] Sodium nitroprusside is an organometallic compound that directly releases its NO ligand apparently in a nonenzymatic process. The nitrovasodilators also differ importantly in their bioavailability, route of administration, doses, elimination half-life, and side effects, but share common characteristics in their releasing NO as their principal bioactive product.[231]

The ability of nitrovasodilators to deliver NO and thereby dilate venous and coronary vessels is exploited in the treatment of hypertension, angina, and heart failure. Prolonged nitrate therapy generally leads to tolerance, characterized by a decreased sensitivity of vascular cells to the nitrovasodilator. Numerous theories have been advanced to account for the observation of tolerance, including vascular tolerance via feedback inhibition, sulfhydryl depletion, increased endothelial production of free radicals including superoxide, neurohumoral reflexes, and alteration in local vascular production of vasoactive compounds.[231] The sulfhydryl hypothesis posits that tolerance is related to depletion of reactive sulfhydryl groups necessary for the enzymatic release of NO from nitrovasodilators.[231] Tolerance could be related to inhibition of NO-cGMP signaling in response to elevated vascular NO levels.[43] At least with respect to nitroglycerin, tolerance has been linked to downregulation of mitochondrial aldehyde dehydrogenase an enzyme reported to be involved in the metabolism of nitroglycerin to release NO.[232]

Importantly, nitroglycerin also induces production of reactive oxygen species, which, in part, blunt the response to vasodilatory agonists while sensitizing the vascular response to endogenous vasoconstricting agents.[232] Overactivation of eNOS by pharmacologic NO donors that ultimately results in BH_4 depletion and eNOS uncoupling has also been suggested as a mechanism to account for nitrate tolerance.[233]

Other Pharmacotherapies that Increase NO Bioavailability

Inhibitors of angiotensin converting enzyme (ACE) exert vasodilatory effects not only by limiting generation of angiotensin II, but also by blocking the breakdown of the key eNOS agonist bradykinin. By inhibiting the catabolism of bradykinin by ACE, ACE inhibitors result in increased endothelial formation of NO in a bradykinin-dependent manner.[234] Likewise, by attenuating the adverse effects of aldosterone on vascular NADPH oxidase-dependent reactive oxygen species formation, mineralocorticoid receptor antagonism is associated with increased vascular levels of bioavailable NO and improved vascular reactivity assessed by flow-mediated vasodilation in humans with hyperaldosteronism.[57,235] HMG-CoA reductase inhibitors are associated with eNOS phosphorylation and increased expression of SIRT1 (see above) in vascular endothelial cells *in vitro*.[236] In preclinical trials, atorvastatin is associated with increased microvascular eNOS mRNA levels, decreased protein nitration (i.e., suggestive of decreased vascular oxidant stress), and improved endothelium-dependent vascular reactivity in normocholesterolemic smokers, suggesting that statin therapy may lead to beneficial effects on vascular function via lipid-independent mechanisms.[237]

CONCLUSION

The distinctive chemical properties and crucial biologic functions of NO place this molecule in a central role for maintaining vascular homeostasis in humans. NO synthesized in the endothelium by eNOS targets sGC in VSMCs and platelets to modulate blood vessel dilation and prevent thrombosis. Additionally, NO also targets leukocytes to prevent cell adhesion in the blood vessel lumen and exerts a favorable effect on endothelial cell senescence. eNOS, the primary NO generating enzyme in the endothelium, itself is regulated by a complex, interlinked network of agonists, calcium transients, and posttranslational modifications including (de)phosphorylation and (de)acetylation. Recently, the discovery of molecular cross-talk between eNOS and key proteins involved in regulating cell metabolism has illuminated novel mechanisms linking bioenergetics with NO biology.

Genetically engineered double- and triple-NOS knockout animal models have further elucidated the roles of iNOS and nNOS as important intermediaries that modulate vascular

function. iNOS appears to be a particularly important player in the hemodynamic responses to sepsis and preeclampsia. Likewise, nNOS assists in vasoneuronal coupling and the regulation of cerebral blood flow, as well as modulating the tone peripheral vessels via nitrergic neurons.

Advancing our understanding of nitrate tolerance and the biochemistry of nitrosothiols in order to develop more sustained, efficient pharmacologic systems of NO delivery are key areas of current investigation. In this way, it is hoped that heightened understanding of NOS molecular regulation and NO chemistry may identify novel treatment targets for patients with vascular disease.

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CHAPTER

41 Blood Vessel Wall in Health and Disease

KEITH R. BRUNT, SONYA HUI, JAGDISH BUTANY, AND MANSOOR HUSAIN

Large arteries and veins are comprised of concentric layers of smooth muscle cells (SMCs) and connective tissue, and a protective inner lining of endothelial cells (ECs). The latter normally functions as a pacifying inner lining that prevents thrombosis and secretes factors that regulate underlying SMC function and phenotype to modulate both vessel diameter and vessel wall structure. When cardiovascular risk factors harm or "activate" the endothelium and impair its normal function, pathogenic processes such as inflammation, thrombosis, SMC proliferation, and abnormal vessel contractility can be initiated. Sustained endothelial dysfunction of this nature is ultimately believed to underlie the earliest stages of atherosclerosis. Similarly, vascular dysfunction can be *the* incipient event of most acquired cardiovascular diseases. This chapter orients the reader to key anatomical and physiologic aspects of the vasculature, from the major conduit vessels to specific clinically relevant vascular beds, with a focus on their cellular composition and function.

BASIC VASCULAR ANATOMY AND HISTOLOGY

All vessels conform to certain common anatomical features. With the exception of capillary beds, the blood vessel wall is organized into three layers, or tunica: the intima, media, and adventitia (FIGURE 41.1).

The *intima* extends from the endothelium to the inner elastic lamina, which is a highly organized tensile matrix. The endothelium and its basement membrane is a dynamically regulated single-cell-thick semipermeable layer interposed between the blood and the rest of the vessel wall or, in the case of capillaries between the blood and tissue parenchyma. The endothelium is the principal site of production for many hemostatic and vasoactive substances such as nitric oxide and prostacyclin (see Chapter 42). Immediately abluminal to the endothelium is a thin layer containing fibroblasts and a fenestrated sheet of elastic fibers, termed the *elastic lamina interna*. This elastic layer membrane is surrounded on both sides by covalently bound loose connective tissue that provides scaffolding for endothelial adherens and integrins, which are proteins that anchor cells to the intimal matrix or to other cells in the intima. In larger vessels, the intima may also contain SMCs. While rarely found in the intima of healthy blood vessels, leukocytes from the blood such as lymphocytes, dendritic cells, or monocyte–macrophages can enter the blood vessel wall at sites of vascular injury or inflammation and contribute to pathologies such as atherosclerosis (see Chapter 89).

In larger blood vessels, the *media* is the thickest layer of the vessel wall and is comprised of several circumferential, spirally aligned SMC layers, each of which can be sandwiched between additional elastic laminae. The general function of the media is to maintain vascular tone (i.e., the state of contraction exerted by SMCs in response to stretch and/or neuroendocrine stimulation). The media of veins is thinner than that of arteries and contains more connective tissue, although major veins such as the inferior vena cava also possess thick medial walls with some longitudinally oriented SMC layers as well. The extracellular composition (e.g., collagen, elastin, and proteoglycan content) of the media varies depending on vessel size, compliance, and muscularity. The medial wall is innervated, and in large arteries with high metabolic requirements, the media is also supplied by small blood vessels known as *vasa vasorum* (see "Vasa Vasorum" below). In large- and medium-sized arteries, the border between the media and adventitia is marked by an external elastic lamina. This border can be difficult to discern as the external elastic lamina disappears in small arteries and is absent in arterioles.

The *adventitia* begins where the media ends and is composed of a complex mixture of mesenchymal and myeloid cells with loose extracellular matrix. In large arteries, the adventitia can be nearly as thick as the media, containing abundant collagen and even elastin fibers. The adventitia contains abundant fibroblasts, as well as SMCs, adipocytes, and leukocytes. It also houses the majority of the *vasa vasorum* (see "Vasa Vasorum" below). The general function of the adventitia is to provide metabolic and structural support for large vessels.

AORTA AND CONDUIT VESSELS

Anatomy of the Aorta

Adult vascular anatomy begins at the aortic root, as blood exits the left ventricle through the aortic valve. The aorta has four noteworthy anatomical segments: (a) ascending aorta (length ~5 cm in humans), (b) aortic arch (~4 cm), (c) descending aorta (1, 2, and 3 are collectively referred to as the *thoracic aorta*), and (d) abdominal aorta. The ascending aorta, just past the cusps of the aortic valve, is also the location of three sinuses: the left, right, and posterior sinuses. The left and right sinuses are the origins of the left and right coronary arteries, respectively. The three sinuses provide eddies that aid in the closure of the proximal cusps of the aortic valve during diastole. The superior curve of the aortic arch contains branches that form the brachiocephalic (becoming the left carotid and subclavian), right carotid, and left subclavian arteries before descending approximately 20 cm left of the midline. Below the diaphragm, the abdominal aorta extends another approximately 15 cm before it terminally branches into two common iliac arteries. As the aorta descends from thorax to abdomen, its diameter narrows from approximately 3.5 to 2.5 cm, as it gives rise to several intervertebral

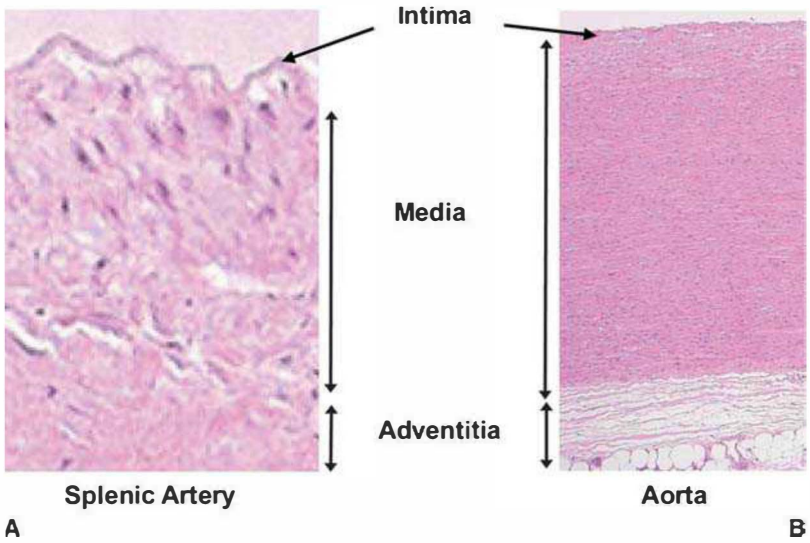

FIGURE 41.1 Tunica of the vessel wall. Cross sections showing the tunica intima, media, and adventitia of a human splenic artery (10×) and human aorta (2.5×), both stained with hematoxylin and eosin (H&E).

arteries in the thorax, and major conduit arteries in the abdomen, namely the celiac, mesenteric, and renal arteries as well as smaller gonadal, lumbar and sacral branches.

Histology of the Aorta

The histology of the aorta is consistent with its function as a major conduit vessel. Its composition is predominately elastic > fibrous > muscular. This differs from resistance arteries, which are muscular > elastic > fibrous. The walls of the aorta and other large arteries contain large numbers of elastic fibers arranged in circumferential lamellar units.[1,2] Each unit consists of two tightly packed elastic lamellae with cells and extracellular matrix contained within. A cross section of the thoracic aorta can reveal as many as 35 to 56 U, whereas the abdominal aorta typically has approximately 28 U.[2,3]

Physiology of the Aorta

To fulfill its functions as the central conductance vessel of the body, the aorta must have the elasticity and strength to stretch during systole, and to recoil during diastole, to correspondingly receive and transmit the entire left ventricular ejection volume and pulse pressure through to the smaller conduit arteries. The pressure pulse of blood created by systolic contraction of the ventricle is cushioned by the compliance of a highly elastic aorta. As the aortic valve closes during diastole, the potential energy stored in the aortic wall is converted into kinetic energy, propelling blood forward into the peripheral arteries with a smooth declination in flow velocity prior to the next systolic contraction.

Pathology of the Aorta

While the aortic endothelium is exposed to both high flow rate and shear stress, the curved geometry of the aorta also results in turbulent blood flow. In the lesser curvature of the aorta, turbulent flow can reduce shear stress, which predisposes the development of atherosclerosis.[4–6,7] Additionally, during ventricular contraction the aorta bears both the circumferential wall tension (from transmural wall pressure) and tensile stress (transmural wall pressure divided by wall thickness), which is exerted perpendicular to the flow of blood. These forces also contribute to aortic remodeling and aneurysm formation that can accompany aortic inflammation and atherosclerosis. Any deficiency in the aortic wall that decreases elasticity will impair compliance and conduit function and can contribute to adverse effects such as aortic dilatation, aortic wall thickening, or even rupture. By contrast, aortic coarctation is a rare congenital anomaly that is associated with aortic constriction, typically at or near the *ligamentum arteriosus*, the adult remnant of the fetal ductus arteriosus (whose function was to allow oxygenated fetal blood from the placenta to bypass the nonfunctioning pulmonary circulation and enter the descending aorta of the fetus from the main pulmonary artery). Aortic compliance can also diminish with age. In this degenerative process, elastic fibers are lost, SMC apoptosis is observed, and extracellular matrix is altered, changing the architecture of the aortic wall. This pathology typically includes intimal thickening and calcification. Although less severe than the mechanical narrowing of aortic coarctation, age-associated aortic degeneration also creates an increased afterload on the heart, causing pressure overload hypertrophy with hypertension. Over time, age-related pathologic alterations to the composition of the aorta may include vessel wall breakdown and aortic ectasia, or dilatation of the aortic wall. Ectasia results in widening, elongation, and uncoiling of the aortic arch, which can so alter the geometry of the aortic root as to limit competency of the aortic valve leaflets and contribute to aortic regurgitation. Accordingly, both diminished or exaggerated aortic wall compliance may eventually affect cardiac function.

As mentioned previously, the aorta is susceptible to turbulent blood flow, endothelial dysfunction, and atherosclerosis (FIGURE 41.2).[8,9] Atherosclerosis of the aorta can in turn lead to aortic aneurysm formation (FIGURE 41.3). Aneurysm, often secondary to atherosclerosis and rarely related to infection, can be: (a) dissecting (FIGURE 41.4), typically caused by cellular necrosis in the aortic media with subsequent tearing of a dissection plane between the lamellar units; (b) saccular, appearing as a bulging "sac" to one side; or (c) fusiform, a circumferential dilatation, in

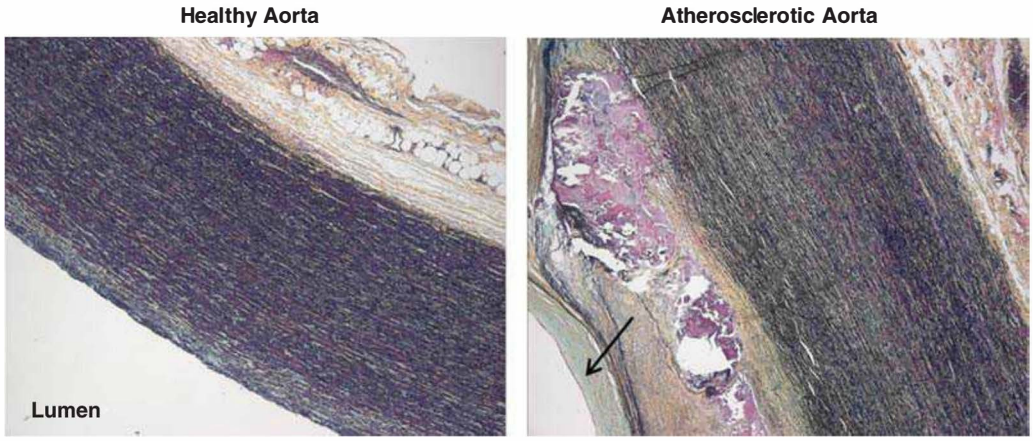

FIGURE 41.2 Human aortic atherosclerosis. Human aortic cross sections (all images 2.5×) stained with Movat pentachrome are shown from a healthy, 61-year-old male **(left panel)** and a 71-year-old female with an advanced atherosclerotic lesion **(right panel)**. Notable features of the complex atheroma include varying/irregular thickness of the plaque, calcification (*purple with Movat pentachrome*), lipid accumulation (*clear spaces*), and neointima formation, indicated by the *arrow*.

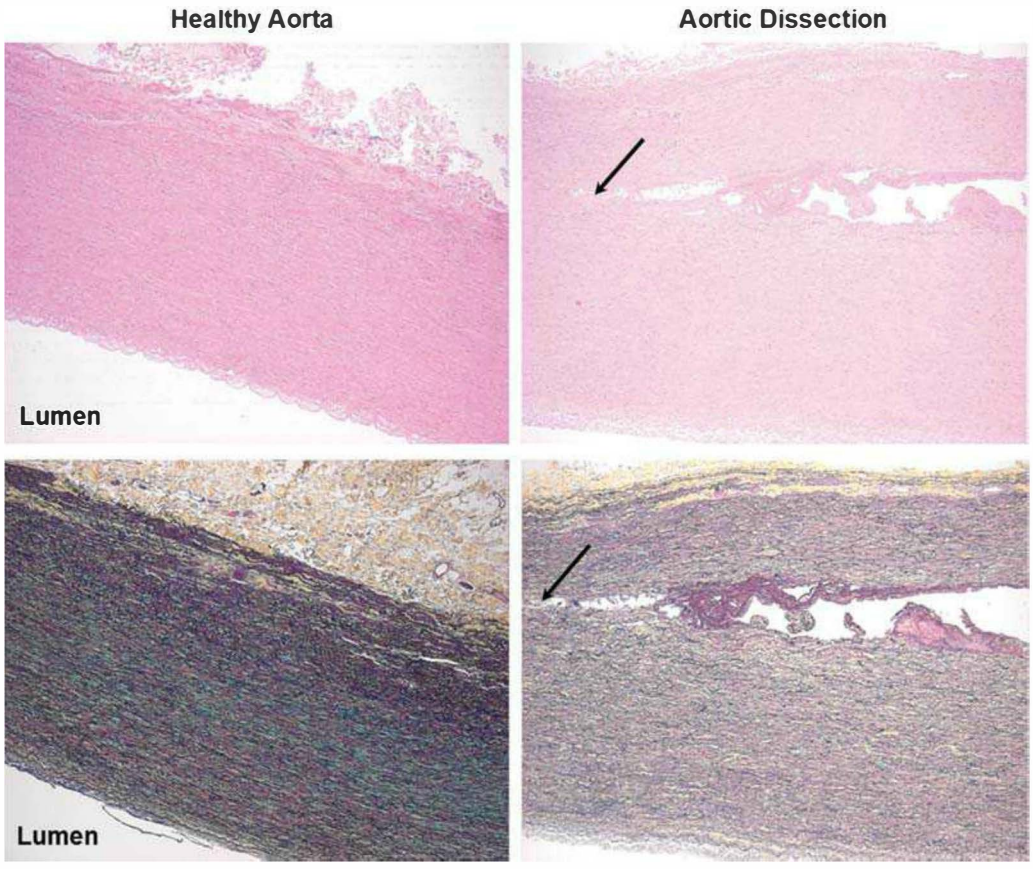

FIGURE 41.3 Histologic images of acute aortic dissection. Human aortic cross sections stained with H&E **(top panels)** and Movat pentachrome staining **(bottom panels)** are shown (**left panel**, 2.5×) and with an aortic dissection (**right panel**, 5×). The site of medial tearing is indicated by the *arrows*. The plane of cleavage usually occurs through the layer of the media in which the vasa vasorum change from radial to circumferential branching.

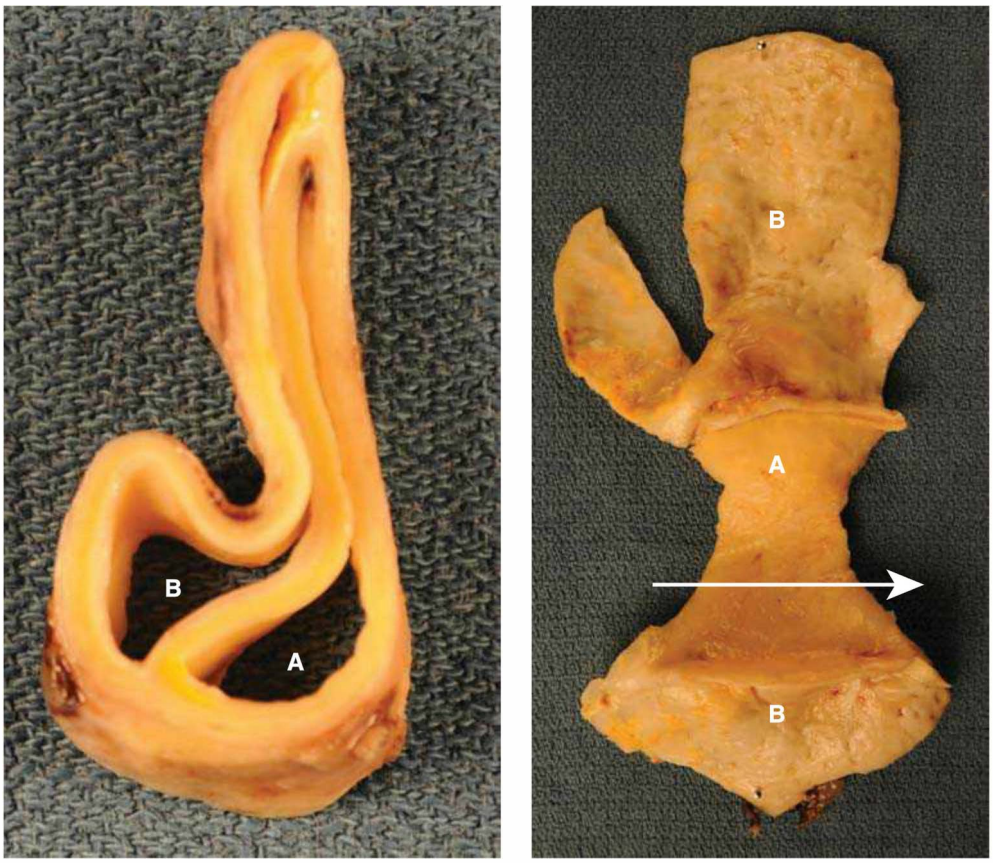

FIGURE 41.4 Gross anatomical images of chronic aortic dissection. Human aortic tissue with chronic aortic dissection, in cross section **(left panel)**, and opened **(right panel)**, with the direction of blood flow indicated by the *arrow*. Both the true lumen (*A*) and the reendothelialized false lumen (*B*) are shown.

the thoracic or abdominal aorta (see Chapter 72). Hypertension is a major contributing factor to aortic aneurysm.[10] Certain vasculitides also predispose to aortic aneurysm including Takayasu arteritis, giant cell arteritis (FIGURE 41.5), and ankylosing spondylitis (see Chapter 100). Trauma, in particular midspeed motor vehicle accidents, can also result in aortic damage, particularly those in which passengers are exposed to sudden decelerations or concussive force trauma, including seat belt injury. Finally, genetic defects of specific extracellular matrix proteins, such as in Marfan disease, are uncommon but important causes of potentially catastrophic aortic pathology.

VASA VASORUM

Anatomy of the Vasa Vasorum

While the inner third of the largest conduit vessels, such as the aorta, and inferior vena cava, is avascular, the outer two-thirds[11] is vascularized by a branched microvascular bed called the *vasa vasorum* (latin "vessel of a vessel"; FIGURE 41.6). The "vasa" can emerge from the lumen, branch up, out and through the adventitial layer (arborize), and weave into smaller vasa throughout the adventitia (and to a lesser degree, the media). However, the

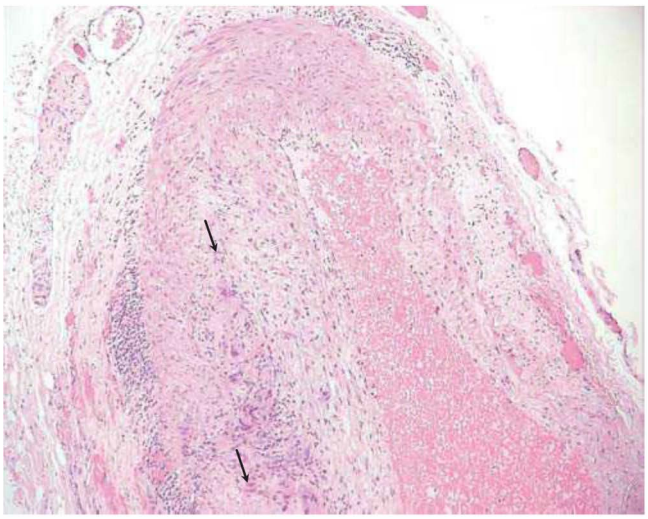

FIGURE 41.5 Giant cell arteritis in the temporal artery. Cross section of a human temporal artery with giant cell arteritis is shown (10×). Multinucleated cell fusions ("giant cells") caused by autoimmune reactions and chronic inflammation are indicated by the *arrows*.

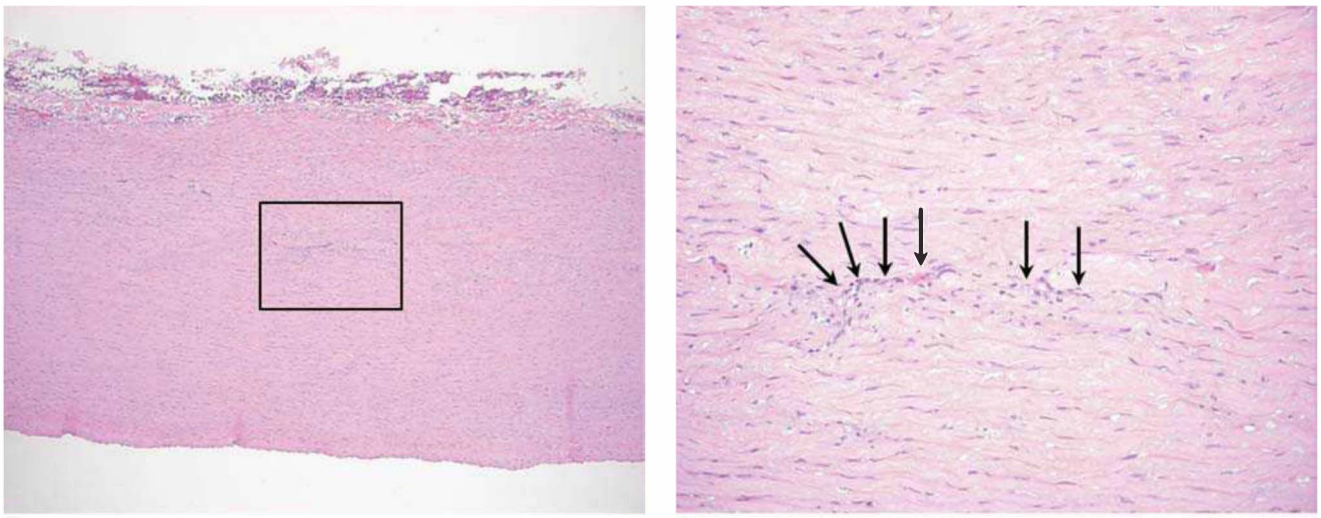

FIGURE 41.6 Vasa vasorum of different vessels. Vasa vasorum are indicated by *arrows* in cross sections of the aorta stained with H&E at **(A)** 5× and **(B)** 10× magnification. In the aorta, vasa vasorum are seen in the media.

vasa can also be a component of more distal arterialization from smaller arteries such as the brachiocephalic artery to ascending aorta, coronary to pulmonary artery, or mesenteric artery to abdominal aorta/inferior vena cava.[12,13] The evidence for both arborized and "nonself" arterialization of vasa arose initially from plastic cast dissections and electron microscopy (EM).[14] However, more recent evidence is based on microanatomical computed tomography and microparticles that assessed both vasa anatomy and blood flow. Large arteries also contain lymph vessels, which can only be distinguished from the vasa through immunocharacterization. Most blood vessels with a cross-sectional thickness of more than 30 to 50 cells possess some form of mural vascularization, invariably more identifiable in adventitia than media. Generally, vasa are limited to the adventitia of vessels with media less than approximately 300 to 500 nm thickness or arteries with <28 lamellae.[15] Diffusion distance is thus a key determinant of vasa. In the adventitia, vasa form capillaries, while in media, they may also form short-distance connections or unique arteriovenous shunts that empty into an adjacent vein.[16] Importantly, vasa are end arteries, or arteries that supply the only perfusion to an anatomical site. As such, reduced flow or occlusion of vasa can result in hypoxia or ischemia of cells within the blood vessel wall.[17]

Physiology of the Vasa Vasorum

Vasa are required to meet the metabolic needs of thicker blood vessels. As such, vasa are governed by the same metabolic cues that regulate angiogenesis in other beds,[17] with vessel wall hypoxia representing a trigger for new vasa formation, increased vasa density,[18,19] and even increases in blood flow.[13] Consistent with this, large energy-consuming arteries are more vascularized in the media than compliant vessels of similar size such as veins.[13] Commensurate with lower vasa density, blood flow through the vasa of veins tends to be lower than in the vasa of arteries.[3,20] However, the more muscular the vein, the greater its metabolic demand and the greater its vasa density and blood flow.[13,21] Compression forces in larger arteries also influence flow, with perfusion through the vasa of carotid arteries occurring mostly after pulse velocity flow.[22] Finally, these vessels are also innervated and also respond to neuroendocrine factors that regulate tone.

Pathology of the Vasa Vasorum

While vasa vasorum appear to play a role in vascular disease, the extent to which their dysfunction is causal or correlative remains unknown. Restricted or occluded flow through the vasa can result in hypoxia or necrosis of the vessel wall.[23] Experiments in dogs have demonstrated that removal or occlusion of vasa caused medial necrosis, reduced vessel compliance,[23] and neointimal formation akin to restenosis.[24] Obstructions of the vasa can also occur as a result of thrombosis, hypertension, or interventions, such as bypass graft surgery or angioplasty, in which vasa may be cut or compressed. Since vasa possess arterial tone, regulated by autocrine and neuroendocrine responses, they are susceptible to constriction from pressor molecules (e.g., angiotensin II or endothelin I) that are typically elevated in hypertension.[16,25] Also, similar to the coronary circulation, vasa blood flow is reduced by compression forces from elevated systemic blood pressure during systolic contraction.[26] Chronic hypertension is associated with impaired vasodilator capacity[27] and can reduce vasa flow, which may partially underlie aortic remodeling in models of chronic hypertension.

Atherosclerotic lesions, particularly in humans, display significant vasa remodeling, with increasingly disorganized microvessel density in proportion to the extent of the lesion. This has broadly led to the hypothesis that vasa play a pathogenic role in atherosclerosis.[28,29] Specifically, thrombotic occlusion of vasa may create acute necrotic events that trigger monocyte invasion and further lipid infiltration.[30,31] Of considerable interest, the vasa of atherosclerotic lesions are anatomically distinct from normal vasa; their architecture resembles that of tumor vessels with increased tortuosity and instability.[32] As such, the

term *vasa plaquorum* has been proposed for vasa supplying atherosclerotic lesions. Vascular lesions in vasa-perfused vessels are not only a source of potent thrombotic and inflammatory stimuli, they also increase arterial wall thickness, and thus reduce oxygen tension in the plaque. Indeed, vasa plaquorum angiogenesis may be initiated by mitogenic stimulation and/or hypoxic demand. However, upon encountering the hostile microenvironment of the plaque itself, vasa plaquorum fail to develop normally; they can be leaky, with fragile endothelium lacking the pericytes (see below) necessary for development of proper tone.[33] Therefore, vasa plaquorum can be a source of intraplaque accumulation of albumin, fibrinogen, hemorrhage, and inflammatory cells. Counteracting these negatives, the vasa plaquorum may also support nutrient flow to the lesion, thereby preventing necrosis.[29] Depending on lesion composition, the vasa may also provide the circulatory basis for plaque regression and vessel remodeling.[34] As such, the role of vasa plaquorum is far from definite.

CORONARY ARTERIES

Anatomy of the Coronary Arteries

The coronary arteries emerge from the left and right aortic sinuses to provide the entire blood supply of the myocardium. The left main coronary artery branches to form the circumflex (Cx) and left anterior descending artery (LAD). The LAD traverses the left ventricle in the anterior interventricular groove, giving off septal perforators and typically two distal diagonal branches as it makes its way toward the apex. The Cx, which lies in the atrial-ventricular (AV) groove, also gives off two descending marginal branches. The right coronary artery (RCA) traverses the descending circumferential aspect of the right ventricle (RV). It branches toward the apex of the RV as the marginal artery, before continuing on to form a dedicated arterialization of the AV node, with the remaining vessel forming the posterior descending artery (PDA) that runs along the posterior interventricular groove toward the left ventricular apex. In approximately 15% of humans, the PDA is formed by a continuation of the Cx rather than the RCA, in which case the coronary circulation is termed "left dominant" (as opposed to the more common "right dominant"). All of the above epicardial arteries give off smaller penetrating branches that perfuse the myocardium from epicardium to endocardium.

The coronary circulation has many small connecting arterial processes termed *collaterals* and *anastomoses* that allow blood to flow between the main epicardial vessels, providing alternative routes of blood flow. The prevalence of collaterals and anastomoses in the coronary circulation undoubtedly reflects the importance of the heart as a vital organ, and the critical importance of redundant perfusion routes, particularly in the event of a coronary vessel occlusion. Having said this, the papillary muscles that tether the AV valves are often vulnerable to ischemic damage and dysfunction with the occlusion of specific coronary territories. The anterior papillary muscle of the left ventricle is perfused by the LAD, whereas the posterior papillary muscle is perfused by the PDA.[35] The atria are vascularized by branches of the AV and sinoatrial (SA) nodal arteries; the SA nodal artery arising most commonly from the RCA.[36–38]

The large coronary veins run in parallel with the coronary arteries (FIGURE 41.7) and connect with venules at right angles to collect deoxygenated blood. The veins ultimately coalesce and form the coronary sinus which traverses through the left AV groove posteriorly to empty into the right atrium. The Thebesian valve at the coronary sinus orifice prevents retrograde flow. Also within the coronary microvasculature, there exists a unique venous system known as *Thebesian veins* that drain blood directly into the cardiac chambers.[39]

Physiology of the Coronary Arteries

The coronary circulation is responsive to both physical (myogenic)[40] and neurohormonal regulation.[41,42,43] Coronary blood flow is a product of aortic pressure, coronary artery resistance, and extravascular compression. The heart produces large intraventricular pressures that compress penetrating myocardial coronary arteries, arresting and potentially reversing arterial flow during systole. By comparison, venous flow is greater during systole.

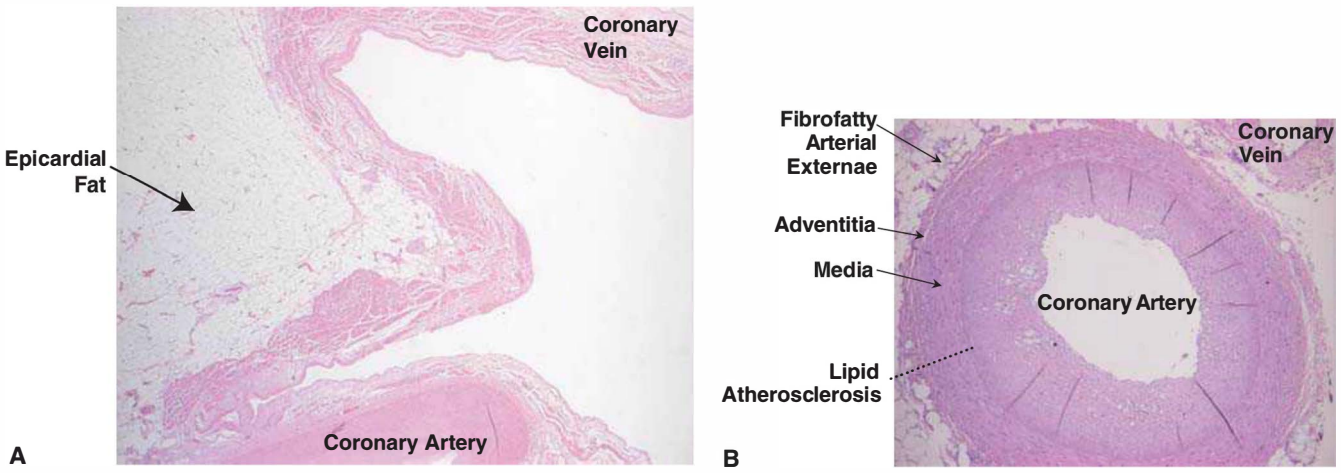

FIGURE 41.7 Coronary arteries from different surfaces of the heart. Images of human coronary arteries stained with H&E are shown from the epicardial (**top panel**, 2.5×) and endocardial (**bottom panel**, 5×) surfaces of the heart.

Heart rate is also a factor in determining coronary flow. At normal resting human heart rates, one-third of the cardiac cycle is systole while two-thirds is diastole, during which coronary flow and myocardial perfusion occurs. However, at elevated heart rates, the diastolic time interval shortens significantly with reduced opportunity for myocardial perfusion. Despite this, the release of metabolites in proportion to increased oxygen consumption can cause profound local coronary vasodilation and dramatically increase diastolic flow. Indeed, as long as the coronary flow reserve has not been compromised (see below), myocardial perfusion can be increased as much as fivefold in response to ventricular myocardial oxygen demand and 15-fold in the atria.[41]

Pathology of the Coronary Arteries

The coronary arteries are critical to cardiac performance. Coronary artery diseases such as atherosclerosis, thrombosis, and/or embolization result in myocardial infarction (MI), which can, in turn, lead to electrical complications such as arrhythmias or sudden death, and/or mechanical complications such as valvular regurgitation, congestive heart failure, or cardiogenic shock.

Coronary artery atherosclerosis is the leading cause of coronary flow impairment.[44] The development of coronary lesions and intraluminal narrowing of the arteries can present clinically as angina pain caused by myocardial ischemia, or infarction following complete vessel occlusion (FIGURE 41.7). The latter is typically an abrupt event caused by plaque rupture and acute occlusive thrombosis.

The location of a coronary occlusion is a major determinant of post-MI survival. More proximal artery occlusions cause larger infarcts and are thus more significant than distal artery occlusions that produce smaller amounts of myocardial damage. Similarly, the duration of coronary occlusion is a major determinant of clinical outcomes. A major revolution in acute coronary care has been the development of rapid pharmacological, interventional, and combined strategies for prompt restoration of coronary patency.

As a consequence of anatomical structure and corresponding increases in turbulent blood flow, the main epicardial coronary artery branch points have a higher propensity for atherosclerosis and plaque rupture.[45–49] While occlusions of arteries supplying the RV and papillary muscles are less common, even small infarcts in these areas can produce disproportionately large clinical effects. For example, both RV and papillary muscle infarcts can result in cardiogenic shock and dramatically diminished survival.

SMALL RESISTANCE ARTERIES

Anatomy and Histology of Small Resistance Arteries

Ranging from 30 to 300 μm diameters, small resistance arteries and arterioles are the end branches of the arterial circulation, lying immediately upstream of tissue capillary beds. Although smaller in diameter, the walls of resistance arteries are proportionately more muscular and less elastic than those of larger conduit vessels. As such, resistance arteries can be distinguished by the presence of several SMC layers in their media, comprising the bulk of their thick, muscular walls.

Physiology of Small Resistance Arteries

In contrast to the elastic, compliant walls of conduit arteries, resistance arteries have muscular walls better suited to their constrictor function. Unlike the large arteries from which they arise, the primary function of resistance arteries is to dynamically regulate tissue perfusion in response to the metabolic needs and physiologic functions of the organs they supply. For example, skeletal muscle resistance arteries dilate in response to the local ischemia caused by increased muscle work. By contrast, resistance arteries within the kidney and lung function to divert blood flow on the basis of glomerular filtration–tubular reabsorption needs and ventilation–perfusion matching, respectively. Overall vasomotor tone of resistance arteries (i.e., total peripheral resistance) is also an important determinant of systemic blood pressure, with blood volume and cardiac output representing the other key factors.[50]

In addition to responding to neurohormonal factors affecting vasoconstriction (e.g., angiotensin and catecholamines) and vasodilation (e.g., nitric oxide and kinins), resistance arteries also possess an inherent autoregulatory ability to respond to sustained increases in transmural pressure with vasoconstriction, a property known as the *myogenic response* (or Bayliss effect). This reversible response serves to establish baseline vascular tone and is believed to maintain consistent blood flow and capillary integrity in the face of varying systemic blood pressures.[51] The myogenic response is believed to be an intrinsic property of resistance artery SMCs, as dissected and cannulated resistance arteries demonstrate myogenic responsiveness *ex vivo*, *in situ*, and in the absence of circulating neurohormonal factors. Of particular interest, specific parameters of the myogenic response have been shown to vary as a function of age, gender, and blood pressure.[52]

Pathology of Small Resistance Arteries

Resistance arteries are susceptible to "arteriosclerosis," or thickening of the arterial wall resulting in decreased elasticity and luminal diameter. Arteriosclerosis has been associated with hypertension, but whether this association is cause or consequence remains uncertain. Indeed, despite high prevalence and decades of investigation, the etiology of hypertension remains a mystery. In approximately 90% of cases, hypertension is "essential," with no single underlying mechanism, and polygenic predisposition.[53–56] Having said this, hypertension can contribute to luminal narrowing (FIGURE 41.8) and predispose vessels to atherosclerosis and dissection.

In addition to age- and gender-related variations in the myogenic response mentioned above, several common clinical conditions have been associated with alterations in the myogenic responsiveness of resistance arteries. These include diabetes,[57] hypertension,[58,59] and heart failure.[60,61] Although the precise molecular mechanisms are not well characterized, studies have implicated the sphingosine-1-phosphate signaling pathway as a major regulator of resistance artery myogenic responsiveness.[62–64] Importantly, in specific animal models, disturbances in myogenic tone have been implicated in the pathophysiology of hypertension and heart failure.[61,65] If these findings translate to the clinic, they may represent novel therapeutic targets for these conditions.

The small resistance arteries can also be affected by a plethora of vasculitides, such as polyarteritis nodosa, microscopic

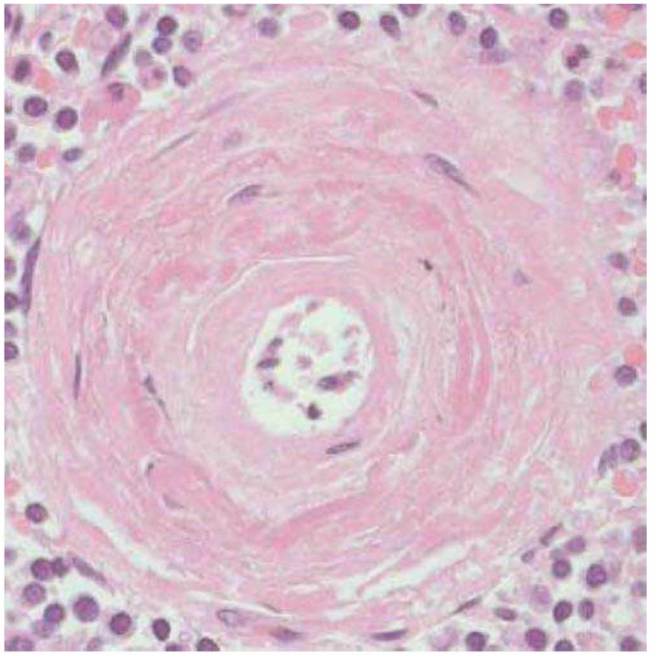

FIGURE 41.8 Smooth muscle hypertrophy in chronic hypertension. Cross section of a human resistance artery stained with H&E from the spleen from a 64-year-old male with chronic hypertension (40×). Hypertrophy is indicated by the few nuclei in the thickened muscle wall.

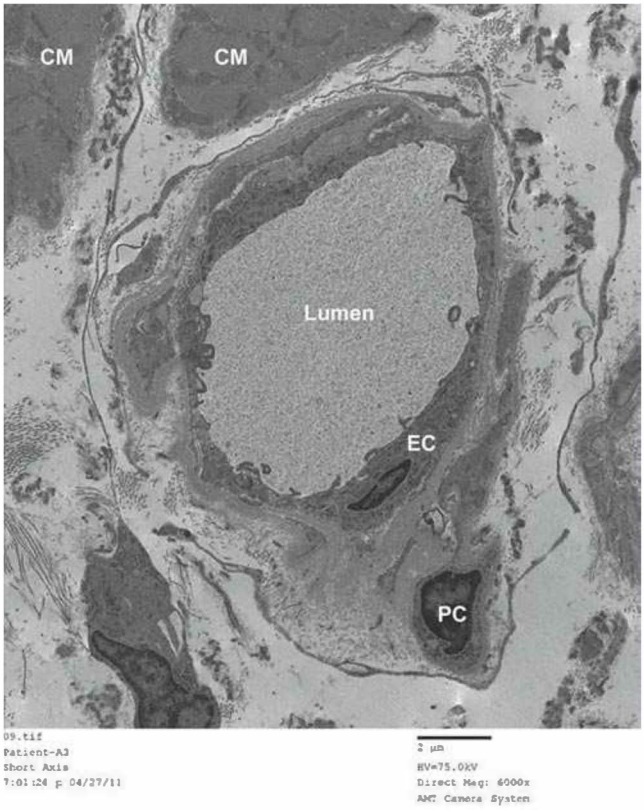

FIGURE 41.9 Cellular structure of the capillary wall. EM image of a pericyte (PC)/endothelial cell (EC) in the capillary wall from human atrial appendage tissue.

polyangiitis, and thromboangiitis obliterans (Chapter 100). Abnormal resistance artery contraction can also result in conditions such as Raynaud phenomenon, in which intensive resistance artery vasospasm leads to cyanosis of peripheral digits. Another example is cerebral vasospasm, in which cerebral resistance arteries profoundly constrict in response to cerebral hemorrhage. The mechanisms underlying these abnormal responses are not fully defined.

CAPILLARIES

Capillary Anatomy and Histology

Capillaries are composed of spirally aligned, circumferential monolayers of ECs that branch off from arterioles into tissue. At the arteriole–capillary junction lie precapillary sphincters, consisting of one or more ring-like SMCs, that can regulate the amount of blood entering the capillary bed.[66] A capillary is made up of at least one circumferential EC, with its two polar ends joined by an adherens junction. As such, the capillary wall can be as thin as 0.1 μm. Capillary beds are typically 3 to 9 μm in diameter, allowing for the serial transit of elastic, biconcave-shaped erythrocytes (diameter ~7 μm, thickness ~2 μm).

The capillary bed is a crisscross of anastomoses that span an average distance of 0.5 to 1 mm between arteriole and venule. The number of capillaries present in a particular tissue is proportional to its metabolic demand or unique function. For instance, heart muscle can have up to 40 times the capillary density per unit area than skin.

A single basal lamina surrounds the capillary, while pericytes, often identifiable by their proximity and larger nuclei as compared to ECs, provide structural, paracrine, and juxtacrine support to the endothelium (FIGURE 41.9). As discussed in detail below, the pericyte is a contractile cell unique to the microcirculation, with a shared EC/SMC profile.[67–69] Pericytes can be thought of as a proxy for vascular SMCs. However, pericytes do not form a continuous, single-celled tunic around capillaries as SMCs do in arterioles, as there are substantial capillary segments completely lacking pericytes. The pericyte is believed to be from the same developmental lineage(s) as vascular SMCs. That being said, it is now well established that vascular SMCs of the various segments of the circulation can have diverse developmental origins.[70,71] Whether pericytes demonstrate similar site-specific origins is unknown. Moreover, it has been shown that endothelial-to-mesenchymal differentiation into pericytes can also occur.[72,73] The transition from capillary to venule can be identified by two factors: the increase in relative diameter and the increase in pericyte number, as pericytes are also present in portions of the postcapillary venule wall.

Capillary Physiology

Capillaries can be thought of as the business end of the microcirculation. They are the principal site of gas, fluid, and metabolite exchange between blood and tissue. Capillary density, flow rate, surface area, and permeability are key factors influencing gas and nutrient exchange. As such, these factors are collectively greater in highly active tissues.[41] Molecular exchange is facilitated by the relative volume-to-surface area ratio in the

capillaries. Accordingly, the capillaries possess the highest cross-sectional area of the vascular system. Exchange occurs principally through simple diffusion (Brownian), as well as advection (Gaussian), and transcytosis.[74,75] Advection describes the process of nonrandom particle exchange, typical of size/charge selective channels or membrane transporters, such as the water transporters aquaporins.[76] Transcytosis is a process common to endothelial barriers that promotes transcellular transport of macromolecules through the intracellular space.[76]

Capillary structure varies between tissues and organs. The endothelium may form a thin continuous layer, as occurs in the lung, benefiting the principal goal of gas exchange. Alternatively, the endothelium may be permeated by small holes, or fenestrae. Fenestrated endothelium is characteristic of vascular beds involved in a high degree of secretion or filtration, including the kidney glomerulus. A third phenotype, the so-called discontinuous endothelium, contains intracellular gaps and is associated with an incomplete basal lamina. Discontinuous endothelium is observed in the sinusoids of the liver, spleen, and bone marrow. The interendothelial cleft functions as a molecular sieve and is the principle pathway for fluid and electrolyte exchange. Compared with other capillary beds, brain ECs have particularly tight junctions, which serve to limit diffusion of substances (particularly hydrophilic macromolecules) between blood and underlying neural tissue, and is supported by both astrocytes and pericytes as part of the blood–brain barrier.[77]

While energy-dependent exchange is important (e.g., transcytosis or ATP-dependent exchangers), the majority of exchange of fluids and small molecules in the capillary bed occurs by passive transport. Passive transport is driven by osmotic, hydrostatic (fluid pressure), and oncotic (proteinaceous and organic constituents) pressures (together comprising the Starling forces), which significantly affect the permeability of the capillary. Starting from the arteriole, relative hydrostatic pressure is higher in the capillary than the osmotic pressure, allowing net fluid transport to the tissue. In the greater capillary bed, pressures and fluid exchanges equalize, allowing the simple exchange of metabolites and gas by diffusion across relative concentration (e.g., glucose, urea) or partial pressure gradients (e.g., oxygen and carbon dioxide). When blood enters the capillary–venule bed, hydrostatic pressure is low in comparison to osmotic pressure, allowing interstitial fluid and metabolites to be drawn up by venous blood. Blood flow in the venous circulation is regulated by different constituents, such as the skeletal muscle, respiratory pumps, resting venous tone, and venous valves (see section on *Physiology of the Veins*). This simplification of microvasculature exchange is important to note as three-dimensional geometry, porosity, matrix composition, and molecular constituents in the abluminal and adluminal sides of the EC membrane can vary substantially in specialized structures such as the blood–brain barrier, kidney glomerulus, liver sinusoids, placenta, muscle, and skin.[76,77,78,79]

In addition to their principal function in metabolic exchange, capillaries can also influence systemic vascular tone through pericyte-dependent regulation of capillary constriction.[68,80] However, our current knowledge of pericyte biology is limited, especially with regard to the intimate structural and functional relationship between pericytes and capillary endothelium. Pericytes are of mesenchymal origin and are more similar to SMCs than ECs. However, they possess molecular markers of both cell types (e.g., vascular cell-adhesion molecule-1, intercellular adhesion molecule-1, inward rectifier potassium channels K_{ir}, platelet-derived growth factor receptor-beta), in addition to their own distinct markers, such as combinations of chondroitin sulfate proteoglycan 4 (NG2), regulator of G-protein signaling 5, vimentin, and/or alpha-smooth muscle actin.[69] In addition to maintaining microvascular tone, pericytes confer endothelial cytoprotection, basement membrane maturation, barrier function, and capillary stability.[81–83] This occurs through direct physical contact with ECs or shared matrix contact via integrin, Notch, and ephrin molecules.[84–87] Pericytes may also contribute to capillary angiogenesis and angiostasis through the secretion of growth factors and proteases,[88,89] as well as inflammation-associated alterations of vascular permeability.[90,91] Moreover, pericytes have also been shown to be involved in ischemia and stress responses. As part of the initial response to acute trauma, pericytes in damaged tissue can respond instantly by constricting capillary blood flow, which is beneficial for hemostasis. However, with ischemic injury, prolonged pericyte constriction can lead to further tissue ischemia.[92,93] For example, middle cerebral artery ischemic reperfusion injury can lead to microvascular constriction such that red blood cells are trapped against the capillary walls, essentially reducing blood flow to zero (so-called *no-reflow phenomenon*).[94]

Capillary Pathology

Physiologic properties of the microvasculature can be altered dramatically with negative impacts on healing, inflammation, and hypoxia. The most common microvascular complications impacting heavily on our society are related to age, diabetes, and inflammation. For instance, age-dependent macular degeneration (AMD) can present as a severe form of microvessel angiogenesis of the choriocapillaris behind the retina. This aberrant growth of capillaries is of unknown etiology but has been correlated with genetic defects, cholesterol levels, and inflammation.[95,96] AMD is the principal cause of blindness in persons over 60 years of age. Diabetic retinopathy is a consequence of glycation stress that primarily affects pericytes, causing apoptosis and breakdown of the capillary barrier function in the retina.[97] This can result in subsequent macular edema and blurred vision. Over time, increased extravascular pressure creates local hypoxia, which stimulates unstable angiogenesis (lacking pericytes).[98–100] The weak microvasculature structure can leak, causing retinal disruption or neovascular glaucoma. Inflammation or autoimmune disruption of microvascular integrity can produce acute and/or life-threatening conditions. For example, Goodpasture syndrome is a form of vasculitis involving autoimmune targeting of the basement membrane, which leads to lung and kidney failure, as these organs have the highest capillary surface area–dependent functions.

VEINS

Anatomy of the Veins

Veins are principally capacitance vessels. Those on the body surface are referred to as superficial veins, as distinct from deep veins that lie in deep-tissue planes and run parallel to arteries of comparable size. Although the venous wall is trilayered, the layers are not as distinguishable as they are in arteries. The venous wall contains a greater accumulation of loose connective tissue, collagen, and elastin fibrils between muscle layers, but less high-tensile

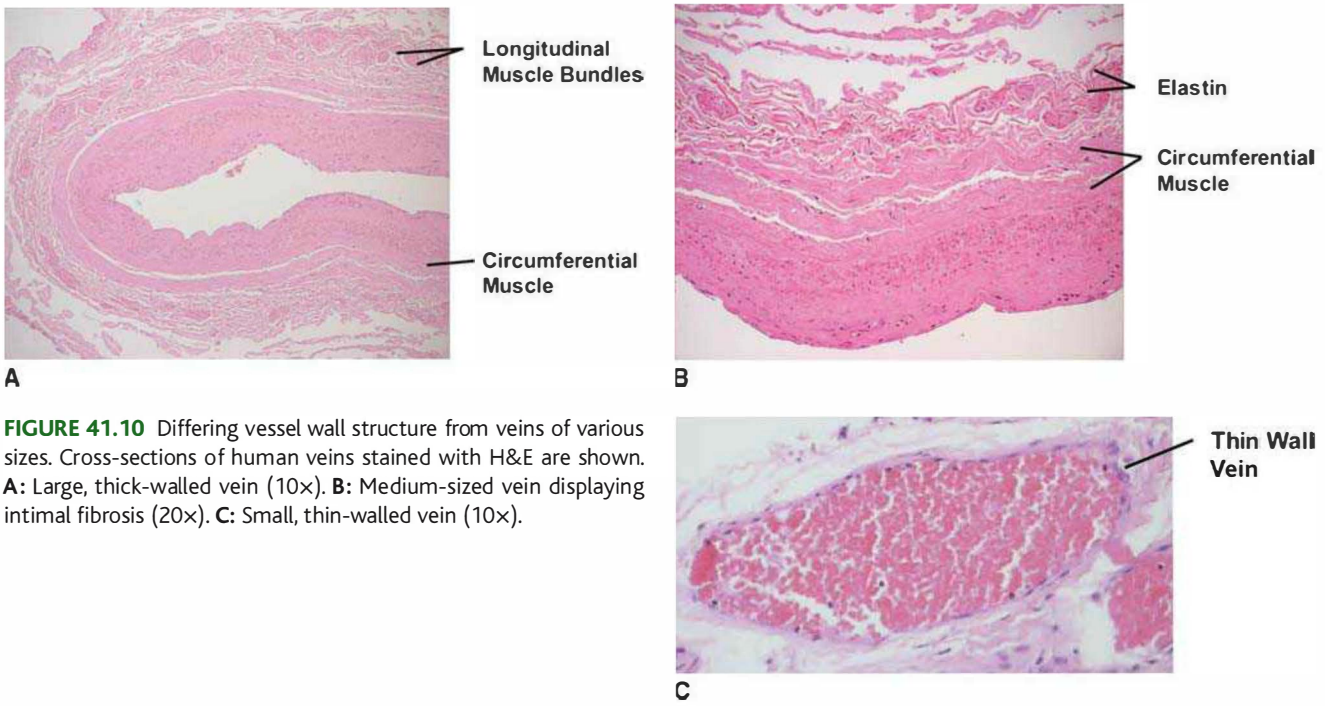

FIGURE 41.10 Differing vessel wall structure from veins of various sizes. Cross-sections of human veins stained with H&E are shown. **A:** Large, thick-walled vein (10×). **B:** Medium-sized vein displaying intimal fibrosis (20×). **C:** Small, thin-walled vein (10×).

elastic laminae, and a decreased smooth muscle-to-fibrocyte ratio (FIGURE 41.10). The head-to-tail circular SMC alignment of arteries is also not as apparent in veins; instead venous SMCs are bundled together around dense extracellular matrix. Larger veins, including the vena cava and saphenous vein, also display some longitudinal alignment of myocyte bundles.

The most important feature of venous anatomy is the presence of intimal folds that act as one-way venous valves. They are typically composed of two flexible, sail-like (bivariate) projections that originate perpendicularly from the inner wall, protrude in parallel with the lumen, and point in the direction of blood flow. Venous valves are covered on both sides by endothelium, accompanied by a base of matrix fibers connected to the venous wall. Larger vein valves may also have fibroblasts, myofibroblasts, or SMCs (near the origin) that function to produce matrix and maintain strength. The latter functions may be impaired with age or a variety of conditions, particularly those associated with elevated venous pressures (right-sided heart failure), venous compression (obesity, pregnancy), or thrombosis.

There is anatomic variability in human deep-vein valves. The femoral veins may have one to six valves, whereas the popliteal only 0 to 4.[101] Though Canadian pathologist Nicholas Popoff first wrote in 1934 that Even very small peripheral digital veins show the presence of valves,[102] there remains a common misconception that valves are absent from smaller veins.[103] In fact, distinct microscopic venous valves are present in veins as small as 20 μm, where they are believed to provide similar function as valves in larger vein or collecting lymphatics. Of interest, large and small veins valves are not universally distributed, but rather occur in areas where pressure, irregular flow, or gravitational backflow is more likely.[103] Venous valve structure is similarly inconsistent. Fenestrated, univariate, or trivariate valves in the coronary veins[104] are a consequence of pressures developed by the heart, whereas no valves, and indeed little or no musculature, exist in the cerebral veins.[105]

Physiology of the Veins

Veins are far more compliant than conduit and resistance arteries. Vein structure is uniquely adapted to low pressure and provides a high capacity for distension. This is a factor that allows veins to serve as the predominant fluid compartment of the circulation. As such, veins act as a reservoir for blood; at any given time, more than 65% of the body's total blood can be found in the venous circulation.[106,107] However, in response to increased cardiac demand, veins are able to contract and actively increase venous blood return, particularly under neuroregulatory control. Moreover, situations requiring increased cardiac output, such as strenuous physical activity, can also increase venous return through mechanical compression of veins. For instance, skeletal muscle contraction "squeezes" the venous circulation (skeletal muscle pump). In combination with venous valves, this action pushes blood toward the heart (see below). Heavy breathing, also a product of exertion, promotes venous return through the respiratory pump. The lowering of intrathoracic pressure reduces the left atria pressure, allowing for greater venous return. Posture and gravity are also factors in venous return. Indeed, cardiac output is regulated by volume and pressure adjustments in the venous circulation by both passive and active physical and neuroendocrine cues.

With regard to venous valves, any reversal of blood flow closes the valves, preventing retrograde blood flow. In this way, venous pressure moves blood toward the heart, even against gravity.

Pathology of the Veins

The venous pathology that most significantly affects quality of life is *deep-vein thrombosis* and subsequent pulmonary

embolism. Predisposing factors include prolonged immobilization, postoperative state, pregnancy, local infection, heart failure, and neoplasia. Prolonged standing can raise hydrostatic pressure over oncotic pressure, gradually increasing interstitial volume in the lower extremities. If this persists for a prolonged period of time, such as in obesity or pregnancy, it can be accompanied by elastin breakdown, and incompetency of the venous valves and ultimately the veins themselves. Arteriovenous fistulas can also contribute to increases in venous pressure in some cases. Vein dilatation by high intraluminal pressure and blood stasis can result in complete venous valve failure, producing *varicose veins*. This condition usually appears on the lower extremities as abnormally dilated and tortuous vein architecture. In addition to trophic skin changes, varicose veins coincide with persistent edema, dermatitis, and can lead to ulceration.

CONCLUSION

As outlined in this chapter, the cellular and matrix components of the blood vessel wall come together to deliver the specialized forms and functions of every compartment of the human circulatory system, from conduit arteries to capillaries and capacitance veins. Together, this highly evolved system enables cardiovascular competence, including the homeostasis of every other organ. From traditional hemodynamics, perfusion physiology, and advanced imaging techniques including EM and *in vivo* imaging, we will continue to gain insights into the pathophysiology of the human vasculature. Future investigations on the molecular mechanisms of the myogenic response, the pathobiology of the vasa plaquorum, and the role of microscopic venous valves may yield particularly new insights into vascular biology.

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DISCLOSURES

The authors declare no competing financial interests.

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CHAPTER

42 Endothelial Cell Function

WILLIAM C. AIRD

The endothelium is an expansive organ, reaching all recesses of the human body. At one time, it was believed that endothelial cells (ECs) were relatively inert, acting as a physical barrier between blood and underlying tissue. It is now well established that ECs are highly metabolically active, and play a critical role in many physiologic processes, including the control of vasomotor tone, the trafficking of leukocytes between blood and underlying tissue, the maintenance of blood fluidity, permeability, angiogenesis, and both innate and adaptive immunity. EC phenotypes display remarkable heterogeneity in structure and function, in space and time, and in health and disease (reviewed in[1–3]). The goals of this chapter are to review the scope and nature of endothelial phenotypes and to apply these principles to an understanding of the endothelium in hemostasis and thrombosis.

ENDOTHELIAL CELL PHENOTYPES

Defining the Endothelium

An interesting question is how do we define the endothelium? Anatomically, the endothelium is the cell layer that lines the inner wall of blood and lymphatic vessels. However, there are examples of vascular mimicry in which other cell types (e.g., trophoblasts) form the inner lining of blood vessels. ECs tend to be extremely flat or thin. In that way, the diffusional path length for gases and solutes across the capillary wall is minimized. However, there are exceptions, including the plump, cuboidal ECs of high endothelial venules (HEV) (reviewed in Ref.[4]). Weibel-Palade bodies, which contain von Willebrand factor (vWF) and P-selectin, are specific to the endothelium, but there is no evidence that these storage granules are present in all ECs. Other ultrastructural features that are characteristic of ECs, such as caveolae, are also observed in other cell types. During development, endothelium arises from mesoderm *via* the differentiation of hemangioblasts and/or angioblasts. However, other cell lineages may transdifferentiate into ECs, and ECs into other lineages. From a molecular perspective, few, if any, genes are both specifically and uniformly expressed in the endothelium. For example vWF is also expressed in megakaryocytes and its expression in the endothelium is patchy. CD31 (also termed platelet–endothelial cell adhesion molecule 1) is also expressed in monocyte/macrophages, and vascular endothelial (VE)-cadherin in trophoblasts and fetal stem cells. Functionally, the endothelium displays a remarkable division of labor, whereby cells from different segments of the vasculature carry out unique activities. Finally, when endothelial-specific promoters are targeted to the mouse genome, they invariably fail to direct expression throughout the endothelium. Rather, they promote expression in specific subsets of ECs (reviewed in[5]). In summary, each of the above definitions—anatomical, structural, functional, and molecular—falls short of fully capturing the endothelium. The "elusiveness" of the endothelium undoubtedly reflects its marked heterogeneity in structure and function. Indeed, far from being a giant monopoly of identical cells, the endothelium is a consortium of smaller enterprises, each uniquely adapted to the needs of the local tissue. In the final analysis, a full understanding of this cell type in health and disease requires a consideration of both the spatial and temporal dynamics of the system.

Scope of Heterogeneity

EC heterogeneity has been described at the level of cell morphology, function, gene expression, and antigen composition. EC phenotypes vary between different organs, between different segments of the vascular loop within the same organ, and between neighboring ECs of the same organ and blood vessel type. *In vivo* proteomic approaches have revealed a striking array of vascular bed-specific phenotypes (reviewed in[6,7]). For example, antibody and subfractionation strategies have been employed to generate monoclonal antibodies that specifically target the caveolae in one vascular bed or another.[8] Others have used phage display peptide libraries to select for peptides that home to specific vascular beds *in vivo*.[9,10] These latter studies have uncovered a vascular address system that allows for site-specific targeting of biologically active compounds, for example, to the endothelial lining of tumor blood vessels.[11]

Structural Heterogeneity

ECs that line straight segments of arteries, but not those lining veins, are oriented along the longitudinal axis of blood flow. Previous studies have demonstrated that flow-dependent alignment of ECs represents reversible endothelial structural remodeling in response to hemodynamic shear stress.[12,13] The transfer of material across the endothelium, a process termed *transcytosis*, is mediated by caveolae and vesiculo–vacuolar organelles (VVOs). Caveolae are 70-nm membrane-bound, flask-shaped vesicles that open to the luminal or abluminal side of the cell. The density of caveolae in capillary endothelium (up to 10,000 per cell) greatly exceeds that in the arteries, arterioles, veins, or venules (reviewed in Refs.[2,14]). An exception is the blood–brain barrier, where the number of caveolae is greatly reduced.[15] VVOs comprise focal collections of membrane-bound vesicles and vacuoles. They are most prominent in venular endothelium.[16] In contrast, clathrin-coated pits, which are involved in endocytosis, are particularly prominent in liver sinusoidal endothelium.

The endothelium of arteries and veins forms a continuous uninterrupted layer of cells, held together by zipper-like tight junctions. The endothelium of capillaries may be continuous, fenestrated, or discontinuous, according to the needs of the underlying tissue. For example, fenestrated endothelium is characteristic of organs involved in filtration or secretion, including exocrine and endocrine glands, gastric and intestinal mucosa, choroid plexus, glomeruli, and a subpopulation of renal tubules. Discontinuous endothelium is similar to fenestrated endothelium, with the exception that the fenestrations are larger in diameter (they may manifest as gaps in the cell) and they lack a diaphragm. Moreover, the underlying basement membrane is poorly formed. Discontinuous endothelium is found in certain sinusoidal vascular beds, including the liver.

Functional Heterogeneity

The endothelium has many functions. In almost every case, the activity is carried out by distinct subsets of ECs within the vascular tree. Below, we discuss three examples of functional heterogeneity: permeability, leukocyte trafficking, and angiogenesis. We review vascular bed-specific hemostasis and thrombosis separately in a subsequent section.

Permeability

Basal and inducible permeability is differentially regulated across the vascular tree. Constitutive flow of material between blood and underlying tissue takes place primarily at the level of capillaries. Transport of fluids and small solutes occurs in between cells (the so-called *paracellular route*), whereas macromolecules are transferred through the cell (the *transcellular route*) *via* caveolae, VVOs, and transendothelial channels. Basal permeability is lower in nonfenestrated vascular beds that are enriched in tight junctions and possess fewer caveolae (e.g., the blood–brain barrier). As evidence for the role of tight junctions in mediating barrier function, mice that null for the tight junction molecule, claudin-5, have a selective barrier defect in the brain.[17] Inducible permeability refers to changes in barrier function that occur in acute or chronic inflammation. These changes take place primarily in postcapillary venules. The extent to which regulated leakiness is mediated by paracellular or transcellular pathways is debated. The predilection for postcapillary venules as a site for inducible permeability may be explained by the relative abundance of VVOs, the relatively low number of tight junctions, and/or high expression levels of agonist-responsive receptors (reviewed in Ref.[2]). Severe inflammation may result in increased permeability in sites other than postcapillary venules, including large veins, arterioles, and capillaries.

Leukocyte Trafficking

The endothelium plays a key role in regulating the trafficking of leukocytes between blood and underlying tissue (hence the metaphor of the endothelium as gatekeeper). Leukocyte trafficking involves a multistep cascade that begins with rolling, followed by firm adhesion and transmigration (reviewed in Ref.[2,18,19,20]). As with transfer of fluids and solutes, transmigration may occur between ECs (the paracellular pathway) or through ECs (the transcellular pathway). Each step in the cascade is mediated by the upregulation of cell adhesion molecules on the surface of the endothelium as well as the expression of chemokines. Binding of endothelial E-selectin and P-selectin to leukocyte carbohydrate-based ligands promotes rolling of leukocytes on the endothelium, whereas binding of endothelial cell adhesion molecules, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 to leukocyte integrins mediates firm adhesion. The mechanisms of transmigration are poorly understood, but involve CD31 and junctional adhesion molecule-1. Similar to inducible permeability, transfer of white blood cells occurs primarily in postcapillary venules. One mechanism underlying this site-specificity is the preferential expression of E-selectin, P-selectin, VCAM-1, and ICAM-1 in the endothelium of postcapillary venules. Under certain conditions, leukocyte trafficking may occur in other segments of the vascular tree, including large veins, arterioles, and capillaries. For example, previous studies suggest that leukocyte sequestration and transmigration in the pulmonary circulation occurs primarily in alveolar capillaries by a rolling- and E-/P-selectin–independent mechanism that involves trapping of poorly deformed activated leukocytes on activated endothelium.[21,22] Similarly, in liver inflammation, the majority of leukocyte adhesion occurs in the sinusoidal endothelium.[23] Another form of leukocyte trafficking is the transmigration of lymphocytes across the specialized postcapillary venules, termed HEV, which are located in secondary lymphoid organs (reviewed in Ref.[24]). Trafficking at these sites occurs constitutively, and is mediated by interactions between lymphocyte L-selectin and its ligand (peripheral node addressin) on HEV ECs. In summary, the "universal cascade" for leukocyte trafficking is, in fact, mediated by spatially and temporally diverse mechanisms.

Angiogenesis

Recent studies have emphasized the existence of structural and functional heterogeneity of ECs during angiogenesis. For example, endothelial sprouts comprise leading-edge tip cells followed by morphologically and functionally distinct stalk cells. The tip cells are enriched in filopodia and in response to guidance cues, they promote the extension of new sprouts and fusion with adjacent sprouting capillaries to form new vascular connections (reviewed in Ref.[25]). This process involves the coordinated activity of Notch and Wnt signaling.

Molecular Heterogeneity

Spatial and temporal differences in the structure and function of ECs ultimately reflect differences in mRNA and protein expression. Endothelial genes may be classified according to whether they are constitutively or inducibly expressed, whether they are specific or not to ECs, and whether they are expressed throughout the endothelium or in subsets of ECs (reviewed in[5]). There are remarkably few endothelial-specific genes that are constitutively expressed throughout the vascular tree. VE-cadherin and Robo4 are two such genes. There are many more examples of endothelial-restricted genes whose constitutive and/or inducible expression is limited to subsets of ECs. For example, several endothelial markers are expressed specifically on the venous versus arterial side of the circulation (reviewed in Ref.[26]). ECs from different arteries, and even from different sites of the same artery, express overlapping but distinct molecular profiles. An example of an inducible, endothelial-restricted gene is E-selectin, which is expressed in activated, but not "resting"

endothelium. In activated endothelium, E-selectin expression is largely confined to postcapillary venules.

Mechanisms of Endothelial Heterogeneity

EC heterogeneity is mediated by one of two proximate mechanisms.[27] First, some site- and time-dependent differences in endothelial properties are governed by differences in the extracellular milieu. Because blood vessels are distributed throughout the body, their endothelial lining is exposed to an enormous variety of tissue microenvironments. In so far as ECs are capable of sensing and responding to their environment, the wide range of signal inputs from one organ to the next is sufficient to generate phenotypic heterogeneity across the vascular tree. When ECs are removed from their native tissue and grown in tissue culture, they become uncoupled from critical extracellular cues and undergo phenotypic drift. For this reason, studies of cultured ECs are fraught with limitations. Second, certain site-specific properties are epigenetically "fixed" and impervious to changes in the extracellular environment. Such properties are mitotically stable, and are thus retained under *in vitro* culture conditions. The relative roles of epigenetic and nonepigenetic forces in mediating phenotypic heterogeneity are not fully understood. A previous DNA microarray study of multiple human EC types grown in culture revealed site-specific signatures even in multiply passaged cells, providing genome-wide evidence for the importance of epigenetics in mediating differential gene expression.[28] A study of ECs from human tonsils revealed that approximately 50% of the vascular bed-specific genes were "washed out" when cultured *in vitro*, implicating a role for *both* the tissue environment and epigenetics in mediating differential gene expression.[29] This conclusion was supported by a DNA microarray study in which the transcriptome of ECs was compared between porcine coronary and iliac arteries. The data revealed that most, but not all, differences observed *in vivo* were lost in multiply passaged cells.[30]

In addition to proximate mechanisms, every biologic trait requires an evolutionary explanation. Proximate explanations (how?) employ traditional approaches of cell biology and molecular biology to determine the anatomy, physiology, and ontogeny (developmental history) of a trait at the level of a single organism. By contrast, evolutionary explanations (why?) draw on the fossil record and comparative morphology and DNA sequences to uncover the phylogeny (evolutionary history) of a trait as well as the fitness advantage that the trait provides at the level of a population or species. Comparative studies have revealed that the endothelium is present in all vertebrate species, but absent in invertebrates. Thus, the endothelium evolved in a common ancestor of the vertebrates following the divergence of the urochordates and cephalochordates some 500-600 million years ago over a period of only 50-100 million years. This same short period witnessed the evolution of a closed circulation, the clotting cascade (see Chapter 2), and acquired immunity.

An interesting question is whether EC heterogeneity, as a trait, appeared early on during evolution of the endothelium or whether it appeared later during vertebrate evolution. One way to address this question is to study hagfish, the oldest extant vertebrate. Any feature that is shared by hagfish and humans may be inferred to have evolved in the ancestral vertebrate. Unlike other vertebrates, hagfish are completely devoid of bone and cartilage. However, similar to the jawed vertebrates, hagfish have a closed circulation lined by endothelium. Importantly, our studies revealed that hagfish endothelium is remarkably heterogeneous at the level of ultrastructure, lectin binding, and function.[31,32] We may conclude from these observations that phenotypic heterogeneity evolved as an early feature of the endothelium. In other words, endothelial heterogeneity is not simply a descriptor of many distinct properties of the endothelium, but rather is itself a core property of the endothelium.

Evolutionary approaches also seek to explain the fitness advantage that a trait confers at a population level. This is a difficult question to definitively answer, but there are two important considerations. One is that the endothelium in traversing the body must provide a broad menu of functions that are adapted to the diverse needs of the underlying tissues. Site-specific differences in function will, in turn, be reflected by structural and molecular heterogeneity. Second, ECs must adapt to different environments simply to survive. For example, ECs in the vasa recta of the inner medulla of the kidney are exposed to profoundly low levels of oxygen, in addition to a hyperosmolar hyperkalemic environment. Although the precise properties of these cells are unknown, it is certain that they are uniquely adapted to this "harsh" environment in ways that render them phenotypically distinct from other types of ECs.

ENDOTHELIAL CELL ACTIVATION AND DYSFUNCTION

The two most common terms used to describe the endothelium in disease are *activation* and *dysfunction*. The term *endothelial cell activation* was introduced in the 1980s when it was observed that numerous inflammatory mediators, including endotoxin, tumor necrosis factor, and interleukin (IL)-1, induced the expression of new antigens (so-called activation antigens) on the surface of cultured ECs, and was correlated with the expression of proadhesive, antigen-presenting, and procoagulant activities (reviewed in[33]). Today, the term *activation* is used more broadly to describe the response of ECs to an inflammatory stimulus *in vitro* or *in vivo* (some investigators in the angiogenesis field employ the term to describe proliferating ECs). The activation phenotype varies according to the nature of the activation agonist, the net signal input, and the vascular bed of origin. Typically, it consists of some combination of a proadhesive and procoagulant surface, increased permeability, and the release of inflammatory mediators. *Activation* should not be confused with *activity*. The normal endothelium is highly active, constantly sensing and responding to cues in its extracellular environment. EC activation describes a phenotype. It does not refer to the cost of that phenotype to the host. In other words, EC activation may be adaptive or nonadaptive.

In contrast, EC dysfunction is by definition maladaptive. The term *dysfunction* was initially applied to the phenotype of the endothelium overlying atherosclerotic lesions in animal models of hyperlipidemia. Today, the term is almost always used to describe abnormalities in endothelium-dependent vasomotor tone in atheromatous conduit arteries. However, because the endothelium is widely distributed, and because it is involved in so many different functions (over and above the control of blood vessel diameter), the term *dysfunction* should be used more broadly to describe situations in which the endothelial

phenotype—whether or not it meets some predetermined definition of activation—represents a net liability to the host.

Assigning liability scores is of course a subjective exercise. An evolutionary biologist might argue that EC dysfunction is most relevant in its effect on an individual's reproductive capacity. A physician would surely expand the meaning of dysfunction to include a far broader spectrum of morbidity. An investigator interested in applying evolutionary principles to an understanding of endothelium in health and disease would point out that the endothelium evolved to a state of maximal fitness in the early ancestral environment, and is not adapted to withstand the rigors of high fat diet, epidemics associated with high density populations, sedentary lifestyle, or old age.

DIAGNOSIS AND THERAPY

The endothelium has remarkable, yet largely untapped diagnostic potential. At the bedside, the endothelium is difficult to assay. It is hidden from view and cannot be inspected, palpated, percussed, or auscultated. Moreover, the endothelium is poorly circumscribed and therefore is not amenable to conventional radiologic imaging. The most commonly used assays for the endothelium are flow studies, which measure endothelium-dependent vasodilation (reviewed in Ref.[34]). Abnormalities in endothelium-dependent flow have been correlated with atherosclerosis and risk for acute coronary syndrome. However, these studies are highly operator dependent, and they measure only a single function of the endothelium. An important goal in vascular biomedicine is to develop novel methods for diagnosing endothelial dysfunction. Such a diagnostic platform may ultimately include some combination of soluble mediators, cell-based assays, and molecular imaging. Dysfunctional ECs release a number of soluble mediators into the blood, including endothelin-1, vWF, tissue-type plasminogen activator (t-PA), soluble thrombomodulin, soluble VCAM-1, soluble ICAM-1, and soluble E-selectin. Cell-based assays include quantification and/or phenotyping of circulating ECs, endothelium-derived microparticles, and late outgrowth endothelial progenitor cells. Finally, molecular imaging holds promise. As an example, phage display has been used to identify a novel VCAM-1–specific cell-internalizing peptide that allows sensitive magnetic resonance imaging of atherosclerotic lesions in mice.[35] It appears likely that clinical events occur minutes, hours, days, or even weeks after initiation of processes that are occurring at the endothelial level. Developing test strategies to identify these processes before their phenotypic expression has great potential.

The endothelium is a highly attractive therapeutic target. It is rapidly and preferentially exposed to systemically delivered agents. Given the capacity of ECs to sense and respond to the local environment, it is hard to imagine that there exists any treatment for any disease that does not affect EC phenotypes in one way or another. From a therapeutic standpoint, the combination of phenotypic heterogeneity and modulability offers both opportunities and challenges. On one hand, the identification and characterization of vascular bed-specific "zip codes" should provide a foundation for site-specific targeting. On the other hand, drugs that lack such specificity are likely to exert mixed effects on the vasculature—with protective effects in some vascular beds and neutral or deleterious consequences in others. This idea is supported by the many knockout models that demonstrate vascular bed–specific phenotypes (reviewed in Ref.[36]). In so far as treatment against one or another target creates a functional knock out of that target, the effects on the vasculature will be similarly heterogeneous. For example, a treatment aimed toward neutralizing P-selectin might reduce leukocyte trafficking in the gastrointestinal tract, but not the liver. A drug that is designed to promote refenestration in liver disease might also induce de novo fenestrations (hence permeability) in vascular beds such as blood–brain barrier, with potentially disastrous consequences.

ENDOTHELIUM IN HEMOSTASIS

An Integrated Model of Hemostasis

Vascular thrombotic disorders are the leading causes of mortality in the Western world. It is noteworthy that most, if not all of these disorders, although consisting of a systemic balance in hemostasis, are associated with local thrombotic phenotypes.[36,37,38] These clinical observations are supported by genetic mouse models, in which the deletion of one or another natural anticoagulant mechanism results in vascular bed-specific fibrin deposition and thrombosis. An important question that arises from these clinical and animal studies is how a systemic imbalance in hemostasis is ultimately manifested by local rather than diffuse vasculopathic lesions. The answer lies, at least in part, in the endothelium.

The endothelium plays a critical role in mediating hemostasis. ECs synthesize many procoagulant and anticoagulant molecules. On the anticoagulant side, they express tissue factor pathway inhibitor (TFPI), heparan, thrombomodulin, endothelial protein C receptor (EPCR), and t-PA. On the procoagulant side, ECs synthesize plasminogen activator inhibitor-1, vWF, protease-activated receptors, and (at least in cell culture) tissue factor (TF). Each of these mediators is differentially expressed across the vascular tree. For example, TFPI is expressed predominantly in capillary endothelium, EPCR in large veins and arteries, endothelial nitric oxide (NO) synthase on the arterial side of the circulation, vWF in veins, t-PA in pulmonary and cerebral arteries, and thrombomodulin in blood vessel types of every caliber in all organs except the brain.[39–41] TF is not detectable in normal endothelium, whereas in a baboon model of sepsis, the gene is upregulated in a subset of ECs in the marginal zone of splenic follicles, and in regions of disturbed flow.[42,43] In response to systemic inflammation, the expression of these various procoagulants and anticoagulants changes in ways that differ between vascular beds. Thus, the picture that emerges is one of heterogeneity layered upon heterogeneity, such that ECs from different sites of the vascular tree employ site-specific "formulas" of hemostatic proteins to maintain blood in its fluid state and to promote limited clot formation when there is a breech in the integrity of the vascular wall (FIGURE 42.1).

The site specificity of endothelial hemostatic function provides a foundation for understanding how systemic imbalances in coagulation are channeled into local thrombotic phenotypes. The liver synthesizes and releases into the circulation a relatively constant amount of precursor serine proteases (zymogens) of the clotting cascade, the structural molecule fibrinogen and certain natural anticoagulant molecules including antithrombin III (ATIII), protein C, and protein S. The bone marrow produces and releases into the circulation a relatively fixed number of monocytes and platelets, cells that are capable of expressing TF and/or promoting clotting reactions. This net output of liver-derived

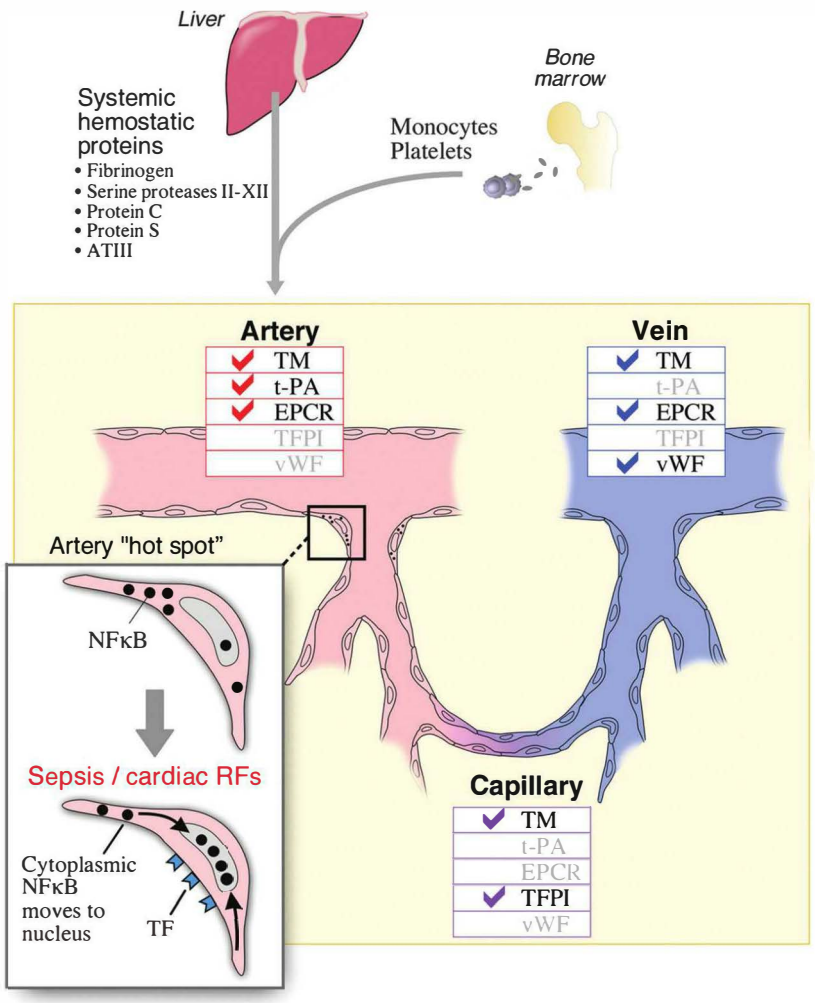

FIGURE 42.1 Endothelium and hemostasis. The liver synthesizes a relatively constant supply of clotting factors (serine proteases II through XII) and fibrinogen. In addition, hepatocytes synthesize several natural anticoagulants including protein C, protein S, and ATIII. The bone marrow produces and releases a relatively constant number of monocytes and platelets. Monocytes are capable of expressing TF, whereas activated platelets provide a cell surface for assembly of clotting reactions. ECs also express procoagulants and anticoagulants. However, the repertoire of EC-derived hemostatic factors varies between vascular beds (shown are reported differences between arteries, veins, and capillaries, though differences also exist between different types of arteries, veins, and capillaries). The systemic mix of liver-derived soluble mediators and bone marrow-derived blood cells is integrated into the unique hemostatic balance of each vascular bed. It follows that changes in the systemic balance (as occurs, e.g., in patients with congenital deficiency of protein C, protein S, or ATIII, or with factor V Leiden) will have different local effects, giving rise to site-specific thrombotic phenotypes. Recent evidence suggests that ECs in regions of disturbed flow in arteries are primed for activation (they have increased levels of nuclear factor-kappaB [NF-κB] in their cytoplasm) and that systemic imbalances (e.g., associated with sepsis or cardiac risk factors) may result in the translocation of NF-κB to the nucleus and increased expression of procoagulants such as TF and adhesion molecules. TM, thrombomodulin; t-PA, tissue-type plasminogen activator; EPCR, endothelial protein C receptor; TFPI, tissue factor pathway inhibitor; vWF, von Willebrand factor (Adapted from Aird WC. Phenotypic heterogeneity of the endothelium. I. Structure, function, and mechanisms. *Circ Res* 2007;100:158–173, with permission).

proteins and bone marrow-derived cells is systemically distributed and integrated into the unique hemostatic balance of each and every vascular bed. Thus, when an alteration occurs in the output of proteins (e.g., protein C or protein S deficiencies, or production of factor V Leiden) or cells (e.g., increased number of activated monocytes in sepsis), the changes affect the hemostatic balance in ways that differ between sites of the vascular tree.

Adding another layer of complexity to the model is the fact that the endothelium may become activated. Moreover, the activation phenotype may vary in different vascular beds. For example, sepsis is associated with widespread, though organ-specific, changes in the extracellular milieu, with subsequent local changes in EC adhesion, permeability, and hemostatic balance.

The value of the above scheme is that it recognizes the involvement of four functionally linked organ systems in mediating coagulation: namely, the liver, bone marrow, cardiovascular system, and endothelium. In this way, we are reminded of the importance of the hepatocyte in synthesizing serine proteases, the two cofactors, fibrinogen, and the natural anticoagulants, ATIII, protein C, and protein S; the critical role of TF-expressing monocytes in initiating coagulation; the participation of the platelet in localizing and perpetuating the coagulation response; and the importance of the EC as a manufacturer of hemostatic factors and regulator of the hemostatic balance.

It is not difficult to reconcile this model with that of Virchow's triad. Two observations have not changed over the past 150 years. First, stasis of blood flow (e.g., secondary to obesity, tumor, congestive heart failure, pregnancy, or immobility) when introduced into the system, may lead to the accumulation of activated clotting factors, reduction in protective hemodynamic forces, and downstream hypoxia—all of which may tip the balance to the procoagulant side. Second, frank disruption or denudation of the endothelium (e.g., as occurs in trauma, surgery, or catheter placement) may result in the exposure of blood to subendothelial adventitial TF and secondary thrombosis. However, two new observations warrant emphasis. First, when referring to blood constituents, we now recognize the importance not only of soluble clotting factors, but also of the cells—not just the platelet, but also the monocyte. Second, we now understand that an intact endothelium may contribute to hemostatic imbalance. In other words, both the structural and functional integrity of the vascular wall are important determinants of thrombotic risk.

The Endothelium and Vascular Bed-Specific Thrombosis

Venous Thrombosis

In the lower extremities, most venous thrombi occur in the pockets of valves (FIGURE 42.2). Three design features render valve pockets particularly vulnerable to thrombus formation. First, ECs lining the valve pocket are exposed to reduced flow velocities and shear stress during the valve cycle.[44] Secondly, the blood in the cusps of the valve is relatively hypoxic.[45] Finally, there is a tendency for leukocytes and platelets to become trapped in the valve pocket.

Venous flow is normally pulsatile. During standing, the venous valves open and close approximately 20 times per minute (reviewed in Ref.[36]). Flow through the valve creates vortices inside the valve pocket, which flush out the blood. Immobilization results in reduced venous blood flow in the lower extremities, with maximal stasis occurring in the valve pockets.

According to Virchow's triad, deep venous thrombosis may occur when there is a reduction in flow velocity or pulsatility (immobilization), when there is a shift in the circulating hemostatic balance (factor [F] V Leiden, G20210A prothrombin mutation, congenital deficiency of ATIII, protein C, or protein S), and/or when there is a loss of vascular wall integrity. Frank structural injury to the valve endothelium is rare, even with surgery.[46] Far more commonly, the intact endothelium becomes activated and dysfunctional, through exposure to stasis, hypoxia, and/or inflammatory mediators. Activated ECs may express increased levels of E-selectin and P-selectin. The selectins in turn bind to their ligands on circulating leukocytes and microparticles, resulting in increased leukocyte–endothelial interactions and TF-mediated initiation of the clotting cascade. There is evidence that TF-mediated activation of clotting precedes platelet activation in venous thrombosis (reviewed in[47]). The source of TF is debated. It is likely derived from circulating hematopoietic cells or microparticles. There is no evidence that TF is expressed in the ECs in the valve pocket. However, it has been proposed that TF may be transferred from microparticles to ECs (reviewed in Ref.[47]).

Thrombosis of intra-abdominal veins occurs with particularly high frequency in patients with myeloproliferative disorders (e.g., essential thrombocythemia and polycythemia vera) and paroxysmal nocturnal hemoglobinuria.[48] Intra-abdominal veins have very few valves compared with veins of the lower extremities. Thus, we must look for other mechanisms to explain thrombosis in this region of the vasculature. ECs lining the intestinal veins are exposed to a very different microenvironment compared with other blood vessels. For example, they experience boluses of absorbed nutrients following meals. Moreover, they are constantly exposed to oral antigens and pathogen-related products. Previous studies have demonstrated that hepatic sinusoidal ECs are immune tolerant and fail to mount an abnormal response to innocuous antigens and endotoxin (reviewed in[3]). It is not known whether the endothelium of mesenteric and portal veins is also immune tolerant. If not, then perhaps these cells are chronically activated and/or express site-specific genes that render them preferentially vulnerable to changes in the circulating blood of patients with myeloproliferative disorders and paroxysmal nocturnal hemoglobinuria. Such changes may include a combination of increased blood viscosity, activated platelets and/or leukocytes, increased microparticles, free hemoglobin, and activated complement. In the case of paroxysmal nocturnal hemoglobinuria, it is interesting to speculate that ECs (perhaps in intra-abdominal veins) are clonally abnormal. As TFPI is a glycosyl phosphatidylinositol-linked protein,[49] ECs derived from the affected clone might be deficient in this anticoagulant.

The vasculature of the adrenal gland is unique in that a large number of arteries feed into a relatively small number of venules *via* medullary sinusoids. As a result of this architecture—which has been referred to as a "vascular dam"–thrombosis of the adrenal vein commonly presents as hemorrhage (reviewed in[36]). The musculature of the adrenal vein is not concentric, but rather is composed of thick longitudinal bundles. It has been suggested that the eccentric arrangement of the muscle in the adrenal vein creates pockets of turbulence and stasis, thus predisposing to thrombus formation. Also, the adrenal vein drains high concentrations of catecholamines, which might be predicted to induce vasoconstriction and activate platelets.

Arterial Thrombosis

Most arterial thrombosis occurs at the sites of atherosclerosis. Branch points and greater curvatures of arteries are normally exposed to disturbed flow. Previous studies have shown that ECs lining these regions of disturbed flow are primed for activation (reviewed in Ref.[50]). This is a classic example of an evolutionary tradeoff. In this case, the fitness advantage a closed circulation must outweigh the disadvantages of regional flow disturbances. In the modern day environment, systemic risk factors for atherosclerosis, including hypertension, hyperglycemia, smoke-related toxins, and hyperlipidemia, may serve to push the primed ECs into a dysfunctional state. Consistent

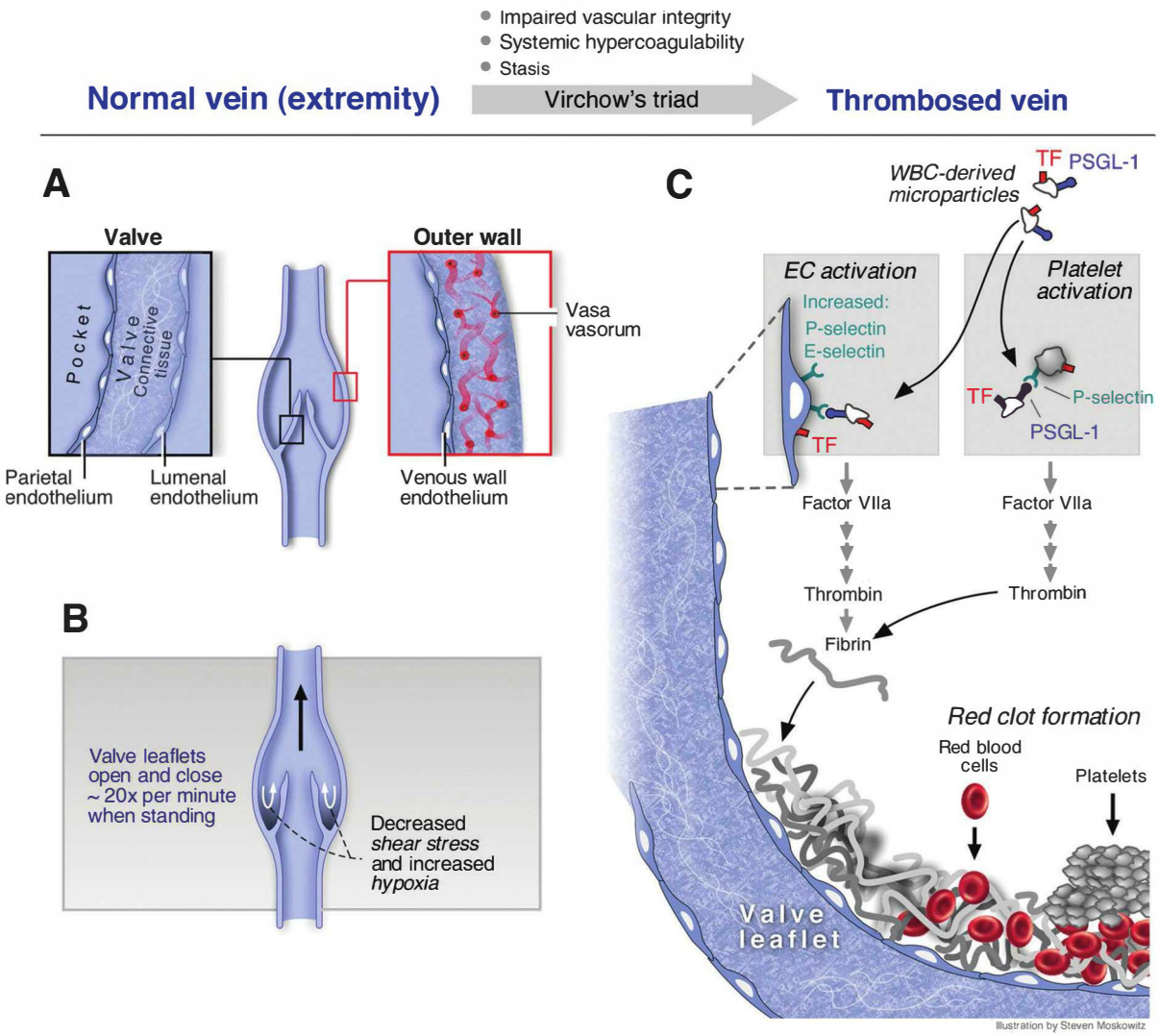

FIGURE 42.2 Venous thrombosis in the lower limbs. **A:** Under normal conditions, the veins of the lower limbs contain a high number of valves, the vast majority of which are bicuspid. The valves consist of a connective tissue core covered on both surfaces (parietal and lumenal) by endothelium. The endothelial linings of the valve leaflets are phenotypically distinct from ECs lining the vein wall (e.g., their density is higher, they are not oriented in the direction of blood flow, and by extension of studies in heart valves and by virtue of their exposure to unique flow patterns, they may demonstrate microheterogeneity in gene expression). In contrast to the vein wall, the valve leaflets do not contain vasa vasorum (and are thus reliant on venous blood oxygen). **B:** The valve cycle is associated with vortical flow behind the valve cusps, which reduces stasis inside the valve pocket. Valve pocket blood is hypoxic relative to blood in the mainstream. **C:** A combination of stasis (e.g., caused by immobilization, congestive heart failure, obesity), systemic hypercoagulability (e.g., associated with congenital deficiency of anticoagulants, factor V Leiden; use of oral contraceptive pills (OCP); pregnancy; or activated platelets), and/or loss of vascular integrity (particularly an activated, as distinct from frankly denuded, endothelium), may lead to TF-mediated initiation of clotting cascade. The thrombus begins as a microscopic nidus of fibrin-red cell collections near the apex of the valve pocket. Alternating layers of fibrin and platelet clumps are characteristic of the propagating thrombus. TF- and P-selectin glycoprotein ligand-1-(PSGL)-positive circulating microparticles bind to P selectin on platelets, or P-selectin or E-selectin on ECs, leading to transfer of TF to the cell and activation of the clotting cascade. (Adapted from Aird WC. Vascular bed-specific thrombosis. J Thromb Haemost 2007;5 Suppl 1:283-291, with permission.)

with this view, ECs overlying atheromatous plaques demonstrate abnormal ultrastructure,[51] increased expression of adhesion molecules,[52] TF and TFPI,[53,54] and reduced expression of thrombomodulin and EPCR.[55] Once the disease process is set in motion, atheromas may compromise the lumen and ultimately lead to occlusive thrombosis.

Arterial thrombi may occur in the absence of atherosclerosis. For example, patients with different types of vasculitis may develop arterial thrombi in characteristic and distinct sites of the arterial tree (reviewed in Ref.[36]). The molecular basis for the site-specific patterns of the vasculitides is likely to involve immune-mediated targeting of spatially restricted antigens or neoantigens

present in the vascular wall, including the macroendothelium and/or endothelium of the vasa vasorum. Essential thrombocythemia is a rare cause of coronary artery occlusion.[56] Essential thrombocythemia-associated arterial thrombus may occur in the absence of atherosclerosis, and is likely to be mediated, in large part, by abnormalities in platelet function. In heparin-induced thrombocytopenia (HIT), the ratio of venous to arterial thrombosis is normally 4:1. However, patients with risk factors for atherosclerosis (i.e., those undergoing open heart surgery), have a higher risk for arterial thrombi,[57,58] suggesting a permissive role for atherosclerosis in HIT-associated arterial occlusive lesions. In contrast to the distribution of atherosclerosis-related complications (coronary > cerebral > lower extremity arteries), HIT-associated arterial thrombi occur most commonly in the aorta and iliofemoral arteries, followed by cerebral and coronary arteries. These differences argue for distinct pathophysiologies underlying atherosclerosis- and HIT-mediated arterial thrombosis.

In general terms, there are some important differences between mechanisms of thrombus formation in veins and arteries. Compared with valve pockets in veins, the loss of vascular integrity (e.g., *via* plaque rupture or fissure) is more common at sites of arterial thrombi. Second, while TF is thought to play a primary role in mediating venous thrombosis, interactions between platelets (particularly GP1b), vWF (or other GP1b ligands) and extracellular matrix (particularly collagen) may be the most important determinant of arterial clots.[59,60,61,62] Finally, the development of arterial thrombosis is not generally increased in patients with thrombophilic mutations, such as FV Leiden or the prothrombin gene G20210A mutation.

Microvascular Thrombosis

Microvessels comprise the vast majority of the surface area of the circulation. Microvascular thrombosis is typically manifest as acute or chronic organ dysfunction. The chronic type is exemplified by such disorders as sickle cell disease and small vessel vasculitis. Acute small vessel occlusion is seen in disseminated intravascular coagulation, catastrophic antiphospholipid syndrome, Trousseau's syndrome, HELLP (hemolysis, elevated liver enzymes, and low platelets), microangiopathic hemolytic anemias (thrombotic thrombocytopenic purpura [TTP] and hemolytic uremic syndrome [HUS]), coumarin-induced skin necrosis, erythromelalgia, and HIT. Although the precise pathophysiology of clot formation is unclear in most of these disorders, the endothelium is likely to play a critical role in mediating their site specificity. For example, in classical TTP and sporadic HUS, platelet-rich thrombi are observed in the microvascular beds of every organ examined with the exception of the liver and lung.[63] Previous studies have demonstrated that plasma from patients with TTP or HUS induces apoptosis of microvascular ECs cultured from many organs, but not the lung.[64] Another major theme in the pathogenesis of TTP is a deficiency in ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motif-13), which mediates proteolytic processing of newly released proadhesive ultralarge vWF multimers from ECs. Mice that are null for ADAMTS13 are not at risk for spontaneous development of TTP phenotype.[65] Indeed, it has been proposed that the pathophysiology of classical TTP and sporadic HUS requires two hits: altered vWF activity (e.g., as might occur with ADAMTS13 deficiency) and site-specific activation and/or apoptosis of microvascular ECs.[66,67]

Patients with polycythemia vera or essential thrombocythemia may present with erythromelalgia (i.e., painful red or blue toes, soles of feet or fingers) (reviewed in Refs.[68,69]). Pathology reveals fibromuscular hyperplasia, narrowing of the lumen, swollen ECs, and platelet-rich thrombi in arterioles. Although untreated erythromelalgia may progress to gangrene, the syndrome is exquisitely sensitive to aspirin therapy. In contrast, warfarin-induced skin necrosis is associated with paresthesias, skin discomfort, and/or discoloration in the regions of the buttocks, breasts, and thighs. Pathology reveals fibrin deposition in the postcapillary venules, and treatment includes the institution of potent and rapidly acting anticoagulants (e.g., heparin). The distribution of lesions in coumarin-induced skin necrosis suggests that the dermal microvascular endothelium is uniquely sensitive to changes in circulating levels of functional protein C. In patients with HIT, warfarin-induced skin necrosis is more commonly observed in the limbs. HIT has also been reported to cause skin necrosis in the absence of coumarin, with thrombosis of venules and capillaries, but sparing of arterioles. The distinct microvascular phenotypes associated with erythromelalgia, coumarin-induced skin necrosis, and HIT underscore the existence of heterogeneity within a single microvascular bed.

SUMMARY

The endothelium is a widely distributed organ system that plays an important role both in physiology and disease. The endothelium has many functions, over and above its well-recognized role in mediating vascular tone. One of these critical activities is maintenance of blood fluidity and repair of injured blood vessels. The endothelium is remarkably heterogeneous in structure and function. Indeed, ECs regulate hemostatic balance by site-specific formulas of anticoagulants and procoagulants. These different equations are sufficient to explain why systemic imbalances in coagulation factors invariably lead to local thrombotic phenotypes. The propensity to develop clots in specific vascular beds is further accentuated by local endothelial dysfunction. An important goal for the future is to identify diagnostic markers that reflect phenotypic changes at the level of individual vascular beds, and to develop therapies that target selected sites of the vasculature.

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CHAPTER

43 Role of the Endothelium in Hemostasis

MICHAEL J. KRISINGER AND EDWARD M. CONWAY

Hemostasis and coagulation, which refer to the arrest of bleeding and the formation of a fibrin clot, respectively, are generally used interchangeably to describe the processes by which blood is maintained in a fluid state in the circulatory system. Normal function of the endothelium, the single cell layer lying at the interface of circulating blood and the underlying tissue, is required for optimal organ function and for maintaining and regulating blood flow to meet the body's dynamically changing demands over a range of pathophysiologic conditions. Rather than playing a passive role in the transport of blood, the endothelium has, in the past half century, become recognized as a central and active player in homeostasis. In response to damaging stimuli, such as trauma or infections, the vascular endothelium, in conjunction with other components of the hemostatic system, serves to rapidly and effectively limit tissue destruction to a local site and to promote healing.

The hemostatic system is continuously in an "on" position, dynamically exhibiting antithrombotic and prothrombotic properties that are delicately balanced to meet local and systemic needs (FIGURE 43.1). Under normal physiologic conditions, natural antithrombotic forces override the prothrombotic ones, limiting platelet adhesion and activation and excess fibrin clot formation, while vasomotor tone is optimized, overall maintaining critical blood flow to ensure organ function. Not unexpectedly, the switches that control hemostatic system activity are tightly regulated. Genetic, environmental, and epigenetic factors that disrupt the exquisite and dynamic balance between prothrombotic and antithrombotic forces are clinically manifest by either bleeding or thrombosis. The major prothrombotic forces are mediated by platelets, monocytes, the clotting cascade, and the vascular endothelium, while the predominant antithrombotic mechanisms center around the endothelium. In this chapter, we explore the major properties of the endothelium in regulating the hemostatic system.

TISSUE FACTOR AND INITIATION OF COAGULATION ON THE ENDOTHELIAL CELL SURFACE

The coagulation system is characterized by the sequential activation of a series of serine proteases, culminating in the generation of thrombin, with subsequent conversion of fibrinogen into an insoluble fibrin clot. The molecular events surrounding initiation of coagulation have been extensively examined and revised since the original description in 1964 of the cascade/waterfall hypothesis of hemostatic system activation.[1] Following vascular injury, tissue factor (TF) is exposed to the circulation and binds to coagulation factor VIIa.[2] When not associated with TF, the factor VIIa that constitutively circulates at low levels has poor enzymatic properties. When complexed with TF, factor VIIa acquires potent catalytic properties and triggers coagulation by converting the zymogens factor IX and factor X to enzymes factor IXa and factor Xa, respectively. The initial factor Xa formed when bound to membrane is sufficient to generate trace (pM) amounts of thrombin from prothrombin. This initial thrombin serves as the primary activator of the zymogen factor XI, the procofactors V and VIII, and importantly platelets, which together provide important amplification feedback. Surface exposure of anionic phospholipids (e.g., phosphatidylserine) is essential in promoting assembly and efficient catalytic activity of the two subsequent membrane-bound enzyme complexes, namely "intrinsic factor Xase" and "prothrombinase." The factor IXa generated from the TF-factor VIIa combines with its cofactor factor VIIIa on the activated platelet membrane to form the "intrinsic factor Xase," which becomes the major activator of factor X. Similarly, the factor Xa formed combines with factor Va on the activated platelet membrane to form "prothrombinase," which converts prothrombin to thrombin.

In what is viewed as the central event in coagulation, the serine protease thrombin finally cleaves fibrinogen to generate fibrin, which is cross-linked by factor XIIIa. The scheme supports the notion that the TF-factor VIIa pathway of blood clotting is the major physiologic mechanism for triggering coagulation and that exposure of TF to the circulation is the critical event that induces the vasculature to acquire a procoagulant phenotype.[3]

TF is a 263–amino acid integral membrane protein, structurally comprised of three domains.[4] The extracellular domain constitutes over 80% of the amino acid sequence of the molecule and provides binding sites for factor VIIa, such that the protease activity of TF-complexed factor VIIa toward its substrates, factors X and IX is dramatically increased. The transmembrane domain of TF is important for optimizing its cofactor function with factor VIIa.[5] The cytoplasmic tail mediates intracellular signals in processes that impact on a range of cellular activities, including cell proliferation, angiogenesis, and inflammation.[6–8]

Expression of TF varies in different organs, with particularly high levels in the heart, lung, brain, testis, uterus, and placenta, and lower amounts in the joints and skeletal muscle.[9] It is found throughout the vasculature, synthesized by adventitial fibroblasts, smooth muscle cells, pericytes, and several hematopoietic and nonhematopoietic cells.[10,11] When stimulated by proinflammatory cytokines, monocytes and macrophages prominently express TF.[10,12] Tumor cells also synthesize TF, which impacts on metastasis formation.[13] In some pathologic conditions, small amounts of TF that are in or on platelets and/or a component of platelet-derived microparticles may also contribute to the overall pool of TF, although the relevance of these sources is not yet clear.[14,15]

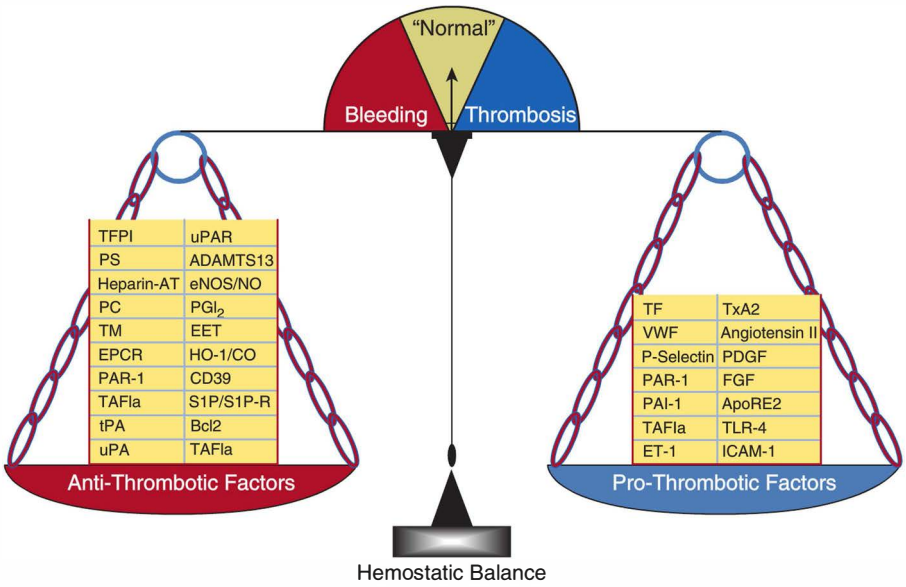

FIGURE 43.1 Balance between endothelial prothrombotic and antithrombotic factors. Relative imbalances in expression of prothrombotic and antithrombotic factors will determine the response to pathophysiologic stresses that may be exhibited by bleeding or thrombosis.

In spite of some conflicting data, most studies concur with the earliest reports that TF is not normally expressed by endothelial cells *in vivo*.[16,17] Indeed, in the absence of injury, functionally active TF is excluded from the intravascular space. However, during disease, vascular endothelial cells may express TF. For example, TF may be prominently expressed by endothelial cells of the pulmonary veins of hypoxic mice with sickle cell anemia,[18] of tumor vessels,[19] and overlying atheromatous lesions.[20] Some investigators have suggested that TF is normally expressed by circulating monocytes but in a functionally inactive "encrypted" form, able to undergo "decryption" during inflammation.[21] Thus, given its prominent perivascular distribution, TF has been represented as a hemostatic "envelope" for the vasculature, readily available to initiate coagulation upon exposure to blood when vascular integrity is compromised by chemical or mechanical injury.[16] Indeed, recent studies suggest that preformed TF-factor VIIa complexes may reside around blood vessels in the absence of injury, allowing even more rapid coagulation activation upon injury.[22]

Numerous extracellular stimuli that are implicated in disorders associated with thrombosis may trigger coagulation *in vivo* by inducing TF expression by most cells exposed to the circulation, including for example endothelial cells, monocytes, platelets, and subendothelial cells (after vessel injury). Among these agonists are proinflammatory mediators, including lipopolysaccharide, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, thrombin, vascular endothelial growth factor (VEGF), infectious agents (e.g., herpes simplex virus, rickettsia, and measles), oxygen free radicals, and low shear stress. This list is not comprehensive, but underlines the fact that the vascular endothelium is constantly exposed to agents that are likely to induce varying degrees of damage with exposure of TF, with constant generation of small amounts of thrombin. Indeed, it has been well established that in normal individuals, there is a persistent low level of activation of coagulation.[23,24] For the TF-factor VIIa triggering reaction to proceed to full-fledged coagulation, the amount of TF exposed and thus the amount of thrombin generated must exceed a threshold to overcome the activity of endogenous natural anticoagulant pathways that otherwise serve to limit thrombin bursts and prevent physiologically excessive thrombotic responses. There are in fact, four dominant natural vasculoprotective mechanisms that regulate thrombin generation and fibrin clot formation. These are tissue factor pathway inhibitor (TFPI), the heparin–antithrombin (AT) pathway, the protein C (PC) anticoagulant pathway, and the fibrinolytic system.

TISSUE FACTOR PATHWAY INHIBITOR

TFPI-1, commonly referred to as TFPI, is the principle inhibitor of the TF pathway, binding to and neutralizing TF-factor VIIa in a factor Xa–dependent manner (reviewed in[25,26]). TFPI is primarily associated (~85%) with microvascular endothelial cells where it is constitutively expressed. Lesser amounts are also found on the surface of monocytes, smooth muscle cells, platelets, mesangial cells, fibroblasts, cardiomyocytes, and plasma.[27,28]

TFPI is a 276–amino acid, 43 kDa, multivalent Kunitz-type serine protease inhibitor[29] that comprises an acidic *N*-terminal region, three Kunitz-type domains, and a basic *C*-terminus. Interaction of TFPI with the endothelial cell surface is achieved via two mechanisms. The *C*-terminal stretch of basic residues of TFPI binds to cell surface glycosaminoglycans.[27] This is therefore heparin releasable and accounts for a two- to four-fold increase in plasma levels after heparin infusion.[30] Most of the cell surface–associated TFPI, which cannot be released by heparin, is bound through a glycosylphosphatidylinositol (GPI) anchor.[31] However, TFPI does not contain a consensus sequence for attachment of a GPI anchor, and thus it is believed that there exists a hitherto unidentified coreceptor that binds to the third Kunitz domain of TFPI.

Alternative pre-mRNA splicing yields at least three isoforms of TFPI in the mouse (α, β, γ) and human ($\alpha\beta\delta$). Only the full-length form (TFPIα or TFPI) contains the third Kunitz domain and the basic *C*-terminal region. The other isoforms contain

two Kunitz domains and the *C*-terminal region. In humans, TFPI is the only one that has been identified at the protein level in tissues.[25] During embryonic development in mice, TFPI and TFPIβ are both widely expressed and at about equal levels. However, adult mouse tissues produce mostly TFPIβ. In contrast to TFPI, TFPIβ has a *C*-terminal GPI anchor attachment sequence, allowing it to directly associate with the cell surface.[31] The role of TFPIγ is unknown. Although it is believed that inhibition of TF-factor VIIa and factor Xa require the third Kunitz and the basic *C*-terminus of TFPI, several *in vitro* studies suggest that TFPIβ may have anticoagulant activity on the cell surface.[32] *In vivo* studies are required to fully assess the relative anticoagulant activity of TFPIβ. Functional data for TFPIδ are lacking. It contains two Kunitz domains and a 12–amino acid *C*-terminus, the latter having no similarity to the other isoforms.

To effectively regulate TF-factor VIIa–induced initiation of coagulation, TFPI must be appropriately localized at the cell surface where vascular injury exposes TF to the circulation. At that site, TFPI is well positioned to prevent small amounts of TF-factor VIIa, formed in response to a minor injury, from unnecessarily generating a large thrombin burst. Indeed, TFPI provides the first natural barrier threshold, without which coagulation would otherwise proceed and amplify in an unregulated fashion (FIGURE 43.2). TFPI utilizes the two *N*-terminal (first and second) Kunitz-type serine protease domains to inhibit the active sites of both factor Xa and TF-factor VIIa. As TF-factor VIIa activates factor X, the second Kunitz domain of TFPI binds to the active site of the newly generated factor Xa that is still associated with the TF-factor VIIa complex, rendering the factor Xa inactive. The flanking Kunitz domains and the basic *C*-terminal region of TFPI enhance their interaction with factor Xa. Protein S (PS), a vitamin K–dependent protein and cofactor in the PC-thrombomodulin (TM) anticoagulant system (see below), also acts as a cofactor for TFPI by interacting with its third Kunitz domain and its *C*-terminus, accelerating inhibition of factor Xa.[33,34] Binding of factor Xa causes TFPI to undergo a conformational change, thereby facilitating subsequent interaction of the TFPI-factor Xa complex via the first Kunitz domain of TFPI, in a Ca^{2+}-dependent manner, with the active site of factor VIIa when bound to TF.[35] The resultant quaternary complex TF/factor VIIa/factor Xa/TFPI lacks both TF-initiated blood coagulation activity as well as protease-activated receptor (PAR)-1/2-mediated cellular signaling activity involved in inflammation. Although the interactions of TFPI with factor Xa and TF-factor VIIa are usually depicted as a two-step process, these effectively occur simultaneously. Indeed, once factor Xa is released from TF-factor VIIa and complexed with factor Va, it cannot any longer be inhibited by TFPI.[36] Thus, the window during which TFPI must function is critically narrow.

As noted above, TFPI has several cellular sources. While endothelial TFPI predominates, it is synthesized by megakaryocytes, and stored in α-granules of platelets, whereupon it may be translocated to the platelet surface upon activation.[37] TFPI is also found in the plasma, the origin of which is primarily endothelial. It circulates in a complex with low-density lipoproteins (LDLs), and comprises various degraded forms, probably generated by proteolysis by a range of enzymes, including thrombin, plasmin, and matrix metalloproteinases.[26] The gamma-carboxyglutamic acid (Gla) domain of PS and the *C*-terminal region of TFPI effectively bind negatively charged phospholipid surfaces and thus localize this negative regulatory system to sites where TF may be exposed for initiation of coagulation, including injured or denuded endothelial cells and activated platelets.[33,34] Nonetheless, delineation of the role of different pools of TFPI and their physiologic site(s) of action require further study.

Not surprisingly, inactivation of the *TFPI* gene in mice results in embryonic lethality[38] due to a consumptive coagulopathy. Heterozygous *TFPI* deficiency in combination with factor V_{Leiden} in mice increases thrombotic susceptibility.[39] The finding that TFPI administration had efficacy in animal models of sepsis prompted phase III clinical trials. Unfortunately, although TFPI dampened activation of coagulation in patients with sepsis, there was no survival benefit.[40] Indeed, the link in humans between TFPI deficiency and thrombotic disease has not been clearly established. There are suggestions that inadequate release of TFPI into the circulation might increase the risk of thrombosis, and only isolated TFPI polymorphisms have been weakly associated with thrombosis.[41] This lack of a clear association is intriguing, as it implies that there may be additional natural anticoagulant mechanisms that compensate for deficiencies in TFPI. It may also mean that moderate deficiencies of TFPI may be incompatible with life.

Similar to TFPI (TFPI-1) in structural architecture, TFPI-2 also has three Kunitz-type domains, but does not inhibit factor Xa and has minimal inhibitory activity for TF-factor VIIa.[42,43] Rather, TFPI-2 expressed by endothelial cells, inhibits trypsin, plasmin, kallikrein, factor XIa, and some matrix metalloproteinases, interacts with complement-binding proteins, and

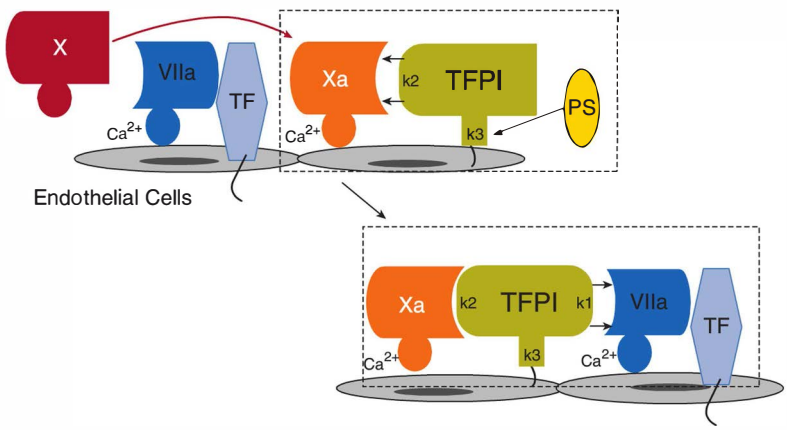

FIGURE 43.2 TFPI-mediated neutralization of factors VIIa and Xa. Localized to the site of injury on the endothelial cell surface, tissue factor pathway inhibitor (TFPI) binds first to factor Xa via its second Kunitz domain (k2), enhanced by protein S (PS) which interacts with the *C*-terminus and the third Kunitz domain (k3) of TFPI. TFPI then undergoes a conformational change, facilitating Ca^{2+}-dependent binding via the first Kunitz domain (k1) and neutralization of factor VIIa which is complexed with tissue factor (TF).

suppresses growth factor–induced proliferation of endothelial cells.[44] Although TFPI-2 may play a role in the coagulation cascade and as a vasculoprotective molecule, it is more likely important in inflammation, atherosclerosis, and tumor cell invasion.[45]

ANTITHROMBIN AND ENDOTHELIAL HEPARIN SULFATE

AT is a circulating serine protease inhibitor (serpin) that neutralizes thrombin, factor Xa and factor IXa,[46] and to a lesser extent the procoagulant factors XIa, XIIa, and TF-factor VIIa. It also exhibits some activity against plasmin, kallikrein, urokinase-type plasminogen activator (uPA), and tissue-type plasminogen activator (tPA). AT is synthesized primarily in the liver and has a molecular weight of approximately 58 kDa. In the circulation, AT exists in a "repressed" conformation that has minimal protease inhibitory activity. In the presence of heparin or heparin sulfate, the neutralizing capacity of AT is accelerated over 500-fold for factor Xa, more than 2,000-fold for thrombin, and approximately one millionfold for factor IXa. Indeed, this is the rationale for using heparin as an anticoagulant drug. Endogenous heparin, however, does not freely circulate in the blood. Rather, endothelial cells express heparin sulfate–containing proteoglycans, including, for example, syndecan-1, -2, -4, glypican-1, betaglycan, CD44, and fibroblast growth factor (FGF) receptor-2, allowing direct interactions with AT,[47,48] as well as with TFPI, thrombin, heparin cofactor II, and tPA. The pattern of sulfation on the repeating disaccharide backbone helps define the binding specificity of each heparin sulfate structure. Although somewhat controversial,[49,50] it is generally believed that the anticoagulant properties of AT are localized to the surface of the endothelium, thereby contributing to the nonthrombogenic properties of the vessel wall. Vascular-bed–specific heterogeneity in the expression of the different heparin sulfate–containing proteoglycans helps to explain the diversity in the anticoagulant properties within different vascular beds.[51,52] In the setting of injury, luminal and subluminal sources of heparin may also activate AT, thereby restricting clot extension.

Heparin-induced activation of AT to a form that rapidly inhibits procoagulant serine proteases occurs via at least two mechanisms (FIGURE 43.3). Through ionic and nonionic interactions, a sequence-specific, negatively charged pentasaccharide within heparin binds to AT with high affinity, thereby inducing allosteric changes in AT that allow it to specifically react with and inhibit factors Xa and IXa. This pentasaccharide is insufficient but necessary to promote inhibition of thrombin by AT. The pentasaccharide must be part of a longer negatively charged polysaccharide of heparin (minimally a 16-mer), which provides a binding site for thrombin. With AT simultaneously bound to the pentasaccharide and a more remote polysaccharide chain, the thrombin molecule thus can be oriented via this heparin bridging mechanism for neutralization by AT. This bridging effect also enhances AT reactivity with factors Xa and IXa for more effective neutralization.[53,54,55] Protease inhibition rates by AT due to heparin sulfate bridging and allosteric changes in AT are estimated to be augmented approximately 2,000-fold and 300-fold, respectively.[56,57] Interestingly, heparin-activated AT is a poor neutralizer of the natural anticoagulant serine protease, activated PC (APC), highlighting the specificity of AT for procoagulant proteases.[58]

AT also plays a role in protecting organisms against inflammatory and infectious disorders, in part via effects on the vascular endothelium. At least in animal models, administration of AT protects against septic shock,[59] although this has not uniformly been observed in human studies.[60] *In vitro*, AT interferes with nuclear factor kappa B (NF-κB) nuclear translocation, thereby suppressing release of cytokines by endothelial cells and the expression of leukocyte adhesion molecules. AT also enhances prostacyclin (PGI_2) release from the vasculature[61] that was protective in a rat model of abdominal sepsis.[62]

The physiologic importance of the heparin-AT mechanism as a natural inhibitor of coagulation is unquestioned. Inherited heterozygous AT deficiency has a reported prevalence that ranges from 1:500 to 1:5,000 and is associated with a significantly increased risk of venous thromboembolic disease.[63,64] Indeed, by the age of 50 years, approximately 50% of patients with AT deficiency will have had at least one thrombotic episode, half of which will be unprovoked.[65] Congenital or inherited deficiencies of heparin have not been reported, suggesting that its absence is not compatible with survival.

THE PROTEIN C SYSTEM

The PC system represents a major physiologically relevant natural anticoagulant mechanism, the function of which depends on the integrity of endothelial cell integral membrane glycoproteins TM and endothelial protein C receptor (EPCR). This system also provides a critical link between coagulation and

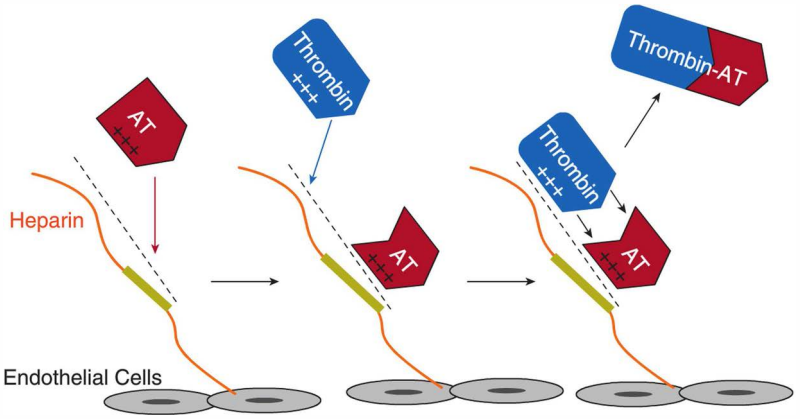

FIGURE 43.3 Heparin-AT neutralization of thrombin. Antithrombin (AT) binds, via nonionic and ionic interactions, to a specific negatively charged pentasaccharide (*green*) within heparin or heparan sulfate molecules on the endothelial cell surface and undergoes a conformational change, allowing it to efficiently react with and inactivate factors Xa and IXa (not shown). For AT neutralization of thrombin, a longer polysaccharide chain provides an additional binding site for thrombin (exosite II), bridging the interaction between thrombin and AT. Once formed, the thrombin–AT complex dissociates from the polysaccharide chain and is cleared from the circulation.

inflammation,[66] highlighting how these biologic processes are spatiotemporally integrated.

PC is a vitamin K–dependent protein, synthesized in the liver that circulates as a two-chain zymogen.[67] APC is a serine protease that is generated by thrombin-mediated cleavage of PC, an event that requires TM, and is indeed amplified over 1,000-fold by its presence[68] (FIGURE 43.4). As the name implies, TM has the remarkable ability to modulate thrombin by allosteric alteration of its active site, converting it from a procoagulant enzyme to an anticoagulant enzyme. This switch in substrate specificity prevents thrombin–TM from cleaving fibrinogen, activating factor V and factor VIII, and triggering platelet activation. PC activation by thrombin–TM is amplified a further 20-fold when PC is bound to EPCR.[69] Platelet factor 4 (PF4) may also accelerate PC activation by inducing allosteric changes in PC that increase its affinity for thrombin-TM.[70] Facilitated by its cofactor PS, APC has potent anticoagulant activities by proteolytically cleaving and inactivating factors VIIIa and Va, which thereby suppresses further production of factor Xa and thrombin[71,72] (FIGURE 43.5). The physiologic importance of this system is underlined by the fact that patients with deficiencies of PC or PS, or who have a mutation in factor V (factor V_{Leiden}) that renders it resistant to inactivation by APC, all have an increased risk of thromboembolic disease[73,74,75] APC also has profound anti-inflammatory properties, mediated by several mechanisms including, for example, suppressing exposure of TF and the release of TNF-α, IL-1β, IL-6, and IL-8, and preventing endothelial expression of leukocyte adhesion molecules.[76–78] Numerous *in vivo* studies have confirmed the important anti-inflammatory properties of APC.[6]

The thrombin–TM complex exhibits additional effects on hemostasis through the activation of the plasma procarboxypeptidase, thrombin-activatable fibrinolysis inhibitor (TAFI). Activation of TAFI by thrombin alone is inefficient but in the presence of TM, is enhanced over 1,000-fold[79] (FIGURE 43.6). The carboxypeptidase activity of activated TAFI (TAFIa), in turn, cleaves *C*-terminal lysine residues from fibrin cleavage products.[80] Fibrin cleavage products, produced by limited plasmin proteolysis of fibrin, accelerate the conversion of plasminogen to plasmin by tPA. Thus, removal of these *C*-terminal lysine residues impedes the degradation of insoluble fibrin, with less incorporation of plasminogen and tPA into the fibrin clot. Accordingly, TM serves an important antifibrinolytic function. TM may also be considered a profibrinolytic factor, since, via APC, it suppresses thrombin generation which

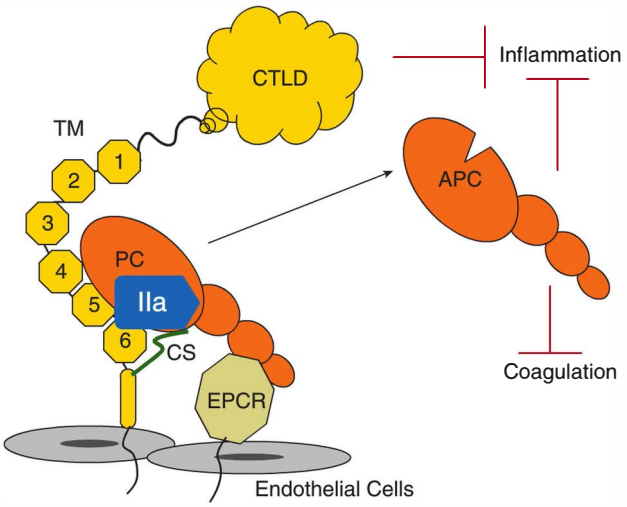

FIGURE 43.4 Activation of PC by thrombin–TM-EPCR. EGF-like 4–6 repeats of thrombomodulin (TM, *yellow*), the chondroitin sulfate (CS) of TM, and the endothelial protein C receptor (EPCR) provide cofactor function for thrombin (IIa)-mediated activation of protein C (PC). Activated protein C (APC) has anticoagulant and anti-inflammatory properties. The C-type lectin-like domain (CTLD) of TM also suppresses inflammation.

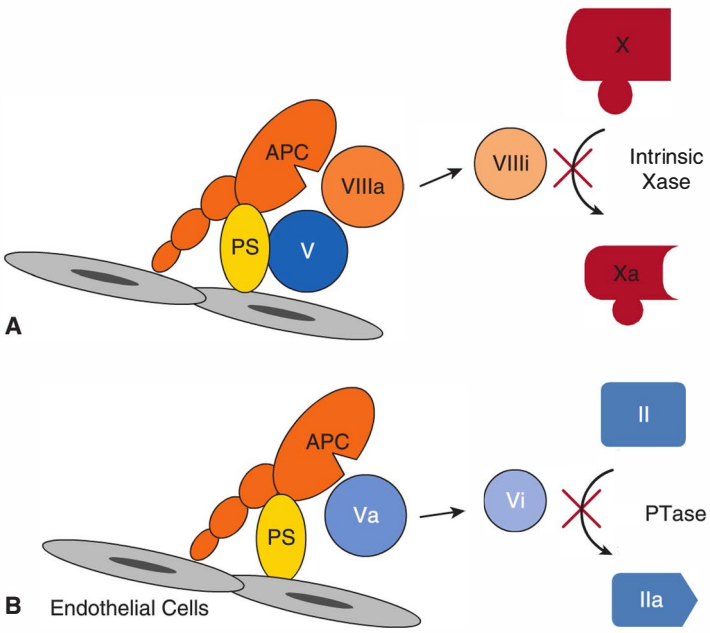

FIGURE 43.5 Anticoagulant properties of APC. When free of EPCR, APC localizes with cofactor protein S (PS) on the surface of endothelial cells or platelets and inactivates factor VIIIa, using factor V as a synergistic cofactor **(A)** or inactivates factor Va **(B)**. This yields, respectively, the inactive species factor VIIIi and factor Vi. Lack of the active cofactors (factors VIIIa and Va) abrogates conversion of factor X to factor Xa by the intrinsic factor Xase complex **(A)**, and prothrombin (II) to thrombin (IIa) by the prothrombinase (PTase) complex **(B)**, respectively.

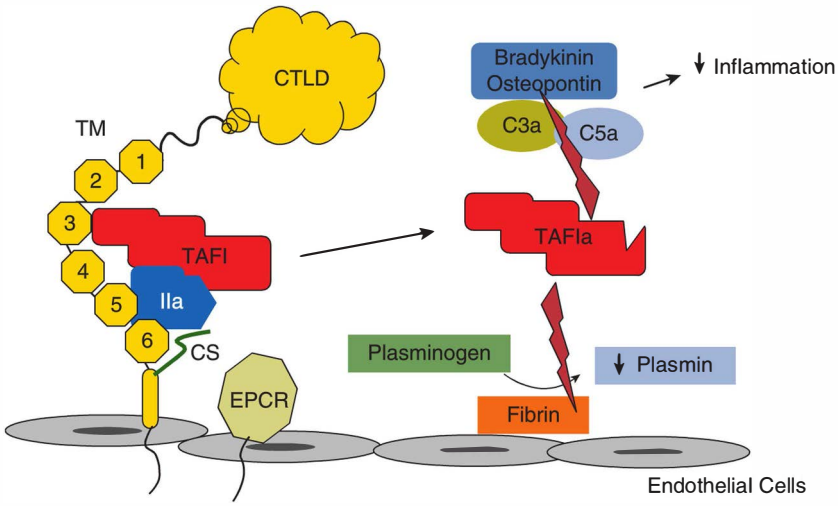

FIGURE 43.6 Activation of TAFI by thrombin–TM. EGF-like 3–6 repeats of TM provide cofactor function for thrombin-mediated generation of TAFIa, which cleaves basic *C*-terminal amino acid residues from several substrates including complement components C3a and C5a, bradykinin, and osteopontin, thereby reducing proinflammatory forces. TAFIa also cleaves fibrinogen, interfering with efficient generation of plasmin from plasminogen.

in turn, down-regulates further TAFIa production. Patients with impaired thrombin generation due to factor XI deficiency exhibit a bleeding tendency that is contributed to by excess fibrinolysis that may be attributed to reduced thrombin–TM-mediated activation of TAFI.[81] Extensive studies have been performed in mice lacking TAFI. However, the *in vivo* significance of TAFIa as a regulator of fibrinolysis remains controversial (reviewed in[82]), although elevated circulating levels of TAFIa in humans may confer a mild increased risk of thrombosis.[83] A role for TAFIa as a natural anti-inflammatory molecule has more recently become of greater interest with recognition of its ability to inactivate the anaphylatoxins C3a and C5a by des-argination.[84] Moreover, the proinflammatory mediators, bradykinin and osteopontin,[85] are also inactivated by TAFIa.

TM, encoded by an intronless gene, is a 557–amino acid, type 1 transmembrane glycoprotein (GP). It is expressed by endothelial cells in all vascular beds. The ectodomain of TM comprises a juxtamembranous serine/threonine-rich domain with potential sites for *O*-linked glycosylation which support the attachment of a chondroitin sulfate that enhances the activation of PC by thrombin–TM,[86] accelerates the neutralization of thrombin by heparin-AT, and facilitates binding of PF4 to PC to increase its activation. The next domain contains six epidermal growth factor (EGF)-like repeats. EGF-like repeats 3, 4, 5, and 6 (EGF3–6) are essential for thrombin-mediated activation of PC and TAFI.[87–90] Via its anion binding exosite I, thrombin binds to EGF5–6. EGF4–6 is required for activation of PC,[91] and EGF3–6 is required for TAFI activation.[92] An aspartic acid residue (Asp349) within EGF4 is required for Ca^{2+}-mediated binding of TM to the gamma-carboxyglutamic acid (Gla) containing domain of PC, and thus for activation of PC by thrombin. Oxidation of methionine 388, which resides between EGF4 and EGF5, abrogates thrombin's ability to activate PC, an event that likely occurs during inflammation when neutrophils are activated. At the *N*-terminus of the molecule, there is a globular *C*-type lectin-like domain.[93] This structure lacks anticoagulant function, but plays a major role in inflammation (FIGURE 43.4). Mice lacking the lectin-like domain of TM are more sensitive to endotoxin challenges and proinflammatory stimuli and exhibit increased tissue accumulation of neutrophils and monocytes, and excess activation of complement,[94] in part by preventing the proinflammatory cytokine high-mobility group box 1 from binding to the receptor for advanced glycosylation end products.[95]

As might be expected, endothelial TM expression is reduced in many prothrombotic conditions. This is achieved via transcriptional and posttranscriptional mechanisms. Thus, for example, shear stress,[96] oxidized LDL,[97] hyperhomocysteinemia,[98] proinflammatory cytokines,[99,100] sepsis,[101] and aging[102] are associated with suppressed TM levels and often enhanced TF exposure, an imbalance that promotes fibrin deposition. Conversely, TM is upregulated by VEGF and statins,[103,104] both of which may be vasculoprotective. Transgenic mice that carry a TM mutation that reduces its cofactor function in thrombin-mediated activation of PC exhibit increased fibrin deposition in the lungs.[105] Total inactivation of the TM gene is incompatible with life, and this is likely the same situation for humans, since no cases of homozygous deficiency have been reported. The clinical significance of TM mutations in venous and arterial thrombotic disease in humans otherwise remains controversial. Since the first report of familial thrombophilia associated with a TM gene mutation,[106] other mutations and polymorphisms in the coding region and the promoter region have been described, and several have been examined *in vitro* with mixed results.[107] In a large prospective analysis of men, the A455V and -1208-1209TTdelTT variants exhibited a small, but not statistically significant increased risk for coronary heart disease.[108,109] In some segments of the population, the Ala25Thr substitution is a risk factor for ischemic heart disease.[110] Mutations in the lectin-like domain and in the serine–threonine region have been associated with an increased risk of developing the thrombotic microangiopathy, atypical hemolytic uremic syndrome.[111] Overall, genome-wide association studies and advances in proteomics should provide more insights into the relevance of TM polymorphisms in human disease.

EPCR is also a transmembrane GP, constitutively expressed by endothelial cells, but more prominently in large vessels.[112,113] EPCR provides a platform for PC so that it may be more accessible for activation by thrombin–TM.[69,114] Lack of EPCR in mice

results in early embryonic lethality with thrombosis[115] while blocking its function postnatally with antibodies accelerates thrombus formation.[116] Its structural similarity to the major histocompatibility complex class 1/cluster of differentiation 1 family of proteins[117] and its expression by dendritic cells[118] suggests that it also likely regulates innate immunity.

EPCR is also a receptor for APC, with similar affinity as for PC. It has been generally accepted that APC interferes with coagulation only when dissociated from EPCR. This has recently been challenged by claims that when docked on EPCR, APC may actually more efficiently inactivate factor Va, particularly when the latter is associated with the endothelial cell surface (reviewed in[119]). Nonetheless, when complexed with EPCR, APC activates endothelial cell PAR-1, inducing many of its anti-inflammatory and vasculoprotective intracellular signals[119,120] (FIGURE 43.7). These include the release of sphingosine-1-phosphate (S1P), expression of the antiapoptotic Bcl-2,[121] augmented endothelial cell proliferation,[122–128] activation of Rac1, inhibition of RhoA and NF-κB signaling, prevention of expression of vascular adhesion molecules,[129,130] and enhanced endothelial barrier function.[131] With reduced expression of EPCR, which occurs in inflammation or injury, excess thrombin cleaves and activates PAR-1 which initiates a proinflammatory response[132] with increased vascular leakage. Although other pathways have recently been implicated,[133,134] EPCR may be considered a master regulator for APC–PAR-1 interactions.

EPCR is also a receptor for plasma coagulation factors VII and VIIa, binding with similar affinities as PC and APC.[135] As noted above, factor VIIa is the key initiating enzyme for the generation of factor Xa and downstream production of thrombin. The EPCR–factor VIIa complex is unable to activate PAR-1 or downstream coagulation factors IX or X, and inhibits the procoagulant activity of factor VIIa–TF.[136] EPCR binding to factor VIIa facilitates internalization of both molecules,[137] possibly providing a clearance mechanism for the procoagulant factor VIIa. These data further support the dual role of EPCR in suppressing inflammation and coagulation.

Both TM and EPCR circulate in the plasma in soluble forms. The literature for soluble TM remains controversial in that there are many fragments that are probably generated by proteolysis of endothelial TM, and the function(s) or the significance of these are not well defined. Soluble EPCR (sEPCR) is generated via alternative pre-mRNA splicing and by proteolytic shedding.[138] sEPCR circulates in normal human plasma, is increased in sepsis,[139] and in patients with the EPCR A3 haplotype. sEPCR binds to the proinflammatory proteinase-3[140–142] and to the integrin Mac-1 receptor on activated neutrophils.[143] The precise role of sEPCR is not known, but patients with the A3 haplotype are at increased risk of venous thromboembolic disease and coronary heart disease.[144–147]

FIBRINOLYSIS

The vascular endothelium is afforded protection from excess fibrin deposition by the action of the fibrinolytic system. The two major activators of fibrinolysis are tPA and uPA. tPA is released primarily by endothelial cells[148] and acts locally at the site of a fibrin clot by catalyzing conversion of plasminogen to plasmin, which in turn degrades fibrin. tPA release from Weibel-Palade bodies where it is stored, is enhanced by desmopressin (DDAVP), thrombin, bradykinin, and endothelin (ET).[149,150] The extent to which uPA contributes to the fibrinolytic response remains incompletely understood.[151] The strongest evidence is derived from genetic studies in mice in which uPA deficiency results in excess fibrin deposition.[152] However, uPA also facilitates cell migration and plays a critical role in tissue repair processes and angiogenesis.[153] uPA and its GPI-linked receptor, uPAR, are minimally expressed by resting endothelial cells, but increase upon stimulation with inflammatory cytokines.[154]

The endothelium also plays an active role in suppressing fibrinolysis. In response to procoagulant and inflammatory stresses,[155] plasminogen activator inhibitor-1 (PAI-1), the major physiologic inhibitor of tPA and uPA, is highly expressed by endothelial cells, thereby dampening the fibrinolytic response. There are two other PAIs, PAI-2 and PAI-3, but their roles in regulating fibrinolysis are less clear. Finally, and as noted previously, when bound to the endothelial cell integral membrane protein, TM, thrombin converts the procarboxypeptidase, TAFI, to its active form, which interferes with fibrinolysis.[80]

The fibrinolytic potential of endothelial cells of different vascular sites varies considerably.[156,157] For example, the absolute and relative amounts of tPA and PAI-1 released after thrombin

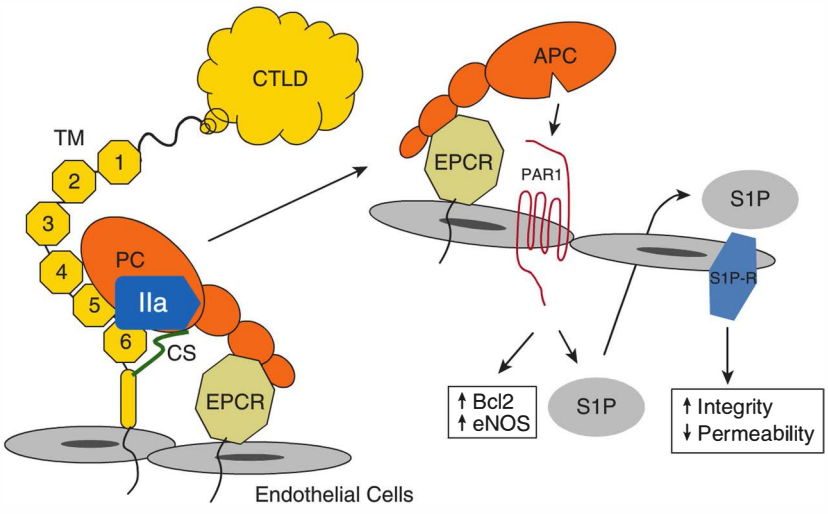

FIGURE 43.7 Vasculoprotective properties of APC. When complexed with EPCR, APC activates endothelial cell PAR-1, inducing anti-inflammatory and vasculoprotective intracellular signals that induce the release of sphingosine-1-phosphate (S1P), expression of Bcl-2, synthesis of endothelial nitric oxide synthase (eNOS), reduction of expression of leukocyte adhesion molecules, and enhancement of endothelial barrier function.

or cytokine stimulation are different with endothelial cells from the umbilical vein *versus* adult saphenous vein. tPA has been localized throughout the bronchial circulation, whereas its expression is much more limited in the pulmonary vasculature, only detected in 7 to 30 μm diameter vessels in the lungs of mice and nonhuman primates.[158] uPA is most prominent in the kidney as compared to many other tissues. And in healthy aortas, tPA is prominently expressed in the vasa vasorum, whereas PAI-1 is found in the aortic endothelium.[159] It is interesting to note that there is little evidence that defects in individual components of the fibrinolytic system (tPA, alpha2-antiplasmin, plasminogen, PAI-1) enhance the risk of venous or arterial thromboembolic disease.[160] This underscores the complexity of these pathways and the compensatory capacity of the endothelium.

PLATELET-ENDOTHELIAL CELL INTERACTIONS

The vascular endothelium has a remarkable capacity, under normal conditions, to prevent circulating platelets from inappropriate recruitment and activation that would otherwise result in vascular occlusion (reviewed in[161]). The intact endothelial monolayer provides a barrier function, separating platelets from subendothelial procoagulant agonists, such as collagen and von Willebrand factor (vWF). Endothelial cells also liberate vasodilatory and platelet-stabilizing factors, such as nitric oxide (NO), PGI_2, and CD39 (see below). In response to injury, the phenotype of the endothelium transforms such that it promotes recruitment, adhesion, activation, and focal aggregation of platelets to form a platelet-rich blood clot. This occurs primarily in the high-flow arterial circulation.[162] In the low-flow venous circulation, platelets play a lesser role, as activated coagulation factors can accumulate on damaged endothelial cell surfaces, allowing for local thrombin generation and fibrin deposition. It is on the venous side that the natural anticoagulant TFPI, heparin-AT and PC systems are most active.

Damaged or denuded endothelium uncovers a matrix to the circulation that is rich in collagen and vWF.[163,164] In response to arterial vascular injury, platelets bind directly to collagen types I and III via platelet GP VI and the integrin $\alpha_2\beta_1$, or indirectly via vWF that is complexed with the exposed collagen. vWF is a multimeric protein that is synthesized in endothelial cells and megakaryocytes and stored in platelet granules and in Weibel-Palade bodies in endothelial cells until released at a basal rate and in a regulated manner, induced by a range of chemical and mechanical agonists.[165,166] There is also a constitutively secreted pool of an uncleaved vWF precursor, the function of which remains unclear.[167] Multimeric vWF from the vessel wall or the circulation forms complexes both with collagen on the exposed vessel wall[168] and with P-selectin. Under high shear conditions, vWF that is immobilized to the endothelium via collagen, undergoes conformational changes,[169] revealing a binding site on its A1 domain that serves as a receptor for capturing fast-flowing platelets via GP Ibα-IX-V. This slows the transit of the rapidly moving platelets long enough to be activated by collagen, ADP (primarily from erythrocytes), and thrombin.[170] The platelets thus form an adherent layer on the damaged endothelium and provide a nidus upon which circulating platelets aggregate and "tether" to join the growing thrombus to establish a hemostatic plug.[171] Serious human disorders underscore the pivotal role of the endothelium in regulating the hemostatic function of platelets. For example, the key role of the endothelium in promoting platelet plug formation is evident from the most common hereditary bleeding disorder, von Willebrand disease, caused by qualitative or quantitative deficiencies in vWF.[172,173] In the rare syndrome, thrombotic thrombocytopenia purpura, the endothelium is believed to contribute to inappropriate thrombus formation by inadequate release of the metalloprotease, ADAMTS13.[174] The size and thus the platelet-aggregating and prothrombotic capacity of vWF multimers is normally held in check by proteolytic processing by ADAMTS13[174,175] that at least *in vitro*, may be released by endothelial cells.[176]

Understanding the role of the endothelium in platelet activation is also uncovering potential therapeutic targets. For example, CD39 is a vascular endothelial ectonucleotidase that hydrolyzes ADP to AMP, limiting platelet adhesion and activation by effectively neutralizing ADP that is released at the injury site by erythrocytes. Overexpression of CD39 in preclinical studies protects against ischemia reperfusion–induced vascular injury and organ damage.[177]

REGULATORS OF VASCULAR TONE

Vascular constriction is a normal physiologic hemostatic response to vascular injury, serving to help restrict blood flow, and orchestrated to occur in concert with formation of the platelet plug. The endothelium plays a pivotal role in regulating vasomotor tone primarily by releasing soluble mediators that cause vascular smooth muscle cells to relax or contract, thereby inducing vessel dilation or constriction, respectively. Many of the key mediators also exert direct or indirect effects on the hemostatic system.

Nitric Oxide

NO is generated by the enzymatic conversion of L-arginine to L-citrulline and is the most potent endothelial cell–derived vasodilator.[178,179] In endothelial cells, endothelial NO synthase (eNOS) is responsible for NO production, and is activated by bradykinin, ADP, shear stress, and proinflammatory cytokines. Endothelial-derived NO has several additional properties, some of which have direct effects on hemostasis.[180] NO interferes with the secretion of vWF, at least in part via S-nitrosylation of key signaling proteins that are required to drive membrane fusion and secretion of prestored vWF from Weibel-Palade bodies.[181] Release of NO from endothelial cells also reduces leukocyte–endothelial cell interactions and inhibits platelet adhesion and aggregation.[182] $eNOS^{-/-}$ mice exhibit increased leukocyte and platelet accumulation in models of inflammation.[183] Antiphospholipid antibodies that are associated with the antiphospholipid syndrome induce thrombosis partly by binding to apolipoprotein E receptor 2 on endothelial cells, inducing intracellular signals that attenuate the function of eNOS and that enhance TF expression.[184,185] Reduced endothelial NO release is also caused by activation of toll-like receptor 4 in response to injury or infection, with concomitant elevated expression of TNF-α, IL-6, and NF-κB, and has thus been linked to thrombosis and with cardiac microvascular endothelial dysfunction after ischemia reperfusion.[186,187]

Eicosanoids

Eicosanoids comprise several compounds that, in response to a range of stimuli, are synthesized from arachidonic acid, whereupon they are released into the extracellular space to act in autocrine and paracrine manners, inducing effects on vascular tone, reactivity, and endothelial cell integrity. The best-characterized and physiologically important products are PGI_2 and epoxy eicosatetraenoic acids (EETs).

PGI_2 is constitutively expressed by quiescent endothelial cells, but is rapidly upregulated by several agents, including thrombin, bradykinin, histamine, and cytokines (e.g., IL-1β, TNF-α).[188] Although the pattern and extent of expression is stimulus dependent, production of PGI_2 declines rapidly after stimulation, exerting potent but short-lived biologic effects. By binding to the so-called I-type prostaglandin receptor, PGI_2 mediates cAMP-dependent signaling pathways that suppress platelet aggregation and leukocyte reactivity, induce vasorelaxation, and antagonize the action of the potent vasoconstrictor and platelet aggregator, thromboxane A_2.[189] PGI_2 may also protect endothelial cells from hydrogen peroxide-induced apoptosis and TF expression via PPARδ pathways.[190,191] Several PGI_2 analogs are being evaluated clinically to treat pulmonary hypertension and intermittent claudication due to peripheral arterial disease.[192]

EETs are a diverse group of compounds that are synthesized by endothelial cells from many, but not all arteries, in response to agonists such as bradykinin or shear stress.[193,194] Their modes of action are complex, but most EETs act as endothelium-derived hyperpolarizing factors that induce arterial relaxation in the presence of NOS and cyclooxygenase inhibitors. In a concentration-dependent and vascular bed–specific manner, EETs suppress endothelial cell expression of proadhesive molecules such as vascular cell adhesion molecule-1, induce release of tPA, interfere with platelet adhesion to damaged endothelial cells, and protect against endothelial cell apoptosis.[195–197] Reversal of endothelial dysfunction via strategies that enhance delivery or expression of EETs represent a potentially viable approach to prevent or treat a range of thrombotic disorders.

Carbon Monoxide

Analogous to the NO/NOS system,[198] heme oxygenase-derived carbon monoxide (CO) plays an important role in hemostasis and vascular endothelial function, promoting vasodilation, suppressing pro-apoptotic and proinflammatory pathways, and interfering with platelet aggregation.[198] CO is endogenously produced in blood from the degradation of heme by the action of inducible and basal microsomal heme oxygenases (HO-1 and HO-2, respectively). Both forms, and possibly a third (HO-3), are found in most, if not all tissues, including endothelial cells and vascular smooth muscle cells, and are important in vasoregulation. In response to injury or stress, endothelial HO-1 is markedly up-regulated,[199] thereby increasing production of CO. Disruption of HO-1 activity in mice increases sensitivity to ischemia–reperfusion injury.[200] Conversely, HO-1 overexpression provides protection against myocardial, renal, and brain ischemia–reperfusion injury.[201,202] The most convincing evidence of an important role of HO-1 in modulating endothelial hemostatic system activity and vascular integrity comes from a child with HO-1 deficiency who developed a thrombotic microangiopathic hemolytic anemia with diffuse vascular endothelial damage and elevated circulating levels of soluble TM and vWF.[203] Taken together, there is strong rationale for considering HO-1 and/or CO as a primary or adjunct therapeutic target for hemostatic disorders associated with vascular endothelial damage.[204]

Endothelin

Countering its vasodilatory properties, the endothelium also produces and secretes several soluble factors that promote vasoconstriction and facilitate platelet activation, adhesion, and thrombus formation during disease. Prominent among these is ET. There are at least three isoforms of ET (ET-1, ET-2, and ET-3), all of which are pleiotropic 21–amino acid peptides that have potent vasoactive properties and that are involved in several pathologic processes (reviewed in[205]). ET-1 is the most abundant form in the vascular system. It is secreted by several cells, particularly in the cardiovascular, endocrine, and reproductive systems. Its inactive precursor, big ET-1, is highly expressed by endothelial cells,[206,207] yielding the mature active form through proteolytic cleavage by membrane-associated endothelin-converting enzymes (ECE-1 and ECE-2) and chymase (reviewed in[208]).[209] ET-1 is released from endothelial cells via 2 pathways; constitutively at low levels from vesicles,[210] and in a regulated manner from Weibel-Palade bodies in response to stimuli[211] such as thrombin, IL-1, oxidized LDL, epinephrine, and hypoxia.[212] Over 75% of ET-1 release is abluminal, while the minority is into the blood, consistent with it having paracrine and autocrine modes of action.

Although ET-1 is recognized as one of the most potent vasoconstrictors, its activities are tightly regulated and partly dependent on the relative expression and distribution of its two G-protein coupled receptors, ETA and ETB, which have largely opposing properties. ETA is highly expressed on vascular smooth muscle cells surrounding arteries, particularly in the lung and kidney, where stimulation by ET-1 triggers vasoconstriction and apoptosis.[213] ETA is also expressed on the surface of endothelial cells in several organs, including the brain, where it regulates blood brain barrier function.[214] On the other hand, ETB is prominently expressed on endothelial cells in veins and in the renal and pulmonary vascular endothelial cells, but is almost undetectable in normal aortic and coronary vessel endothelial cells.[213] Activation of ETB by ET-1 limits the vasoconstrictor response mediated via ETA by promoting the endothelial cell release of vasodilators such as NO and PGI_2, and by removing ET-1 from the circulation, probably by endocytosis.[208,215,216]

There is little evidence that ET-1 has direct effects on hemostasis. However, several lines of evidence indicate that with endothelial dysfunction associated with injury, inflammation, or aging, ET-1 facilitates progression to a thrombotic phenotype by enhancing TF expression,[217] platelet adhesion and activation, and leukocyte–endothelial cell interactions.[218] Several preclinical and clinical studies, using either ETA-selective or dual ETA/ETB pharmacologic blockade have demonstrated efficacy in preserving organ function, at least in part by improving vascular tone, endothelial integrity, and function.[205]

Other endothelial cell–derived molecules that cause vasoconstriction and play a role in the hemostatic response to injury include, for example, angiotensin II,[219] platelet-derived growth factor,[220] and FGF-1 and FGF-2.[221]

Complement

Protection from injury requires simultaneous and coordinated activation of coagulation to restrict bleeding and innate immunity to limit pathogen invasion. Several lines of evidence indicate that these two systems are linked via the endothelium, and the data are leading to new therapeutic strategies. The complement system, a major component of innate immunity, comprises over 30 soluble and membrane-bound proteins.[222] Three complement activation pathways converge to transform plasma factor C3 into C3b, with generation of the C3 and C5 convertases, release of the anaphylatoxins C3a and C5a, and formation of the C5b-9 membrane attack complex which promotes lysis of the pathogen. P-selectin that is translocated to the surface of thrombin-activated endothelial cells and platelets, acts as a receptor for C3b[223] which in turn, facilitates a localized and coordinated thrombotic and innate immune response at the site of injury. Endothelial cells also express receptors for the anaphylatoxins C3a and C5a, the latter of which triggers the release of vWF multimers from Weibel-Palade bodies, thereby promoting the deposition of platelet-rich fibrin clots. Indeed, inhibitors of C5 and the receptor for C5a have shown therapeutic efficacy in some clinical and preclinical trials for the thrombotic microangiopathy, atypical hemolytic uremic syndrome,[224] and for cerebral ischemia–reperfusion injury.[225] C5b-9 complexes are also deposited *in vivo* on endothelial cells. Although they are usually removed by vesiculation from the quiescent, undamaged endothelial cell membrane before lysis occurs, C5b-9 can at least transiently facilitate an influx of calcium into the cell, stimulating heterotrimeric G-proteins which upregulate TF and more vWF secretion.[226] C5b-9 containing vesicles that are shed from the endothelial cell surface provide docking sites for factors Va and Xa which support prothrombinase assembly.[227,228] The *in vivo* significance of the C5b-9 complex in endothelial hemostatic system activity remains incompletely understood, although it likely plays a role in thrombotic syndromes involving complement fixing antiendothelial antibodies.[229]

ENDOTHELIAL CELL DIVERSITY AND ITS IMPACT ON HEMOSTASIS

Endothelial cells are plastic and heterogeneous, anatomically and functionally, with differences reflected during growth and development, in response to specific stresses, and based on their spatial distribution in different organs (e.g., lung, liver, brain, pancreas, eye) and the types and calibers and locations of vessels (e.g., vein, lymphatic, artery, arteriole, capillary).[230,231] This heterogeneity is reflected in hemostasis most notably by site-specific and age-dependent differences in bleeding and thrombosis.[232] For example, patients with hemophilia tend to bleed into their joints, whereas those with primary platelet function defects suffer primarily from mucosal bleeds. Hemolytic uremic syndrome mainly affects the kidneys. Patients with heterozygous deficiency of PC, PS, or AT develop lower limb clots, yet homozygous PC deficiency and warfarin-induced skin necrosis cause diffuse microvascular thrombosis. Atherothrombotic disease has an early predilection for coronary, carotid, and femoral arteries. Finally, aging is a major determinant of both venous thromboembolic disease and of atherothrombotic disease. Investigators are only just starting to study the mechanisms by which endothelial heterogeneity impacts on hemostasis. However, viewing the endothelium in this context is critical for understanding the pathogenesis of disorders associated with excess bleeding and/or thrombosis, and for the strategic design of more effective diagnostics and therapeutics.

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CHAPTER

44

Coagulation-Independent Signaling of the Extrinsic Coagulation Pathway

WOLFRAM RUF

Tissue and vascular injury requires a rapid hemostatic response that is critically dependent on the tissue factor (TF)-initiated extrinsic coagulation pathway. TF triggers coagulation by forming a catalytic cofactor–enzyme complex with coagulation factor VIIa (VIIa), converting factor X to Xa, and thereby initiating the common downstream coagulation pathway, activating platelets, and converting fibrinogen to fibrin. TF is required for embryonic development in the mouse,[1–3] and no deficiencies of TF have been reported in humans. The central role of TF–VIIa complex formation for postnatal hemostasis is underscored by the highly similar phenotypes of genetically engineered mice in which TF or VII expression levels are severely reduced. These mice develop cardiac microbleeds, progressive fibrosis, and heart failure.[4] Expression of TF in a variety of cell types in mouse and man[5,6] supports a role in providing a "hemostatic envelope" around organs and at boundaries to the environment, but TF also has broader functions beyond providing hemostasis and maintaining vascular integrity.

Expression of TF by leukocytes and blood-derived monocytes initiates coagulation that is pivotal for intravascular sequestration of microorganisms in host defense[7] and for fibrin formation supporting cell-mediated immune responses in extravascular locations.[8,9] Conversely, excessive activation of the TF procoagulant pathways drives disseminated coagulation and lethality in sepsis.[10] However, TF is also a direct regulator of myeloid cell-dependent inflammation.[11,12] Thus, the concept is emerging that TF-initiated coagulation and direct cell signaling cooperate to protect from infection and regulate innate immune cell function.

TF expression by nonhematopoietic cells also has nonhemostatic functions. TF is synthesized by smooth muscle cells where it can initiate acute intravascular thrombosis following vessel wall injury.[13] In addition, TF is upregulated by growth factor stimulation and promotes smooth muscle cell migration and intima hyperplasia.[14,15,16–18] TF expressed by smooth muscle cells or endothelial cells has been implicated in angiogenic processes by coagulation-independent pathways.[19,20,21,22,23] TF is expressed by epithelial cells in the gastrointestinal tract and plays a pivotal role in coagulation activation and inflammatory cell recruitment in experimental colitis.[24] TF is distinctly expressed in the basal layers of epithelial barriers in the skin, and reduced wound healing in VII-deficient mice indicates roles for the TF–VIIa complex in stimulating epithelial migration and wound closure.[25]

The association of TF with cell adhesion integrin receptors,[26,27] cytoskeletal structures and cell–cell contacts,[28–30] and a promigratory G protein–coupled receptor, the protease-activated receptor (PAR)2 provides molecular connections by which TF can support regenerative tissue repair as well as cancer progression. This chapter reviews the basic mechanisms of TF-initiated cell signaling and our current understanding as to how these noncoagulant roles of the TF pathway support physiologic and pathologic processes.

MOLECULAR CELL BIOLOGY OF TF

TF is evolutionary related to the cytokine receptor family, and the TF extracellular domain shares the cytokine receptor architecture of two fibronectin type III domains aligned in an elongated manner with an angled and rigid intramodule interface.[31–34] However, the TF cytoplasmic domain lacks the typical cytokine receptor tyrosine kinase recognition motifs. Instead, a Ser/Thr-Pro phosphorylation site that is the target for unidentified Pro-directed kinases is highly conserved in all mammalian species.[35] Phosphorylation of the canonical Ser258 in the human TF cytoplasmic domain requires prior protein kinase C–dependent phosphorylation of a more amino-terminal Ser–Xxx–Lys motif[35–37] and is regulated by palmitoylation of a conserved membrane proximal Cys residue[36,38] (FIGURE 44.1). The phosphorylated TF cytoplasmic domain has a more compact structure, and phosphorylation might thus prevent or promote the binding of intracellular adaptor proteins supporting TF trafficking.[39]

The TF extracellular domain mediates ligand interactions with each of the structural domains of VIIa. The docking of the protease domain leads to allosteric activation of catalytic activity of the zymogen-like protease VIIa,[40] whereas interactions of the VIIa light chain with the carboxyl-terminal module of TF confer high-affinity binding and stable TF–VIIa complex formation necessary for efficient activation of macromolecular substrates X and IX. The details of these procoagulant interactions of TF have been elucidated at the molecular level.[41–44] On cell surfaces, a substantial portion of TF is not involved in the initiation of coagulation, although these pools of TF bind VIIa and activate its catalytic activity. Procoagulant TF, as found on activated monocytes, has high affinity for VIIa (K_D < 1 nM), but noncoagulant cell surface pools of TF are only saturated at higher concentrations of VIIa.[45–47] Chemical cross-linking and the binding of substrate-dependent inhibitors[48] or antibody reactivity[49] indicate that the noncoagulant pools of TF have a distinct tertiary or quaternary structure. Heterogeneity of cell-surface TF is also evident from functional dose–response curves, demonstrating that higher concentrations (5 to 20 nM) of VIIa are required to saturate pools of TF involved in direct TF–VIIa signaling.[49,50,51]

Multiple factors contribute to the functional and structural heterogeneity of cell-surface–expressed TF. Negatively charged surfaces containing phosphatidylserine increase the measured

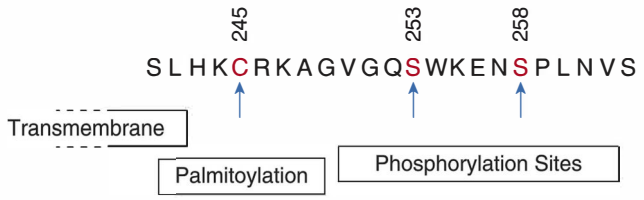

FIGURE 44.1 Features of the human TF cytoplasmic domain.

affinity of VIIa for TF in purified systems.[52,53] The cellular lipid environments surrounding TF could alter TF structure[54] or increase VIIa affinity through contacts of the VIIa Gla domain. TF is found in phospholipid-rich as well as glycosphingolipid-rich raft microdomains and caveolae of the cell membrane. The dynamic trafficking of TF between subcellular locations is a critical determinant for TF procoagulant activity.[55–58] TF cell-surface expression is regulated by basolateral sorting in endothelial and epithelial cells,[59] and basolateral sorting and formation of cell–cell contacts influences cell-surface procoagulant activity and changes TF glycosylation.[49,60]

Human TF has three extracellular *N*-linked glycosylation sites that to a variable degree determine the overall carbohydrate content of the molecule.[61–63] Whereas secretion of recombinant soluble TF extracellular domain is markedly reduced by mutation of the most amino-terminal *N*-linked glycosylation site,[61] overexpression of full-length TF in commonly used cell lines did not reveal reduced procoagulant activity following mutation of any of the TF glycosylation sites.[63,64] However, biochemical comparison of highly purified TF from natural and recombinant sources demonstrated that recombinant TF has less complex, high mannose carbohydrates, while natural TF recovered from a particular thromboplastin reagent contains only hybrid, highly fucosylated, and sialylated sugars.[65] Natural TF has higher specific procoagulant activity, indicating that these complex carbohydrates significantly modulate TF activity. Recent experiments in mice show that TF glycosylation either marks or is required for cell-surface expression of TF in epithelial cells *in vivo*. A rapid change in TF glycosylation in intestinal epithelial cells occurs following colonization of the small intestine with microbiota, and the resulting cell-surface expression of TF triggers coagulation, procoagulant PAR1 signaling, and adaptive angiogenesis.[66]

The carbohydrate composition of proteins is determined by intracellular trafficking and recycling routes. Ligand binding of VIIa to TF induces internalization and degradation, but a significant portion of procoagulant TF recycles back to the cell surface with its ligand VIIa associated.[67] TF–VIIa endocytosis and the subsequent mobilization of additional pools of TF from the Golgi are regulated by the activation of PAR2.[68,69] In addition, the TF–VIIa–Xa ternary coagulation initiation complex is inhibited on cells by TF pathway inhibitor (TFPI). TFPI is anchored to the cell surface by a glycosylphosphatidyl-inositol (GPI) lipid attachment, either by direct protein modification of the splice variant TFPIβ or potentially by binding to an unidentified GPI-anchored receptor in the case of TFPIα.[56,70–72] TFPIβ is mainly responsible for regulating TF procoagulant function and represents the major GPI-anchored form in endothelial cells in culture.[73,74] TFPI is coexpressed in most tissues with an androgen-induced, raft localized protein that regulates TFPI expression and inhibitory activity.[75] GPI-anchored TFPI directs the TF ternary complex to caveolae and initiates a slow recycling pathway that eventually restores in part both TF coagulant activity and TFPI inhibitory control on the cell surface.[56,76] Thus, binding of VIIa or formation of the ternary coagulation initiation complex are not static cell-surface events, but promote cellular trafficking and internalization of TF.

TF function in thrombosis and cell signaling is under the control of thiol–disulfide exchange pathways.[49,77] *In vitro* and *in vivo* evidence supports roles for protein disulfide isomerase (PDI)–mediated thiol exchange in regulating TF procoagulant activity, microparticle release, and thrombosis.[49,78–81] The TF extracellular domain has a surface exposed Cys186–Cys209 disulfide bond with a redox potential indicative of a functional, allosteric disulfide that can undergo reversible reduction and oxidation.[77,82] Mutational breaking of this disulfide impairs procoagulant activity,[83] but an intact disulfide is not required for direct TF–VIIa cell-signaling activity that is supported by a TF mutant with a single, unpaired Cys186 with a dose–response curve indistinguishable from wild TF.[49] Precise mechanisms of breaking the allosteric disulfide on cells remain to be elucidated, and it is still debated whether thiol modifications of TF itself is the predominant regulator of cellular procoagulant function.[84,85] Evidence that cell-surface PDI is associated with TF on cells expressing a TF–PAR2 signaling complex indicates that disulfide exchange is a cellular pathway that can switch certain cellular pools of TF to a selective signaling receptor.[49]

TF-SIGNALING COMPLEXES AND CORECEPTORS CONTRIBUTING TO PROTEOLYTIC PAR ACTIVATION

Groundbreaking studies by Hans Prydz and colleagues demonstrated that the upstream coagulation proteases VIIa and Xa elicit cell-signaling events that are dependent on their proteolytic activities, but independent of thrombin-mediated activation of PARs.[86,87] VIIa in addition to TF-expressing cells activates several signaling cascades and transcriptional responses, as documented subsequently in various experimental systems.[88–93] TF–VIIa signaling prevents apoptosis and anoikis (cell death following loss of adhesion).[94,95] Several of these TF–VIIa signaling events, including the activation of mitogen-activated protein kinase (MAPK) pathways and the upregulation of kinase cascades promoting protein synthesis, were found to be independent of the TF cytoplasmic domain in overexpression systems.[91,96]

The intracellular signaling elicited by VIIa was reminiscent of thrombin signaling through the G protein–coupled receptor PAR1, but a candidate PAR receptor that is cleaved by TF–VIIa remained elusive.[97] Reconstitution experiments clearly demonstrated that TF–VIIa signals through the thrombin-insensitive PAR2.[98,99] Experiments with blocking antibodies or knockdown approaches confirmed the critical role of PAR2 as a signaling receptor for TF–VIIa in normal and transformed cells.[51,100,101] Similarly, PAR2 emerged as the receptor that mediates signaling of Xa,[102–104] but Xa also cleaves PAR1 directly and independent of thrombin generation.[105] The structure–function analysis of PAR2 cleavage by TF–VIIa identified hotspots for binding of PAR2 in the VIIa catalytic cleft and enabled the design of PAR2 variants that are selectively activated by TF–VIIa, but not Xa.[106] Thus, PAR2 is a common signaling receptor for upstream coagulation proteases of the TF pathway, but distinct coreceptors

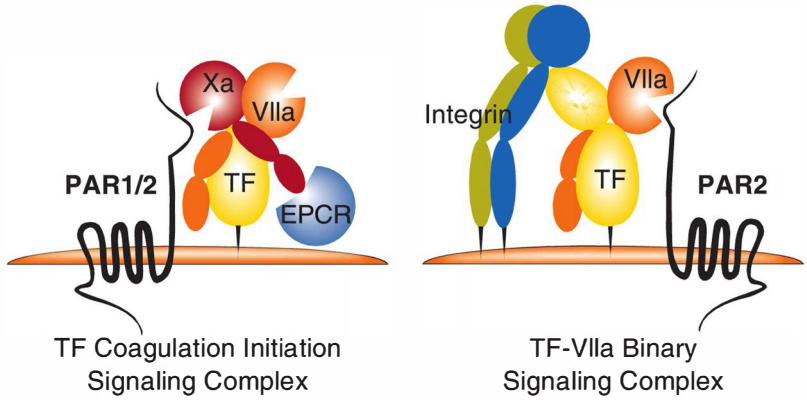

FIGURE 44.2 Schematic overview of the proteolytic TF-signaling complexes and associated coreceptors.

further diversify the signaling responses induced by proteolytic activation *via* TF–VIIa or Xa (FIGURE 44.2).

The combined addition of VIIa and X to TF-expressing cells is a potent stimulus for PAR2 signaling that occurs concomitantly with coagulation activation.[98,99] Although high concentrations of VIIa elicit cell signaling independently of Xa generation, it is the nascent product Xa in the high-affinity TF–VIIa coagulation initiation complex that primarily cleaves and activates PAR1 or PAR2.[49,99] Cell-surface–anchored TFPI not only regulates the procoagulant function but also signaling of the ternary TF–VIIa–Xa complex *via* PARs.[107] Thus, initiation of coagulation is mechanistically coupled with cell-signaling events and controlled by the physiologic inhibitor of the TF pathway. This principle similarly operates in the anticoagulant pathway in which the thrombomodulin–thrombin complex generates activated protein C (APC).[108] Initiation of the anticoagulant pathway is supported by Gla domain–mediated binding of PC to the endothelial cell protein C receptor (EPCR),[109] which subsequently supports APC-mediated cytoprotective PAR1 signaling.[110,111]

A broader role for EPCR is emerging, because EPCR binds not only APC but also VIIa with similar affinity,[112,113] and EPCR has been implicated in Xa-mediated PAR1 activation in overexpression systems.[114] The low affinity of X/Xa for EPCR relative to VIIa and APC[115] suggested a preferred interaction of TF–VIIa with EPCR. However, mutations of the VIIa Gla domain that severely reduce EPCR binding were without effect on PAR cleavage by either the binary or the TF–VIIa–Xa ternary complex.[116] In contrast, signaling of the ternary TF–VIIa–Xa complex was strictly dependent on EPCR, in both human and mouse cells,[116,117] indicating that EPCR is involved in functional interactions with Xa, rather than VIIa. These initial data indicate that EPCR is a cosignaling receptor for both procoagulant and anticoagulant protease complexes, a concept that requires further exploration *in vivo*.

An entirely different class of receptors participates in signaling of the binary TF–VIIa complex. On epithelial cell surfaces, VIIa at concentrations required to elicit cell signaling promotes the association of TF with integrin heterodimers, foremost integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ involved in basal membrane anchoring and epithelial cell migration.[27] An anti-TF antibody was identified that has no appreciable anticoagulant activity and no effect on VIIa binding to TF on cells. This antibody prevents VIIa-induced association of TF with integrins and potently inhibits TF–VIIa binary complex–induced chemokine production, but not signaling of the TF–VIIa–Xa ternary complex.[27,49] Thus, distinct surface pools of TF are involved in the noncoagulant and coagulation-associated cell-signaling events of the TF pathway. Whereas TFPI controls signaling of the ternary TF–VIIa–Xa complex, it is currently unknown whether signaling of the binary TF–VIIa complex is similarly regulated by protease inhibitors that can neutralize the TF–VIIa complex, including GPI-anchored TFPI,[118] antithrombin,[119,120] or plasminogen activator inhibitor 1.[121]

TF–VIIa promotes transcriptional responses in epithelial cells. In noncancerous cells, the TF–VIIa signaling-induced transcriptional program indicates roles in wound healing and tissue repair.[88,93] Extracellular matrix–regulated TF–VIIa signaling in tumor cells elicits an even broader array of antiapoptotic proteins, angiogenic regulators, chemokines, and growth factors for myeloid cells.[122] These mediators are predicted to change the cellular composition and vascular organization of the tumor microenvironment.

TF SIGNALING IN THE REGULATION OF CELL MIGRATION

Coagulation-independent functions of TF in cell adhesion and migration were uncovered by the demonstration that immobilized ligands for TF, including VIIa and TF antibodies, promote spreading of TF-positive cells.[28] Similarly, cells expressing the TF–VIIa binary complex bind matrix- and potentially cell-associated TFPI to engage in proadhesive interactions.[123] Chimeric receptors with an unrelated extracellular domain demonstrated a critical role for the TF cytoplasmic domain in the induction of actin polymerization and colocalization of the actin-binding protein filamin A at sites of TF-induced cell adhesion points.[28] TF ligation induces cell migration through activation of the small GTPase Rac and the p38 MAP kinase pathway.[124] Smooth muscle cell migration toward inactive VIIa is markedly reduced in mice lacking the TF cytoplasmic domain ($TF^{\Delta CT}$ mice).[125] Consistently, intima hyperplasia after injury is impaired in $TF^{\Delta CT}$ mice[125] or mice deficient in smooth muscle cell TF.[15]

Although these functions of the TF cytoplasmic domain were uncovered in experiments that utilized inactive extracellular ligands for TF, proteolytic signaling of TF-bound VIIa promotes promigratory PAR2 signaling[101] and the phosphorylation of the TF cytoplasmic domain.[126] PAR2 recruits the intracellular adaptor protein β-arrestin.[127] β-arrestins are best known for their role in receptor internalization and desensitization, but the recruitment of β-arrestin to PAR2 targets and activates the MAP

kinase extracellular signal–regulated kinase to pseudopodia of migrating cells[128] and promotes breast cancer migration through the cofilin pathway that is crucial for cytoskeleton reorganization.[129–131] TF is closely associated with the cell adhesion machinery, because the TF extracellular domain is a ligand for several integrin heterodimers of the β_1 integrin family as well as the integrin $\alpha_v\beta_3$.[26] In noncancerous cells, VIIa promotes TF coprecipitation with these integrin heterodimers under cell lysis conditions that preserve lipid-raft structure.[27] TF expression results in a marked, TF cytoplasmic domain–dependent reduction of integrin $\alpha_3\beta_1$-dependent migration of melanoma cells on the extracellular matrix protein laminin 5. However, addition of VIIa reverses this inhibitory effect by inducing TF cytoplasmic domain phosphorylation.[26] Thus, the TF cytoplasmic domain regulates the function of integrins that interact with the extracellular domain of TF.

In mesenchyme-derived smooth muscle cells and fibroblasts, TF–VIIa–PAR2 signaling synergizes with platelet-derived growth factor (PDGF) BB-induced migration by Src kinase-dependent crossphosphorylation of the PDGF receptor.[20,132] This promigratory response also requires the TF cytoplasmic domain for efficient cell migration.[133] In cancer cells, TF–VIIa–PAR2 signaling–stimulated production of the promigratory chemokine interleukin-8 (IL-8) plays additional essential roles in promoting cell migration and invasion.[51] Thus, VIIa is crucial for outside-in signaling that regulates TF cytoplasmic domain function and promigratory effects of PAR2 signaling.

TF-DEPENDENT SIGNALING PATHWAYS IN ANGIOGENESIS

Much of our understanding of the functional contribution of TF-signaling pathways to physiologic and pathologic processes is derived from results in genetic mouse models. Deletion of the TF gene revealed an important role for TF in developmental angiogenesis.[1] In the absence of TF, pericyte recruitment and maturation of the yolk sac vasculature is reduced in a phenotype that is very similar to the loss of PAR1 signaling in endothelial cells.[134,135] TF on both smooth muscle and endothelial cells contributes to the formation of vascular structures in an *in vitro* coculture assay.[19] TF expression in endothelial cells upregulates the chemokine CCL2, a gene induced by endothelial PAR1 signaling.[111] While pericyte recruitment to sprouting vessel *in vivo* is critically dependent on PDGF-BB signaling,[136] CCL2 may enhance smooth muscle cell recruitment to newly formed endothelial tubular structures.[19] However, cell type–specific deletion of TF from either endothelial or smooth muscle cells is compatible with normal embryonic development, indicating that residual TF expression due to incomplete gene deletion or functional redundancy of vessel wall cell-derived TF is sufficient to generate proteases to locally trigger PAR1 signaling.[13,137] Consistently, TF-deficient mice that are reconstituted with a human TF transgene to approximately 1% of normal activity also develop normally with no apparent embryonic vascular abnormalities.[138]

The concept that TF is not a *bona fide* initiator of angiogenesis but rather a modulator of angiogenic processes is further supported by studies of postnatal angiogenesis. Deletion of the cytoplasmic domain of TF is dispensable for embryonic angiogenesis,[139] but altered postnatal angiogenesis is found in $TF^{\Delta CT}$ mice. Colonization of the gut with microbiota induces broad adaptive changes, including angiogenesis in the small intestine that improves nutrient uptake. Vascular remodeling in the small intestine following microbial colonization is prevented by anti-TF treatment and requires the TF cytoplasmic tail downstream of PAR1 signaling in epithelial cells.[66] In this context, TF-PAR1 signaling increases the expression of the proangiogenic angiopoietin 1 (Ang1) and stimulates a paracrine signal to expand and remodel the intestinal villi vasculature.

In contrast, the postnatal development of the retina superficial vascular plexus occurs faster in $TF^{\Delta CT}$ relative to wildtype mice.[21] Increased vascular sprouting was also seen from $TF^{\Delta CT}$ relative to wildtype aortic rings *in vitro*. This phenotype is dependent on PAR2 and requires VIIa and PDGF-BB, suggesting that the TF cytoplasmic domain in vessel wall cells counteracts *de novo* sprouting angiogenesis. Further studies confirmed *in vivo* that the proangiogenic phenotype of $TF^{\Delta CT}$ mice is dependent on PAR2 and growth factor signaling.[140] The oxygen-induced retinopathy model mimics the hypoxia-driven angiogenesis observed in premature infants. In this model, $TF^{\Delta CT}$ mice revascularized areas of central vasoobliteration significantly faster than wildtype controls. The enhanced angiogenesis was abolished by genetic deletion of PAR2, but not of PAR1, as well as the tyrosine kinase inhibitor Gleevec, which blocks PDGF-BB receptor signaling, or a specific inhibitor of the TF-VIIa complex, NAPc2.[140] NAPc2 has also direct effects on host angiogenesis in tumor models, and these effects are not recapitulated by coagulation blockade with direct inhibitors of Xa.[141] These data implicate vessel wall TF signaling in the regulation of hypoxia-driven angiogenic processes *in vivo*.

TF localization to endothelial cells is observed in pathologic angiogenesis in tumors and endometriosis.[22,142] TF is not necessarily synthesized by the endothelium, because recent data point to important roles of alternatively spliced TF (asTF) in the regulation of angiogenesis. The TF mRNA can be spliced in two distinct gene products, full-length TF with a transmembrane anchor and cytoplasmic domain, or asTF that lacks exon 5 encoding an extracellular region involved in substrate X binding. Instead asTF has a unique carboxyl-terminus translated from an alternative reading frame of exon 6 with a concomitant deletion of the transmembrane domain.[143] A genetic mouse model expressing normal levels of human full-length TF, but no asTF, has no spontaneous bleeding or developmental abnormalities, excluding that asTF is a significant contributor to developmental angiogenesis.[144] In contrast, overexpression of asTF by tumor cells promotes angiogenesis[145] and purified asTF induces endothelial cell sprouting *in vitro* and *in vivo* independent of ligand VIIa binding.[146] Proangiogenic effects of asTF are mediated by ligation of endothelial cell-expressed integrins $\alpha_6\beta_1$ and $\alpha_v\beta_3$. Both asTF and the unspliced TF extracellular domain share a binding site tentatively localized by antibody inhibition studies to an amino-terminal region in proximity to the VIIa-binding site.[146,147]

Integrin-dependent endothelial activation by asTF also upregulates leukocyte adhesion molecules and enhances monocyte–endothelial interactions.[148] This pathway is likely distinct from TF-dependent transmigration and reverse migration of monocytes that is blocked by an antibody localized to an epitope that is not contained in the asTF sequence.[149] The endothelium has the capacity to take up TF^+ microparticles released from stromal,[150] immune,[151] or tumor cells.[152] Full-length TF transfer can induce changes in endothelial function through integrin ligation[153]

or promote PAR2-dependent signaling in hypoxic endothelial cells.[152] These paracrine transsignaling pathways of asTF or TF^+ microparticles may occur locally as part of the TF-dependent regulation of angiogenesis or wound healing.[23,154] Our understanding of these mechanisms is currently incomplete, and the roles of alternative ligands for TF, including plasminogen and its proteolytic fragment angiostatin, have not been studied *in vivo*.[155–157]

TF SIGNALING IN CANCER PROGRESSION

TF is expressed by multiple cell types in the tumor microenvironment, but tumor cell–expressed TF is a key marker for poor outcome and tumor progression.[158] TF expression in cancer cells is caused by tumor-specific oncogenic events,[159–161] and tumor cell TF expression significantly enhances tumor growth through TF cytoplasmic domain–dependent regulation of angiogenesis.[162,163] Oncogenic transformation or hypoxia also cause ectopic synthesis of VIIa[164,165] and upregulate PAR2,[164,166] endowing tumor cells with an autonomous upstream coagulation signaling pathway that can operate prior to tumor angiogenesis-induced vessel leaks and extravasation of coagulation factor from the blood. A comprehensive gene profiling study in breast cancer cells[122] compared gene induction of TF–VIIa–PAR2 with the direct activation of the thrombin receptor PAR1. This approach identified highly selective cancer cell responses to TF–VIIa–PAR2 signaling, including induction of proangiogenic chemokines and growth factors (IL-8, CXCL1, and vascular endothelial growth factor C) and immune regulators such as granulocyte macrophage colony stimulating factor (CSF2) and macrophage colony stimulating factor (CSF1).

The identification of an antibody that selectively blocks human TF–VIIa–PAR2 signaling enabled unique approaches to specifically address the roles of TF signaling in tumor growth and angiogenesis. Aggressive clinical breast cancer is characterized by an upregulation of TF and PAR2.[166] Tumor growth of a TF–PAR2 signaling–competent breast cancer cell line was inhibited by signaling blockade selectively on tumor cells, whereas an anticoagulant anti-TF antibody was without significant effects.[27] TF–VIIa signaling blockade reduced angiogenesis, supporting *in vitro* studies that tumor cell TF–VIIa–PAR2 signaling is proangiogenic. Treatment with this antibody also induced similar tumor growth inhibition in glioblastoma[167] and melanoma,[27] although some of these tumors are also sensitive to coagulation blockade.[168] Thus, tumor cell–expressed TF initiates significant proangiogenic signaling though PAR2 activation, even in the context of triggering the coagulation cascade in the tumor microenvironment.

Genetic approaches further demonstrated tumor-promoting roles of TF–PAR2 signaling. The mammary tumor virus promoter-driven expression of the polyoma middle T antigen (PyMT) results in spontaneous development of breast cancer in mice that mimics important aspects of human breast cancer progression. PAR1 deficiency had no effect on the incidence, growth, and metastasis to the lungs of breast cancer in the PyMT model.[169] In contrast, $PAR2^{-/-}$ mice displayed a significant delay in the transition from adenomas to invasive carcinoma. PyMT $PAR2^{-/-}$ early tumors were less vascularized, showed lower levels of the chemokine CXCL1, and had less abundant macrophages relative to wildtype tumors. Reconstitution of PAR2 in PyMT $PAR2^{-/-}$ cells directly showed that tumor cell, rather than host PAR2 signaling, supports breast cancer development.[170] Consistent with the clinical observation that PAR2 expression and TF cytoplasmic domain phosphorylation is closely correlated with cancer recurrence and that TF cytoplasmic domain phosphorylation is reduced in PyMT-$PAR2^{-/-}$ tumors,[166] $TF^{\Delta CT}$ mice showed delayed spontaneous breast cancer development similar to $PAR2^{-/-}$ mice in this oncogene-driven model.[170] In a different model of cancer development, the $Apc^{Min/+}$ mouse, which models colon cancer development in familial adenomatous polyposis, inhibiting TF–VIIa signaling and coagulation with the inhibitor NAPc2, also significantly reduced the number of intestinal tumor nodules that spontaneously develop.[171] Thus, TF-dependent upstream coagulation signaling is an important pathogenetic event in tumor progression.

TF–PAR2 SIGNALING IN INFLAMMATION AND METABOLIC DISORDERS

TF in monocytes and macrophages is induced by toll-like receptor agonists (lipopolysaccharide [LPS]), oxidized lipoproteins, and the inflammatory cytokine interferon γ and suppressed by anti-inflammatory cytokines, including IL-4 and IL-10 that promote alternative macrophage activation.[172,173] While roles of TF in promoting disseminated intravascular coagulation in the most extreme form of acute inflammation, that is sepsis, are well appreciated, TF signaling more broadly and directly regulates the function of mononuclear phagocytes. VIIa induces proinflammatory cytokines in endotoxin-stimulated monocytes *in vitro*,[174] which may explain the reduced inflammatory responses of endotoxin-challenged mice with very low levels of VII.[175] VIIa induces reactive oxygen species production in monocytic cells, and this effect is dependent on the TF cytoplasmic domain.[176] Mice that lack the TF cytoplasmic domain, but retain the cytosolic palmitoylation site ($TF^{\Delta CT}$ mice), are protected from inflammation and lethality in endotoxemia models,[177,178] and the TF cytoplasmic domain regulates MAPK activation in LPS-stimulated macrophages.[178]

TF not only plays a role in acute, but also in chronic inflammation. Cutaneous delayed-type hypersensitivity requires macrophage TF^8 and is significantly attenuated in $TF^{\Delta CT}$ mice.[179] In animal models of arthritis, blocking TF with active site–inhibited VIIa decreases synovial thickness as well as cartilage damage,[180] and both PAR1 and PAR2 contribute to arthritis severity.[181,182] Arthritis severity is similarly reduced in $TF^{\Delta CT}$ mice, and reduced macrophage inflammation is observed in these experiments.[183] $TF^{\Delta CT}$ mice and PAR2-deficient mice are similarly protected from antiphospholipid antibody–induced pregnancy complications.[12] Thus, TF and PAR2, in many cases dependent on functional crosstalks with the TF cytoplasmic domain, play important roles in the regulation of inflammation.

Chronic macrophage inflammation in the visceral adipose tissue is important for the development of type 2 diabetes and the metabolic syndrome associated with obesity in mouse models.[184] TF expression is increased in the adipose tissue in genetic models of obesity relative to nonobese mice,[185] and circulating TF is detectable at increased levels in the blood of patients with signs of low-grade inflammation and insulin resistance.[186] TF is also upregulated in models of diet-induced obesity and specifically expressed on macrophages in the inflammation-prone visceral adipose tissue of mice. Adipose tissue macrophages of $TF^{\Delta CT}$ and PAR2 knockout mice express reduced levels of inflammatory cytokines.[11] In addition, acutely blocking macrophage TF signaling rapidly attenuates inflammation and reduces

insulin resistance in obese mice. Similarly, reduced levels of TF in hematopoietic cells have beneficial effects on hepatic inflammation and steatosis in mice fed a proatherogenic diet.[187,188] Surprisingly, $TF^{\Delta CT}$ or PAR2 mice also developed less obesity.[11] VIIa association with adipocyte-expressed TF directly influences insulin signaling and suppresses adiponectin, a systemic regulator of insulin resistance and energy expenditure.[11] These data provide initial evidence that TF is not only a prothrombotic mediator in the complications of obesity and metabolic disorders, but rather a significant contributor to the development of these diseases.

THERAPEUTIC PERSPECTIVES

These studies document noncoagulant roles of upstream coagulation proteases and TF in regenerative processes, angiogenesis, cancer, inflammation, and metabolic diseases. In several of these physiologic and pathologic contexts, TF initiates coagulant responses that provide the platform and support the full biologic complexity of multicellular interactions, for example in inflammatory lesions or the tumor microenvironment. Nevertheless, prototypic antibody reagents that selectively block direct TF signaling are effective in scenarios where coagulation inhibitors also have partial efficacy.[11,27,167,168,189,190] Thus, TF signaling makes significant and independent contributions to pathologic processes. The elucidated basic mechanisms of TF signaling identify distinct targets to interrupt these pathways, including the interaction of TF with integrins, catalytic activities of the TF-associated proteases VIIa or Xa, and the activation of PAR2 as the central receptor for proteolytic signaling by upstream coagulation proteases. Considering the broad biologic functions of PAR2, PAR2 antagonists may not specifically target TF-signaling pathway. Our current basic understanding of TF signaling further predicts that direct inhibitors of Xa will not provide the same antisignaling effects as inhibitors that block the activity of VIIa. Crucial next steps are to evaluate the expanding clinical experience with direct inhibitors for Xa and thrombin in the context of these basic research findings and to explore the clinical profiles of direct VIIa inhibitors for their efficacy to attenuate disorders with potential direct TF-signaling contributions.

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CHAPTER

45 Heparin and Vascular Proteoglycans

DOUGLAS M. TOLLEFSEN AND LIJUAN ZHANG

Heparin is a sulfated glycosaminoglycan isolated from mammalian tissues that are rich in mast cells. When administered intravenously, heparin binds to the plasma protein antithrombin (previously known as antithrombin III), causing it to rapidly inhibit proteases of the intrinsic and common coagulation pathways. This interaction produces a potent anticoagulant effect. Endogenous heparin-like molecules appear to be involved in the inhibition of coagulation within normal blood vessels and may have a variety of other biologic functions. Although heparin is an effective agent for the treatment of venous thromboembolic disease, unstable angina, and acute myocardial infarction, it occasionally produces life-threatening bleeding or triggers an immune reaction, causing thrombocytopenia associated with venous and arterial thrombosis.

HISTORICAL OVERVIEW

The term *heparin* was used by Howell[1] in 1923 to describe an aqueous extract of canine liver that inhibited coagulation of blood *in vitro*. These extracts later were shown to consist of mixtures of sulfated polysaccharides containing uronic acid and glucosamine.[2] The suggestion that heparin might be used to treat thromboembolism was initially greeted with skepticism because of the fear that patients given heparin would bleed to death. According to Jaques,[3] this fear was allayed by a dramatic demonstration conducted by Charles Best at the University of Toronto in 1937:

> Best asked me [Jaques] to anesthetize a dog, prepare it for laparotomy, and inject heparin intravenously. When the most illustrious surgeons in the world arrived, Best explained what I had done (a blood sample which I had taken was incoagulable). Best then picked up a scalpel and handed it to Dr. Balfour with the request that he make a midline incision. The surgeons, properly dressed in their morning suits, jumped back, whereupon Best made the incision and I applied haemostats to two tiny bleeding points and showed a clean dry incision. Within months of this demonstration, surgeons all over the world took up heparin enthusiastically.

Canadian and Swedish investigators reported success in the use of heparin to treat recurrent thrombosis and pulmonary embolism in 1939.[2,4] The indications for heparin were soon expanded to include vascular surgical procedures, extracorporeal circulation, and prophylaxis of thromboembolism.

Despite the ensuing widespread clinical use of heparin, its mechanism of action remained obscure for several decades. As early as 1895, "thrombin" had been observed to lose activity gradually when added to defibrinated plasma or serum[5]; this inhibitory potential became known as the progressive antithrombin activity. In 1939, Brinkhous et al.[6] showed that heparin was effective as an anticoagulant only in the presence of a nondialyzable plasma component that they termed *heparin cofactor*. These early observations were linked in 1968, when Abildgaard[7] reported that a single protein (i.e., antithrombin) isolated from human plasma possessed both progressive antithrombin and heparin cofactor activity. Rosenberg and Damus[8] purified larger quantities of antithrombin, enabling a more detailed physicochemical characterization of the protein. Subsequently, a second protein with heparin cofactor activity was purified from plasma by Tollefsen et al.[9] and termed *heparin cofactor II* (HCII).

In the sections that follow, we discuss the structure, biosynthesis, and isolation of heparin and heparan sulfate (HS); the synthesis of anticoagulantly active heparan sulfate proteoglycans (HSPGs) by endothelial cells; the physiologic role of the heparin–antithrombin system; some of the nonanticoagulant actions of heparin; the synthesis of anticoagulant heparin analogs; and the interaction of HCII with glycosaminoglycans. A discussion of the structures and mechanisms of action of antithrombin and HCII is presented in Chapter 18.

STRUCTURE AND BIOSYNTHESIS OF HEPARIN AND HEPARAN SULFATE

Heparin is a highly sulfated polymer built on a backbone of alternating D-glucuronic acid and *N*-acetyl-D-glucosamine residues[10,11] that is widely distributed in the animal kingdom, being found in both vertebrates and invertebrates.[12] In mammals, heparin is initially synthesized in mast cells as a heparin proteoglycan in which multiple heparin chains, each containing 100 to 150 disaccharide units, are covalently linked to a core protein called *serglycin*.[13,14] Once generated, the heparin proteoglycan is degraded by three different types of lysosomal enzymes: (i) proteases that cleave the polypeptide chain; (ii) endoglycosidases that cut the heparin chain between glucuronic acid and glucosamine residues; and (iii) exoglycosidases that remove monosaccharide units from the nonreducing ends of heparin oligosaccharides.[15] The degraded products are then stored in the secretory granules of mast cells, which are widely distributed in a variety of organs, including the liver, heart, lungs, kidneys, and intestine.

Mammalian cells also synthesize HSPGs that consist of core proteins with covalently attached HS chains of 50 to 150 disaccharide units.[16] Both heparin and HS chains exhibit structural diversity, which arises from various patterns of *N*-sulfation, *N*-acetylation, and *O*-sulfation of the disaccharide units. The distinction between heparin proteoglycan and HSPGs is based on (i) the structure of the core protein; (ii)

the location of the glycoconjugate within the cell; and (iii) the relative amounts of *N*-acetylglucosamine and glucuronic acid present in the carbohydrate chains. In particular, the heparin core protein is small and relatively simple in structure with extended runs of Ser–Gly attachment sites,[13,14,17] whereas HS core proteins are large and more diverse in structure[18] with short runs of Ser–Gly or closely spaced Ser–Gly attachment sites[19]; heparin proteoglycan is found within mast cell granules, whereas HSPGs are located on cell surfaces or in the surrounding matrix[20]; and heparin contains larger amounts of *N*-sulfate, *O*-sulfate, and iduronic acid, whereas HS is less extensively modified.

Biosynthesis of a heparin or HS proteoglycan occurs in the Golgi apparatus and is initiated by attachment of a linkage region tetrasaccharide containing one xylose, two galactose, and one glucuronic acid to the serine hydroxyl group of a Ser–Gly (or Ala) dipeptide in the core protein.[10,11] The formation of heparin/HS chains depends on nearby acidic or hydrophobic residues or close spacing of Ser–Gly sites.[19–21] In humans, the heparin core protein serglycin contains an extended run of eight Ser–Gly repeats, in which virtually all of the Ser residues become modified with the linkage region tetrasaccharide. The tetrasaccharide is synthesized by the sequential action of four glycosyltransferases (xylosyltransferase, galactosyltransferase I, galactosyltransferase II, and glucuronyltransferase I) that use uridine diphosphate (UDP) sugars as their substrates. The structures of serglycin and its linkage region are depicted in FIGURE 45.1.

Immediately after formation of the linkage region tetrasaccharide, α-*N*-acetylglucosaminyltransferase I adds an *N*-acetylglucosamine residue and thereby commits the nascent chain to the heparin/HS biosynthetic pathway (see FIGURE 45.2).[11] Linkage region tetrasaccharides on other core proteins can be modified at this stage by β-*N*-acetylgalactosaminyltransferase I, resulting in biosynthesis of chondroitin sulfate or dermatan sulfate chains.[20] The heparin/HS chain is assembled by the alternate incorporation of glucuronic acid and *N*-acetylglucosamine from the corresponding UDP sugars. This is accomplished through the action of copolymerases that are members of the *EXT* gene family having β-glucosaminyltransferase II and α-*N*-acetylglucosaminyltransferase II activities. Thereafter, this simple copolymer is modified by a concerted process that leads to different modifications in various regions of the glycosaminoglycan chain.

First, glucosamine residues are partially *N*-deacetylated, and the exposed amino groups serve as acceptors of sulfate groups in a transfer reaction with 3'-phosphoadenosine-5'-phosphosulfate (PAPS). These two reactions are carried out by a bifunctional enzyme, *N*-acetylglucosamine *N*-deacetylase/*N*-sulfotransferase (NDST), which is composed of a single polypeptide chain. Four isoforms of human NDST (NDST-1, -2, -3, and -4) have been identified, differing in their expression in various tissues and in their relative *N*-deacetylase and *N*-sulfotransferase activities.[22] Mice that lack NDST-2 do not synthesize mast cell heparin but produce HS,[23,24] whereas mice that lack NDST-1 produce undersulfated HS and die at birth of lung failure.[25,26] At this stage of biosynthesis, the nascent heparin or HS polymer contains stretches of *N*-sulfoglucosamine interspersed with unmodified *N*-acetylglucosamine residues.

Next, glucuronic acid residues are variably epimerized to iduronic acid by glucuronyl C5-epimerase. Then the iduronic acid residues and, to a lesser extent, glucuronic acid residues are partially *O*-sulfated at the C2-position. Finally, the glucosamine moieties are *O*-sulfated to a variable extent at the C6- and the C3-positions. Epimerization and *O*-sulfation occur only in regions of the polymer that contain

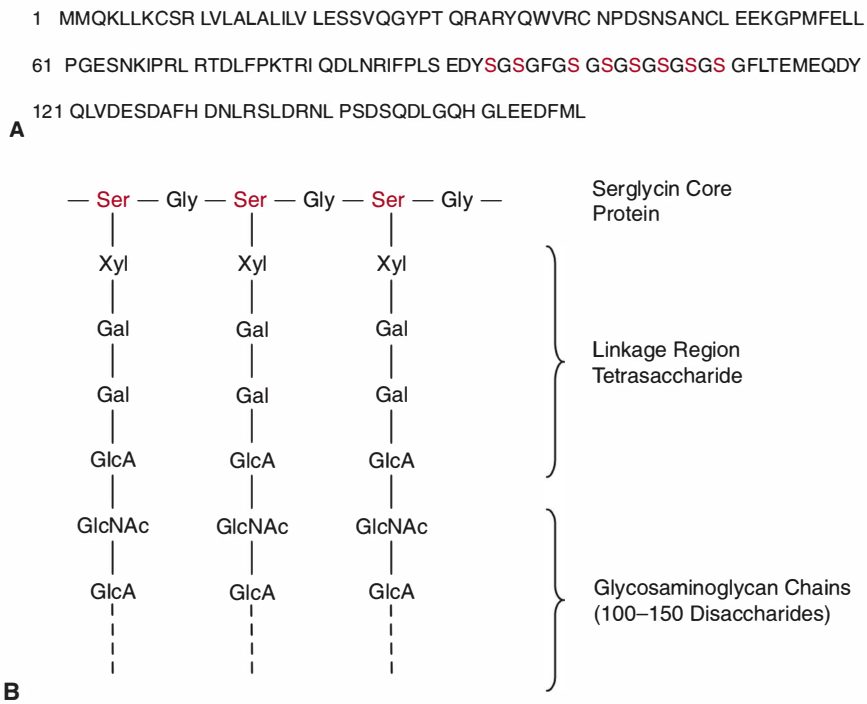

FIGURE 45.1 Heparin proteoglycan structure. **A:** Amino acid sequence of the human serglycin core protein. The *red*-colored residues comprise the glycosaminoglycan attachment sites. **B:** Structure of the linkage region. Ser, serine; Gly, glycine; Xyl, xylose; Gal, galactose; GlcA, glucuronic acid; GlcNAc, *N*-acetyl-glucosamine.

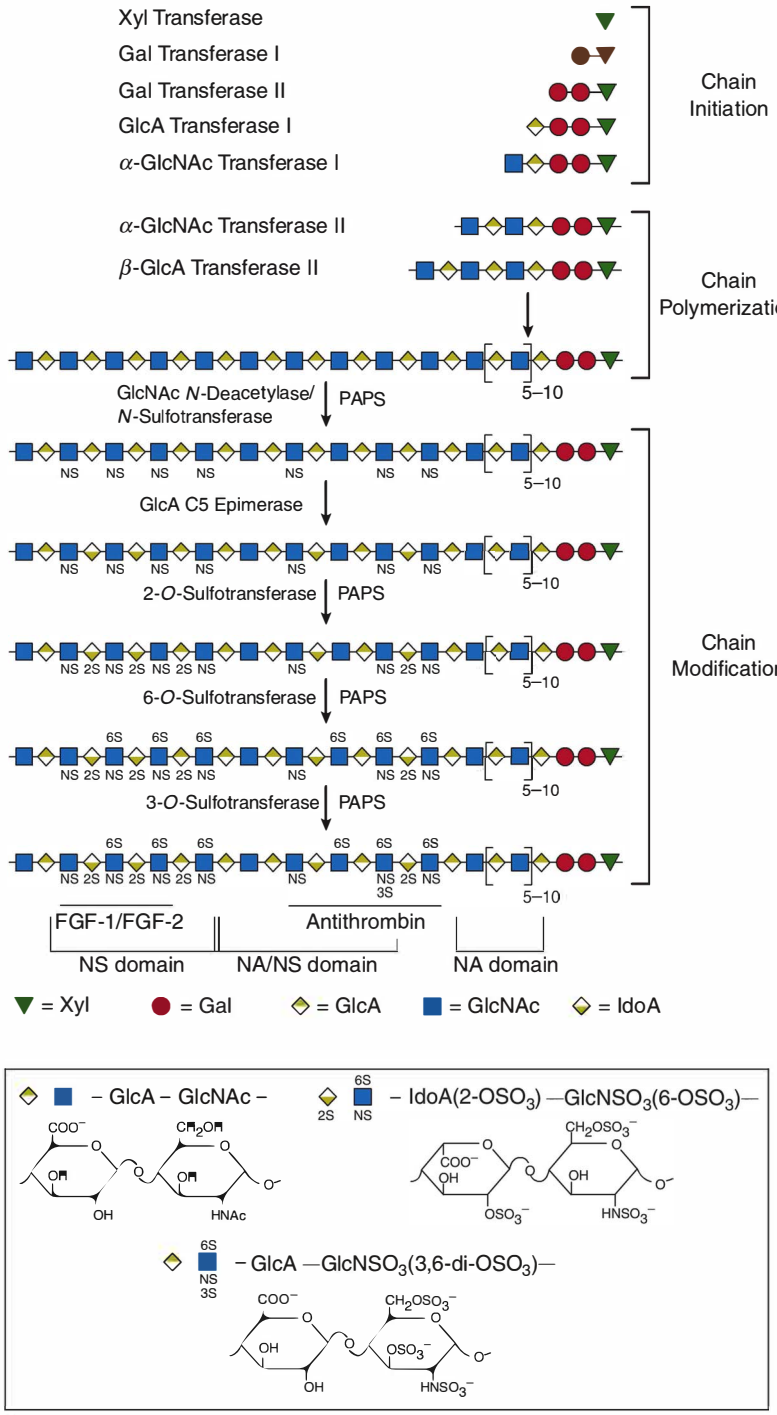

FIGURE 45.2 Heparin/HS biosynthesis. The symbols are defined by the structures shown below the scheme. Representative tracts of contiguous *N*-acetylated disaccharide units (NA domains), contiguous *N*-sulfated sequences of variable length (NS domains), and alternating *N*-acetylated and *N*-sulfated units (NA/NS domains) in HS are indicated. Heparin is predominantly composed of a single, extended NS domain. Regions that have been implicated in binding of fibroblast growth factors FGF-1/ FGF-2 and antithrombin are shown. IdoA, iduronic acid; PAPS, 3′-phosphoadenosine-5′-phosphosulfate. Other abbreviations are defined in the legend to FIGURE 45.1. (Reprinted from Esko JD, Lindahl U. Molecular diversity of heparan sulfate. *J Clin Invest* 2001;108:169–173, with permission.)

N-sulfoglucosamine, but these modifications are incomplete. As a result, a wide variety of oligosaccharide structures can be formed, each having the ability to interact with a different protein (e.g., antithrombin) or class of proteins (e.g., fibroblast growth factors [FGFs] or viral capsid proteins).[10] One HS 2-*O*-sulfotransferase, three HS 6-*O*-sulfotransferases, and six HS 3-*O*-sulfotransferases have been identified in humans.[22] Multiple isoforms make possible tissue-specific and developmentally regulated expression of the corresponding enzyme activities. Because the enzymatic properties of the isoforms may differ in subtle ways, their selective expression may be responsible for unique oligosaccharide sequences found in heparin/HS in different tissues and different animals. For example, 3-*O*-sulfotransferase-1 (3-OST-1) is primarily responsible for sulfation of the C3-position of glucosamine in the sequence GlcA-GlcNS, which gives rise to the high-affinity antithrombin-binding site (discussed in the subsequent text).

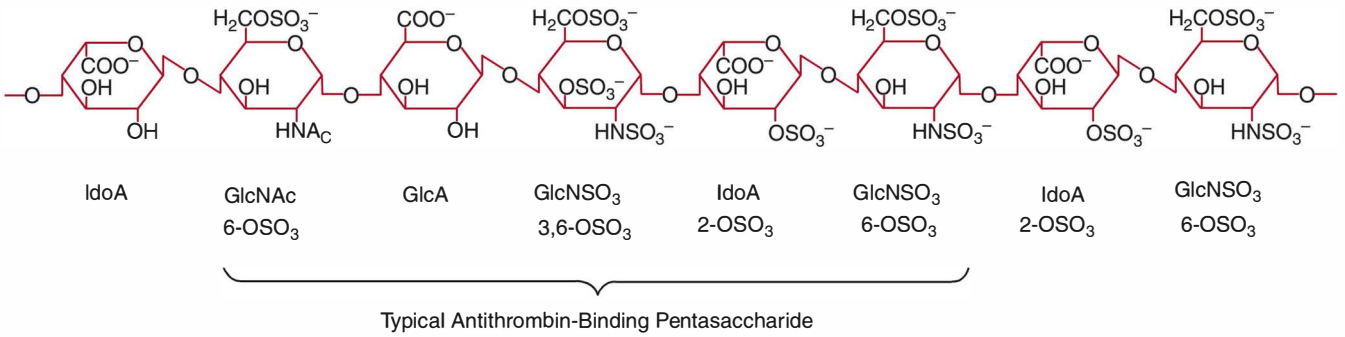

FIGURE 45.3 Representative region of a heparin chain containing a typical antithrombin-binding sequence. IdoA, iduronic acid; GlcA, glucuronic acid; GlcNAc, *N*-acetyl-glucosamine.

ANTITHROMBIN-BINDING SEQUENCES IN HEPARIN

A segment of a heparin chain containing an antithrombin-binding site is depicted in FIGURE 45.3. It is important to emphasize that this sequence is only one of many possible arrangements of uronic acid and glucosamine moieties found elsewhere in the polymer. As progress was being made in defining various aspects of the biosynthesis, linear sequence, and three-dimensional configuration of heparin and HS, it was tacitly assumed that heparin may exhibit numerous alternative structures equally capable of interacting with antithrombin.[27] This assumption was shown to be incorrect in 1976, when several groups demonstrated that only approximately 30% of commercial heparin is able to bind to antithrombin but that the bound fraction is responsible for virtually all of the anticoagulant activity.[28–30]

These findings led to investigations designed to determine the structural differences between active/high-affinity (AT+) and inactive/low-affinity (AT−) heparin species.[27] Rosenberg et al.[31] showed that oligosaccharides produced from high-affinity heparin were enriched in a tetrasaccharide sequence that contained glucuronic acid and *N*-acetylglucosamine. Leder[32] isolated an enzyme that removes sulfate from the 3-*O* position of glucosamine, and Lindahl et al.[33] showed that this substituent is critical for high-affinity binding of heparin to antithrombin. These observations were confirmed and extended by Choay et al.[34,35] who synthesized a series of pentasaccharides that are able to catalyze the factor Xa–antithrombin reaction. The contributions of individual substituents within the antithrombin-binding pentasaccharide have been evaluated by comparing the avidity of synthetic oligosaccharides for antithrombin.[36] On the basis of these data, the relative importance of individual residues is shown in FIGURE 45.4. The presence of an *N*-sulfate in place of the *N*-acetyl group on the first glucosamine residue increases the affinity for antithrombin approximately twofold. Similarly, the 2-*O*-sulfate and 6-*O*-sulfate groups on the fourth and fifth residues of the pentasaccharide increase the affinity but are not

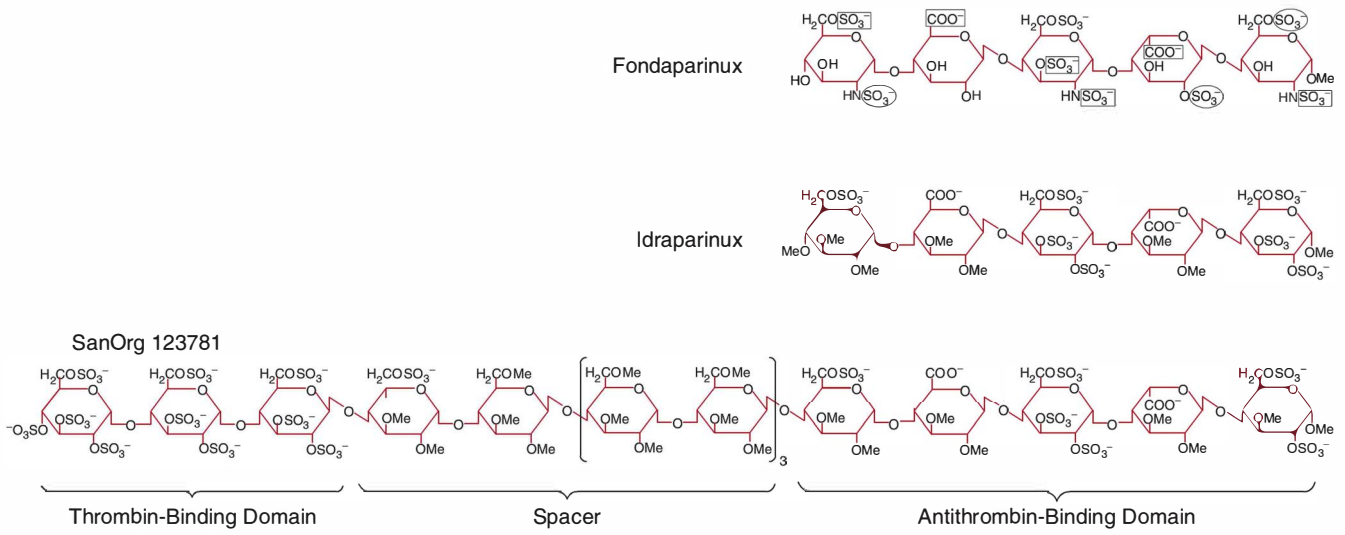

FIGURE 45.4 Synthetic heparin–like oligosaccharides. Sulfate and carboxylate groups required for high-affinity binding to antithrombin are indicated by *boxes* in the structure of fondaparinux. Sulfate groups that are not essential but that contribute to high-affinity binding are *circled*.

absolutely required for high-affinity binding. Therefore, some variability in the structure of heparin at these sites is compatible with antithrombin binding and anticoagulant activity. In contrast, certain structures are critical for antithrombin binding, including the presence and orientation of carboxylate groups on the two uronic acid residues, the 6-*O*-sulfate on the first glucosamine, the 3-*O*-sulfate and *N*-sulfate on the middle glucosamine, and the *N*-sulfate on the last glucosamine of the pentasaccharide.

Although synthetic heparin pentasaccharides that bind to antithrombin with high affinity accelerate the factor Xa–antithrombin interaction, they do not significantly increase the rate of neutralization of thrombin or other hemostatic enzymes. Isolation of oligosaccharides derived from partial chemical depolymerization of heparin revealed that molecules at least 16 monosaccharide units in length are required to accelerate both the thrombin–antithrombin and factor Xa–antithrombin interactions.[37] For inhibition of factor Xa to occur, it is sufficient to form a binary complex between heparin and antithrombin that leads to a conformational change in the reactive site loop of the inhibitor. To facilitate thrombin inhibition, however, longer heparin chains must bind simultaneously to thrombin and antithrombin to form a ternary thrombin–heparin–antithrombin complex. These differences in mechanism of inhibition explain why smaller heparin molecules can inhibit factor Xa while failing to inhibit thrombin. The inhibitory mechanism of antithrombin is discussed in more detail in Chapter 18.

SYNTHESIS OF ANTICOAGULANTLY ACTIVE HEPARAN SULFATE PROTEOGLYCANS BY ENDOTHELIAL CELLS

Mast cells were once thought to be the only site of synthesis of anticoagulantly active heparin. These cells are located beneath the endothelium and sequester heparin within their secretory granules,[38] where it forms crystalline arrays with cationic molecules such as histamine and neutral proteases. Ordinarily, heparin is not released from mast cells into the circulation; one exception may be patients with systemic mastocytosis, in whom small amounts of heparin can appear in the bloodstream and slightly prolong the activated partial thromboplastin time.[39] Therefore, mast cell heparin is unlikely to affect hemostasis under normal circumstances. These considerations led to the suggestion that endothelial cells might synthesize HSPGs endowed with the critical oligosaccharide structure required to bind and activate antithrombin.[40] In parallel investigations of the structure of anticoagulantly active mast cell heparin, HSPGs synthesized by endothelial cells were shown to contain a small subpopulation (~1%) of HS that binds antithrombin with high affinity (HS^{AT+}) and accelerates inhibition of thrombin and factor Xa by antithrombin *in vitro*.[41] These studies suggested that HS^{AT+} might be a major physiologic modulator of hemostasis.

Early investigations revealed that HS is present in the aorta and other vessels and exhibits trace amounts of anticoagulant activity.[42,43] It proved difficult, however, to rule out the possibility that mast cells were the source of this material. Direct evidence that endothelial cells are able to generate anticoagulantly active HSPGs was eventually obtained by Marcum and Rosenberg,[44] who studied cloned microvascular endothelial cells isolated from the rat epididymal fat pad. They showed that polysaccharides extracted from these cells possessed antithrombin accelerating activity that could be completely eliminated by treatment with purified *Flavobacterium* heparinase. Virtually all of the heparin-like material could be harvested by a brief exposure of the endothelial cells to dilute trypsin, which suggested that these components are located on the cell surface. Furthermore, metabolic labeling of the polysaccharides with [^{35}S]sulfate, followed by affinity fractionation with antithrombin and structural analysis, revealed the presence of oligosaccharide structures typically found in anticoagulantly active commercial heparin. Similar studies conducted with cloned bovine aortic endothelial cells gave virtually identical results and indicated that the level of anticoagulantly active HS species present would correspond to approximately 50,000 antithrombin-binding sites per endothelial cell.[45] This estimate of surface-bound anticoagulantly active polysaccharide was in excellent agreement with the data generated by characterization of the interactions of antithrombin with bovine aortic tissue sections.

Two major endothelial cell core proteins that are endowed with anticoagulantly active HS chains have been characterized. Kojima et al.[46] isolated intact HSPGs from rat microvascular endothelial cells using a combination of ion-exchange chromatography, affinity fractionation with antithrombin, and gel filtration in denaturing solvents. The anticoagulantly active HSPGs bound to antithrombin ($HSPG^{AT+}$) constituted approximately 5% of the total HSPGs and were endowed almost entirely with anticoagulantly active HS chains, whereas the anticoagulantly inactive HSPGs that did not interact with antithrombin ($HSPG^{AT-}$) represented approximately 95% of the total HSPGs. The two types of HS chains had the same molecular mass of approximately 25 to 30 kDa, and the anticoagulantly active HS chains possessed a single antithrombin-binding region. The core proteins of $HSPG^{AT+}$ and $HSPG^{AT-}$ were isolated after treatment with *Flavobacterium* heparitinase and purification by ion-exchange chromatography. Peptide mapping studies suggested that $HSPG^{AT+}$ and $HSPG^{AT-}$ contained the same two major core proteins with molecular masses of 50 and 30 kDa.

The primary sequences of internal peptides derived from the HSPG core proteins were used to clone two cDNAs from a rat microvascular endothelial cell library.[47] The first cDNA constituted a previously unidentified species, termed syndecan-4 (ryudocan), whereas the second cDNA represented the rat ortholog of syndecan-1. The latter molecule was a known proteoglycan that was originally thought to be limited in its distribution to epithelial cells. The two cDNAs encode integral membrane proteins of 202 amino acids (syndecan-4) and 313 amino acids (syndecan-1), which have extraordinarily homologous transmembrane and intracellular domains but very distinct extracellular regions. Syndecan-4 has three potential glycosaminoglycan attachment sites in its extracellular domain, whereas syndecan-1 has five. Both molecules are expressed at high levels (0.1% to 0.5% of total mRNA) in primary endothelial cells, primary smooth muscle cells, and primary fibroblasts. The possibility that slightly different core proteins direct the synthesis of $HSPG^{AT+}$ and $HSPG^{AT-}$ was excluded by stably expressing an epitope-tagged syndecan-4 in cells that synthesize anticoagulantly active and anticoagulantly inactive HS chains; after isolation of the proteoglycan with a specific antibody against the epitope, both HS^{AT+} and HS^{AT-} were found to be present.[48] This finding suggested that the presence of HS biosynthetic enzymes rather than expression of specific core proteins determines the fine structure of the HS chains. Further studies

suggested that in addition to syndecan-1 and syndecan-4, glypican-1[49] and perlecan[50] synthesized by endothelial cells also bear anticoagulantly active HS chains.

The HS biosynthetic pathway generates limiting amounts of $HSPG^{AT+}$ with regions of defined structure that contain the antithrombin-binding site; this pathway also produces the more abundant $HSPG^{AT-}$ with regions of varying structure that carry out other biologic functions. Analyses of cell mutants, created by overexpression of the syndecan-4 core protein or by chemical mutagenesis, revealed that anticoagulant HS generation requires a component present in limiting amounts.[51,52] To identify this component, a cell-free system was developed by Shworak et al.[53] in which wild-type microsomes were incubated with radiolabeled HSPG precursor extracted from mutant cells blocked during $HSPG^{AT+}$ generation; coincubation of these two extracts resulted in the production of large amounts of $HSPG^{AT+}$. Furthermore, in many different cell types, the concentration of this "microsomal conversion activity" correlated with the cell's ability to generate $HSPG^{AT+}$. Treatment of microsomal and cell surface HSPGs from wild-type cells with excess microsomal conversion activity transformed up to 35% of total HSPGs into $HSPG^{AT+}$. This extent of conversion was much greater than the normal levels of cell surface $HSPG^{AT+}$, which average approximately 1% to 5% of total HSPGs.[53] These investigations provided evidence that a microsomal component limits transformation of small amounts of a HSPG precursor population into $HSPG^{AT+}$.

The assay described in the preceding text was then used to isolate the limiting component, which proved to be the long-sought glucosaminyl 3-OST-1.[54,55] This enzyme usually is present in trace amounts as compared to other HS biosynthetic enzymes. When 3-OST-1 is no longer limiting, the cell's capacity to generate HS^{AT+} is determined by the abundance of HS^{AT+} precursors.[56] *In vitro* 3-*O*-sulfation with purified 3-OST-1 was used to tag the regions of the HS^{AT+} precursors destined to become antithrombin-binding sites and allow the HS^{AT+} precursors to be captured. The tagged regions were then structurally examined.[57] It was shown that six 3-*O*-sulfation sites existed per HS^{AT+} precursor chain. At least five out of the six 3-*O*-sulfate-tagged oligosaccharides in HS^{AT+} precursors bound antithrombin, whereas none of 3-*O*-sulfate-tagged oligosaccharides from HS^{AT-} precursors bound antithrombin. When treated with low pH nitrous acid or heparitinase, 3-*O*-sulfate-tagged HS^{AT+} and HS^{AT-} precursors exhibited clearly different structural features: They had different uronic acid epimerization and *O*-sulfation patterns around the 3-*O*-sulfate acceptor sites. 3-*O*-sulfate-tagged HS^{AT+} hexasaccharides were antithrombin affinity purified, sequenced by chemical and enzymatic degradation, and shown to have structures compatible with the antithrombin-binding sequence illustrated in FIGURE 45.3. The differences in structure surrounding potential 3-*O*-sulfate acceptor sites in HS^{AT+} and HS^{AT-} precursors suggested that these precursors might be generated by different concerted assembly mechanisms in the same cell.[57]

ANATOMIC LOCATION OF ANTICOAGULANTLY ACTIVE HEPARAN SULFATE PROTEOGLYCANS

The anatomic location of $HSPG^{AT+}$ was investigated by de Agostini et al.,[58] who perfused normal rat aorta for short periods of time with ^{125}I-antithrombin and observed that the labeled protein was bound to the aortic subendothelium (see FIGURE 45.5). Quantitation of the data revealed that only approximately 1% of the bound ^{125}I-antithrombin was associated with the luminal surface of the endothelium and that only small amounts were associated with smooth muscle cells or connective tissue deeper in the vessel wall. The labeled antithrombin was also noted to bind to regions around capillaries, especially in the subendothelium associated with the basal lamina/basement membrane. Perfusion of the aorta with ^{125}I-antithrombin–labeled

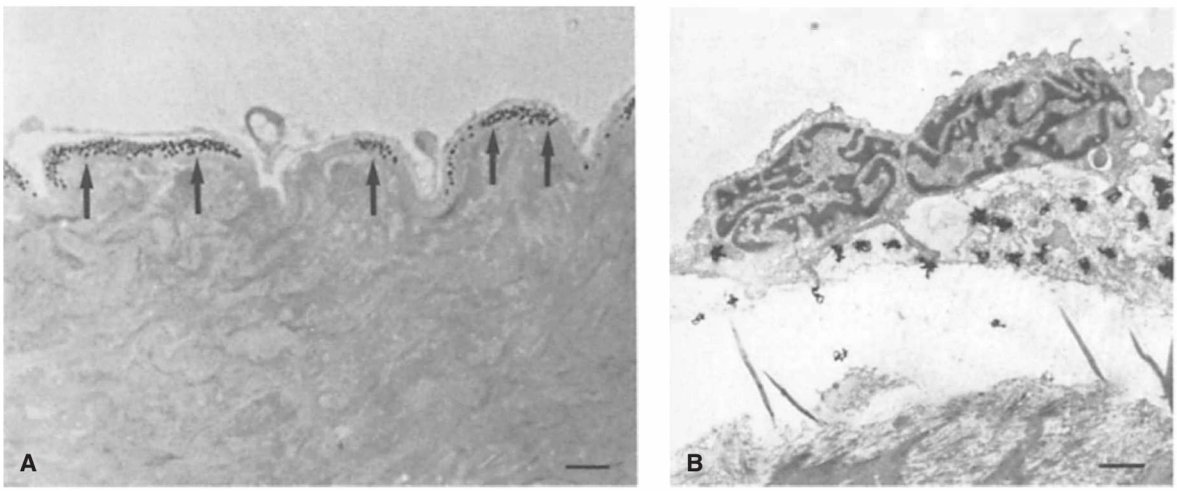

FIGURE 45.5 Autoradiography of normal rat aortas perfused with ^{125}I-antithrombin. **A:** Light microscopic autoradiograph. Intense labeling is present in the basement membrane beneath the endothelial cells (*arrows*). Bar, 10 μm. **B:** Electron microscopic autoradiograph. Labeling is again evident beneath the endothelium. Bar, 1.0 μm. (Reprinted from de Agostini AI, Watkins SC, Slayter HS, et al. Localization of anticoagulantly active heparan sulfate proteoglycans in vascular endothelium: antithrombin binding on cultured endothelial cells and perfused rat aorta. *J Cell Biol* 1990;111:1293–1304, with permission.)

bovine serum albumin produced no detectable binding, which indicated that the binding of ^{125}I-antithrombin within the aorta was not due to nonspecific interactions or trapping of the labeled protein. To demonstrate that the ^{125}I-antithrombin specifically interacted with HS chains, the vasculature was perfused with *Flavobacterium* heparinase before perfusion with labeled antithrombin. This treatment effectively prevented any binding of ^{125}I-antithrombin to all regions, including the subendothelium, while leaving the endothelial layer intact.

These data were consistent with the known synthesis of HSPG^{AT+} by endothelial cells and suggested that the proteoglycans are released from these cells and accumulate predominantly in the basement membrane. This observation suggested two possible ways in which coagulation system activity could be regulated by blood vessel wall HSPG^{AT+}. On the one hand, the small amounts of luminal HSPG^{AT+} could be in a critical position to bind and activate antithrombin and thereby regulate hemostatic activity at the blood–vessel wall interface. The much larger quantities of abluminal HSPG^{AT+} could serve as a potential reservoir that could be brought into play with extensive damage to the overlying endothelium. On the other hand, plasma antithrombin may have relatively free access to the much greater amounts of HSPG^{AT+} that accumulate on the abluminal surface of the endothelial cells, as suggested by studies that document the permeability of the endothelial cell layer.[59] Indeed, the presence of ^{125}I-antithrombin bound to subendothelium after *ex vivo* perfusion suggests that this region is readily accessible to antithrombin. The accessibility of subendothelial HSPG^{AT+} would also explain kinetic tracer data, which suggest that 10% to 20% of antithrombin partitions to an extravascular compartment.[60] These observations imply that coagulation proteases might be inhibited by antithrombin bound to HSPG^{AT+} in the subendothelium.

Marcum et al.[61] obtained evidence that intact blood vessels possess HSPGs that stimulate the activity of plasma antithrombin. In their experiments, rat hindlimb preparations were perfused with purified thrombin at a constant level, and purified antithrombin was infused at several different concentrations. The amount of thrombin–antithrombin complex formed within the vasculature was estimated by a specific radioimmunoassay for the interaction product. The rate of complex formation was enhanced 19-fold in the animal with respect to the uncatalyzed rate *in vitro*. The antithrombin-stimulating activity detected in the hindlimb vasculature appeared to be due to an HSPG, because the rate of thrombin–antithrombin complex formation was reduced to the uncatalyzed rate by preinfusion of the vasculature with *Flavobacterium* heparinase. Furthermore, when the hindlimb preparation was perfused with antithrombin chemically modified at Trp49 to decrease its affinity for HS, complex formation also occurred at the uncatalyzed rate. Finally, when purified human platelet factor 4 (PF4) was added to the perfusion stream along with antithrombin, thrombin inhibition occurred at the uncatalyzed rate. These data suggested that PF4, after being released from activated platelets, may play a role in thrombogenesis by neutralizing anticoagulantly active vascular HSPGs. Similar results were obtained with mice genetically deficient in mast cells, strongly suggesting that mast cell heparin plays little or no role in this phenomenon.[62]

In summary, it appeared likely that a small fraction of plasma antithrombin is normally bound to HSPG^{AT+} associated with endothelial cells of the blood vessel wall. It was unclear whether the functionally important HSPGs are primarily the small amounts present on the luminal surface of the endothelium, or the much larger quantities located in the subendothelial region, or both. In either case, the endothelial HSPGs could interact with antithrombin to produce an antithrombotic effect at blood–surface interfaces where coagulation proteases are commonly generated. Therefore, antithrombin would be in a position to protect surfaces lined by normal endothelium against thrombus formation. Furthermore, the catalytic nature of anticoagulantly active HS species would ensure the continual regeneration of the nonthrombogenic properties of the endothelial cell layer.

THE PHENOTYPE OF 3-*O*-SULFOTRANSFERASE-1–DEFICIENT MICE

As discussed in the preceding section, a critical limiting factor in the biosynthesis of HS^{AT+} is the presence of 3-OST-1, which creates the high-affinity antithrombin-binding motif by modification of selected regions within the HS polymer.[54,55] 3-OST-5 can also generate HS^{AT+}, although much less efficiently than 3-OST-1.[63] Therefore, 3-OST-1 is likely to be the major enzyme responsible for generation of HS^{AT+} *in vivo*. Mice lacking the gene that encodes 3-OST-1 (*Hs3st1*) were generated by HajMohammadi et al.[64] to evaluate the biologic role of HS^{AT+}. The absence of 3-OST-1 enzyme caused large reductions (75% to 98%) in HS^{AT+} levels in most tissues as determined by the ability of HS purified from these tissues to accelerate inhibition of factor Xa by antithrombin. Unexpectedly, removal of most HS^{AT+} did not alter the basal rate of fibrin generation in 3-OST-1–deficient mice, nor did it lead to enhanced fibrin deposition in the lungs under hypoxemic conditions known to produce a strong procoagulant stimulus. To evaluate the possibility that HS^{AT+} plays a role only after endothelial injury, which might allow direct contact of the blood with the subendothelial matrix, mice were investigated in a carotid arterial injury model in which application of ferric chloride to the adventitia rapidly induces endothelial denudation leading to thrombosis. However, *Hs3st1*$^{-/-}$ and *Hs3st1*$^{+/+}$ mice had similar thrombotic occlusion times and equivalent postinjury levels of thrombin–antithrombin complexes, despite the virtual absence of HS^{AT+} in the uninjured carotid artery of the *Hs3st1*$^{-/-}$ mice as determined by ^{125}I-antithrombin binding.

Because 3-OST-1–deficient mice lack an obvious procoagulant phenotype, hemostatic balance does not appear to be tightly correlated with HS^{AT+} levels. Whereas *Hs3st1*$^{-/-}$ mice appeared normal on a mixed C57BL/6 × 129S4/SvJae genetic background, intrauterine growth retardation and early postnatal lethality were observed in *Hs3st1*$^{-/-}$ mice backcrossed into the C57BL/6 background. Although the cause of neonatal mortality in these mice is unclear, histologic examination did not reveal evidence of focal thrombosis as seen in antithrombin-deficient embryos,[65] which further suggests a lack of association between 3-OST-1 and antithrombin activity. The unexplained phenotype of *Hs3st1*$^{-/-}$ mice raises the possibility that HS^{AT+} participates in a novel biologic function(s). Indeed, recent analyses of *Drosophila* deficient in the orthologous enzyme, 3-*O*-sulfotransferase-B, suggest a critical role in Notch signaling pathways.[66] Novel functions might even occur outside of the vascular system, because 3-OST-1–derived HS^{AT+} is produced in several nonendothelial cell types, including

fibroblastic cell lines,[67] ovarian granulosa cells,[68] embryonal carcinoma cells,[57] alveolar type II cells,[69] and glomerular epithelial cells.[70]The lack of a prothrombotic phenotype in 3-OST-1 knockout mice challenges the concept that HS^{AT+} plays a major role in hemostasis. However, unprovoked thrombosis occurs in humans or mice homozygous for antithrombin mutations that reduce the inhibitor's affinity for HS.[71,72] These findings suggest that HS binding is required for proper antithrombin function *in vivo*. How, then, could one explain the 3-OST-1 knockout data?

First, the residual 2% to 20% anticoagulant HS activity in the 3-OST-1–deficient mice likely reflects 3-*O*-sulfation by 3-*O*-sulfotransferase isoforms other than 3-OST-1. As indicated in the preceding text, 3-OST-5 might play an important role if hemostatic balance requires only low levels of HS^{AT+}.[63] Because the plasma antithrombin concentration (2 to 5 μM) greatly exceeds the dissociation constant of antithrombin for HS^{AT+} (~15 nM), small amounts of HS^{AT+} might always be saturated with antithrombin and thereby exert a basal anticoagulant tone. Assessment of this possibility will require deletion of the gene for 3-OST-5 and perhaps other isoforms that may be responsible for the synthesis of the HS^{AT+} present in *Hs3st*$^{-/-}$ mice.

Second, HS^{AT+} may not be responsible for the anticoagulant properties of HS *in vivo*. Scully et al.[73] reported that HS that failed to bind to a column of immobilized antithrombin (generally defined as HS^{AT-}) accelerated the factor Xa–antithrombin reaction by 1,100-fold, although much higher concentrations were required in comparison with high-affinity HS (HS^{AT+}). They proposed that the nonthrombogenic property of blood vessels is due to acceleration of the factor Xa–antithrombin interaction by the greater mass of vascular HS^{AT-} rather than by the much smaller proportion of HS^{AT+} molecules. If this hypothesis is true, one would predict that defective HS synthesis would lead to a procoagulant phenotype. To complicate matters, HS interacts with a great variety of proteins other than antithrombin, and multiple functions have been attributed to HS throughout embryonic development, including interactions with the FGF, Wnt, transforming growth factors-β (TGF-β), and hedgehog (Hh) signaling pathways.[74] Targeted disruption of *EXT1*, one of the genes responsible for HS chain polymerization, resulted in the loss of HS, disruption of gastrulation, and embryonic lethality before embryonic day 8.5.[75] Conditional knockouts of *EXT1* or other HS biosynthetic genes in vascular tissue at later stages of development could be more informative with regard to the hemostatic properties of HS but have not yet been achieved. Knockout of the syndecan-4 gene (*Synd4*) suggests that HS might be involved in anticoagulation of the placenta.[76] Syndecan-4, a transmembrane protein bearing both HS^{AT+} and HS^{AT-} chains, was detected in the fetal vessels of the placenta by *in situ* hybridization and immunohistochemical staining. During late gestation, degeneration of fetal vessels in the placental labyrinth was more extensive in *Synd4*$^{-/-}$ embryos than in wild-type controls and was associated with deposition of fibrin(ogen). These findings suggest that the absence of syndecan-4 produces a deficit in placental anticoagulation.

Third, chondroitin sulfate or dermatan sulfate in the vessel wall might compensate for the reduction in anticoagulant HS activity in the 3-OST-1 knockout mouse. For example, the chondroitin sulfate moiety on thrombomodulin enhances the reactivity of thrombomodulin-bound thrombin with antithrombin,[77,78] and dermatan sulfate may promote inhibition of thrombin by HCII in the carotid arterial injury model, as discussed in the subsequent text.[79]

Fourth, HS^{AT+} synthesized by 3-OST-1 might serve an anticoagulant role in a distinct subset of tissues or under pathologic conditions not yet examined.[64] For example, $HSPG^{AT+}$ is synthesized by ovarian granulosa cells and secreted into the developing follicle in response to gonadotrophins.[80] In this location, $HSPG^{AT+}$ is thought to interact with antithrombin and other serpins (i.e., protease nexin-1 and plasminogen activator inhibitor-1) to prevent fibrin formation in the follicular fluid surrounding the oocyte.[81,82] During ovulation, the HSPGs are released from the follicle and may have anticoagulant activity in the oviduct or uterus.

Fifth, unmasking of an overt procoagulant effect might require a second genetic defect in addition to 3-OST-1 deficiency. For example, the combination of factor V_{Leiden} and protein Z deficiency produces a much greater procoagulant phenotype than does either of the two deficiencies alone.[83]

PREPARATION OF HEPARIN FOR CLINICAL USE

Pharmaceutical-grade heparin is usually isolated from mammalian tissues such as the lung or intestinal mucosa. Preparation of the polysaccharide involves homogenization of tissues; treatment of the resultant material with proteolytic enzymes, such as papain, or extraction at elevated pH and temperature; differential precipitation of complex sugars with quaternary ammonium salts such as cetylpyridinium chloride; and chromatography of the substances on anion exchange resins such as QAE-Sephadex.[84] The purified products behave as single glycosaminoglycan chains with molecular weights varying from approximately 3,000 to 35,000, with a mean of approximately 12,000. If oxidizing agents have been avoided during the purification procedure, approximately 15% to 30% of the polysaccharide chains remain covalently linked to fragments of the core protein. The remainder have free uronic acid or glucosamine-reducing groups. This suggests that the polysaccharide species are generated by cleavage of the larger individual polysaccharide chains of the heparin proteoglycan at three to five separate sites. In most commercial preparations of heparin, oxidizing agents are used late in the isolation procedure to "bleach" the polysaccharide. This treatment appears to be responsible for the absence of protein linkage regions within these preparations. Low molecular weight heparins are prepared by controlled depolymerization of standard heparin.[84]

In 2007 and 2008, certain lots of standard heparin from a single manufacturer were associated with an excess of anaphylactoid reactions and death of at least 149 patients after intravenous administration. Oversulfated chondroitin sulfate[85] and other oversulfated glycosaminoglycans,[86] which mimick the anticoagulant activity of heparin, were identified as contaminants in the affected heparin lots. Further work demonstrated that the oversulfated contaminants activate the contact system,[87] leading to generation of vasoactive mediators such as bradykinin and complement components C3a and C5a, and that they induce cell signaling events through a variety of growth factor receptors.[88] Oversulfated glycosaminoglycans have also been implicated in the pathogenesis of heparin-induced

thrombocytopenia (HIT)[89] and may explain the increased incidence of HIT observed in patients exposed to contaminated heparin.[90]

SYNTHETIC HEPARIN–LIKE ANTICOAGULANT DRUGS

In the early 1980s, the total chemical synthesis of a pentasaccharide related to the antithrombin-binding domain in heparin was achieved in more than 60 individual steps by chemists at Sanofi and Organon.[36] Extensive structure-activity studies using a variety of other synthetic oligosaccharides, as well as nuclear magnetic resonance (NMR) and x-ray crystallography, were subsequently performed.[91] On the basis of these studies, a concerted drug development effort was undertaken to develop the synthetic pentasaccharide fondaparinux, which has proven to be safe and effective in human trials and is now used for prevention and treatment of venous thromboembolism[92] (FIGURE 45.4). Fondaparinux mediates factor Xa inhibition by antithrombin but does not cause thrombin inhibition because of its short polymer length. In fact, fondaparinux has a stronger affinity for antithrombin and a higher anti-Xa activity in comparison with the most common antithrombin-binding pentasaccharide structure in porcine heparin. These differences have been attributed to the first glucosamine residue in fondaparinux being *N*-sulfated, whereas that of porcine heparin is *N*-acetylated. Fondaparinux does not interact significantly with platelets or with plasma proteins other than antithrombin and therefore has predictable pharmacokinetic behavior. Because it does not interact with PF4, fondaparinux is unlikely to trigger HIT.[93] A derivative (idraparinux; FIGURE 45.4) with a much longer half-life than fondaparinux (120 vs. 16 hours) has also been evaluated in clinical trials, but, lacking an antidote, it was deemed to present an unacceptable risk of bleeding. An idraparinux–biotin conjugate (idrabiotaparinux), which can be cleared rapidly from the circulation by intravenous injection of avidin, is undergoing clinical evaluation.[94,95]

A synthetic polymer that could mimic heparin's full anticoagulant activity (i.e., inhibition of both thrombin and factor Xa) would need to have both an antithrombin-binding domain and a thrombin-binding domain. Thrombin binds nonspecifically to highly sulfated heparin oligosaccharides and to other anionic polymers. Therefore, heparin analogs have been synthesized to contain a highly sulfated oligosaccharide linked to the nonreducing end of an antithrombin-binding pentasaccharide through a neutral spacer region.[96] In these compounds, *N*-sulfate groups were replaced by *O*-sulfates, and hydroxyl groups were alkylated, thereby greatly simplifying the synthetic process. The resulting compounds, one of which is depicted in FIGURE 45.4, catalyzed inhibition of thrombin by antithrombin and were 5- to 10-fold more potent than standard heparin in *in vivo* models. This is probably because these compounds interact much less avidly with basic plasma proteins including PF4, thereby leaving more molecules available for binding to thrombin and antithrombin. More importantly, these compounds seem to have little effect on platelets; they do not activate platelets in the presence of plasma from patients with HIT and do not affect the bleeding time of rats while still inhibiting thrombosis. Recently, more complicated constructs that include a fondaparinux analog, a benzamidine-based direct thrombin inhibitor, and biotin have been synthesized.[97] Enzymatic and chemoenzymatic methods have also been employed to synthesize heparin-like anticoagulants. Rosenberg's group has synthesized a series of potent anticoagulant heparin analogs by using a capsular bacterial polysaccharide (heparosan) obtained from *Escherichia coli* K5 and different mammalian-expressed HS sulfotransferases and epimerase with yields in the microgram range.[98–100] This approach is currently limited by the high cost of the enzymes and the low yield compared to commercial heparin production.

Lindahl et al.[101] have been able to generate gram amounts of "neoheparin" with antifactor Xa and antithrombin activities similar to those of heparin using chemoenzymatic modifications of heparosan. However, it is unknown whether the significant amounts of nonnaturally occurring 3-*O*-sulfated GlcA and IdoA residues in the neoheparin molecule might create undesirable side effects *in vivo*.

NONANTICOAGULANT ACTIONS OF HEPARIN AND HEPARAN SULFATE

Heparin and HS have a wide variety of biologic actions that are independent of its anticoagulant effects. As one of the most acidic biopolymers found in nature, heparin interacts with hundreds of proteins such as growth factors, cytokines, chemokines, extracellular matrix components, protease inhibitors, proteases, and lipases.[102] HS has been implicated in the control of many processes in multicellular organisms, including cell division, adhesion, spreading, migration, chemoattraction, inflammation, axon guidance, matrix assembly, lipoprotein uptake, extracellular proteolysis, tumor metastasis, angiogenesis, body size, body weight, and uptake of bacteria and viruses.[10,18,74,103–107,108,109,110] In this section, we briefly consider a few examples of the nonanticoagulant effects of heparin and HS.

Effects on Platelets

Heparin administration commonly produces an immediate, mild reduction in the platelet count, which is thought to result from enhanced platelet aggregation.[111] This phenomenon, termed *nonimmune heparin-associated thrombocytopenia*, probably results from direct interactions between heparin and platelets.[112] Heparin binds in a saturable and specific manner to approximately 2,500 sites on the platelet surface.[113,114] The affinity of heparin for platelets increases with the size and charge density of the polymer. Dissociation constants of 1.3 and 0.3 μM have been determined for heparin molecules having molecular weights of approximately 3,000 and 15,000, respectively.[115] Although it has been reported that the $\alpha_{IIb}\beta_3$ integrin (fibrinogen receptor) interacts with heparin,[116] the binding sites for heparin on platelets have not been identified with certainty.

When added to citrate-anticoagulated platelet-rich plasma, heparin alone weakly stimulates platelet aggregation and potentiates platelet activation induced by adenosine 5'-diphosphate or other agonists.[117–121] These effects are probably not artifacts of decreased ionized calcium produced by the citrate anticoagulant, because heparin also enhances platelet aggregation induced by collagen in hirudin-anticoagulated whole blood.[122] Low molecular weight heparin is less able to stimulate platelet aggregation than is unfractionated heparin,[112,117,119–121,123–125] consistent with the relative binding affinities of these polymers.[115] Furthermore, heparin species with low affinity for antithrombin or fibronectin are more active, presumably because these proteins

competitively inhibit binding of heparin to platelets.[112,124] The mechanism by which heparin enhances platelet function is unknown, in part, because inconsistent effects of heparin on aggregation of washed platelets have been observed.[112,123,124,126,127] Nevertheless, it is clear that the mechanism is distinct from that of HIT, which is triggered by antibodies that bind to neoepitopes on PF4–heparin complexes and activate platelets by interaction with their Fc receptors.[128]

Heparin may also interfere with platelet function and thereby contribute to the hemorrhagic side effects of the drug. In the presence of antithrombin, heparin blocks activation of platelets induced by thrombin.[123,129] Heparin also inhibits binding of von Willebrand factor to platelets, reducing adhesion of platelets to the subendothelium of an injured vessel.[130,131] These effects may cause prolongation of the bleeding time in patients receiving heparin.[132,133]

Inhibition of Selectins

The selectins (L-, P-, and E-selectin) are integral membrane proteins involved in cell adhesion interactions, including lymphocyte homing, inflammation, and tumor metastasis.[134] L-selectin is expressed constitutively on leukocytes, whereas P-selectin is rapidly translocated from secretory granules to the cell surface upon activation of platelets or endothelial cells. E-selectin expression is induced more slowly in endothelial cells by proinflammatory agonists. Selectins bind to carbohydrate structures related to the sialyl–Lewis^x tetrasaccharide; these structures are components of glycoproteins found on the surface of leukocytes, activated endothelium, and mucinous adenocarcinoma cells. Heparin and HS also interact with L-selectin and P-selectin (but not E-selectin) and inhibit binding of these proteins to sialyl-Lewis^x at concentrations less than those typically used for anticoagulation.[135] In mouse models, heparin produces a potent anti-inflammatory effect, which appears to be caused by blockade of L- and P-selectins; modified heparins that lack anticoagulant activity are also effective in these models.[136]

Experimental studies suggest that heparin inhibits tumor cell proliferation, migration across the endothelial barrier, invasion of tissues, and angiogenesis.[137,138,139] Furthermore, clinical studies have documented a survival benefit for certain patients with cancer receiving heparin.[140] Although the mechanisms responsible for these effects are unknown, selectin-mediated cellular interactions have been implicated in the hematogenous phase of tumor metastasis. For example, adhesion of activated platelets expressing P-selectin to mucin-bearing tumor cells is blocked by heparin.[141] In a mouse model in which human colon adenocarcinoma cells were injected intravenously, tumor cell–platelet aggregates accumulated rapidly in the pulmonary vasculature, and tumor foci were detectable in the lungs 6 weeks later. A single dose of heparin administered 30 minutes before injection of the tumor cells markedly decreased the number of metastatic foci at 6 weeks.[141] Subsequent studies showed that nonanticoagulant heparin derivatives blocked metastasis by interference with selectins or inhibition of heparanase, a tumor endoglycosidase that degrades HS in the extracellular matrix.[142]

Selectin–mucin interactions also may trigger migratory thrombophlebitis (Trousseau syndrome) in patients with mucinous adenocarcinomas.[143] In wild-type mice, injection of mucins extracted from human colon carcinoma cells led to formation of platelet-rich microthrombi in the lungs. Thrombus formation was inhibited by heparin but not by hirudin and therefore appeared to be independent of thrombin generation. Furthermore, thrombus formation was largely absent in mice deficient in either P-selectin or L-selectin. Although the mucins did not activate platelets suspended in buffer or plasma, platelet activation occurred when mucins were added to whole blood. Platelet activation by mucins *in vitro* was inhibited by heparin and did not occur in blood from L-selectin–deficient mice. These observations are consistent with a model in which soluble carcinoma mucins first bind to L-selectin on leukocytes, stimulating release of an unidentified platelet agonist. The activated platelets then bind to mucins via P-selectin and become cross-linked to form aggregates or bind to P-selectin glycoprotein ligand-1 on neutrophils or monocytes to signal production of thromboxane A_2 or tissue factor. Heparin could block some or all of these selectin interactions. This mechanism may explain the clinical observation that heparin is generally more effective than vitamin K antagonists for treatment of Trousseau syndrome.

Complement, Antiphospholipid Antibodies, and Fetal Loss

Heparin is used to prevent fetal loss in patients with the antiphospholipid syndrome.[144] Injection of pregnant mice with human IgG containing antiphospholipid antibodies caused fetal resorption at a rate of more than 40%; in comparison, approximately 10% of fetuses were absorbed in mice given normal human IgG. Experiments with gene-targeted mice showed that activation of both the classical and alternative complement pathways is necessary to produce fetal loss in this model.[145] Fetal loss was prevented by treatment with unfractionated or low molecular weight heparin but not by fondaparinux or hirudin.[146] *In vivo* and *in vitro* experiments showed that heparin inhibited activation of complement factor C3, as well as deposition of C3b on the surface of placental trophoblasts. Therefore, the ability of heparin to inhibit complement may explain its beneficial effect in pregnant patients with antiphospholipid antibodies[147]; heparin may also inhibit the inflammatory response that normally follows complement activation by blockade of P- and L-selectins, as described in the preceding section.

Microbial Adherence and Invasion

A wide variety of bacteria, parasites, and viruses adhere to HSPGs on cultured cells *in vitro* and are displaced from the cell surface by soluble heparin.[107] In some cases, binding of the microbe to an HSPG is thought to mediate internalization or tissue tropism. Although some of these interactions may be nonspecific, specific adhesion molecules that bind to heparin have been identified on the surfaces of pathogens such as *Plasmodium falciparum, Trypanosoma cruzi, Bordetella pertussis*, human immunodeficiency virus, and herpes simplex virus (HSV).[148] One of the best-studied examples is HSV-1. This virus contains two envelope glycoproteins, gB and gC, that are responsible for reversible adhesion of the virus to HSPGs on target cells. A third viral glycoprotein, gD, then interacts with one of several other cellular proteins to mediate fusion of the viral envelope with the cell membrane, resulting in entry of the viral nucleocapsid into the cytoplasm. Although the first proteins found to interact with gD did not have attached glycosaminoglycan chains, HSPGs with

HS chains modified by a specific 3-*O*-sulfotransferase (3-OST-3) also bind to gD.[109] Therefore, HSPGs may be sufficient for adhesion and entry of HSV-1 into susceptible cells.

Growth Factor Signaling

HSPGs serve as coreceptors for a variety of soluble ligands, including FGFs, vascular endothelial growth factors, glial cell line–derived neurotrophic factor, hepatocyte growth factor, TGF-β, CC and CXC chemokines, and various cytokines.[149,150] An example that has been well studied is the interaction of FGF2 with the receptor tyrosine kinase FGFR1. Efficient signal transduction by FGFR1 does not occur in cells that are unable to synthesize HS or cells treated with enzymes that degrade HS,[151,152] but addition of soluble heparin to these cells restores the signaling pathway. Heparin or an HS chain of syndecan or glypican on the cell surface appears to facilitate the formation of a complex containing two FGF monomers and two FGF receptors, which leads to receptor dimerization and signaling. Although crystallographic studies have identified potential heparin-binding sites in both FGF and its receptor, the precise orientation of heparin in the signaling complex is unknown. Heparin oligosaccharides with a minimum of 12 sugar residues are required for FGF signaling. Binding to FGF requires *N*-sulfation of glucosamine and iduronic acid 2-*O*-sulfate, whereas binding to the receptor also requires 6-*O*-sulfation of glucosamine (FIGURE 45.2).[153]

Anti-Inflammatory Activity of Antithrombin

High concentrations of antithrombin (10 to 20 μM) inhibit nuclear factor-κB (NF-κB) activation in cultured monocytes and endothelial cells, thereby interfering with expression of various proinflammatory and procoagulant genes (e.g., interleukin-6, tumor necrosis factor-α, and tissue factor) that are regulated by NF-κB.[154] The anti-inflammatory effect appears to depend on interaction with cell surface HSPGs, because the effect is abolished by chemical modification of antithrombin's heparin-binding site, by the presence of free heparin, or by enzymatic removal of HS from the cell.[155] Intravenous infusion of antithrombin has been reported to ameliorate experimental models of sepsis, disseminated intravascular coagulation, and ischemia/reperfusion injury.[156] Although a large phase III trial of intravenous antithrombin in patients with severe sepsis failed to demonstrate a reduction in mortality at 28 days,[157] subgroup analysis showed a possible survival benefit in those patients who did not concomitantly receive heparin.[158]

Functions of Heparan Sulfate in Development

Direct evidence implicating proteoglycans in development was obtained initially from studies with *Drosophila*, in which mutations at the *dally* (*division abnormally delayed*) locus cause morphologic abnormalities in the wings, eyes, antennae, and other tissues.[74,108] The *dally* gene and its homolog *dlp* (*dally-like protein*) encode HSPG core proteins homologous to vertebrate glypicans, which are attached to the plasma membrane by glycosyl phosphatidylinositol linkers. Subsequent studies revealed that *dally* or *dlp* mutations affect signaling pathways that involve known HS-binding growth factors, including decapentaplegic (Dpp), Hh, and wingless (Wg).[159] Mutations that affect development in *Drosophila* have also been discovered in genes involved in glycosaminoglycan biosynthesis. These genes include *sugarless*, which encodes a UDP-glucose dehydrogenase homolog required for synthesis of UDP-glucuronic acid; *sulfateless*, which encodes an *N*-deacetylase/*N*-sulfotransferase homolog; and *tout-velu*, which encodes an HS-specific copolymerase homologous to EXT1. Mutations in these genes appear to affect pathways involving the growth factors Dpp, Hh, Wg, and FGF. Enzymes that determine specific sulfation patterns in HS are also important in development. For example, down-regulation of *Drosophila* 3-*O*-sulfotransferase-B by RNA interference causes neural abnormalities due to defects in Notch signaling pathways.[66] The mechanisms by which HSPGs affect these growth factors are not entirely clear. HSPGs may serve as coreceptors that facilitate activation of signaling receptors on the cell surface as in the case of FGF, they may control diffusion of growth factors from their sites of production and thereby assist in formation of morphogenic gradients in tissues, or they may be involved in stabilization or clearance of the growth factors or their receptors.

Mutations that affect vertebrate HSPGs also cause developmental abnormalities.[108] For example, Simpson-Golabi-Behmel syndrome is a human disorder caused by mutations in glypican-3. This syndrome is characterized by overgrowth of many somatic tissues, including heart, kidneys, and bone, as well as increased susceptibility to Wilms tumors and neuroblastoma. A murine knockout of glypican-3 produced a similar phenotype. Other examples include mutations in the HS copolymerase genes *EXT1* and *EXT2*, which cause familial exostoses (cartilaginous tumors of the growth plates of bones), and targeted mutations in the murine HS 2-*O*-sulfotransferase gene, which lead to renal agenesis as well as eye and skeletal defects. Many other examples exist in which specific biosynthetic modifications of HS chains appear to be important for particular aspects of development.[160]

Lipoprotein Metabolism

HSPGs bind to lipoprotein lipase and anchor this enzyme to the luminal surface of endothelial cells, where it hydrolyzes triglycerides in circulating lipoprotein particles.[18] The lipoprotein particles also interact with endothelial HSPGs through their apolipoprotein B or E components. Interestingly, lipoprotein lipase is synthesized by myocytes and adipocytes, and transport of the enzyme from the basal to the luminal side of the endothelial cell appears to require HSPGs on the basal surface of the cell.[161] Intravenous administration of unfractionated or low molecular weight heparin displaces lipoprotein lipase from the endothelium into the circulation, from which the enzyme is cleared by hepatic receptors.[162] By this mechanism, repeated exposure to heparin may contribute to the elevated serum triglyceride levels observed in patients undergoing hemodialysis and play a role in the development of atherosclerosis.

HSPGs are expressed by endothelial cells that line the hepatic sinusoids and the underlying hepatocytes and are present in the perisinusoidal space (space of Disse).[163] Genetic data indicate that the HSPG syndecan-1 acts as an independent receptor for the clearance of triglyceride-rich lipoproteins in the liver.[164] Moreover, HS chains of syndecan-1 bind to plasma triglyceride-rich lipoproteins. Triglyceride-rich lipoprotein uptake depends less on overall charge of the HS chains than on uronic acid 2-*O*-sulfation[165] and glucosamine *N*-sulfation,[166] with glucosamine 6-*O*-sulfation making little contribution.[163] These data suggest that some familial human dyslipidemias may

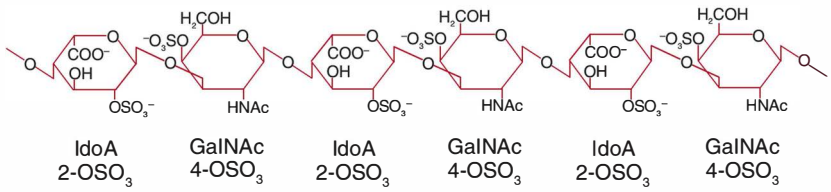

FIGURE 45.6 Structure of a dermatan sulfate hexasaccharide with high affinity for HCII. IdoA, iduronic acid; GalNAc, *N*-acetylgalactosamine.

be explained by polymorphisms in genes encoding syndecan-1 and enzymes involved in HS biosynthesis.

ACTIVATION OF HEPARIN COFACTOR II BY DERMATAN SULFATE

The rate of inhibition of thrombin by HCII increases more than 1,000-fold in the presence of heparin, HS, or dermatan sulfate.[167] The HCII–thrombin reaction is also stimulated by a variety of other sulfated polyanions, several of which have been studied as potential antithrombotic agents. They include naturally occurring compounds such as chondroitin sulfate E[168] and fucoidan[169] as well as semisynthetic polyanions such as dextran sulfate,[170] pentosan polysulfate,[171,172] and sulfated bis-lactobionic acid amide.[173]

HCII is unique in its ability to be stimulated by dermatan sulfate and binds to a minor subpopulation of dermatan sulfate oligosaccharides.[174] The high-affinity binding site for HCII in porcine skin dermatan sulfate is a tandem repeat of three iduronic acid 2-*O*-sulfate → *N*-acetylgalactosamine 4-*O*-sulfate disaccharide subunits (see FIGURE 45.6). The HCII-binding site in dermatan sulfate from tissues other than porcine skin may also include iduronic acid → *N*-acetylgalactosamine 4,6-*O*-disulfate subunits.[175–177] Unlike antithrombin, HCII binds nonspecifically to heparin oligosaccharides at least four monosaccharide units in length.[178]

Hexasaccharide sequences with high affinity for HCII are present at low abundance in dermatan sulfates of mammalian origin.[174] Dermatan sulfates with the same backbone structure but with different patterns of sulfation are present in ascidians (marine invertebrates commonly known as sea squirts). Ascidian dermatan sulfates have a high content of iduronic acid 2-*O*-sulfate residues, but they differ in the pattern of sulfation of the *N*-acetylgalactosamine units. For example, the *N*-acetylgalactosamine residues of *Halocynthia pyriformis* are predominantly 4-*O*-sulfated,[179] whereas those of *Ascidia nigra* are quantitatively 6-*O*-sulfated.[180] Dermatan sulfates stimulate thrombin inhibition by HCII with the following relative potencies: *H. pyriformis* > mammalian >> *A. nigra*.[179,180] These results indicate that the ability of dermatan sulfate to activate HCII is not simply a function of the overall charge density of the polymer, because the total sulfate contents of the ascidian dermatan sulfates are similar; instead, activity appears to require the presence of *N*-acetylgalactosamine 4-*O*-sulfate residues.

Mice with targeted deletion of the gene for HCII have been produced independently in two laboratories.[79,181] He et al.[79] found that HCII$^{-/-}$ mice develop normally and have no evidence of spontaneous thrombosis despite the complete absence of HCII antigen and dermatan sulfate–dependent thrombin inhibitory activity in their plasma. Subsequently, Aihara et al.[181] reported that homozygosity for the HCII–null allele is embryonically lethal and suggested that the more severe phenotype of their knockout could be due to minor differences in genetic background. Both groups found that a decreased level of circulating HCII promotes the accumulation of smooth muscle cells in the arterial intima after mechanical injury, and Vicente et al.[182] reported that intravenous administration of dermatan sulfate (but not heparin) during the first 48 hours after injury markedly inhibits neointima formation in HCII$^{+/+}$ mice but not in HCII$^{-/-}$ mice. Both groups also noted that HCII deficiency promotes atherosclerosis in apolipoprotein E (apoE)–null mice fed a cholesterol-rich diet.[181,182] These observations are consistent with experimental work implicating thrombin in the pathogenesis of neointima formation and atherosclerosis.[183] Furthermore, human studies have demonstrated an inverse correlation between plasma HCII levels and in-stent restenosis[184,185] or atherosclerosis,[186–188] although in one study baseline HCII levels did not predict the subsequent occurrence of symptomatic coronary artery disease.[189] HCII deficiency also increases the severity of angiotensin II–induced cardiomyopathy (concentric remodeling and cardiac fibrosis) in mice.[190]

He et al.[79] discovered that thrombi form more rapidly in the carotid arteries of HCII$^{-/-}$ mice in comparison to wild-type mice following photochemically induced endothelial injury. Subsequent experiments suggested that the antithrombotic effect depends upon interaction of HCII with dermatan sulfate in the vessel wall.[191] Thus, intravenous injection of either wild-type recombinant HCII or a variant with low affinity for HS (K173H) corrected the abnormally short thrombosis time of HCII-deficient mice, while a variant with low affinity for dermatan sulfate (R189H) did not. Immunohistochemical studies demonstrated that dermatan sulfate is present predominantly in the adventitia of the mouse carotid artery, whereas HS is found mainly in the intima and media. When HCII was incubated with frozen sections of the mouse carotid artery, it bound to the adventitia, and binding was abolished by pretreatment of the section with chondroitin B-lyase, which specifically cleaves glycosidic linkages in dermatan sulfate. HCII was undetectable in the wall of the uninjured carotid artery, but it became concentrated in the adventitia following endothelial injury. These results support the hypothesis that HCII interacts with dermatan sulfate in the vessel wall after disruption of the endothelium and that this interaction regulates thrombus formation *in vivo*.

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CHAPTER

46 Endothelial Cell–Blood Cell Interactions

KLAUS LEY

Interactions between monocytes, platelets, and endothelial cells are intimately involved with and are of key importance for the regulation of hemostasis, thrombosis, inflammation, and atherosclerosis. Although other cells such as neutrophils and T lymphocytes participate, the monocyte–platelet–endothelial cell axis has emerged as a predominant factor in vascular disease and thrombosis. This chapter introduces the molecular basis of the adhesive interactions among the three cell types, explores the crosstalk between hemostasis, thrombosis, and inflammation, and discusses the pathophysiologic significance of these cell–cell interactions with an emphasis on vascular disease.

CELLULAR AND MOLECULAR BASIS

Monocytes

Monocytes are myeloid cells produced in the bone marrow. Although originally thought to be a homogeneous population, it has become clear that in human and mouse blood at least two subpopulations of monocytes exist.[1,2] One type is characterized by a more inflammatory phenotype while the other type can migrate along the vascular endothelium.[3] Dendritic cells are the most important antigen-presenting cells of the immune system. Both dendritic cells and monocytes are derived from macrophage-dendritic cell progenitors in the bone marrow.[4,5] In mice, a committed blood precursor for dendritic cells (pre-DC) has been described, which is distinct from monocytes.[6] Both "inflammatory" and "resident-type" monocytes express adhesion molecules, chemokine receptor, and cytokine receptor molecules that support interaction with platelets and endothelial cells (Table 46.1). It is unclear whether the human blood monocyte subsets correspond to the mouse subsets.[7,8]

Most adhesion molecules are integral transmembrane cell surface molecules that can bind to the same (homotypic), or other cell surface molecules (heterotypic), or molecules in the extracellular matrix. Most adhesion molecules provide mechanical strength to the interaction between cells by mechanically linking the extracellular environment to the intracellular cytoskeleton through reversible interactions. Many if not all cell adhesion molecules also transduce signals into the cells upon engagement by extracellular ligands.

Monocyte Adhesion Molecules

Integrins

Integrins are transmembrane $\alpha\beta$ heterodimers that bind many extracellular matrix proteins and certain immunoglobulin-like adhesion molecules to other cells.[9,10] Most integrins require conformational activation to support binding. The mechanisms of integrin activation have been studied in detail for $\alpha_V\beta_3$,[11] an integrin also expressed on monocytes, and lymphocyte function-associated antigen 1(LFA-1),[12] an integrin highly expressed on monocytes and other leukocytes. Activation of $\alpha_x\beta_2$[13] and $\alpha_4\beta_1$ integrins[14] has also been studied. Both are expressed on blood monocytes at low and high levels, respectively. Integrin activation is probably initiated by the binding of the intracellular cytoskeletal adaptor molecules talin and kindlin-3 to the integrin β chains,[15,16] which causes extension of the extracellular domain that exposes the ligand-binding site and greatly increases the on-rate of ligand binding (FIGURE 46.1A). This process is called inside-out signaling, because the change in the extracellular domain of the integrin is brought about by intracellular processes. The mechanism of activation-induced conformational change is thought to apply fairly generally to many, if not all integrins.[10] However, the regulation of affinity by reducing the dissociation of the ligand from the integrin is much less clear. Although conformational changes in the ligand-binding domain have been reported,[17] it is not clear whether these changes are directly linked to integrin extension or could be regulated separately.[14]

Integrins may first undergo some initial conformational change (probably extension) through inside-out signaling, followed by ligand binding, which then causes outside-in signaling that leads to full activation and strong binding[10,18,19] (FIGURE 46.1B). However, not all experimental data are consistent with this model.[20] In addition, integrins can also rearrange in the plasma membrane to cluster and form patches, which results in enhanced binding (FIGURE 46.1C). This rearrangement and clustering probably happens after ligand binding, results in increased binding of multivalent ligands, and is called avidity change. It does not result in increased affinity for the monovalent ligand.[21] Under *in vivo* conditions, cell activation probably results in a combination of integrin affinity and avidity increase. Although this can be tested by looking at monovalent versus polyvalent ligand binding, the role of avidity and affinity regulation remains controversial.[10,18,21]

Of the 24 known integrins, mature blood monocytes express only a few. The most abundant monocyte integrin is $\alpha_4\beta_1$ integrin or very late antigen-4 (VLA-4) (CD49d/CD29). It is composed of a 150 kDa α_4 chain that can undergo proteolytic cleavage and a noncovalently associated 130 kDa β_1 chain. $\alpha_4\beta_1$ integrin is preferentially expressed on cell surface projections that are often called microvilli, but resemble ridges rather than true villous processes.[22] This position is believed to facilitate the interaction of $\alpha_4\beta_1$ integrin with its ligands under conditions of flow. The most important ligands for $\alpha_4\beta_1$ integrin include

Table 46.1

Cell surface molecules on monocytes relevant to cell–cell interaction

	"Inflammatory" Monocytes		"Resident" Monocytes			
Molecule	**Mouse**	**Human**	**Mouse**	**Human**	**Main Function**	**Secondary Function**
CD11a/CD18 ($\alpha_L\beta_2$, LFA-1)	+	+	+	+	Inducible adhesion to ICAM-1, 2	Reduces rolling velocity (neutrophils)
CD11b/CD18 ($\alpha_M\beta_2$, Mac-1)	+	+	+	+	Inducible adhesion to C3bi, ICAM-1, 2, fibrinogen	Phagocytosis
CD11c ($\alpha x\beta 2$)	–	+	–	+	Inducible binding to VCAM-1	Complement binding
CD14	–	+	–	low	LPS coreceptor	
CD16 (FcγRIII)	+	–	+	+	Fc receptor for IgG	
CD64 (FcγRI)		+		–		
CX3CR1	low	+	high	high	Binds CX3CL1, activates monocytes	May mediate adhesion
CCR1	n.d.	+	n.d.	–	Binds chemokines CCL3, 5, 7, 14, 15, 16, 23; causes activation	Chemokine binding causes arrest of rolling cells
CCR2	+	+	–	–	Binds chemokines CCL2, 7, 12, 13, causes activation, chemotaxis	Soluble CCL2 can cause monocyte arrest
CCR4	n.d.	+	n.d.	–	Binds chemokines CCL17, 22	
CCR5		–		+		
CCR7	n.d.	+	n.d.	–	Binds chemokines CCL19, 21	Promotes mature DC migration to lymphatic organs
CXCR1 (IL-8 receptor)	–	low	–	–	Binds chemokines, mainly CXCL8	
CXCR2	n.d.	+	n.d.	–	Binds chemokines CXCL1, 2, 3, 5, 6, 7, 8	Chemokine binding causes arrest
CXCR3	n.d.	–	n.d.	–	Binds chemokines CXCL9, 10, 11	Promotes type 1 inflammation
CXCR4	n.d.	low	n.d.	+	Binds CXCL12, induces arrest	Release from bone marrow
Ly-6C (Gr-1)	+	–	–	–	No known function	
CD49b ($\alpha_2\beta_1$, VLA2)	+	n.d.	–	n.d.	Inducible binding to collagen	
CD49d ($\alpha_4\beta_1$, VLA4)	+	n.d.	+	n.d.	Inducible binding to VCAM-1, fibronectin	Monocyte arrest on atherosclerotic lesions
CD62L (L-selectin)	+	n.d.	–	n.d.	Binds PSGL-1, secondary tethering	Binds PNAd, monocyte rolling in lymph nodes
CD162 (PSGL-1)	+	+	+	+	Binds P-selectin on platelets, endothelial cells, microparticles	Binds L-selectin for secondary tethering, binds E-selectin
Myeloperoxidase		high		Low		
MHC-II		low		High		
Tissue factor	Inducible, but unknown in which subsets				Initiator of coagulation	Signaling into monocyte
PECAM-1 (CD31)	Most or all				Transendothelial migration	Monocyte activation

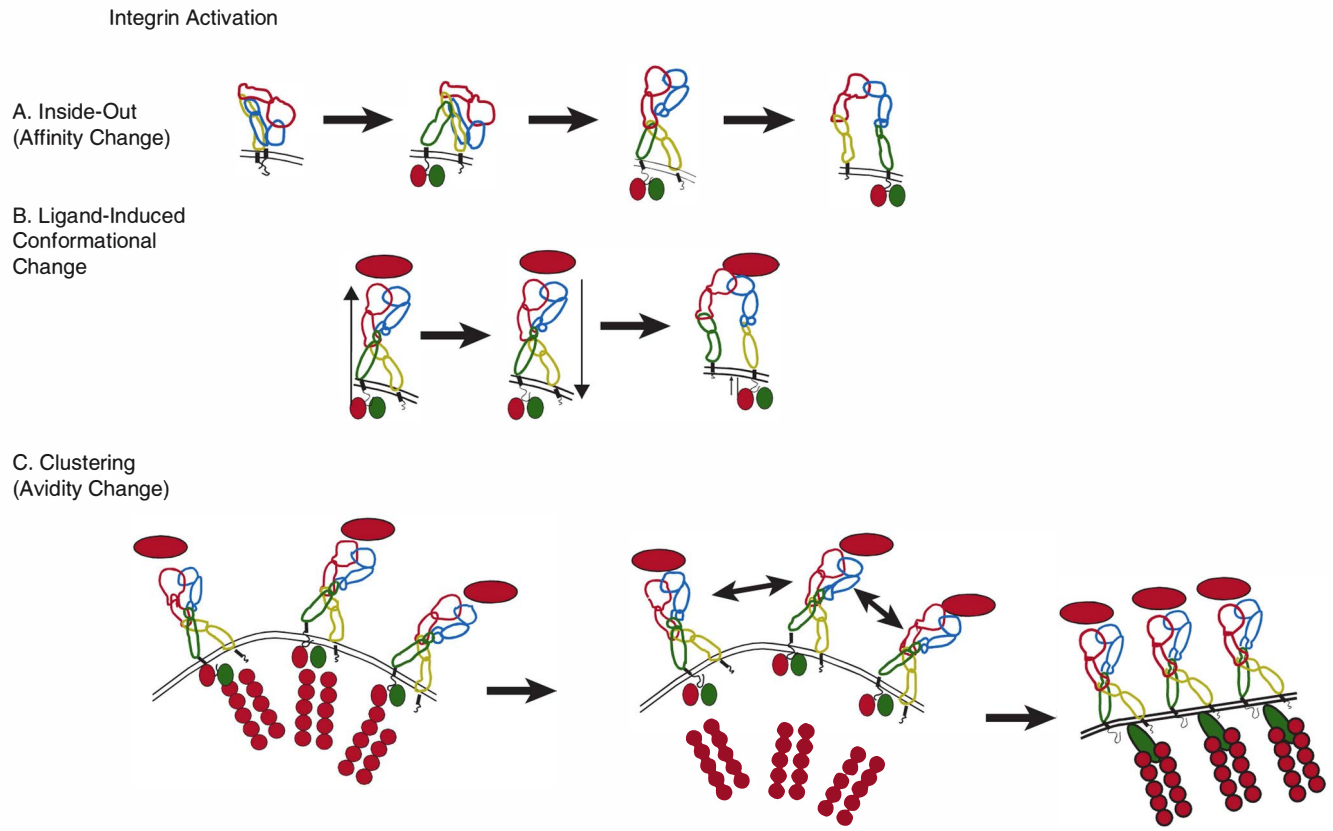

FIGURE 46.1 Regulation of integrin ligand binding activity. **A**: Integrin affinity regulation by conformational changes in the α and β chains (*red-yellow and blue-green, respectively*), resulting in increased availability and accessibility of ligand binding sites (third diagram) and, through an additional activation step, increased affinity for monovalent ligands (last diagram). During conformational activation, talin and kindlin-3 (*green and red ellipses*) bind to the integrin β cytoplasmic tail, causing the integrin "legs" to move apart. **B**: Ligand-induced activation and outside-in signaling. After activation as in (A, inside-out signaling, *arrow up*), ligand (*red ellipse*) binding to integrin induces outside-in signaling (*arrow down*) and bond maturation. **C**: Integrin avidity regulation by lateral mobility/clustering. Transient release of integrins from cytoskeletal anchorage (actin filaments, represented as *strings of circles*) allows integrin rearrangement and clustering in the plane of the cell membrane, resulting in increased avidity for multivalent ligands. It is not known whether the release occurs at the level of talin-actin binding. Integrins bind actin through talin, and various other linker proteins (not shown).

the vascular cell adhesion molecule 1 (VCAM-1) on endothelial and other cells (Table 46.2) and the heparin-binding region of alternatively spliced fibronectin expressed in the extracellular matrix and on the luminal surface of inflamed endothelial cells.[23] Like other integrins, $\alpha_4\beta_1$ integrin can probably undergo conformational activation.[14,24,25] This process of affinity regulation can be triggered by monocyte activation, for example, through chemokines. Gene-targeted mice lacking either α_4[26] or β_1[27] are not viable. Blocking $\alpha_4\beta_1$ with a monoclonal antibody or a peptide based on the fibronectin sequence ILDV reduces atherosclerosis in mice,[28] suggesting that $\alpha_4\beta_1$ is important in monocyte recruitment to atherosclerotic lesions.

The integrin $\alpha_M\beta_2$ (CD11b/CD18) is also known as Macrophage-1 or Mac-1. Mac-1 antibodies were some of the earliest monocyte-macrophage specific antibodies described. Although Mac-1 is also expressed on neutrophils, most of its function seems to be related to monocyte-macrophages. First, Mac-1 participates in monocyte adhesion to various substrates including endothelial cells. Second, Mac-1 is an important receptor for the complement and is also known as complement receptor 3 (CR3). Mac-1 binds complement C3bi and is critically involved in phagocytosis of complement-coated bacteria and particles. Mac-1 engagement promotes a proinflammatory response, including a respiratory burst with vigorous oxygen radical production, actin polymerization, induction of nitric oxide synthase, and shape change. Interestingly, under flow conditions such as those achieved in flow chambers *in vitro*, or isolated perfused vessels *ex vivo*, Mac-1 does not appear to contribute to monocyte adhesion to endothelial cells.[29] Mac-1 has been shown to lower the rolling velocity of neutrophils,[30] but the role of Mac-1 in monocyte rolling has not been studied. Mice lacking Mac-1, prepared by targeting the gene for α_M, are viable, fertile, and healthy under vivarium conditions.[31] There is no evidence that these mice are protected from atherosclerosis, but their response to vascular injury is blunted.[32] Like all β_2 integrins, Mac-1 has an I-domain with homology to the von Willebrand factor (vWF) A-domain inserted in its α subunit, which contains the ligand-binding site. Mac-1 binds many other ligands

Table 46.2

Ligands for monocyte and platelet integrins

Integrin	Ligands	Function
$\alpha_4\beta_1$	VCAM-1	Adhesion to endothelial cells
	Fibronectin	Adhesion to extracellular matrix
Mac-1	Complement C3bi	Phagocytosis of opsonized particles
$\alpha_M\beta_2$	Coagulation factor Xa	Assembly of prothrombinase complex
	Fibrinogen	Bridging between monocytes and platelets
	Intercellular adhesion molecule-1 (ICAM-1)	Adhesion to endothelial and other cells
	Intercellular adhesion molecule-2 (ICAM-2)	Adhesion to endothelial cells and platelets
	Denatured collagen	Migration, phagocytosis?
	Denatured albumin	Migration, phagocytosis?
	Leishmania GP63	Uptake of leishmania
	Bortedella FHA	Uptake of Bortedella
	Fibronectin	Adhesion to extracellular matrix
CD11c/CD18	Complement C3bi	Phagocytosis of opsonized particles
$\alpha_x\beta_2$	VCAM-1, ICAM-1	Monocyte adhesion
	Fibrinogen	Bridging between monocytes and platelets
LFA-1	Intercellular adhesion molecule-1 (ICAM-1)	Adhesion to endothelial and other cells
$\alpha_L\beta_2$	Intercellular adhesion molecule-2 (ICAM-2)	Adhesion to endothelial cells and platelets
$\alpha_V\beta_3$	Vitronectin	Bone remodeling?
	Entactin	Unknown
	L1	Unknown
$\alpha_{IIb}\beta_3$	Fibrinogen	Binds immobilized Fg without activation
	Fibronectin	Induced by GPIb binding to vWF
	vWF	Promotes CD40L shedding
	Vitronectin	
	CD40L	
$\alpha_2\beta_1$	Collagens	On platelets, requires activation by GPVI

including fibrinogen and coagulation factor X (Table 46.2). Mac-1 is thought to be involved in assembling prothrombinase on the monocyte surface and may be able to support platelet binding to monocyte through a fibrinogen bridge between $\alpha_{IIb}\beta_3$ on platelets and Mac-1 on monocytes.[33] Although Mac-1 deficient mice have no obvious defect in hemostasis, Mac-1 could participate in monocyte activation and the delivery of tissue factor to sites of thrombosis.[34] Human, but not mouse monocytes also express a closely related integrin, $\alpha_x\beta_2$, which is also a complement receptor, alternatively known as CR4. Abundant α_x expression is found on dendritic cells. Like Mac-1, $\alpha_x\beta_2$ binds the intercellular adhesion molecule 1 (ICAM-1), C3bi, and denatured proteins,[35] but in addition VCAM-1 also.[36]

The LFA-1, or $\alpha_L\beta_2$ integrin (CD11a/CD18) is expressed on all leukocytes including monocytes. While LFA-1 is responsible for lymphocyte arrest, the sudden stopping of rolling cells upon activation[37] and participation in neutrophil arrest under flow, little is known about its function in monocytes. LFA-1 binds to cell surface immunoglobulins including InterCellular Adhesion Molecules ICAM-1 and-2, and has no known extracellular matrix ligands. Mice lacking LFA-1 were prepared by targeting the gene for αL[38] and are viable, healthy, and fertile under vivarium conditions. There are no reports of these mice having altered thrombosis, hemostasis, or atherosclerosis. Like the other β_2 integrins, LFA-1 has an I-domain and undergoes extensive conformational changes of the extracellular domain upon activation.[12]

The integrin $\alpha_V\beta_3$ is expressed on blood monocytes at a low copy number. Its expression increases upon differentiation to osteoclast-like cells. This integrin was initially called leukocyte response integrin[39] because it participates in inducing the respiratory burst associated with NADPH oxidase activation and oxygen free radical production in neutrophils. Ligands for $\alpha_v\beta_3$ integrin include vitronectin, entactin, and possibly the immunoglobulin-like adhesion molecule L1. Gene-targeted mice lacking α_V are not viable, whereas mice lacking β_3 have a defect in both $\alpha_V\beta_3$ on monocytes, neutrophils, and proliferating endothelial cells, and $\alpha_{IIb}\beta_3$ on platelets, which share the common β_3 subunit. The phenotype of these mice is dominated by the platelet defect (Glanzmann thrombasthenia-like), and these mice also have osteosclerosis, suggesting defective osteoclast function.[40] $\alpha_V\beta_5$ integrin is also a vitronectin receptor. Monocytes express $\alpha_2\beta_1$ integrin, a collagen receptor, at the mRNA and protein levels. $\alpha_5\beta_1$, a fibronectin receptor, $\alpha_{10}\beta_1$, a collagen receptor, and $\alpha_E\beta_7$, a receptor for E-cadherin, are found at the message level, but functional data have not been published.

Immunoglobulins

Blood monocytes express many immunoglobulin-like molecules. Of importance for this chapter is ICAM-1, because it supports homotypic aggregation of monocytes via LFA-1 and Mac-1 and because it can bind fibrinogen.[41] Mice with hypomorphic mutations in the ICAM-1 gene or lacking ICAM-1 entirely have no overt defect in hemostasis or thrombosis, but are somewhat protected from atherosclerosis in the C57BL/6 and apolipoprotein E knockout models.[42] However, these mice also lack ICAM-1 on endothelial cells, smooth muscle cells, lymphocytes, and many other cells. ICAM-2 and ICAM-3 are found in monocytes at the message level, but have no known function in monocytes.

Platelet endothelial cell adhesion molecule 1(PECAM-1) (CD31) is a homotypic adhesion molecule expressed on blood monocytes and has an important role in transendothelial migration and in monocyte activation.[43,44] Monocyte PECAM-1 interacts with PECAM-1 on endothelial cells during transmigration. PECAM-1-deficient C57BL/6 mice have no apparent defect in leukocyte and monocyte transmigration, demonstrating that PECAM-1-independent pathways of transmigration exist.[45]

Other immunoglobulin-like molecules expressed on monocytes include major histocompatibility complex (MHC) class II, which is important in antigen presentation, but is not fully induced until monocytes differentiate to macrophages. CD83 is also expressed and has costimulatory functions in macrophages, but no known function in blood monocytes.

Selectins and their Ligands

L-selectin (CD62L) is expressed on "inflammatory" blood monocytes and most other leukocytes. Its most important function is in lymphocyte homing to secondary lymphatic organs,[46] but it is also involved in inflammation.[47] Like the other selectins, L-selectin can mediate leukocyte rolling, a passive motion of leukocytes down a vessel wall driven by the blood flow and the forces exerted on the loosely attached cell. During rolling, molecular bonds form at the leading edge and continually break at the trailing edge of the cell, allowing the leukocyte to stay in contact with the endothelium without actually stopping[48] (FIGURE 46.2). Rolling is thought to serve to "scan for" inflammatory stimuli, and rolling cells can stop (arrest) in response to appropriate stimuli.[18,49] On neutrophils, L-selectin is expressed on the tips of microvilli[50] and can be rapidly shed upon cell activation by a protease-dependent mechanism involving tumor necrosis factor-α-converting enzyme (TACE) (ADAM-17).[51] Although L-selectin has been shown to support monocyte rolling on L-selectin ligands in flow chambers, it is not known whether L-selectin–mediated monocyte rolling on endothelial cells serves a physiologic function. Endothelial ligands for L-selectin known as peripheral node addressins are expressed in endothelial cells of lymphoid organs, but L-selectin ligands on endothelial cells outside lymphoid organs have not been identified. It is tempting to speculate that monocyte L-selectin may enhance monocyte recruitment to atherosclerotic lesions by nucleating monocyte-monocyte interactions through binding

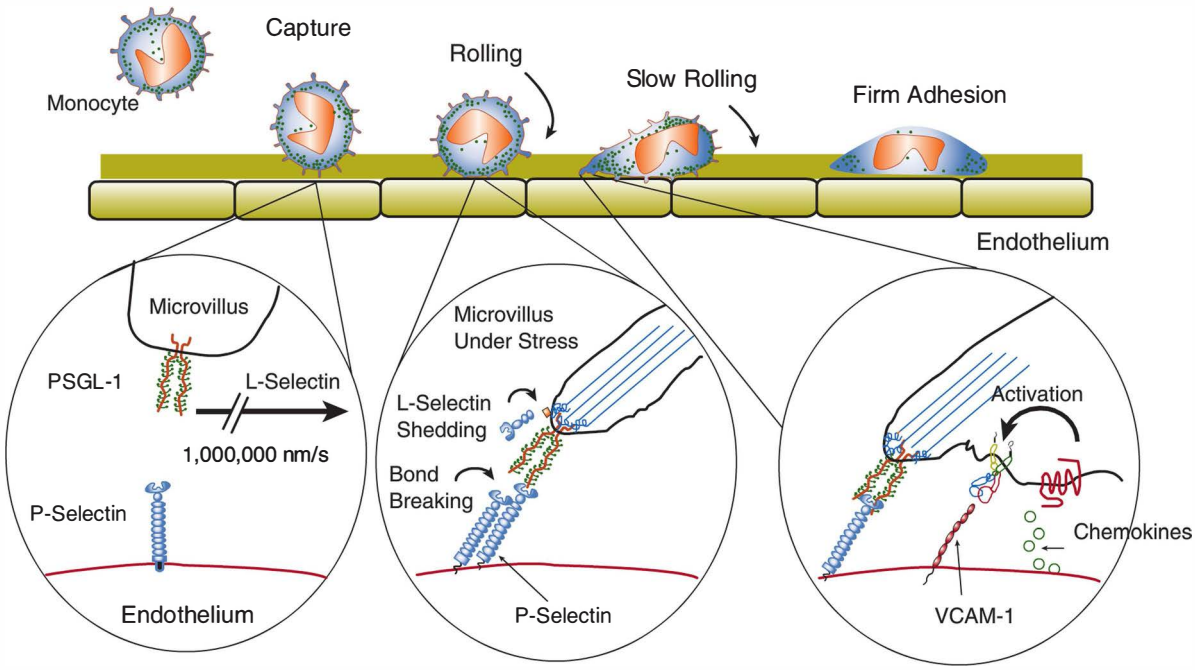

FIGURE 46.2 Sequence of monocyte capture, rolling, slow rolling, and adhesion on endothelial cells. Flow from left to right, endothelial surface layer shown in *green*. Primary capture or tethering is initiated by monocyte PSGL-1 binding to endothelial P-selectin (*left insert*). Note high velocity of monocyte (1 mm/s). The *middle insert* shows a P-selectin/PSGL-1 bond at the trailing edge of a rolling monocyte. Applied stress induces faster bond breakage and may also activate cleavage of L-selectin. The *right insert* shows VLA-4/VCAM-1–dependent bond required for slow rolling. Monocyte activation via surface-immobilized chemokine binding to chemokine receptor resulting in integrin affinity upregulation and firm binding to endothelial ligands such as VCAM-1 (shown here).

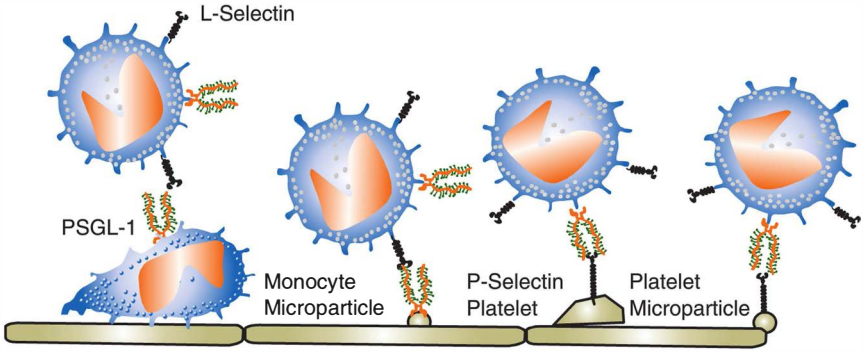

FIGURE 46.3 Secondary capture or secondary tethering. Monocytes can be captured by binding to other, already adherent monocytes or other leukocytes **(left)** or to monocyte-derived microparticles **(middle)**. Both require L-selectin on the circulating and PSGL-1 on the adherent monocyte or microparticle. Monocytes can also adhere to platelets or platelet-derived microparticles **(right)**, which requires monocyte PSGL-1 and platelet P-selectin.

to P-selectin glycoprotein ligand-1 (PSGL-1, CD162, see below) in a process called secondary capture[52] (FIGURE 46.3). Secondary capture is initiated when a vessel wall-adherent monocyte or monocyte-derived microparticle exposes PSGL-1 to other monocytes that pass by in the free stream. These cells can transiently bind to the adherent monocyte through L-selectin and then attach to the endothelium downstream from the nucleation site. While this process is certainly plausible and has been observed to occur in mouse aortas,[53] no direct evidence has been provided to support its importance in atherosclerosis, thrombosis, or hemostasis.

The most important selectin ligand on monocytes is PSGL-1, (CD162). PSGL-1 is somewhat of a misnomer, because PSGL-1 also binds L- and E-selectins with similar affinity as P-selectin.[54] All monocytes, like most leukocytes, express PSGL-1 protein on the cell surface, but not all cell surface-expressed PSGL-1 can bind selectins. PSGL-1 binding to selectins is regulated by a series of glycosyltransferases and sulfotransferases required to make it a functional selectin ligand (Table 46.3). Blood monocytes express fucosyltransferase VII, core2 *N*-acetylglucosaminyltransferase-I, and at least one sialyltransferase. Therefore, PSGL-1 on monocytes is constitutively active and can bind all three selectins. PSGL-1 is a covalent homodimer and, like VLA-4 and L-selectin, is expressed on microvilli.[55]

Recently, PSGL-1 has been shown to be of key importance for the delivery of tissue factor to sites of thrombosis.[56,57] PSGL-1-expressing microparticles, presumably derived from monocytes, deliver tissue factor to sites of thrombosis in a PSGL-1 and P-selectin-dependent process. Apparently, the generation of these microparticles can be induced by soluble P-selectin[58] in a process that requires PSGL-1 expression. Taken together, PSGL-1 is one of the most important molecules connecting inflammation with hemostasis and thrombosis. PSGL-1 deficient mice show reduced inflammatory responses in many models and have a remarkable defect in tissue factor recruitment to sites of vascular injury and thrombosis.[56,57] The importance of PSGL-1 is underlined by epidemiologic evidence that humans expressing PSGL-1 alleles that encode shorter isoforms (fewer tandem repeats) are relatively protected from cardiovascular disease.[59] The shorter versions of PSGL-1 support less monocyte-platelet adhesion.

Recently, P-selectin was also found to be expressed on peritoneal macrophages[60] and in foam cells found in the neointima

Table 46.3

Glycosyl- and sulfotransferases relevant to selectin binding

Enzyme	Abbreviation	Involved in Ligands for:	Expression
Fucosyl transferase-VII	FucT-VII	L, E, P	myeloid, act. T cells, HEV
Fucosyl transferase-IV	FucT-IV	L, E, P[a]	myeloid, some HEV
core2 $\beta_{1,6}$ glucosaminyl transferase	C2GlcNAcT-I	L, P	myeloid, act. T cells B cells
sialyl 3-galactosyltransferase	ST3Gal	L, E, P	ubiquitous
$\beta_{1,4}$ galactosyl transferase	$\beta_{1,4}$GalT-I	P[b]	ubiquitous
tyrosine protein sulfotransferases	1, 2 TPST-1, 2	L, P	ubiquitous
HEC-glucosaminyl sulfotransferase	HEC-GlcNAc6ST	L	HEV, chronically inflamed ECs

[a]Only in myeloid cells, not T cells.
[b]Not yet tested for E, L; probably involved for L.
From Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. *Nat Rev Immunol* 2004;4:325–335.

formed after vascular injury in atherosclerotic mice.[61] There is no evidence for P-selectin expression on blood monocytes.

Integrin-Associated Molecules

The urokinase plasminogen activator receptor (UPAR) is expressed on blood monocytes and sharply upregulated by activation. UPAR associates with $\alpha_1\beta_2$, $\alpha_M\beta_2$, and various other integrins. UPAR coexpression increases the affinity of integrins and can change ligand specificity.[62] Tetraspanins have also been reported to associate with integrins and change their activation status.[63] A third integrin-associated molecule is CD47, which is associated with leukocyte activation and transendothelial migration.[64] These integrin-associated cell surface glycoproteins can change integrin affinity for ligand, ligand specificity, and serve signaling functions.[65,66]

Monocyte Chemokines

Chemokines are small peptides that bind to G-protein-coupled receptors on monocytes, lymphocytes, neutrophils, platelets, and many other cells.[67] Most chemokines are classified according to the location of two conserved pairs of cysteins that either do (CXC), or do not (CC) have an intervening amino acid. Chemokines are named for their ability to induce a chemotactic response, that is, initiate migration of a receptor-bearing cell from areas of lower to areas of higher concentration of chemokines. However, chemokines have many other important functions. Some, but not all can induce rapid integrin activation by inducing conformational changes and/or clustering of integrins (see above). When chemokines act on rolling leukocytes, such integrin activation often results in arrest, converting the rolling motion to firm adhesion.[49,68] Inflammatory chemokines such as interleukin-8 (CXCL8) also induce degranulation of secondary and tertiary granules in neutrophils and monocytes, and induce a respiratory burst resulting in production of oxygen-derived free radicals. Stromal cell-derived factor-1α (SDF-1α, CXCL12) has homeostatic functions in the bone marrow where it supports progenitor cells and hematopoiesis. Other chemokines participate in the organogenesis of lymphatic organs.

In the context of this chapter, we will discuss chemokines elaborated by monocytes, platelets, and endothelial cells. These chemokines can have autocrine, paracrine, and remote effects. Monocytes express low levels of CCL2, 3, 4, 20 and CXCL8, which are heavily and immediately upregulated upon activation. CCL7 and 8 as well as CXCL9 and 10 show only modest upregulation. By contrast, CCL5 is downregulated in activated monocytes. CCL22 (macrophage-derived chemokine [MDC]) is an inflammatory chemokine that shows low expression on monocytes, but is induced by macrophage differentiation. CCL2 (monocyte chemotactic protein-1[MCP-1]), CCL3 (macrophage inflammatory protein 1 alpha (MIP-1α)), CCL4 (MIP-1β), CCL7 (MCP-3), CCL8 (MCP-3) and CXCL8 (interleukin-8) are considered proinflammatory chemokines, because they can activate neutrophils and monocytes. Injection of these chemokines into experimental animals results in a rapid and significant accumulation of monocytes and/or neutrophils. CCL20 (liver activation regulated chemokine [LARC]), MIP-3α) is considered a constitutive chemokine that was first described to be expressed in the intestine and skin. Its function in monocytes is not known. CXCL9 (Mig) and CXCL10 (IP-10) are considered classical T-helper1 chemokines that are proinflammatory and can attract effector T lymphocytes by binding to their CXCR3 receptor. Although CCL5 is considered an inflammatory chemokine, it is constitutively expressed in many organs including the lung and in some blood cells, predominantly platelets.[69] Overall, it is clear that monocytes produce proinflammatory chemokines that can sustain and increase inflammation, attract more monocytes, neutrophils, and inflammatory T cells. In the context of this chapter it is interesting to note that monocytes can synthesize several chemokines including CCL3, CLL5, and CCL22 that can help activate platelets and promote platelet aggregation in the presence of suboptimal concentrations of agonist. Platelets express the corresponding chemokine receptors CCR1, 3, and 4 (Table 46.4).

Monocyte Chemokine Receptors

Chemokine receptors are G-protein-coupled receptors that transduce signals via phospholipase C (PLC) and phosphatidylinositol-3-kinase (PI3K). CCR2 is the main receptor for CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), and CCL13 (MCP-4). MCP-1 was the first monocyte-specific chemoattractant to be discovered. Atherosclerosis-prone mice lacking CCL2[70] or CCR2[71] are about 50% protected from atherosclerosis in all models tested. CCR2 is not only important in monocyte recruitment to sites of inflammation, but also crucial for their release from the bone marrow,[72] and CCR2-deficient mice have reduced circulating monocyte numbers. Monocyte exit from the bone marrow compartment is mainly controlled by the CCR2 ligand CCL7 (MCP-3).[73] Beyond CCR2, monocytes express many other chemokine receptors, including CCR1, 5, CXCR1, 2, and 4, and CX3CR1. CXCR1 and 2 are of special interest, because they elicit no chemotactic response in monocytes, but can very effectively activate monocyte integrins to promote arrest.[29] In fact, CXCR2 on monocytes is the first example of a "pure" arrest chemokine receptor, because it only induces arrest of rolling monocytes but no other responses like chemotaxis. CCR1 is upregulated in activated monocytes and stays expressed in macrophages, which suggests possible autocrine effects of CCR1 chemokines such as MIP-1α and RANTES, which are also produced by monocytes. CCR1 is the receptor responsible for RANTES-induced monocyte arrest.[74] CCR5 is also expressed on monocytes, another receptor for the chemokines RANTES, MIP-1α and MIP-1β. RANTES has been shown to induce platelet–monocyte and monocyte–endothelial interactions.[69,75] CCR7 is not highly expressed in monocytes, but induced upon macrophage differentiation. CCR7 binds and responds to the secondary lymphoid tissue-expressed chemokines CCL19 and CCL21, attracting macrophages (and dendritic cells) to lymph nodes and Peyer patches. CXCR4 is very highly expressed on resting monocytes and downreguated upon activation. It stays low in macrophages. CXCR4 is believed to be involved in retaining monocytes in the bone marrow, but is also a very effective arrest chemokine[76] (Table 46.1). The fractalkine receptor CX3CR1 is highly expressed on "resident-type" blood monocytes and is involved in controlling monocyte development and survival.[77]

Endothelial Cells

Endothelial cells line all blood and lymph vessels. Endothelial cells are specialized by organ and even by vessel size (large artery, arteriole), or position in the circulatory system

Table 46.4

Cell surface molecules on platelets relevant to cell-cell interaction

Molecule	Main Function	Secondary Function
GPIbα	Binds vWF, mediates adhesion under high shear	Binds P-selectin
GPIIb/IIIa	Promotes platelet aggregation by binding fibrinogen	Binds monocyte Mac-1
GPVI	Main platelet receptor for collagen	
CD16	Obligatory co-receptor for GPVI	Binds Fc
P-selectin	Binds PSGL-1 on monocytes, microparticles, neutrophils	Anchors vWF
vWF	Binds GPIbα on other platelets, binds P-selectin	
ICAM-2	Binds LFA-1 on monocytes, neutrophils, lymphocytes	
CCR1	Receptor for MIP1α, RANTES	Augment responses to low-dose ADP
CCR3	Receptor for RANTES, eotaxin	
CCR4	Receptor for MDC	
CXCR4	Receptor for SDF-1α	
CD40L	Binds CD40 on monocytes and endothelial cells	GPIIb/IIIa on platelets
FcγRIIa	Binds Fc portion of IgG	Activates platelets

(arteriole, capillary, venule). The endothelial cell properties and molecules mentioned below apply to endothelial cells in a general sense. Most data are derived from experimental systems using cultured human umbilical vein endothelial cells.

Endothelial Cell Adhesion Molecules

Endothelial cells express a host of constitutive and inducible adhesion molecules, some chemokines, and some chemokine receptors (Table 46.5).

P-Selectin

In the context of this chapter, P-selectin is probably the most important endothelial adhesion molecule. As a member of the selectin class of adhesion molecules, it mediates leukocyte rolling (see FIGURE 46.2). P-selectin is prepackaged in Weibel-Palade bodies of endothelial cells, where it is intimately associated with vWF.[57] From Weibel-Palade bodies, P-selectin can be transported to the luminal surface by exocytosis induced by mild inflammatory stimuli. Endothelial P-selectin, once surface-expressed, can

Table 46.5

Cell surface molecules on endothelial cells relevant to cell-cell interaction

Molecule	Main Function	Secondary Function
P-selectin	Leukocyte/monocyte rolling, binds PSGL-1	Platelet rolling
E-selectin	Leukocyte/monocyte slow rolling, binds PSGL-1 and unknown ligand	leukocyte activation
PNAd	Lymphocyte rolling in HEV, binds L-selectin	
ICAM-1	Leukocyte/monocyte adhesion, binds LFA-1, Mac-1	binding Fg
ICAM-2	Leukocyte/monocyte adhesion, binds LFA-1	
VCAM-1	Monocyte adhesion, binds $\alpha_4\beta_1$	slow rolling
Fibronectin	monocyte adhesion, binds $\alpha_5\beta_1$, $\alpha_V\beta_3$ and $\alpha_4\beta_1$	
vWF	interaction with GPIb-V-IX on platelets	
GPIbα	interaction with vWF	binds Fg, Fn
CCL5 (RANTES)	monocyte arrest through CCR1	chemotaxis
CXCL1 (GRO-α)	monocyte arrest through CXCR2	chemotaxis
PSGL-1	Leukocyte rolling in intestinal microvessels	

also support platelet interactions with the endothelium.[78] Once platelets are activated, it is platelet and not endothelial P-selectin that is responsible for platelet–endothelial interactions.[69,75] Mice lacking P-selectin are about 50% protected from atherosclerosis[79] and show almost no neointima formation in response to vascular injury.[80] Experiments in bone marrow chimeric mice show that in both spontaneous atherosclerosis[81] and injury-accelerated neointima formation,[82] it is mainly the platelet and not the endothelial P-selectin that is responsible for inflammatory cell recruitment and neointimal growth. In addition to cell surface expression, both endothelial and platelet P-selectin can also be released into the plasma by alternative splicing and by a poorly understood shedding mechanism.[83]

Most of the knowledge about endothelial P-selectin function was derived from mouse experiments. However, the regulation of endothelial P-selectin expression is different in mice and primates, where P-selectin shows less sustained expression during inflammation.[84] It is possible that the function of human P-selectin is not quite as important as that of mouse P-selectin. There is some evidence that E-selectin is important for atherosclerosis in humans,[85] but less so in mice.[42] Human monocytes roll on human endothelial cells through P-selectin under shear. Human PSGL-1, like its mouse counterpart, can transmit proinflammatory and prothrombotic signals into monocytes.[86,87]

E-Selectin

Activated endothelial cells express E-selectin, which binds PSGL-1[88,89] and other unidentified ligands.[90] E-selectin ligand-1[91] and CD44 have been proposed as relevant E-selectin ligands on neutrophils.[92] Functionally, E-selectin ligands are heavily dependent on glycosylation by fucosyl transferase-VII and at least one sialyltransferase,[93] but unlike PSGL-1 binding to P-selectin, they do not require modification by core2GlcNAcT-I,[94] or sulfyltransferases. Like the other selectins, E-selectin supports leukocyte and monocyte rolling on inflamed endothelial cells *in vitro* and *in vivo*. E-selectin–dependent rolling is typically slower than P- or L-selectin–dependent rolling, suggesting that cells rolling through E-selectin can more closely scan the endothelial surface for activating signals. Neutrophils rolling on E-selectin induce the extended, but not high affinity conformation of LFA-1,[20,95] which is required for slow rolling. Endothelial E-selectin does not support platelet interactions, but very efficiently binds neutrophils and monocytes. E-selectin is required for an additional activating signal[96] that can arrest rolling neutrophils[97] and possibly monocytes. This signal is independent of Gαi-coupled receptors such as chemokine receptors.[20] E-selectin–deficient mice have no spontaneous defect in inflammation, platelet aggregation, or thrombus formation, suggesting that E-selectin function in mice overlaps with other rolling and activation molecules. Indeed, mice lacking both P- and E-selectin have a severe defect in neutrophil recruitment to sites of inflammation and show spontaneous pathology and altered hematopoiesis.[98,99] In humans, a "hyper-adhesive" polymorphism in the E-selectin gene correlates with an increased incidence of myocardial infarctions,[85] suggesting that E-selectin may be involved in atherosclerosis in primates.

ICAM-1

Endothelial cells constitutively express ICAM-1 (see above). Endothelial ICAM-1 expression is increased by inflammatory cytokines such as IL-1-β or tumor necrosis factor-alpha (TNF-α) and further enhanced in the presence of interferon-gamma (IFN-γ). Endothelial ICAM-1 is thought to be the major ligand supporting the binding of the β_2 integrins LFA-1 and Mac-1, but other ligands exist.[30] One of these other ligands is ICAM-2, a molecule with two extracellular immunoglobulin domains that is constitutively expressed on the endothelial surface and platelets, and is not upregulated by inflammatory mediators.

VCAM-1

Most endothelial cells express VCAM-1 upon cytokine stimulation. VCAM-1 is an immunoglobulin-like cell adhesion molecule with six to seven immunoglobulin domains. VCAM-1 is the most important endothelial ligand for VLA-4 ($\alpha_4\beta_1$ integrin), and intimately involved with monocyte recruitment to sites of inflammation and atherosclerosis. VCAM-1 expression is localized in areas at risk for atherosclerosis and precedes lesion formation.[100] Mice deficient in VCAM-1 are not viable because of developmental defects, but endothelial cell-specific knockouts and mice expressing a low level of a mutated VCAM-1 that lacks one of the VLA-4 binding sites have been prepared. These VCAM-1 hypomorphic mice are dramatically protected from atherosclerosis is all models tested.[101,102]

PECAM-1

Endothelial cells constitutively express CD31 (PECAM-1) and CD99, both of which have been implicated in monocyte transmigration.[103] C57BL/6 mice lacking CD31 have no transmigration defect, but on other genetic backgrounds, the migration defect is evident. Other endothelial-expressed adhesion molecules include junctional adhesion molecule (JAM)-1, 2, and 3.[104] They may function in monocyte and leukocyte transmigration.

Other Adhesion Molecules

Some specialized endothelial cells express adhesion molecules in an organ-specific manner. This includes mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed by high endothelial venules of the mesenteric lymph node and Peyer patches,[105] E-selectin expressed in resting skin endothelial cells,[106] and peripheral node addressin (PNAd) expressed in high endothelial venules of peripheral lymph nodes.[107] These region- and organ-specific adhesion molecules will not be discussed further, because they have not been reported to be involved in platelet–monocyte–endothelial interactions.

The endothelial adhesion molecules mediating interactions with platelets are not well defined. Endothelial-expressed P-selectin can support platelet rolling,[108] but interaction of activated platelets with endothelial cells is dependent on platelet P-selectin.[75] The endothelial adhesion receptors responsible for binding P-selectin are unknown. Activated endothelial cells secrete vWF as a very large multimer (ultralarge vWF), which can form long adhesive strings that can capture platelets by binding to platelet GPIbα.[109] The fluid drag force on the attached platelets stretches out vWF multimers, exposing a cleavage site that allows a plasma protease, a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS-13), to cleave the multimers into shorter oligomers of vWF that are released into the plasma.[110] This seems to be an important source of plasma vWF.

Endothelial Cell Chemokines and Chemokine Receptors

Endothelial cells can express a number of proinflammatory chemokines, most notably of the IL-8 family.[111] The endothelial isoform of IL-8 is slightly different from the monocyte-expressed isoform,[112] but there are no known functional differences. IL-8 is immobilized on the endothelial cell surface, where it is in a strategic position to activate and arrest monocytes and neutrophils that may be rolling by. Although endothelial cells synthesize little MCP-1 or RANTES, RANTES can be delivered by rolling platelets and be immobilized on the endothelial surface.[69,75] Immobilized RANTES triggers monocyte arrest, whereas MCP-1 immobilization is less efficient and triggers monocyte arrest in some,[113] but not all vascular beds.[75] The ability of endothelial cells to produce other chemokines has not been tested systematically. Endothelial cells express the decoy chemokine receptor, the Duffy antigen receptor for chemokines (DARC), which promotes transport of inflammatory chemokines across the endothelial barrier and thus helps transmigration.[114] Endothelial cells may also express CCR2, the main receptor for MCP-1.[115] Lung endothelial cells express CXCR2, which regulates permeability and leukocyte migration.[116] The role of endothelial CCR2 and CXCR2 for interactions with platelets and monocytes has not been studied.

Platelets

Platelets are nonnucleated disks (2 × 0.5 μm) derived from megakaryocytes. They are abundant in blood (about 300,000/μL) and primarily responsible for hemostasis.

Platelet Adhesion Molecules

P-Selectin

Platelets express large amounts of P-selectin, which is stored in α secretory granules (Table 46.4). Upon activation, platelet P-selectin is translocated to the cell surface by exocytosis. In fact, platelet surface P-selectin is a hallmark of platelet activation. P-selectin-expressing platelets are found in patients with stable and unstable angina pectoris, myocardial infarction, and other cardiovascular diseases.[117] P-selectin is also found on the surface of a subset of blood platelets in apolipoprotein E–deficient mice,[75] a standard model of atherosclerosis. Platelet P-selectin is not only a marker of, but also an active participant in vascular disease. Injecting activated platelets into apolipoprotein E–deficient mice exacerbates the size of atherosclerotic lesions.[75] Similarly, preventing platelets from interacting with the vascular endothelium reduces atherosclerotic lesion size.[118] L- and E-selectin are not expressed in platelets.

Platelet Integrins

Platelets express several integrin adhesion molecules. The most abundant, best known, and possibly most important of them is integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). Even in the resting state, GPIIb/ IIIa can bind immobilized fibrinogen and fibrin. This integrin rapidly acquires ligand-binding activity upon platelet activation.[119] $\alpha_{IIb}\beta_3$ is well documented to change its conformation and express neoepitopes related to the ligand-binding site.[19,120] Platelets from patients or mice that do no express GPIIb/IIIa (Glanzmann thrombasthenia) show defects in platelet aggregation and clot retraction.[40,121] vWF, vitronectin, and fibronectin bind GPIIb/IIIa through Arg-Gly-Asp (RGD) sequences, fibrinogen binds through Ala-Gly-Asp-Val (AGDV), and soluble CD40 ligand (sCD40L) uses a Lys-Gly-Asp (KGD) sequence. In the context of this chapter, GPIIb/IIIa binding of fibrinogen is of particular interest, because it may provide a means to promote platelet–monocyte interactions through Mac-1, which can also bind fibrinogen.[122] Blocking GPIIb/IIIa by antibodies or small molecules is a successful clinical treatment for coronary artery disease.[123] In addition to inhibiting platelet adhesion and aggregation, the various GPIIb/IIIa antagonists also reduce formation of the proinflammatory sCD40L from platelets. Interestingly, deficiency in GPIIb/IIIa does not confer protection from atherosclerosis.[124]

Other platelet integrins include $\alpha_5\beta_1$, which binds fibronectin and the collagen receptor $\alpha_2\beta_1$ (GPIa-IIa). Knockout mice have been prepared for both of these integrins, but show no detectable platelet defect. Humans lacking $\alpha_2\beta_1$ have a mild bleeding tendency and decreased platelet adhesion to the subendothelium.[125] $\alpha_2\beta_1$ appears to be activated by inside-out signaling secondary to GPVI binding (see below).

Collagen Receptors

The main platelet collagen receptor is GPVI, a 339 amino acid immunoglobulin-like molecule with similarity to the natural killer receptors.[126] GPVI ligation causes platelet activation and thus initiates hemostasis. It associates through its transmembrane domain with the Fc Receptor gamma (FcRγ) chain, which has an immunoreceptor tyrosine-based activation motif (ITAM) domain[127] for signaling. There is some evidence that GPVI resides preferentially in so-called lipid rafts, domains of the platelet membrane that are rich in sphingolipids and cholesterol.[128] Lipid rafts can merge and possibly give rise to platelet-derived microparticles (see below). A congenital defect in GPVI causes a mild bleeding tendency.[129] There is no evidence that GPVI is involved in platelet interactions with other cells.

GPIb, the main platelet receptor for vWF, is associated with GPV and IX in a GPIbα/Ibβ/V/IX complex,[130] also known as CD42bα/CD42bβ/CD42d/CD42a. All four glycoproteins belong to the leucine-rich repeat family. GPIb/V/IX mediates platelet adhesion to vWF under high shear. GPIbα also has binding sites for P-selectin, Mac-1, thrombin, and coagulation factors XI and XIIa. Deficiency of GPIb-V-IX in humans causes Bernard-Soulier syndrome, a relatively severe bleeding disorder. GPIb-V-IX binding to vWF triggers intracellular signals that lead to activation of GPIIb/IIIa. Injecting *Apoe*$^{-/-}$ mice with an antibody to GPIbα reduces the size of atherosclerotic lesions,[118] suggesting that platelet GPIbα interaction with endothelial vWF may have proinflammatory consequences.

Fc Receptors

Platelets express one Fc-gamma receptor, FcγRIIa. Upon cross-linking by intact immunoglobulin G (IgG), FcγRIIa relocates to lipid rafts and activates platelets through src-family kinases and adapter proteins. Fc γRIIa associates with GPIb.[128]

von Willebrand Factor

Platelet α-granules contain vWF, which is rapidly secreted upon activation and forms long strings that may be anchored to platelets by P-selectin. Platelet vWF is very large and very thrombogenic. Deficiency in vWF protects from atherosclerosis in mice.[131]

Platelet Chemokines and G-Protein Coupled Receptors

Platelet Chemokines

Platelets constitutively and abundantly express the chemokines CXCL4 (platelet factor 4, PF4) and RANTES (CCL5). Both are important in mediating monocyte–platelet–endothelial cell interactions. RANTES is deposited by transiently interacting platelets on inflamed endothelium *in vitro*,[69] and on atherosclerotic endothelium and monocytes *in vivo*,[75] where it promotes monocyte arrest and activation, most likely through CCR1.[74] PF4 is of importance in macrophage differentiation.[132] PF4 forms heterodimers with CCL5 (RANTES), and these heterodimers are very efficient at promoting monocyte adhesion.[133] On endothelial cells, PF4 binds to and activates a splice variant of CXCR3.[134]

Platelet Chemokine Receptors

Platelets express the chemokine receptors CCR1, 3, 4, and CXCR4 (Table 46.4). These receptors bind proinflammatory cytokines and SDF-1α, respectively. Chemokine receptor ligation can augment platelet responses to low doses of agonists.[135] These chemokine receptors are modifiers rather than initiators of responses, but are nevertheless important in the cross-talk between inflammation, platelet aggregation, hemostasis, and thrombosis (see below).

Other G-Protein Coupled Receptors

The most important activating G-protein–coupled receptors on platelets belong to the protease-activated receptor (PAR) and adenosine diphosphate (ADP)-receptor (P2Y) families. Of the four known PARs, platelets express PAR-1 and 4 in humans, and PAR-3 and 4 in mice. These receptors are responsible for thrombin-induced platelet activation, a prominent event initiating platelet aggregation. The PAR receptors bear a tethered ligand that becomes available to bind to and activate the receptor after cleavage of the receptor *N*-terminus by a specific protease such as thrombin.[136] Details of PAR receptors on platelets are covered in Chapter 33. Platelets also express G-protein–coupled receptors for ADP, most prominently, P2Y1 coupled to Gαq, and P2Y12 coupled to Gαi. P2Y12 is the molecular target of platelet aggregation inhibitors of the clopidogrel-type. In the context of atherothrombosis and vascular disease, ADP may be released from red and other blood cells, triggering platelet aggregation. *Per se*, ADP is not a very strong stimulus for platelet activation, but may synergize with other stimuli such as low-dose thrombin, or chemokines. Blocking P2Y12 also reduces formation of CD40L. Other platelet GPCRs include receptors for epinephrine and thromboxane A2, a cyclooxygenase product that amplifies platelet aggregation. Some GPCRs, including PAR-1, are preferentially found in lipid rafts.[128]

Platelet Cytokines

The most important cytokine produced by platelets is the TNF-like CD40 ligand (CD40L), which has both prothrombotic and proinflammatory effects.[119] CD40L is a type II transmembrane protein that binds CD40 on antigen-presenting cells and GPIIb/IIIa on platelets. GPIIb/IIIa binding promotes cleavage ("shedding") of CD40L by an unknown metalloproteinase in response to activation by collagen or thrombin. sCD40L also has proinflammatory and prothrombotic effects. Almost all CD40L in blood is on platelets, although some lymphocytes also express CD40L. Mice lacking CD40L have a thrombosis defect and are protected from atherosclerosis. sCD40L accumulates in stored platelet concentrates, and elevated levels of serum sCD40L are associated with thrombotic diseases, acute coronary syndrome, rheumatoid arthritis, lupus, and other inflammatory diseases. Platelets also contain the platelet-derived growth factor (PDGF), a cytokine-like growth factor for smooth muscle and other cells, and transforming growth factor-β (TGF-β), which is important in wound healing, fibrosis, and lymphocyte differentiation.

Small Molecules Secreted by Platelets

Thromboxane A2 (TXA2) and serotonin are powerful vasoconstrictors released by activated platelets. Both bind to receptors on smooth muscle and endothelial cells. TXA2 binding to thromboxane receptor (TP) is an important amplifier of platelet aggregation. TXA2 is a cyclooxygenase-1 product, an enzyme that is inhibited by aspirin.

Microparticles

Blood contains a host of microparticles with diameters between 50 nm and 1 μm that can support platelet–monocyte–endothelial interactions.[137] Most (about 90%) microparticles in normal human plasma are derived from platelets,[138] but others come from monocytes, endothelial cells, smooth muscle cells, and other cells. Platelet-derived microparticles are very prothrombotic by providing a phosphatodylserine-rich surface on which coagulation factors can assemble.[139] Monocyte-derived microparticles are a major source of tissue factor that initiate intravascular coagulation during thrombus formation.[137] The adhesion molecules used by microparticles to interact with endothelial cells, monocytes, and platelets are most likely derived from the cells from which the microparticles were generated. Indeed, the delivery of tissue factor to platelet aggregates has been shown to require PSGL-1 on monocyte-derived microparticles, and P-selectin on activated platelets.[56,57] Intravascular thrombosis can also be initiated by coagulation factor XII.[140]

Plasma and Extracellular Matrix Proteins Relevant to Cell–Cell Interactions

Although platelet–monocyte–endothelial cell interaction is often studied in tissue culture media, these adhesive interactions occur naturally in blood, which is itself a rich source of other adhesive proteins. Plasma fibrinogen can bind to many cell adhesion receptors, including Mac-1, $\alpha_V\beta_3$ integrin, and ICAM-1 on monocytes, activated GPIIb/IIIa on platelets, and ICAM-1 on endothelial cells. Fibrinogen has multiple binding sites per molecule that can bridge between platelets, endothelial cells, and monocytes. Fibrinogen-deficient mice have a surprisingly mild bleeding defect[141] and demonstrate that fibrinogen is not strictly necessary for hemostasis.

Plasma contains vWF, most of which exists as dimers and oligomers. vWF is required for platelet adhesion under high shear stress. vWF deficient mice,[142] and humans have a significant bleeding disorder (von Willebrand disease). By binding to platelet GPIb-V-IX, vWF promotes platelet–endothelial interactions. Plasma also contains fibronectin, an adhesive protein that can bind to integrins $\alpha_5\beta_1$ and $\alpha_V\beta_3$ on endothelial cells and

supports endothelial cell interaction with monocytes (through binding of $\alpha_4\beta_1$) and platelets (through binding to $\alpha_{IIb}\beta_3$). Mice lacking fibronectin are not viable.[143] Upon coagulation, fibrin is formed from fibrinogen, forming a tight meshwork that can entrap all classes of blood cells including poorly adhesive red blood cells. Fibrin shares some of its adhesive properties with fibrinogen. Immobilized fibrin can bind GPIIb/IIIa even in its nonactivated state.

When in the course of vascular injury the subendothelial matrix is exposed, various collagens, laminin, fibronectin, vWF, and other adhesive molecules become exposed. Collagen and vWF are very important for platelet binding. These interactions are treated in Chapters 28–30.

CELL–CELL INTERACTIONS

Shear Rate and Shear Stress

To understand the mutual interactions among platelets, monocytes, and endothelial cells, their attachment must be studied under shear flow conditions, because such conditions exist in almost all situations where these interactions occur in the flowing blood. Shear flow in the circulatory system is usually a pressure-driven flow through a series of distensible tubes. Owing to the physical principle of the no-slip condition at the fluid/solid interface, the fluid particles very near the vessel wall (and near each blood cell) do not move relative to that surface. In tube flow, there is a gradient of flow velocities such that the highest velocity is reached in the center of the vessel. The rate of change of flow velocity between adjacent fluid layers is called the shear rate, and has units of meters per second (velocity) divided by meters (distance between layers), or s^{-1}. The change in velocity between adjacent layers of fluid is associated with a drag force (Newtons) that is calculated per unit surface area (square meters), which is defined as the shear stress (in N/m^2 or Pascals). The cgs unit, dyn/cm^2, is also used for shear stress, and 10 dyn/cm^2 is equal to 1 Pa. The ratio between shear stress and shear rate is the viscosity, which is measured in Pas. An older unit, centipoise, is often used, where 1 centipoise is equal to 0.001 Pas. In blood, the viscosity is not constant across the cross section of a blood vessel, but changes as a function of radial position and hematocrit.[144] The shear stress and shear rate along the vessel wall, the wall shear rate, and wall shear stress, are most relevant for cell interactions with the vessel wall. When only one shear stress or shear rate is reported without a qualifier, this usually refers to the wall shear stress or wall shear rate.

Endothelial Surface Layer

Under normal conditions, the luminal aspect of most endothelial cells is covered by a thick layer of extracellular matrix material called the glycocalyx. The thickness and composition of this layer probably varies between vascular beds, vessel sizes, and physiologic conditions, but most reports place its thickness between 0.3 and 0.6 μm.[145] This layer contains proteoglycans, glycosaminoglycans, and hyaluronic acid. The mechanical properties of this layer are such that there is almost no fluid flow through this layer along the vessel surface.[146] This means that the wall shear stress and wall shear rate on the surface of the endothelium of healthy blood vessels is nearly equal to zero.[147] All known endothelial adhesion molecules are buried deep within this layer and hardly accessible to adhesion molecules on monocytes, platelets, and other cells in the flowing blood. However, as monocytes and other white blood cells pass through and exit from small capillaries, they can compress the endothelial surface layer sufficiently to initiate adhesive interactions and start rolling.[148] In large vessels such as the aorta, carotid artery, or coronary arteries, where cells do not exit from capillaries, it is probably necessary that the endothelial surface layer is degraded before productive leukocyte–endothelial cell interactions can occur.[149] It is not known whether cultured endothelial cells maintain a surface layer of similar size and composition as that found *in vivo*, although there is indirect evidence that they express some hydrodynamically relevant layer on their luminal surface. The glycocalyx layer is thinner in areas of disturbed flow, endothelial dysfunction, or under inflammatory conditions.[150]

Cell–Cell Interactions Under Shear

To study cell–cell interactions under shear, several *in vitro* and *in vivo* methods have been developed. A defined shear stress and shear rate can be applied to cells in suspension using a plate-and-cone apparatus, which produces a uniform shear throughout the fluid.[151] A modification of this apparatus is the rheoscope, constructed of two counter-rotating transparent cones, which allows the direct visualization of cell–cell interactions at a defined shear rate and shear stress.[152] A very popular device is the parallel plate flow chamber,[153] which combines excellent visibility due to its optical properties with versatile use of adhesion molecules, cultured endothelial cells, and other substrates. Leukocyte–endothelial and platelet–endothelial interactions have also been studied using capillary tubes,[154] which mimic the radial symmetry of the flow profile in most blood vessels *in vivo*, but have limited visibility due to optical diffraction. In a traveling capillary apparatus, the microscope stage is moved at a speed equal and opposite to that of the cell suspension flow such that the interacting cells appear to remain stationary.[155] Other adhesion assays include the radial flow chamber[156] and the graded shear stress flow chamber.[157] Recently, autoperfused flow chambers have been developed that allow the study of leukocyte and platelet interactions with adhesion molecules in their natural blood environment.[158,159] This flow chamber was miniaturized so that studies in mouse blood became possible without returning the blood to the mouse.[160] Microfluidic devices allow control of the local site density of adhesion molecules.[161] In so-called static adhesion assays, adhesion occurs in the absence of shear stress, but nonadherent cells are washed off with an undefined shear stress. Such assays are not suitable for studying blood cell interactions with each other, or with endothelial cells. In some tissues and species, monocyte interaction can be studied by intravital microscopy. Relevant to this chapter, monocyte and platelet interactions have been studied in the mouse aorta,[162] carotid artery,[163] and femoral artery (Huo and Ley, unpublished) *in vivo*.

Monocyte–Endothelial Interactions

Monocytes may encounter the endothelium in two fundamentally different situations. In the microcirculation, the initiation of monocyte interactions with the endothelium has not been studied systematically, but the process is probably similar to that described

Table 46.6

Endothelial–monocyte interactions

Endothelial Cell	Monocyte	Remarks
P-selectin	PSGL-1	Rolling, monocyte activation?
VCAM-1	$\alpha_4\beta_1$	Slow rolling, adhesion
Fibronectin CS-1	$\alpha_4\beta_1$	Adhesion
ICAM-1	LFA-1, Mac-1	Adhesion strengthening?
ICAM-2	LFA-1	
E-selectin	Unknown ligand	Slow rolling?
CXCL1, 2, 3, 8	CXCR1, 2	Monocyte activation

for neutrophils.[164] In postcapillary venules, most leukocyte–endothelial interactions are initiated at the point where leukocytes enter the venules from capillaries. Since capillary diameters are smaller than leukocyte diameters in most organs, leukocytes must deform to pass, and in the process probably squeeze down the endothelial surface layer, which brings them in close contact with the endothelial adhesion molecules. Once engaged, these adhesion molecules (Table 46.6) support stable rolling, during which the endothelial surface layer is probably continuously flattened. The layer appears to recover after the leukocyte has passed. In large vessels such as arteries, this mechanism is not operative because the vessel is much wider than the monocyte. In these cases, most monocyte–endothelial interaction is initiated by secondary capture.[53]

Monocyte Capture

In large vessels such as arteries, it is likely that the endothelial surface layer is damaged or reduced in thickness during the initiation of atherosclerosis or other forms of vascular inflammation. Monocyte interaction is largely P-selectin/PSGL-1 dependent.[163] In these larger vessels, secondary capture is very important.[53] Secondary capture or secondary tethering describes the transient interaction between a free-flowing blood cell and an already adherent blood cell that can greatly facilitate the initiation of monocyte interactions with the endothelial wall. Although this process was originally described as an interaction between like cells,[154] monocyte capture onto the endothelial surface of inflamed arteries can also be initiated by platelets that transiently or stably interact with the vessel wall[75] (FIGURE 46.3). In this case, the interaction requires platelet P-selectin and monocyte PSGL-1. When a monocyte is captured through interaction with another monocyte, secondary capture probably requires PSGL-1 on the adherent monocyte and L-selectin on the incoming monocyte. Secondary capture does not allow predictions about whether or how rolling along the endothelium will ensue. There is evidence that interaction with platelets is more important for monocytes than for other leukocytes.[165]

Monocyte Rolling

In mouse models, monocyte rolling is almost entirely dependent on P-selectin expressed by the inflamed, or atherosclerotic endothelium and PSGL-1 on the monocytes. Monocyte rolling is slowed by transient $\alpha_4\beta_1$/VCAM-1 interactions.[166] In human atherosclerotic lesions, E-selectin expression has been described.[167] Monocytes express E-selectin ligands, which remain to be identified at the molecular level. With the exception of high endothelial venules in secondary lymphatic organs,[113] monocyte rolling in small microvessels has not been studied systematically.

Monocyte Arrest

Rolling *per se* is not sufficient to allow monocyte recruitment. Monocyte arrest is commonly triggered by specific arrest chemokines, chemokines that are deposited on the endothelial surface and can signal integrin activation by binding to G-protein–coupled receptors while the cell is rolling by. Identified arrest chemokines for monocytes include CXCL1, 2, and 3, all of which bind to CXCR2, and CCL5, which mediates arrest by binding to CCR1. In the human system, IL-8 (CXCL8), which binds to CXCR1 and CXCR2, can also act as an arrest chemokine.[168] CXCL1, 2, 3, and 8 are synthesized by inflamed endothelial cells, while CCL5 is deposited by platelets that transiently interact with the vessel wall.[69,75] Soluble CCL2 (MCP-1) can also trigger monocyte arrest, but the relevance for monocyte arrest of endothelial-bound CCR2 has not been demonstrated outside lymph node venules.[29,113] The arrest function of many chemokines for monocytes has not been investigated, but it is likely that other chemokines may be able to trigger arrest. Of note, the arrest and chemoattractant functions of chemokines do not necessarily correlate with each other.[169] Monocyte arrest does not require a change in intracellular free calcium ("calcium flux") or activation of MAP kinase.[169]

When a rolling monocyte is exposed to an arrest chemokine, its surface integrins are activated by inside-out signaling. In adhesion assays conducted in atherosclerotic vessels under shear flow conditions, monocyte arrest is triggered by $\alpha_4\beta_1$ integrin binding to VCAM-1,[29] but an arrest component through β_2 integrins has been described in a flow chamber system using cultured endothelial cells.[168] While it is clear that monocyte arrest is integrin dependent, the detailed molecular mechanisms can currently only be inferred from studies in other cells (see above). In addition to affinity regulation, integrins also cluster in the plane of the monocyte membrane, giving rise to patches of integrins with increased apparent affinity for multivalent ligands (avidity regulation, FIGURE 46.1). The lateral movement of integrins requires their temporary release from cytoskeletal constraints and subsequent reattachment to cytoskeletal elements, an event that is unlikely to occur within milliseconds. A third possibility is that monocyte integrins undergo some limited conformational change, followed by ligand binding and outside-in signaling that leads to rapid bond maturation.[18] The exact contribution of affinity and avidity regulation during monocyte arrest is controversial and awaits further experimentation.

Outside-In Signaling

Once α_4 and β_2 integrins are activated and engage their respective ligands, they transmit signals into the adherent monocyte in a process called outside-in signaling.[170] These signals probably help the adhesive contact to mature, initiate cell crawling on the endothelial surface, and initiate transmigration into the subendothelial space. In neutrophils,[164] but not monocytes,[169]

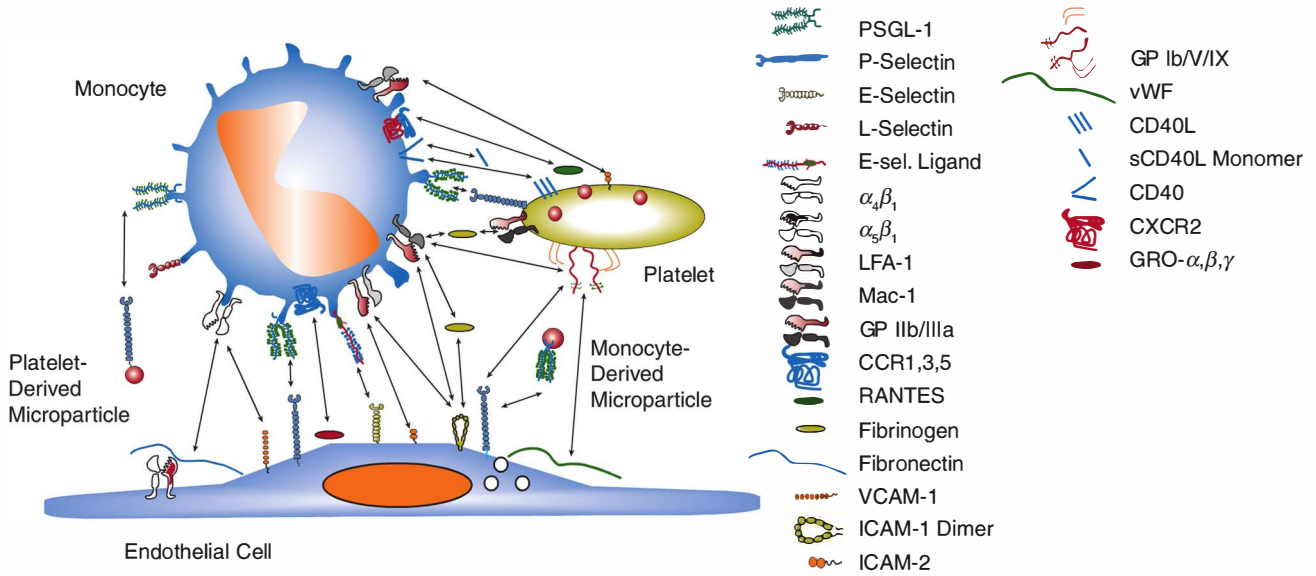

FIGURE 46.4 Monocyte–platelet–endothelial interactions. Each arrow shows a molecular interaction, legend for molecules indicated in figure. Monocyte- and platelet-derived microparticles are also shown. See text for details.

a gradual increase in intracellular free calcium has been shown during rolling, with a massive increase in intracellular free calcium upon arrest.

Cytoskeletal interactions of integrins are likely to be important not only for outside-in signaling, but also for lending mechanical strength to the bonds between the adherent cells and the vascular endothelium. Many of the relevant monocyte–endothelial cell interactions in the arterial circulation occur in the presence of very high shear forces. α_4 integrins attach to VCAM-1 and fibronectin. β_2 integrins attach to endothelial ICAM-1, ICAM-2, and other ligands. Although arrest of monocytes under moderate wall shear stress of 3 dyn/cm^2 was attributed to α_4 integrins and did not require β_2 integrins,[29] it is likely that β_2 integrins contribute to bond strengthening, migration, and transendothelial migration.

Platelet–Monocyte Interactions Under Shear

Platelet–monocyte interactions are most commonly studied in plate-and-cone devices, which produce a uniform shear rate and shear stress throughout the cell suspension.[171] It is clear that most if not all monocyte–platelet adhesion is mediated by P-selectin on the activated platelet surface and PSGL-1 on the monocyte. That this interaction occurs *in vivo* is demonstrated by the presence of P-selectin–dependent platelet–monocyte aggregates in patients with coronary heart disease.[172] The abundance of these aggregates correlates with the incidence of unstable angina, myocardial infarction, and stroke.[117] Platelet–monocyte aggregates are also found in the blood of apoE-deficient mice on a proatherogenic diet, but not in control mice.[75,173]

Monocyte interactions have also been studied in flow chambers coated with platelet monolayers. This model is most relevant to the adhesion of monocytes at sites of vascular injury, where a platelet monolayer forms almost immediately. Monocyte interaction with platelet monolayers is strictly P-selectin and PSGL-1 dependent and shares many similarities with monocyte–endothelial cell interactions, with the exception that P-selectin expression on monolayers of activated platelets is probably higher than on endothelial cells, and the rolling velocity is consequently lower. Monocytes can adhere to platelets via an interaction of monocyte Mac-1 with plasma fibrinogen and platelet GPIIb/IIIa.[174] Platelets express ICAM-2, a known ligand for LFA-1, which is expressed on monocytes (FIGURE 46.4). The relative importance of these other pathways *in vivo* is not known. P-selectin/PSGL-1 interactions seem to dominate platelet–monocyte interactions (Table 46.7).

Platelet–Endothelial Interactions Under Shear

Platelets can roll on and adhere to endothelial cells through at least two mechanisms. One requires P-selectin expression on inflamed endothelial cells and results in platelet rolling in microvessels.[108] In this interaction, endothelial P-selectin binds to a ligand on platelets, most likely GPIbα.[175] Another mechanism for platelet–endothelial cell interactions is found in large arteries and appears to be relevant for the process of atherosclerosis.[75] It requires P-selectin expression on platelets, which interact with an unknown ligand on endothelial cells (Table 46.7). A third candidate mechanism for platelet attachment to endothelial cells is dependent on platelet GPIbα interacting with vWF, which is stored in Weibel-Palade bodies of endothelial cells and can be released as an ultralarge vWF upon activation.[109]

Enhanced Monocyte Adhesion by Platelets and Microparticles

Given these extensive interactions, the monocyte–platelet–endothelial cell system can be viewed as a synergistic proinflammatory and prothrombotic triad. Platelets enhance monocyte

Table 46.7

Platelet interactions with endothelial cells (left) and monocytes (right)

Endothelial Cell	Platelet	Monocyte	Remarks
Fibronectin	GP IIb/IIIa	Mac-1	IIb/IIIa with Mac-1 through Fg
CD40	CD40L	CD40	Activates EC and monocytes
P-selectin	GP Ib V IX	Mac-1	Also binds vWF
		ICAM-1	Homotypic aggregation
	ICAM-2	LFA-1	Function not demonstrated
Unknown	P-selectin	PSGL-1	GPIbα on EC controversial
		L-selectin	Secondary capture
Proteoglycans	RANTES	CCR1, 3, 5	Activates monocytes
CXCR3b	PF4	unknown	Activates ECs and monocytes
VEGF-R	VEGF		Activates EC

Table 46.8

Proinflammatory and prothrombotic molecules on platelets, monocytes, and endothelial cells

Platelets
Prothrombotic
vWF, IIb/IIIa, fibrinogen
CD40L, sCD40L: induces MCP-1, IL-6, IL-8 in monocytes, E-selectin, VCAM-1, ICAM-1 in endothelial cells
Serotonin, thromboxane A2
Proinflammatory
RANTES (CCL5), platelet factor 4 (CXCL4), CCL5-CXCL4 heterodimers
P-selectin
sCD40L, CD40L
VEGF
Monocytes
Prothrombotic
Tissue factor, Mac-1
Proinflammatory
Chemokine receptors CCR2, CXCR1, CXCR2, CCR1, CCR5, CX3CR1, Mac-1, LFA-1, ICAM-1, chemokines
CD40
Endothelial cells
Prothrombotic
vWF
Proinflammatory
P-selectin, E-selectin, VCAM-1, ICAM-1, ICAM-2, CXCR2

binding to inflamed endothelium, which is critically important in atherosclerosis.[75,118] Conversely, monocytes rolling on endothelial cells can deliver platelets that are attached to them.[172] Since each of these cell types brings critical components of the hemostasis, coagulation, and inflammation system, it is easy to see how the mutual interaction of these cells greatly enhances both inflammation and thrombosis (Table 46.8). The intimate interaction between platelets, endothelial cells, and monocytes results in enhanced monocyte recruitment (FIGURE 46.5), and accelerated atherosclerosis.[75]

Tissue Factor and Monocyte-Derived Microparticles

Tissue factor initiates coagulation through the extrinsic pathway (see Chapter 11). Although tissue factor is expressed in many extravascular cells, its expression is highest on activated monocytes. Monocytes continuously shed microparticles ranging in size from 0.05 to 1 μm. Monocyte-derived microparticles lack metabolic functions, but are enriched in tissue factor and many other monocyte components.[56,137] Microparticles of both platelet[176] and monocyte origin are abundant in human and mouse plasma, and their number can increase during disease states.

Some[177] if not most of the "plasma" tissue factor is in fact contained in monocyte-derived microparticles.[56,137] This source of tissue factor is available for incorporation into platelet aggregates, where it initiates coagulation. Monocyte-derived microparticles express PSGL-1, and activated platelets express P-selectin. Since the P-selectin/PSGL-1 interaction is critical for microparticle recruitment to sites of thrombosis, P-selectin–deficient mice[178] and PSGL-1 deficient mice[179] both have a reduced propensity for intravascular thrombus formation with little evidence of an overt bleeding disorder. The formation of monocyte-derived particles can be induced by soluble P-selectin,[179] which has been proposed to be a useful mechanism for delivering procoagulant activity to hemophilia patients.

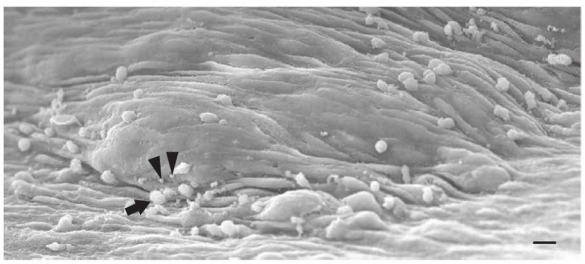

FIGURE 46.5 Monocyte and platelet adhesion in an atherosclerotic apoE-/- mouse carotid artery. Monocytes (*arrow*) and platelets (*arrowheads*) preferentially adhere in the shoulder region of this atherosclerotic plaque. Sample taken 30 minutes after IV infusion of activated mouse platelets. Scale bar indicates 10 μm. (Scanning electron micrograph prepared by Jan Redick and Yuqing Huo.)

CROSSTALK BETWEEN INFLAMMATION AND HEMOSTASIS

Monocyte Activation by Interaction with Platelets

Activated platelets can bind to monocytes and deposit chemokines such as CCL5, which cause an increase in $\alpha_4\beta_1$ integrin avidity (lateral clustering) and affinity (conformational change).[69,75] Platelet-induced deposition of PF4 can also induce monocyte differentiation into macrophages.[132,180] Disrupting heterodimer formation between CCL5 and PF4 reduces monocyte recruitment to atherosclerotic lesions.[133] Platelet CD40L, either surface-expressed or secreted, interacts with monocyte CD40, and causes monocyte activation.[119] Chemokine-induced L-selectin shedding on monocytes could potentially limit the inflammatory response. Chemokines can also induce a secretory response in monocytes, including release of specific, secretory, and tertiary granules. Monocyte degranulation releases a wide variety of proinflammatory mediators and enzymes.

Proinflammatory Effects of Coagulation Products

Not only does the inflammatory response enhance the coagulation response (discussed above), but the converse is also true. Many coagulation products are proinflammatory, including fibrin degradation products. Thrombin has strong proinflammatory effects, as has the binding of coagulation factor X to monocyte Mac-1.[181]

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CHAPTER

47 Leukocyte Function and Contribution to Disease

EDITH VAN DE VIJVER AND TACO W. KUIJPERS

Inflammation is a tightly regulated process initiated after tissue injury or infection. The main function of inflammation is to eliminate the pathogenic insult and remove damaged tissue, with the aim of restoring tissue homeostasis. The concerted action of professional phagocytes—neutrophils, monocytes, and macrophages—is crucial to the effective elimination of intruders and cell debris.

Neutrophils are short-living cells that are recruited early in the inflammatory response. Upon binding of cytokines, which originate either from microorganisms or from surrounding cells, neutrophils transmigrate through the vascular wall toward the infected tissue. The opsonized pathogen is bound and phagocytosed by the neutrophil, whereupon it is killed by mediators such as toxic granular proteins or reactive oxygen species (ROS). These inflammatory mechanisms are potentially harmful to the host, with the risk of inducing sepsis. Therefore, neutrophil activation has to be tightly controlled by several negative regulation mechanisms.

Inflammation also shifts the hemostatic mechanisms in favor of thrombosis. Multiple mechanisms are at play, including upregulation of tissue factor leading to the initiation of clotting and amplification of the clotting process by augmenting exposure of cellular coagulant phospholipids. Moreover, a decrease in the activity of natural anticoagulant pathways takes place, which is particularly targeted toward downregulation of the activated protein C (APC) pathway through multiple mechanisms. The decreased function of the natural coagulation pathways appears to play a role in dampening inflammatory responses. The dual roles of the neutrophil in inflammatory responses and hemostasis are highlighted in this chapter.

DEVELOPMENT AND TURNOVER

About 60% of all nucleated cells in the bone marrow belong to the myeloid series. Neutrophils originate from myeloid progenitor cells, and they mature in the bone marrow in about 2 weeks. During maturation and until activation of neutrophils, it is important that the neutrophils are retained in the bone marrow. Interactions between chemokine receptor CXCR4 on neutrophils and its ligand CXCL12, which is constitutively expressed by bone marrow stromal cells, play a crucial role in this process. Disruption of this interaction by administering CXCR4 antagonist AMD3100 results in neutrophilia due to increased neutrophil release from the bone marrow.[1]

During myelopoiesis, which includes the development of granulocytic as well as monocytic lineages, the myeloid-specific growth factors, granulocyte colony-stimulating factor (G-CSF) and granulocyte-monocyte-CSF (GM-CSF), play an important role.[2] G-CSF and GM-CSF exert a dual effect: a boost in cell survival as well as the induction of myeloid development through an increase in the number of colony-forming units. The effect of G-CSF is transmitted to the myeloid cell through the G-CSF receptor. Mice deficient in either G-CSF or its receptor have a reduced neutrophil count (~20% of normal).[3,4]

Normally, <2% of all neutrophils in the body are circulating in the blood. When released from the bone marrow, neutrophils normally have a short life span (6 to 10 hours) in the bloodstream.[5] Thereafter, they move to the extravascular tissues and within hours undergo spontaneous apoptosis. Indeed, neutrophils are predisposed to cell death by apoptosis. This process prevents the cytotoxic contents in neutrophil granules from being released into the surrounding tissues, and it facilitates the elimination of dead neutrophils by tissue macrophages.

The exact molecular mechanisms underlying neutrophil apoptosis are unknown, although members of the Bcl-2 protein family and caspases have been shown to be involved.[6] At the level of mitochondria, these two groups of proteins are intimately connected: Bcl-2 homologues govern the activity of caspases by exerting their effect through regulation of mitochondrial function.[7] Proapoptotic Bcl-2 proteins, such as Bax, redistribute from the cytosol to the mitochondria to disturb the mitochondrial membrane integrity by forming channels, which facilitates the subsequent release of cytochrome *c* and the activation of Apaf-1 and downstream caspases. The family of caspase proteases executes cleavage of specific targets, which then leads to cell disassembly and death. Among these proteases, caspase-3 stands out for the large number of substrates that it destroys, including nuclear proteins, cytoplasmic structures, and cytoskeletal elements.

In the clinic, survival of both the immature myeloid progenitor cells and mature neutrophils can be extended by delaying apoptosis through the action of a wide variety of agents, including G-CSF and GM-CSF. During infections, the release of neutrophils into the circulation is accelerated, and these cells are also among the first to leave the blood vessels by extravasation and to migrate toward the site of infection.

EXTRAVASATION AND MIGRATION

During inflammation, circulating neutrophils migrate to the site of infection following a gradient of chemotaxins in a process called chemotaxis. Chemotaxins may be derived from either the infected tissue or local complement activation, or directly from the pathogens themselves, and diffuse within the tissue into the local vasculature. These gradients of chemotaxins recruit the neutrophils in interplay with factors expressed on the

luminal side of blood vessel endothelial cells locally.[8] Many of the chemotaxins involved in granulocyte movement are small proteins of about 60 to 100 amino acids, very homologous in structure. The major family of chemotaxins, known as the chemokine superfamily (see Chapter 46), consists of over 30 different chemotactic molecules, which are produced by host cells in response to inflammation, injury, hypoxia, or other forms of stress. Most chemokines can be classified into α (CXC) and β (CC) chemokines, distinguished by the presence or absence of a single amino acid between the first two of four conserved cysteines.[9] The γ (single C) and δ (CX_3C) classes of chemokines have only recently been coined, each with as yet one member. As exemplified by CXCL8—the first CXC member described—most CXC chemokines activate neutrophils, whereas the CC chemokines act toward various lymphocyte subsets, monocytes, eosinophils, or basophils. CXCL8 is also referred to as interleukin-8 (IL-8).

In addition to the chemokines, chemotactic peptides are released by infecting microorganisms, for example, formyl-methionyl-leucyl-phenylalanine, and the host complement system, for example, the split product of activated C5 (C5a). Cell membrane-derived lipid mediators, such as platelet activating factor, are also strong chemoattractants. For each of these agents, specific receptors on the neutrophil plasma membrane exist. These receptors, as well as those for the chemokines, belong to the seven-span superfamily of G-protein–coupled receptors (GPCRs), which are integral membrane proteins with seven transmembrane domains. Ligand specificity is created by differences in the extracellular domains. The intracellular domains interact with trimeric guanidine triphosphate (GTP)-binding proteins, enabling a link with signal transduction pathways leading to a wide range of functional responses, the complexity of which is still largely unclear.

Cells following the chemotaxin gradient toward the site of infection have to leave the bloodstream in a process called extravasation (FIGURE 47.1). Extravasation is a multistep process involving adhesion molecules in which chemotaxins function as activating agents or (pro-) inflammatory mediators. The first step of extravasation consists of initial contact between endothelial cells and neutrophils marginated by the fluid flow of the blood. L-Selectin (CD62L) on leukocytes plays a role herein, contacting several cell adhesion molecules on endothelial cells. Within the local environment of an inflammatory tissue reaction, the endothelium begins to express the adhesion molecules P-selectin (CD62P) and later on E-selectin (CD62E) (see Chapter 46).[10] The low-avidity interaction of these selectins with their ligands on the opposite cells forces the neutrophils to slow down and make a rolling movement along the vessel wall.

In contrast to the low-avidity binding of neutrophils to selectins, the final step of firm adhesion and subsequent migration depends on stable interaction between integrins on the neutrophil and their ligands on the endothelial cells. For this process, the integrins need to be "activated," that is, change their conformation into one with high binding avidity for their ligands, which is described in more detail in Chapter 46. Integrins consist of heterodimeric proteins of an α-chain covalently associated with a β-chain.[11] One particular β-chain may associate with one of various α-chains. As a consequence, several integrin receptor subfamilies exist. The β_2 integrin receptor subfamily is only expressed on leukocytes and comprises four different heterodimeric proteins, each of which contains a different α subunit: that is, $\alpha_L\beta_2$ (LFA-1; CD11a/CD18), $\alpha_M\beta_2$ (CR3; CD11b/CD18), $\alpha_X\beta_2$ (gp150,95; CD11c/CD18), and $\alpha_D\beta_2$ (CD11d/CD18). The β_2 integrins bind to adhesion molecules on endothelial cells (intercellular adhesion molecule [ICAM]-1 and ICAM-2) and to several complement factors. The main β_2 integrin on neutrophils is CR3.

In contrast to rolling and adhesion of neutrophils, the mechanisms that mediate migration through venular walls are less well understood. However, there is now good evidence that besides ICAM-I and -II junctional molecules such as PECAM-1 (CD31) and the junctional adhesion molecule (JAM) family (JAM-A, -B, and -C) play a major role.[12,13]

As mentioned above, slowly rolling neutrophils are able to recognize concentration differences in a gradient of chemotaxins and to direct their movement toward the source of these

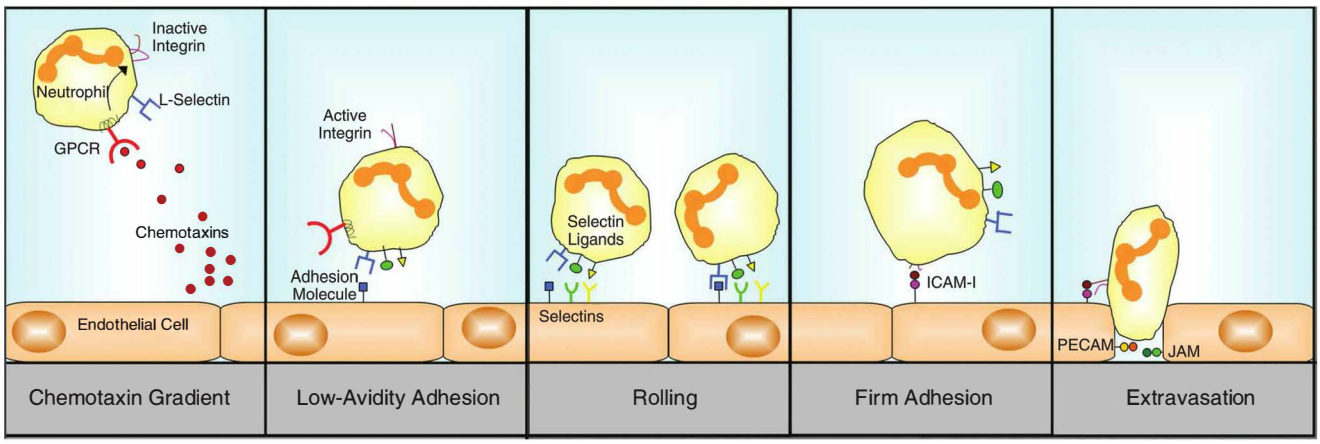

FIGURE 47.1 Extravasation of neutrophils. Neutrophils migrate in the direction of a gradient of chemotaxins, released at the site of infection. Upon initial contact with the endothelial cells through L-selectin, neutrophils slow down and interact with endothelial cell selectins while rolling along the vessel wall. Subsequently, integrins on the neutrophil are activated and bind to adhesion molecules on the endothelial cells, which results in stable adhesion. Binding to PECAM, JAMs, and other adhesion molecules guides neutrophils through the endothelial layer in the direction of the infection.

agents. Although the details of this process remain unknown, the gradient mostly likely causes a difference in the number of chemotaxin receptors on either side of the cell, thereby inducing the cytoskeletal rearrangements needed for movement. Since adhesion molecules such as the β_2 integrins are essential for the connections with the tissue cells or with the extracellular matrix proteins, these connections must be formed at the front of the moving neutrophils and broken at the rear end.[8,14] Moreover, for continued sensing of the chemotaxin gradient, the chemotaxin receptors on the neutrophil must be freed from their ligand for repeated usage. This occurs through internalization of the ligand–receptor complex, intracellular disruption of the connection, and transport of the free receptor to the front of the cell, followed by reappearance of the free receptor on the leukocyte surface. Within the infected tissue, the chemotaxin gradient persists, and leukocyte migration is maintained.

INTRACELLULAR SIGNALING

This complex process of cytoskeletal remodeling and cellular movement is controlled by several small GTPases of the Rho family and their guanine-nucleotide exchange factors (GEFs). In neutrophils, it has been shown that, in particular, Rac-2 is crucial in the signaling leading to cytoskeletal remodeling and cellular motility. Deficiency in Rac-2 or its upstream regulator PI3Kγ results in failure to chemotax and to degranulate upon GPCR activation. P-Rex1, a GEF for Rac, is believed to be regulated by G$\beta\gamma$ and PtdIns(3,4,5)P3 signaling and thereby links GPCRs and PI3Kγ activity to Rac-dependent neutrophil responses.[15]

Several other GEFs, for example, DOCK2 and the Vav proteins, are also important for cell movement. Upon GPCR stimulation, neutrophils deficient for DOCK2 show impaired chemotaxis due to loss of polarization of the cell.[16] Vav1/3 knock-out neutrophils, on the other hand, show defective β_2 integrin-induced responses, which are specifically required for stable adhesion.[17]

Recent studies have suggested that GTPase Rap1 is also involved in neutrophil adhesion. We were the first to describe a syndrome of combined leukocyte adhesion deficiency and platelet dysfunction despite normal integrin expression, designated as LAD-1/variant syndrome.[18,19] In a boy with a similar clinical syndrome, then named LAD-III, Rap1 activity was found to be absent.[20] Therefore, the Rap1 regulation by CalDAG-GEF1 was suspected to be defective in these clinical pictures.[21] However, recently we and others showed mutations in *FERMT3*, encoding a hematopoietic structural protein called kindlin-3, as the genetic cause for this syndrome. Kindlin-3 acts as a switch to activate the integrins similar to what was known for talin at the time.[22,23] Whether and how exactly these two proteins interact is still unclear.[24]

IL17-PRODUCING CELLS AS A DRIVING FORCE BEHIND NEUTROPHILIA

Neutrophil release from the bone marrow into the blood stream has been extensively studied, and depends to a great extent on cytokines.[25] An important group of cytokines in this respect is the IL family, which is mainly produced by activated lymphocytes and macrophages. ILs stimulate other immune cells to differentiate and proliferate, and can induce fever. The identification of ILs, as well as discoveries of their receptors, and their production have markedly changed the way in which we study immunologic processes. Through the study of the role of IL-23 in autoimmunity, an alternative T cell subset was discovered: the adaptive Th17 cell, whose expression is promoted by both chronic inflammation and tissue damage.[26] The Th17 cell was identified as a cell that induces an inflammatory response by producing IL-17, IL-21, and IL-22, and is thereby clearly distinct from the well known Th1 and Th2 subsets. Furthermore, Th17 cells have been associated with autoimmune diseases.[27]

Interestingly, the IL-17–mediated immune pathway is induced within 4 to 8 hours following epithelial cell injury or activation of pattern recognition receptors (PRRs), which is too short to allow the development of Th17 cells. *In vivo* models of infection have shown that several innate cell subsets, among which unconventional $\gamma\delta$ T cells, cooperate to promote this immediate IL-17–mediated response. Studies of extracellular pathogens that target the lungs showed that IL-23 was a main stimulator of innate IL-17 production.[28] Innate IL-17–producing cells activate epithelial cells to release granulopoietic factors such as G-CSF, thereby recruiting large numbers of neutrophils crucial for effective and rapid control of bacterial and fungal pathogens. Furthermore, IL-17 synergizes with other inflammatory cytokines, such as IL-1, IL-6, and tumor necrosis factor (TNF)-α.

It is notable that IL-17 often works cooperatively with IL-22, during homeostasis as well as during pathogenic insults. In the airways, IL-17 and IL-22 have been shown to work together to promote the secretion of human β-defensin-2, β-defensin-3, and calgranulin by bronchial epithelial cells, essential for killing of bacterial and fungal pathogens.[29] A defect in this pathway may explain the recurrent skin and lung infections in hyper-IgE syndrome caused by dominant-negative mutations in STAT3, a transcription factor required for Th17 cell development. Synergistic effects of IL-17 and IL-22 may also play a role in the gut mucosa, on the induction of antimicrobial proteins by gastrointestinal epithelial cells. Hereby these cells, known as Paneth cells, limit dissemination of commensal bacteria that penetrate a disrupted epithelial barrier. These innate and adaptive memory immune cells can be assumed to act in concert to produce a basal level of IL-17 and IL-22, which then maintain a constitutive level of antimicrobial proteins.[30] At the same time, phagocytes are generated and attracted to guarantee a first line of defense.

RECOGNITION OF MICROBIAL-DERIVED INFLAMMATORY SIGNALS

Neutrophils bind a wide variety of pathogens and commensal microorganisms, including gram-positive and Gram-negative bacteria, viruses, and fungi. Binding occurs by affinity of neutrophil receptor proteins for common patterns, the pathogen-associated molecular patterns (PAMPs), on the surface of the pathogen. The receptors for PAMPs are called PRRs, and are expressed on the surface of or intracellular in many effector cells of the innate immune system (such as macrophages, dendritic cells, neutrophils) as well as epithelial cells. Whereas immune cells respond vigorously to invading microorganisms, the epithelial linings have to discriminate more selectively to avoid a response to the commensal flora in the guts, the oropharynx, or on our skin. The most common family of PRRs are the Toll-like

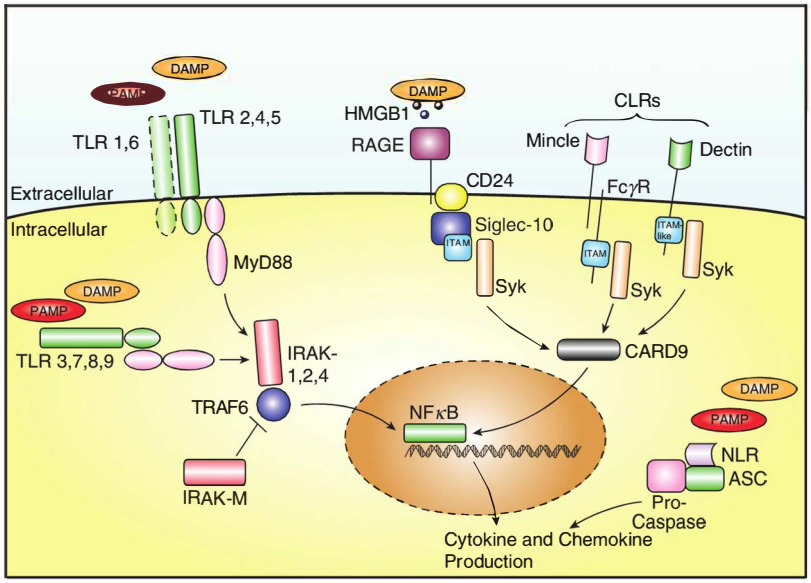

FIGURE 47.2 Pattern recognition receptors (PRRs). Several PRRs recognize pathogen-associated molecular patterns (PAMPs), for example, Toll-like receptors (TLR2/TLR1, TLR2/TLR6, TLR3,-4,-5,-7,-8,-9), C-type lectin receptors (CLRs; Dectin-1,-2, Mincle), and NLRs (NOD-like receptors; NOD1,2). DAMPs bind to carriers like HMGB1 and bind to RAGE as well as to certain TLRs. TLR3 and TLR7 are not expressed on neutrophils.

receptors (TLRs), which upon ligand binding recruit adaptor molecules to their intracellular signaling domain (FIGURE 47.2).[31] This interaction leads to the activation of several kinases that ultimately results in the activation of transcription factor NF-κB and immune-responsive genes and the release of cytokines, for example, TNFα. The TLRs recognize a wide variety of ligands, including lipopeptides by TLR2/TLR1 and TLR2/TLR6 and bacterial lipopolysaccharide (LPS) and flagellin by TLR4 and TLR5. Besides these extracellular pathogen fragments, microbial RNA and DNA are detected by TLR3, 7 and 8, and 9, respectively, all located inside the host cell.

Neutrophils express all TLRs, except TLR3 and TLR7.[32,33] However, in comparison with monocytes and macrophages, TLR2 and TLR4 are expressed at relatively low levels (in addition to the TLR2/TLR1 and TLR2/TLR6 dimers). This may explain partially why the concentration of LPS required to activate neutrophils is about 100- to 1,000-fold higher than that needed for monocytes.

Most TLR family members have a conserved intracellular signaling motif, the so-called TIR domain. This signaling motif, which is also found in the intracellular domain of the IL-1 receptor (IL-1R), is responsible for NF-κB activation/translocation after TLR or IL-1R ligand binding, and is an essential signaling pathway for IL-1β and TNFα secretion.[34] All TLRs signal through MyD88 and Mal, which interact with various IL-1 receptor-associated kinases (IRAKs). However, both TLR3 and TLR4 can also activate cells through a MyD88-independent pathway in which TRIF (TIR-domain-containing adapter-inducing interferon-beta) and TRAM (TRIF-related adaptor molecule) play a role. This can result in phosphorylation and activation of IRF3 or IRF7, which in turn activates a wide range of proinflammatory genes, such as interferon-β. These pathways are specifically active in monocytes and subtypes of dendritic cells. Although mRNA for both TRAM and TRIF is expressed in neutrophils, MyD88-independent signaling in neutrophils does not exist. LPS-responsiveness is strictly IRAK-4 dependent.[33]

Apart from the TLRs, another family of important surface-expressed PRRs is the transmembrane C-type lectin receptors (CLRs or CLECs).[35] The most studied CLR members to date are Dectin-1 (CLEC7a), detecting certain yeast species, and its close homologue Dectin-2 (CLEC4n), which recognizes several fungi. All CLRs are activating members of the myeloid-specific Ig superfamily.

The activating members of this family stimulate cells by virtue of cytoplasmic immunoreceptor tyrosine–based activation motifs (ITAMs) in their cytoplasmic tails or in the tails of associated molecules. Tyrosine phosphorylation of these molecules permits binding of phosphotyrosine kinases such as Syk and ZAP-70. Next, tyrosine phosphorylation of these kinases allows binding of tyrosinephosphatases such as SHP1 and 2, which results in activation of NF-κB and the subsequent secretion of cytokines and chemokines. Contrarily, the inhibitory members of the family contain an immunoreceptor tyrosine–based inhibitory motif (ITIM), which has the potency to decrease NF-κB activity.

A third CLR, CLEC4e (also known as Mincle), was also recently shown to recognize fungal pathogens, thereby inducing an intracellular signaling cascade that, besides phosphotyrosine kinases, involves adaptor protein CARD9.[36] This ultimately leads to the production of proinflammatory cytokines and chemokines, including TNFα, IL-6, CXCL1 (GROα), and CXCL2 (MIP-2), which stimulate the recruitment of neutrophils to infected tissues.[35]

In addition to extracellular pathogen recognition by TLRs and CLRs, intracellular recognition of microbial derivatives by host cells can also mount an appropriate immune response. PAMP recognition by these cytosolic NOD-like receptors (NLRs) results in assembly of protein complexes called inflammasomes.[37] Only some NLRs are present in neutrophils, and their role may be limited in this cell type. Macrophages and DCs are considered to be the main cell type in which the NLR proteins function as cytoplasmic or intracellular PRRs. The ubiquitously expressed NOD1 senses diaminopimelic acid–containing peptidoglycan present in Gram-negative bacteria, whereas epithelial and myeloid NOD2 sense the muramyl dipeptide present in most organisms. The cytosolic inflammasomes may contain different members of the NLR protein family, suggesting that

they have their own specificity, which in many instances has not yet been identified.[38]

DAMAGE-INDUCED INFLAMMATORY SIGNALS

In addition to recognizing PAMPs, the immune system has evolved to recognize endogenous danger signals or, by analogy, damage-associated molecular patterns (DAMPs), such as single-stranded DNA and extracellular ATP or nucleosomes, many of which are released by dying or necrotic cells (FIGURE 47.2). Recognition of DAMPs contributes to inflammation in a noninfectious sterile setting. One of the best known endogenous alarm signals is high-mobility group box 1 protein (HMGB1), which is a nuclear protein that modulates chromatin accessibility. When cells undergo necrosis, HMGB1 is released and plays an important role in initiating inflammation upon binding to several extracellular DAMPs, for example, DNA, RNA, or nucleosomes from the necrotic cell, as well as IL-1β and LPS.[39] These complexes between HMGB1 and DAMPs can interact with several receptors on macrophages, including HMGB1 receptor RAGE (receptor for advanced glycation end products) as well as TLR2, TLR4, TLR9, and IL-1R. This interaction induces macrophages to synthesize proinflammatory cytokines, such as TNFα and IL-6, as well as phagocyte-attracting chemokines, such as CXCL8 (IL-8), CCL3 (MIP-1α), and CCL4 (MIP-1β), thereby promoting the recruitment of neutrophils. Besides HMGB1, RAGE can also bind to several members of the calcium-binding S100 family of proteins and various RNA and DNA ligands. Not only HMGB1 but also the homologous HMGB2 and HMGB3 proteins have been reported to function as universal sentinels for nucleic acid–mediated activation of innate immune responses. The relative contribution of intracellular and extracellular HMGBs in this response remains to be determined.

Heat shock proteins (HSPs) and several cytokines, including IL-1α and IL-33, have also been shown to be involved in damage signaling. HSP exposed on dying cells serves as an immunostimulator, whereas cells dying under conditions of no HSP exposure are rapidly cleared without an immune response. It is unclear, however, whether HSPs function as DAMPs or that they are carriers of DAMPs, like HMGB1, and signal as a HSP–DAMP complex.[40] IL-33 is constitutively present in the nucleus of endothelial and epithelial cells, and is released upon damage together with HMGB1.[41] Unregulated IL-33 activity leads to activation of T-helper type 2 cells, mast cells, dendritic cells, eosinophils, and basophils, ultimately leading to increased expression of cytokines and chemokines that define the disease. Although widely studied in allergy and infectious models, the role of IL-33 in DAMP-associated inflammation has not yet been studied in great detail.[42]

NEGATIVE REGULATION MECHANISMS

Excessive inflammation can cause severe conditions like autoimmunity or (after infection) sepsis, a serious systemic disorder with a high mortality rate. Therefore, innate immune responses must be finely regulated, and underlying mechanisms are now being examined extensively. Several autoinflammatory mechanisms have been reported so far. In the case of PRR signaling, several negative regulators of TLR-mediated signaling pathways have been proposed.[43] First, overactivation of TLR pathways is prevented by IRAK-M. Whereas IRAK-1, -2, and -4 have stimulatory roles, IRAK-M prevents dissociation of activating IRAK-1 and IRAK-4 from MyD88 and subsequent formation of the activating IRAK-TRAF6 complexes. IRAK-M$^{-/-}$ cells exhibit increased cytokine production upon TLR/IL-1 stimulation, as well as increased inflammatory responses in mice to bacterial infection. Thus, IRAK-M negatively regulates TLR signaling and innate immune homeostasis by preventing, in particular, IRAK-4 signaling. Expression in myeloid cells of IRAK-M seems restricted to macrophages.[44] Several other cytoplasmic proteins are also implicated in these TLR-related counteractive processes.[45–47]

Other important regulators of the balance between pro- and anti-inflammatory signals are several members of the previously mentioned Ig superfamily. Certain subfamilies, for example, the KIRs (killer immunoglobulin-like receptors), ILTs (immunoglobulin-like transcripts), and SIRP (signal regulatory proteins), consist of ITAM- as well as ITIM-containing members in which the latter perform a counterregulatory role.[48] KIRs and at least two ILTs are known to recognize different members of the human leukocyte antigen (HLA) class I of proteins. SIRP1α, being a negative regulator, protects against inflammation upon binding hematopoietic self-antigen CD47 (integrin-associated protein).

Furthermore, to induce different outcomes of trauma and infection, the signaling pathways downstream of DAMPs and PAMPs are not identical.[39] It has recently been shown that the DAMPs, HMGB1 and HSPs, but not PAMPs, can interact with inhibitory molecule CD24, a glycosylphosphatidylinositol (GPI)-anchored membrane protein. CD24 binds sialic acid–binding immunoglobulin-like lectin-10 (Siglec-10), which contains an ITIM and is an inhibitory member of the previously mentioned Ig superfamily.[49] The CD24 and Siglec-10 receptor complex appears to provide a molecular brake for endogenous danger signals without compromising responses to infectious agents.

Another molecule that may distinguish between DAMP and PAMP pathways is thrombomodulin, a cell-surface glycoprotein expressed on vascular endothelial cells that has anti-inflammatory as well as anticoagulant functions. Thrombomodulin binds HMGB1 and promotes its cleavage, thereby blocking its interaction with RAGE and thus reducing its proinflammatory potential.[50]

Thrombomodulin is also able to downregulate the complement system. The role of complement in innate immunity is known for its association with the susceptibility of certain infections and the involvement in direct clearance of microorganisms and immune complexes.[51] To prevent unwanted complement activation and deposition on the host's own cells, complement activation is controlled by a number of regulatory complement proteins, among which is complement factor H (CFH).[52] Heterozygous mutations in the genes encoding CFH and thrombomodulin may both result in the clinical trials of nonimmune hemolytic anemia, thrombocytopenia, and renal impairment, known as atypical hemolytic-uremic syndrome (HUS).[53] It implicates that—apart from CFH and its cofactor CFI—also thrombomodulin is able to facilitate complement inactivation.

During tissue infiltration, negative regulation of neutrophils is performed through CD200 receptor (CD200R). CD200R is expressed by infiltrating neutrophils and monocytes, and engages its ligand CD200, which is a glycoprotein exposed by many opposing tissue cells. Hereupon, CD200R restrains tissue-damaging activity by associating with the

SH2-containing inositol phosphatase (SHIP), consistent with a role in downregulation of myeloid activity.[54]

IgG RECEPTORS BRIDGE INNATE AND ADAPTIVE IMMUNITY

Besides their importance for the primary innate immune response, neutrophils play an important role in adaptive immunity. Complement factors and IgG antibodies bind to microorganisms in a process called opsonization, whereupon these complexes can be bound by neutrophils. The proximity of the Fc regions of the IgG antibodies promotes direct binding of the opsonized microorganisms to the Fcγ receptors FcγRs (FcγRs) on the neutrophils. There are three main classes of FcγRs: FcγRI, FcγRII, and FcγRIII, which are all members of the Ig superfamily.[55] Each has its characteristic IgG-binding avidity, functional properties, and expression profile among hematopoietic cells.

The activating members of the FcγR family, that is, FcγRIa, FcγRIIa, FcγRIIIa, and FcγRIIIb, contain ITAMs or associate with ITAM-containing signaling molecules, whereas a single inhibitory Fcγ receptor, FcγRIIb, contains an ITIM. The balance between activating and inhibitory IgG receptors largely depends on the cell type and the expression level of the various receptor subtypes.

Further complexity arises from the fact that each FcγR is encoded by various genes, some of which can undergo alternative splicing. For instance, FcγRII is encoded by three genes (*FCGR2A*, *FCGR2B*, and *FCGR2C*). Recently, we have found evidence for the functional expression of FcγRIIc, encoded by *FCGR2C*,[56] which was previously assumed to be a pseudogene.[55] More genomic variation was demonstrated by the presence of copy number variation as well as a gene-dosage effect on the functional expression of FcγRIIc, FcγRIIIa, and FcγRIIIb.[57,58] We and others have indicated the presence of such variation in cohorts that suffer from systemic forms of autoimmunity, vasculitis or hematologic disease, indicating potential contribution to the susceptibility, and course of clinical disease in autoinflammation or infection.[58,59]

DEGRANULATION

After phagocytosis of a microorganism, or in its close proximity, intracellular granules in the neutrophils fuse with the plasma membrane or phagosomal membrane, thereby releasing the contents out on the pathogen. Granule constituents are traditionally considered as potent antimicrobial peptides and proteolytic enzymes. These proteins assist in the killing and digestion of microorganisms, but are also potentially harmful to the host if released inappropriately. The granules exocytose only after considerable cell activation, which is a fail-safe mechanism preventing unnecessary injury.

Neutrophils contain different subsets of granules: azurophil, specific and gelatinase granules, and secretory vesicles (FIGURE 47.3).[60] The azurophil granule proteins are synthesized at the promyelocytic stage only. Specific granule proteins are synthesized at the myelocyte stage, with an increasing amount of gelatinase being formed at the later metamyelocyte and early-band cell stage, after which granule formation stops. The so-called secretory vesicles are believed to be formed by endocytosis and fuse with the plasma membrane most readily, to some extent already during standard purification steps.[61]

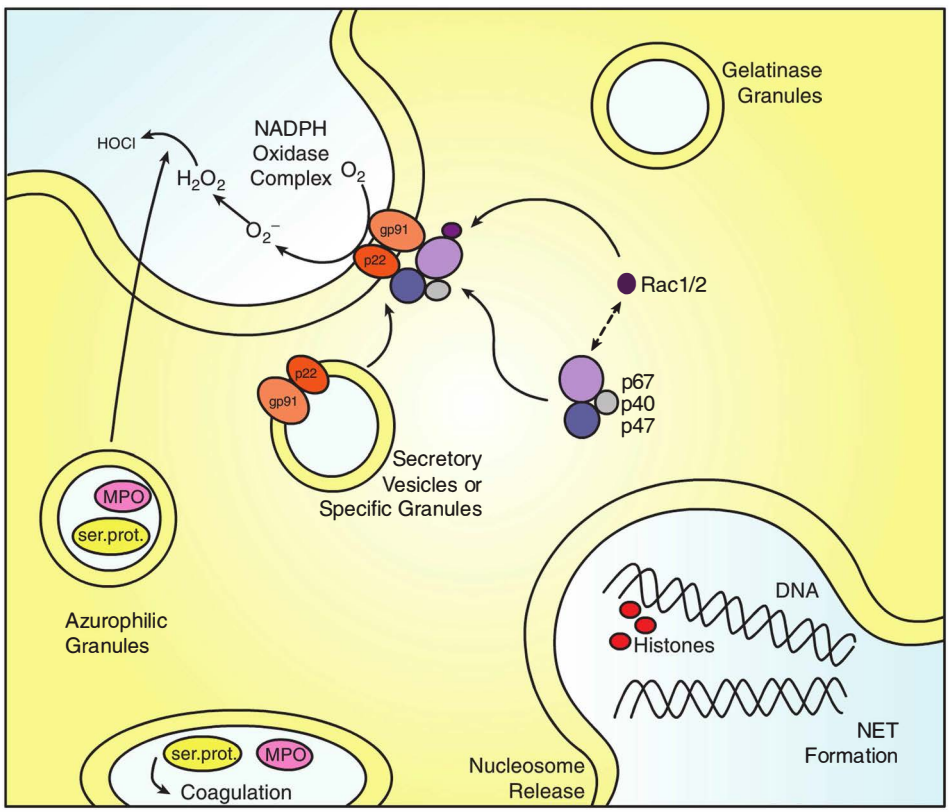

FIGURE 47.3 Neutrophil degranulation and nucleosomes' release. Neutrophils contain several granule subsets (i.e., azurophilic, gelatinase and specific granules, and secretory vesicles) as well as nucleosomes, which release cytotoxic compounds. Nearby or after phagocytosis of pathogens, cytoplasmic p67, p40, p47, and Rac1/2, together with gp91 and p22 from secretory vesicles or specific granules, form the NADPH oxidase complex, which generates ROS. Azurophilic granules release serine proteases such as elastase, which are cytotoxic as well as capable to induce a simultaneous coagulatory response. Release of nucleosome components results in pathogen trapping in NETs and damage signaling.

The specific granules support cell movement in the tissues, and the azurophilic granule proteins are for killing and degrading ingested material. Moreover, not all proteins released from the specific granules act proinflammatory. Also several protease inhibitors, such as secretory leukocyte protease inhibitor, α_1-antitrypsin, and cystatins, are released from the specific granules of neutrophils.[60] These soluble proteins may help to respond rapidly to the environment and at the same time minimizing tissue destruction by, for instance, secreting inhibitors of its own proteases.

Recent data have revealed that some granule proteins, previously believed to be specific to neutrophils, are also expressed in a variety of other cell types, for instance in epithelial cells of skin and mucosal barriers. The important difference is that epithelial cells make these antibacterial peptides and proteins only when an infection or inflammation is established, whereas neutrophils have these antibacterial proteins stored in granules ready for use without any delay when needed.

KILLING ACTIVITY

Simultaneous with degranulation, an oxidase enzyme complex (flavocytochrome b_{558} subunits p22-*phox* and gp91-*phox*) located in the membrane of secretory vesicles and specific granules is activated to generate ROS needed in the killing process (FIGURE 47.3). This NADPH oxidase complex is activated in the plasma membrane or the phagosome membrane by fusion of these vesicles and granules with the membrane and by translocation of a number of activity-regulating proteins from the cytoplasm (p40-*phox*, p47-*phox* and p67-*phox*).[62] The enzymatic subunit is gp91-*phox*. This protein consists of a membrane-spanning *N*-terminus, containing two heme moieties, and a hydrophilic, probably cytosolic, *C*-terminus, which contains one FAD group and one NADPH-binding domain. The other flavocytochrome b_{558} subunit, p22-*phox*, serves to stabilize gp91-*phox* in the membrane of specific granules and secretory vesicles and in the phagosomal membrane of activated cells.

The proteins p40-*phox*, p47-*phox*, and p67-*phox* form a complex in the cytosol of resting cells. This complex unfolds during cell activation, in concert with activation of small GTPase Rac2 (in neutrophils) or Rac1 (in macrophages).

Upon interaction of the cytosolic components with flavocytochrome b_{558}, the enzyme complex starts to generate superoxide (O_2^-). NADPH donates electrons, which are passed on within gp91-*phox* from the NADPH-binding site to FAD, then to the two heme groups, and finally to the edge of the protein that faces the phagosome or the extracellular space. At that site, the electrons are attached to molecular oxygen, thus generating superoxide. Superoxide spontaneously dismutates into hydrogen peroxide (H_2O_2), which may then react with chloride ions to form hypochlorous acid (HOCl), in a reaction catalyzed by myeloperoxidase. This enzyme is an abundant constituent of neutrophil azurophilic granules, and is released upon cell activation into the phagosome and into the extracellular space (FIGURE 47.3). HOCl is very toxic for a broad range of microorganisms, but is rather short-lived. However, it can react with primary and secondary amines, and thus gives rise to *N*-chloramines, some of which are very stable, microbicidal agents. Under normal, phagocytosing conditions, neutrophils convert more than 75% of their superoxide into HOCl and *N*-chloramines, and thus create a highly toxic environment within the phagosomes and in the cell surroundings.[63] Patients with a defect in the NADPH oxidase system suffer from a syndrome called chronic granulomatous disease, characterized by recurrent, serious infections with bacteria, yeasts, and fungi.[64]

Besides phagocytosis of pathogens and the secretion of cytotoxic compounds, there is a third way of defense against pathogens: the formation of neutrophil extracellular traps (NETs) (FIGURE 47.3).[65,66] When neutrophils are overwhelmed by pathogens, the necrotic cells release DNA and histones that form long net-like stretches, which, together with large amounts of granular antimicrobial enzymes, are capable to bind, disarm, and kill pathogens extracellularly. The granular cytotoxins are concentrated within the NETs, thereby minimizing damage to the host cells. In addition to their role in pathogen killing, the NETs may serve as a physical barrier that prevents further spreading of the pathogens.

The role and contribution of cell death in NET formation have been recently reviewed. The involvement *in vivo* of NETosis remains unclear to date. Moreover, *in vivo* discrimination between fibrin and NETs is not as easy as once thought.[67] NETosis or the passive leakage of DNA from dying cells may well contribute to the inflammatory process, as will be described below for nuclear proteins including HMGB1 but also for DNA-associated histones.

SYSTEMIC INFLAMMATION AND DAMAGE CONTROL

As mentioned above, overactivation of the inflammatory system can induce severe conditions like sepsis. In humans, sepsis is defined as suspected or proven infection plus a systemic inflammatory response syndrome (e.g., fever, tachycardia, tachypnea, and leukocytosis).[68] Severe sepsis is defined as sepsis with organ dysfunction (hypotension, hypoxemia, oliguria, metabolic acidosis, thrombocytopenia). Septic shock is defined as severe sepsis with hypotension, despite adequate fluid resuscitation. This situation of septic shock represents a combination of an inflammatory response and ischemia/reperfusion injury in which both PAMPs and DAMPs are taking their toll. Septic shock and multiorgan dysfunction are the most common causes of death in patients with sepsis. The incidence of severe sepsis is estimated to be between 70 and 150 cases per year per 100,000 population. The mortality rates associated with severe sepsis and septic shock are around 30% and 60%, respectively.

Several inhibitors of inflammatory pathways have been suggested as a potential cure for sepsis. However, antibodies toward cytokines, such as anti-LPS, anti-TNFα, and IL-1-antagonism, failed to inhibit septic shock in humans. Up to now, none of the TLRs have been targeted to bend inflammation or infectious disease in the human setting.

One of the major causes of severe damage and host mortality during sepsis might be the release of nuclear components of necrotic cells. As mentioned above, extracellular localization of nucleic protein HMGB1 induces inflammation, and this might be a positive feedback loop causing a switch from local inflammation to sepsis. In mice, injection of nucleus-derived HMGB1 is lethal.[69] These observations raise the possibility that nuclear contents in the circulation might indicate severe damage and contribute to mortality in the host. In concordance with these results, inhibition of HMGB1 has been shown to inhibit sepsis remarkably. In mice, antibodies against HMGB1 inhibit LPS lethality and reduce lung inflammation following airway LPS

exposure. The activity of HMGB1 under these conditions seems to depend on RAGE, since RAGE-deficient mice are also protected from endotoxemia-induced lethality. Although widely expressed, RAGE expression on the endothelium appears to be essential and sufficient to mediate lethality.[70]

Thus, antagonists of HMGB1 may be highly effective to avoid unwanted damage, even when administered at a later time point in the inflammatory response.[71] In humans, the late application of a blocking agent is the only realistic option that matters. However, the major problem until now is the lack of high-affinity monoclonal antibodies and the involvement of HMGB1 protein homologues in the DAMP-mediated responses.

HMBG1 is not the only nuclear protein involved in sepsis or septic shock. Release of nucleosomes during necrosis results in increased serum concentration of histones H3 and H4, which have a high cytotoxic potential.[72] Histones kill endothelial cells *in vitro* and mimic the symptoms of sepsis, including organ failure, when injected into mice. Antibodies against histone 4 (H4) protected mice in three different models of sepsis: injection of LPS, injection of TNFα, and a cecal ligation and puncture (CLP) model.

An alternative approach was reported recently; this concerned blockade of a survival and proliferation signaling molecule, sphingosine-1 phosphate kinase (SPHK1).[73] SPHK1 expression was found to be highly upregulated in neutrophils and macrophages stimulated with various bacteria or bacterial components, including LPS. SPHK1 blockade in two mouse models of sepsis—the first involving intraperitoneal injection of a lethal dose of LPS and the second involving CLP to trigger polymicrobial sepsis—showed 100% survival. A small inhibitor of SPHK1 was 100% effective if given 2 hours after CLP, with a much reduced efficacy (10%) if given 12 hours after CLP. However, combined treatment of the inhibitor and broad-spectrum antibiotics to treat patients with sepsis had synergistic effects, extending the time window for treatment and resulting in better outcomes than either drug alone, which is most welcome in medical practice.

To date, the most promising drug trial for critically ill septic shock patients consisted of the early administration of APC, although the efficacy is still relatively limited.[68,74] APC effectively degrades the toxic extracellular histones. It targets also protease-activated receptor-1 (PAR1) and endothelial cell protein C receptor, which are essential for protection in a mouse sepsis model.[72]

Despite its known role as a natural anticoagulant, APC appears to have important anti-inflammatory functions. Recombinant human soluble thrombomodulin has also been identified as anti-inflammatory and a successful drug toward sepsis in humans. Thrombomodulin is also most prominently known as an anticoagulant.[75] The high potency of these anticoagulants to treat sepsis implicates that the coincidence of inflammation and coagulation potentiate each other considerably in sepsis-induced host damage. However, for the same reason, the disadvantage of sepsis treatment with these proteins is an increased risk of bleeding.

SYNERGISM OF INFLAMMATION AND COAGULATION

Clinical data give multiple implications for the coincidence of inflammatory and coagulation problems. First, the majority of critically ill patients with a systemic inflammatory response have coagulation abnormalities. These abnormalities may vary from a mild thrombocytopenia to thrombosis and bleeding, known in its most severe form as disseminated intravascular coagulation (DIC). In a consecutive series of patients with severe sepsis, the mortality of patients with DIC was 43%, compared with 27% in those without DIC.[76] Furthermore, several studies have shown the importance of IL-6 in the initiation of coagulation activation and the role of TNFα and IL-1 in the regulation of physiologic anticoagulation.

All together, there is a clear simultaneous induction of inflammation and coagulation, suggesting that this synergism is beneficial for the inflammatory response. In line herewith, recent studies have confirmed that activation of coagulation by neutrophils results in "trapping" of bacteria in small blood vessels.[77] During coagulation, the production of blood clot components such as fibrin is increased, which allows formation of NET-like structures, thereby preventing tissue damage during killing of pathogens.

Inflammation can induce the procoagulatory response through several pathways. The most prominent pathway involves tissue factor. Tissue factor is a cell surface procoagulant glycoprotein, expressed by extravascular tissues and conspicuously absent from most cells that come in contact with blood, that is, endothelial cells, red blood cells, lymphocytes, neutrophils, and platelets. After injury, blood is exposed to tissue factor, which results in a procoagulant pathway involving factor VIIa, IX, X, and Xa and induces generation of thrombin from prothrombin. One key function of thrombin is to convert fibrinogen into fibrin, a major component of blood clots. During infection, the fibrin forms barriers that restrict microbial dissemination. In addition, thrombin itself is also involved in inflammation, as it is chemotactic to neutrophils and monocytes and stimulates the release of IL-1 and CXCL-8 (IL-8). Furthermore, thrombin-activated endothelial cells or platelets express P-selectin, thereby facilitating neutrophil rolling and adhesion to platelet-bound fibrinogen in the hemostatic plug.

Although the general view of hemostasis has been that tissue factor only becomes exposed after vessel wall injury, it can as well have its origin within the vessels. Resting monocytes have very little tissue factor activity, but several inflammatory mediators, such as LPS, IL-1, IL-6, and TNFα, for example, during sepsis, can induce tissue factor synthesis in monocytes (see Chapter 46).[76,78] Furthermore, leukocyte-derived tissue factor–bearing microvesicles may fuse with activated platelet membrane and have thereby emerged as a possible contributor to normal hemostasis and thrombosis.[79,80]

Tissue factor antigen is present in most cells, but it is functionally inactive. The physicochemical basis for activation of tissue factor is unknown. Phosphatidylserine has been proposed as a possible mediator, based on the fact that addition of phosphatidylserine to tissue factor–containing vesicles enhances its activity. Phosphatidylserine, like other anionic phospholipids, is normally sequestered on the inner leaflet of the membrane bilayer.

Besides the stimulatory linkage between inflammation and coagulation through tissue factor, several inhibitory pathways are also involved. The physiologic inhibitor of tissue factor is tissue factor pathway inhibitor (TFPI), which is synthesized by endothelial as well as by other cells. TFPI is degraded by serine proteases like elastase, which are released from neutrophil granules (FIGURE 47.3). The previously mentioned extracellular

histones bind TFPI and serve as a platform for its degradation by elastase. In mice, infusion of a mutant TFPI protein resistant to proteolysis by elastase results in decreased bacterial clearance, showing the crucial role of tissue factor and the subsequent generation of fibrin in host defense.[77]

Next to TFPI, other anticoagulants are the previously mentioned APC and thrombomodulin. Thrombomodulin binds to thrombin and inhibits its role in coagulation. This heterodimeric thrombomodulin/thrombin complex converts protein C into APC. As mentioned above, APC and thrombomodulin have been recently discovered as potent drugs in the treatment of septic shock.

In addition to synergism between inflammatory and coagulatory pathways, neutrophils and platelets undergo physical interactions. Activated platelets attract neutrophils and have increased P-selectin expression, thereby allowing formation of neutrophil–platelet clots.[81] Similarly, arthrosclerotic plaques can be the pathologic substrate for binding neutrophils in the pathogenesis of acute arterial thrombotic events. Furthermore, activated platelets induce nucleosome release, which results in TFPI degradation and a loop that increases coagulation even further. This pathway also stimulates the formation of NETs.

Thus, neutrophils and platelets interplay at several layers and the outcome should be controlled by a fine balance between pro- and anticoagulatory and inflammatory signaling. Overactivation of any of these pathways may result in an accumulated response and may trigger sepsis as well as (myocardial) infarction and stroke.

CONCLUSION

Neutrophils are a major leukocyte subset when it comes to degradation of pathogens. Several stimulatory pathways as well as negative regulatory mechanisms participate to tune this inflammatory response and to prevent damage to the host. However, severe systemic inflammation and septic shock cannot always be avoided. Interestingly, severe inflammation often occurs in parallel with coagulatory problems. In turn, inflammatory response may cause coagulation. The coincidence of these symptoms has led to the discovery of many synergistic pathways, including, for example, tissue factor, and the anticoagulatory proteins TFPI, thrombomodulin and APC, and several cytokines. These findings are now being implemented in the treatment of sepsis as well as coagulation problems, and major improvements in these therapies can therefore be expected in the near future.

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CHAPTER

48

Laboratory Markers of Endothelial Cell Activation and Dysfunction

EDUARD SHANTSILA, GREGORY Y.H. LIP, AND ROBERT P. HEBBEL

The diverse functions of the vascular endothelium are of critical importance in maintaining physiologic homeostasis, yet they also are participants in a great variety of disease processes.[1,2] These functions include provision of the physical and biologic interface between blood and tissue, integration of disparate signals and coordination of responses, governance of hemostatic balances, regulation of vasomotor tone, management of vascular permeability, participation in immune and inflammatory processes, transduction of mechanosensitivity signals, and operation as an autocrine/paracrine/endocrine signaling network. Yet, the endothelium retains the flexibility to exhibit regional and local variations in these functions as required. Moreover, the endothelial cell lies at the interface of these multiple physiologic functions and thereby fundamentally links them. Illustrative examples of this include the frequent associations of coagulation activation and of vasoregulatory disturbance with inflammatory states.

ENDOTHELIAL CELL ACTIVATION AND DYSFUNCTION

Each of these component biologies exhibits quantitative and qualitative variation (albeit with substantial interdependence), so endothelial phenotype potentially can exist in a complex continuum of states. Nevertheless, the traditional description of the endothelium as being "quiescent," "activated," or "dysfunctional" is still conceptually useful in biomedicine. In actuality, however, the normal state of the endothelium is anything but quiescent; rather it contributes active homeostatic adjustments for maintenance of physiologically optimal states, an example being its vigilant husbandry of vasomotor tone. Its normal phenotype is anti-inflammatory, antithrombotic, and antiadhesive.[1,2]

The term "activated" endothelium generally refers to its state of response—often localized or regional—to a challenge as part of the host response to disease. Typically, such states are adaptive and organized to be of benefit to the host. Examples are the endothelial cell's participation in the normal inflammatory response, adjustment of hemostatic balance to staunch bleeding or favor fibrinolysis, and its assumption of an angiogenic phenotype as required for wound healing and placentation.[1,2] Some activation responses occur rapidly (e.g., shedding of surface adhesion and hemostasis molecules, retraction, decryption of tissue factor [TF], and release of P-selectin, von Willebrand factor [vWF], and monocyte chemoattractant protein-1), while others require gene transcription and protein synthesis (expression of E-selectin, intercellular adhesion molecule [ICAM]-1, vascular cell adhesion molecule [VCAM]-1, plasminogen activator inhibitor [PAI]-1, TF, and platelet activating factor).

In contrast, "endothelial dysfunction" is typically used to describe states in which the extant endothelial biology is harmful to the patient, typically by developing phenotypes with features that are the converse of the normal state. Examples include the counterproductive roles of a dysfunctional endothelium in atherosclerosis,[3] chronic renal failure,[4] reperfusion injury physiology,[5] sickle cell anemia,[6] gestational disorders,[7] diabetes,[8] and sepsis.[9] Notably, these all involve widespread, not just localized, endothelial dysfunction, which is pathologic and has escaped from normal, delicate, adaptive control. Not coincidentally, each of these disorders includes disturbance of hemostatic systems and elevated risk for untoward clinical events, particularly thrombosis,[10] because an endothelial dysfunctional state potentially perturbs all arms of Virchow triad.

The difficulty arises in attempting to distinguish between activation and dysfunction. From the perspective of the cardiovascular literature, a functional deficiency of endothelial nitric oxide synthase (eNOS) is the sine qua non of endothelial dysfunction, as documented by experimental and physiologic studies.[11] Indeed, the eNOS knockout mouse provides the paradigmatic experimental model of endothelial dysfunction and demonstrates that this leads to deficient endothelial-dependent vasodilation, hypertension, increased thrombosis, atherosclerosis, and stroke.[12] Accompanying defects include loss of the nitric oxide (NO) inhibitory effect upon platelet aggregation, leukocyte adhesion, smooth muscle cell proliferation, and expression of some surface molecules (e.g., VCAM-1 and TF). In human biomedicine, however, eNOS is not absent but rather becomes functionally inadequate and uncoupled so that it produces superoxide. This state is promoted by disparate conditions, for example, inflammation, oxidative stress, substrate/cofactor deficiencies, elevated levels of the competitive inhibitor asymmetric dimethylarginine (ADMA), oxidized lipids, aberrant wall shear stress, perturbation by microparticles (MP), and others.

However, this eNOS-based definition of endothelial dysfunction is perhaps too restrictive, as the majority of clinical information about it is derived from atherosclerosis and its risk factors and comorbidities, in which deficient NO bioavailability is paramount. Yet, there are various dysfunctional states where it perhaps need not be.[10] Endothelial dysfunction additionally involves various mixes of multiple activation-related imbalances leading to a proinflammatory and prothrombotic phenotype, permeability barrier dysfunction, and oxidative stress in addition to disturbed vasoregulation. While inflammation in general, with its attendant oxidant generation, is a major cause of eNOS dysfunction,[13] it remains possible that defective endothelial-dependent vasodilation may not be a universal feature of endothelial dysfunction. Indeed, from the hemostasis perspective, endothelial dysfunction is typically regarded to be

the provisional interpretation if the patient is put at increased risk for clinical harm.[10]

Regardless of the definition, these changes provide the basis for the available laboratory testing of endothelial status. Interpretation of these is complicated by the continuum of activation states, and the ability of biomarkers to distinguish between activation and dysfunction is by no means unambiguous. This chapter discusses the four types of available laboratory testing that have sufficient endothelial specificity so as to be useful. Physiologic testing provides the gold standard of evidence for endothelial dysfunction from the cardiovascular perspective. Blood biomarkers and their associations with disease have received the most attention. Circulating MP, although still emerging as a tool, perhaps offers the most potential promise for revealing pathobiologically useful information about the endothelium *in situ*. Circulating endothelial cells (CECs) are still in development as a potentially useful tool. Cells popularly referred to as "endothelial progenitor cells" (EPCs) cannot be recommended as a biomarker of endothelial dysfunction. Predictably, most clinical information on validity and utility of these biomarkers is derived from the atherosclerosis context. However, that is advantageous since there is a wealth of information regarding all aspects of atherosclerosis, in particular its state of clinically inapparent, subtle inflammatory involvement that is tightly associated with endothelial dysfunction.

PHYSIOLOGIC ASSESSMENT OF ENDOTHELIAL FUNCTION

Dynamic regulation of vascular tone by the endothelium is realized by its production of substances having either vasoconstrictive or vasodilatory properties, the balance of which (along with other inputs) governs vascular hemodynamics. The major endothelial-derived vasodilator is NO,[14,15] which additionally contributes to several other aspects of vascular health, as noted above. In aggregate, these exert antiatherogenic effects.[16] Because endothelial dysfunction typically involves impaired endothelial-dependent vasodilatation, its investigation can provide useful information even if clinical focus is not upon vasoregulation *per se*. Indeed, physiologic assessment is the only method that directly identifies endothelial dysfunction.

Invasive Assessment

Angiography—the Gold Standard

Direct angiographic measurement of the change of coronary artery diameter, blood flow, and vascular resistance in response to an intracoronary infusion of acetylcholine (ACH) is considered to be the gold standard in endothelial function assessment.[17,18] Normal epicardial and microvascular coronary arteries respond to this by an increase in diameter and blood flow. In subjects with endothelial dysfunction, ACH causes paradoxical vasoconstriction and a decrease in coronary blood flow due to the disruption of NO-dependent vasodilation.

Plethysmography. Venous occlusion plethysmography records changes in the forearm volume after occlusion of blood circulation to the hand. Thereby, changes in vascular resistance after intravascular administration of vasoactive drugs can be assessed as a measure of endothelial function. The method is accurate and reproducible, but it is semi-invasive and labor intensive, thus limiting its clinical utility.[19,20]

Noninvasive Assessment

Imaging

Noninvasive assessment of coronary artery endothelial function can be performed using positron emission tomography imaging or contrast ultrasound by measuring the change in myocardial blood flow in response to pharmacologic (e.g., adenosine, dipyridamole) and/or physiologic (cold pressor test) stimuli. This technique permits calculation of coronary flow reserve as a parameter reflecting vascular smooth muscle and endothelial contributions to microcirculatory vascular function.[21]

Peripheral Arteries

Several methods have been developed for the assessment of peripheral artery endothelial function as a surrogate marker of coronary arterial endothelial dysfunction. Indeed, measurement of the vascular response to reactive hyperemia after arterial compression has become the most popular approach for the assessment of it. Flow-mediated dilation (FMD) tests conduit artery function, while peripheral artery tonometry (PAT) tests microvascular function.

The FMD response is recorded by brachial artery ultrasound imaging (FIGURE 48.1). This comprises the current noninvasive gold standard method, one that is extensively validated, has a solid biologic foundation, and correlates with coronary endothelial function.[22,23] Yet, it is very operator-dependent, exhibits diurnal variation, and requires substantial experience in order to achieve reproducible results.[24] Nonetheless, it is the evaluative tool that has been most widely deployed and for which the most data exists. PAT, on the other hand, is virtually observer-independent and is performed using a system that calculates a reactive hyperemia index based on the contralateral finger serving as control. The device has been approved by the U.S. FDA and has been validated in different populations. PAT also correlates with coronary endothelial dysfunction and FMD, and assessment of vascular health using it has been demonstrated to be feasible in outpatient settings.[25,26] Therefore, it is emerging as a potential clinical tool to identify patients at high risk for future cardiovascular events (CVE).[26] The relative limitation of the latter approach is the high cost of consumables.

Controls

It is essential that these approaches be accompanied by similar evaluation of endothelial-independent vasodilation (e.g., in response to a NO donating drug) so that the meaning of observations is not misinterpreted.

Clinical Utility of Vasoregulatory Assessment

Endothelial dysfunction is understood to be the earliest detectable vascular abnormality in the natural history of atherosclerosis and atherothrombosis and, therefore, is believed to have predictive value.[27] It is closely associated with the presence of traditional cardiovascular risk factors.[28] For example, even in the absence of known atherosclerosis abnormal FMD is observed in smoking, hypercholesterolemia, obesity, diabetes, and hypertension, and FMD deterioration parallels accumulation of such risk factors (FIGURE 48.2). This has been interpreted as supporting the concept that the endothelium serves as the ultimate integrator of all cardiovascular risk inputs (environmental, genetic,

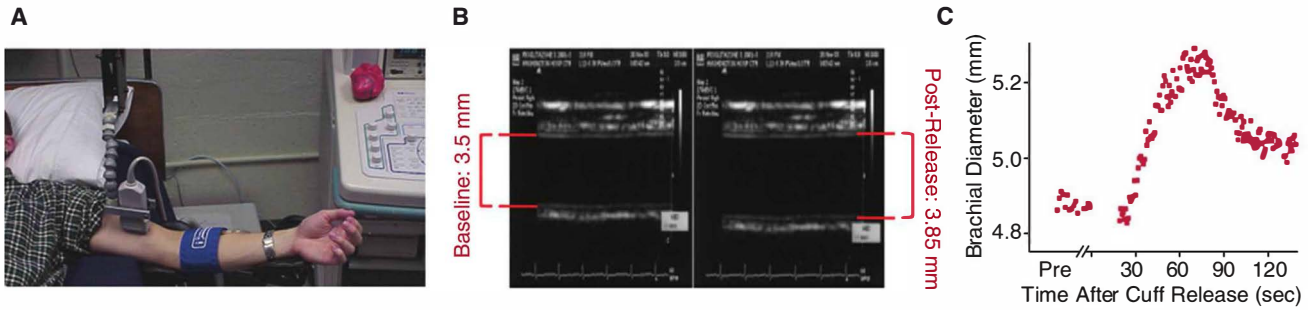

FIGURE 48.1 Measurement of FMD at the brachial artery. **A:** Placement of the ultrasound probe proximate to the site of transient arterial occlusion. (Kindly provided by Dr. Aaron Kelly, Pediatrics, University of Minnesota). **B:** Signal output of arterial wall diameter during rest **(left)** and postocclusion hyperemia **(right)**. (Reproduced from Barac A, et al. Methods for evaluating endothelial function in humans. *Hypertension* 2007;49:748–760, with permission from Wolters Kluwer Health.) **C:** Representative results from the multiple measures over time for a given patient. (Reproduced from Corretti A, et al. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery; a report of the International Brachial Artery reactivity task force. *J Am Coll Cardiol*, 2002;39:257–265, with permission from Elsevier.)

unknown) and, thus, provides a meaningful way to identify overall risk even in patients not having classical atherosclerotic risk factors.[18,29]

Endothelial dysfunction identified by aberrancy of endothelial-dependent vasodilation predicts future disease progression and is independently associated with future CVE.[18,29,30] In established coronary artery disease (CAD), it can predict future acute vascular events.[18,29] The major extant issue regarding the clinical applicability of FMD is that virtually all data are from cohort studies, such that applicability to the individual patient has not been robustly and prospectively studied. Yet, lending support to the belief that it meaningfully reflects biologic endothelial dysfunction is the improvement of FMD by therapeutic interventions that improve cardiovascular prognosis[32] such as statins,[18] weight loss,[33] smoking cessation,[34] and control of hyperglycemia[35] and hypertension.[36]

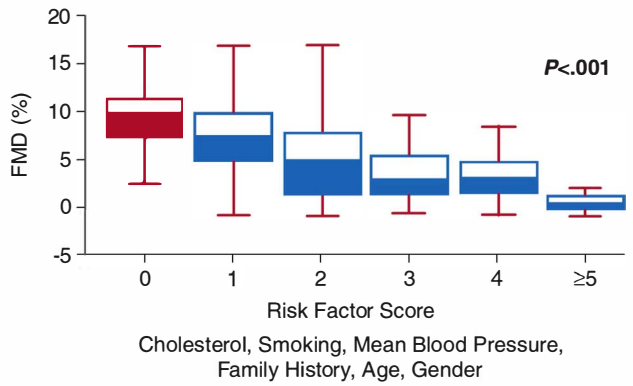

FIGURE 48.2 Inverse relationship between FMD and accumulating coronary risk factors in asymptomatic adults. Presence of hypercholesterolemia, smoking, hypertension, family history, age, and gender are represented by a proportionate, composite risk score. (Adapted from Celermajer DS, et al. Endothelium-dependent dilation in the systemic arteries of asymptomatic subjects relates to coronary risk factors and their interaction. *J Am Coll Cardiol* 1994;24(6): 1468–1478, with permission from Elsevier.)

Aside from atherosclerotic disease, abnormal FMD was observed in patients who had previously developed idiopathic venous thrombosis,[37] as well as in those with antiphospholipid antibody syndrome.[38] In patients with systemic lupus erythematosus, FMD has been observed to be[39] and to not be[40] abnormal, although blood biomarkers of endothelial dysfunction do tend to be abnormal (see below).

Caveats

Evaluation of FMD has been performed mostly in chronic conditions in which endothelial dysfunction would be strongly expected given the clinical context. Its potential value in many nonatherosclerotic disease settings, for example, sepsis, is a largely unstudied possibility, and interpretative caution is necessary, as FMD can be transiently diminished by unrelated concurrent situations (e.g., an acute viral illness).[31] Abnormal FMD has interestingly been reported in some unexpected situations, for example, migraineurs,[41] Alzheimer disease,[42] children of hypertensive parents,[43] rheumatoid arthritis,[44] and sarcoidosis.[45] It is reported to be alleviated by aromatherapy for night-shift workers.[46] It is not yet clear whether such observations reflect the substantial issues regarding test performance[47] or its specificity or the nascence of biomedical understanding of some pathophysiologies. The major source of variability in FMD measurement is between subjects, and the normal range is sufficiently large that definitive identification of endothelial dysfunction in an individual patient is difficult.

SOLUBLE MARKERS OF ENDOTHELIAL DYSFUNCTION

Direct, specific, and quantifiable detection of endothelial surface molecule expression on the activated/dysfunctional endothelium *in vivo* in clinical settings eventually will likely be achieved through engineering advances in reporter nanoparticles and imaging technologies. In lieu of such definitive testing, states of endothelial activation or dysfunction currently are often inferred from the increased levels of the soluble forms of these endothelial-derived molecules in the blood.

General Cautions

Substantial caveats apply to examination of circulating biomarkers. Of those used to probe endothelial cell function, only E-selectin is endothelial-specific; all others can be derived from other cellular sources and, therefore, reflect the biology of those as well. The usual reported levels of blood biomarkers derived from endothelial cells are based on plasma samples that do not distinguish between their truly soluble versus MP-bound forms. Levels of biomarkers in human disease overlap with their normal ranges, so data on their clinical instructiveness often have been derived from comparison of ends of the value spectrum (e.g., lowest versus highest quintiles). Additionally, virtually all studies rely upon single measurement at any given point of time, so that they are affected by normal variability, including diurnal variation for some. Although patients with documented endothelial dysfunction (by FMD) tend to have blood elevations of endothelial-derived biomarkers, there has been no comprehensive examination of the robustness of their independent association with documented endothelial dysfunction.

In general, these issues render biomarker measurements more useful for large patient studies than for application to the individual patient. Moreover, some biomarkers appear to retain sufficient functional characteristics so as to actively influence disease pathobiology. Thus, clinical correlates with biomarker elevations potentially can be different in different disease contexts. Nonetheless, they have been widely used, and some do have predictive value.

Endothelial Adhesion Molecules

The vascular endothelium exhibits carefully orchestrated modulation of its surface to govern its coordinated interactions with circulating blood cells. This involves multiple adhesion molecules, cytokines/chemokines and other mediators.[48] The principal endothelial molecules are P- and E-selectins that mediate initial tethering/capture/rolling, and VCAM-1/ICAM-1 that enforce stronger and stationary adhesion. Experimental studies have revealed that increased endothelial expression of such molecules is a typical and participatory feature in the development of atherosclerosis.[49–52] For example, knockout of either E- or P-selectin renders mice less susceptible to atherosclerosis, and knockout of both has additive benefits.[53,54]

Of note, however, blood levels of endothelial adhesion molecules do not necessarily quantitatively reflect the phenotype of the endothelial surface *in situ* because the levels of these soluble forms mostly reflect activation of the differing mechanisms causing their specific shedding (i.e., a cleavage event, shedding of MP, or release of an alternatively spliced variant). Thus, biomarker elevations in blood can occur in parallel with reciprocal diminished expression on the cell surface, and sometimes are seen even in the context of their concurrently diminished transcription. Due to such regulated shedding, their soluble forms (sP-selectin, sE-selectin, sVCAM-1, sICAM-1) are typically—but variably—increased in states of endothelial activation and dysfunction, and they have often been used to presumptively identify such states.

So, within the data set from a study focused on a specific disease context, a higher level of soluble endothelial adhesion molecules can be associated with worse endothelial dysfunction (as documented by FMD),[55,56] although this discrimination can require the within-study empirical identification of a high/low cutoff point. Conversely, soluble adhesion molecules can be elevated in active vascular disease absent abnormal FMD, as reported for lupus patients,[57] emphasizing that such elevations can reflect endothelial activation without actual (apparent) dysfunction, as narrowly defined by abnormal FMD. The resulting levels of these molecules tend to track together,[58] so sorting out their independent predictive value for future trouble even in the context of endothelial dysfunction can only be done via large studies (Table 48.1).

Endothelial Selectins

E-selectin has the greatest degree of endothelial specificity, it is not constitutively expressed normally, and it becomes expressed on the endothelial surface upon activation in the inflamed endothelium.[50] Yet, sE-selectin has been disappointing as a biomarker for prediction of future CAD in prospectively evaluated healthy subjects.[58] It may or may not be useful to indicate injury responses or cardiac disturbances.[59–61] Of interest, sE-selectin was observed to be elevated in lupus vasculopathy patients having thrombotic risk.[62]

P-selectin is stored in Weibel-Palade bodies and is constitutively expressed on endothelial cells at extremely low levels. It is also expressed in megakaryocytes and is stored in the alpha granules of platelets. Its surface expression on the endothelium and appearance in the blood (perhaps due to both cleaved and alternatively spliced forms[63]) can robustly increase in response to varied stimuli,[50] so plasma sP-selectin often parallels endothelial activation/dysfunction. However, utility vis-à-vis endothelial status is negated by the nonspecificity of its origin (i.e., from platelets also). Elevated sP-selectin can add confirmatory information in acute thrombosis and has predictive value in terms of recurrent deep vein thrombosis and future venous thromboembolism in patients with and without cancer.[64] However, a large prospective trial did not support any value for sP-selectin in predicting future CVE in patients with CAD[58] (Table 48.1).

Endothelial Cell Adhesion Molecules

The elaboration of soluble forms of endothelial CAMs by inflammatory cells confers some ambiguity upon interpretation of their typically elevated levels in inflammatory disease contexts. Endothelial VCAM-1 plays a prominent role in initiation and progression of atherosclerotic lesions.[49,50,65,66] Yet elevated sVCAM-1 levels had no predictive value for subsequent development of CAD or events (CVE) in prospective studies of healthy men[58,67,68] (Table 48.1). On the other hand, acute elevations predictably occur in acute coronary syndromes (ACS) in which higher levels may identify those having increased risk for future adverse CVE.[69,70] In type 1 diabetics, higher sVCAM-1 levels were associated with subsequent development of retinopathy, albuminuria, and cardiovascular disease.[71]

Endothelial ICAM-1 plays a similar pathogenetic role in atherosclerosis and is similarly released by nonendothelial cells in inflammatory states.[49–51] Yet, elevated sICAM-1 levels do appear to have value for assessing endothelial dysfunction in the atherosclerosis context (Table 48.1), in which they are tightly associated with levels of both C-reactive protein (CRP) and vWF.[58] Three large prospective studies of healthy men demonstrated that the highest elevations of sICAM-1 predict future CAD or CVE, independent of CRP or traditional risk factors.[58,67,72] Oddly, a large prospective study of healthy women found that

Table 48.1

Predictive value of circulating endothelial-derived adhesion molecules for cardiovascular disorders

Author [Citation]	Subjects	Follow-up	Predictive Value
Elevated Soluble E-Selectin (sE-Selectin)			
Malik [58]	5661 HM	16 y	Low for CAD
Elevated Soluble P-Selectin (sP-Selectin)			
Malik [58]	5661 HM	16 y	Low for CAD
Elevated Soluble VCAM-1 (sVCAM-1)			
Luc [67]	9758 HM 50-59	5 y	None for CVE
De Lemos [68]	14916 HM	9 y	None for CVE
Malik [58]	5661 HM	16 y	Low for CAD
Postadzhiyan [70]	75 after ACS	6 mo	Good for CVE
Elevated Soluble ICAM-1 (sICAM-1)			
Luc [67]	9758 HM 50-59	5 y	Good for CVE
Ridker [72]	14916 HM	9 y	Good for CVE
Malik [58]	5661 HM	16 y	Low for CAD
Jenny [76]	HS ≥ 65		Good for CVE
Albert [73]	23984 HW	10 y	Low for CAD None for CVE
Ray [80]	1164 after ACS	2 y	Mod for CVE
van der Harst [83]	87 after CABG	7.6 y	Good for CVE
Marcinkowski [81]	107 after MI	18 mo	Good for CVE
Postadzhiyan [70]	75 after ACS	6 mo	Good for CVE

ACS, acute coronary syndromes (acute MI or unstable angina); CABG, coronary arterial bypass graft; CAD, coronary artery disease; CVD, cardiovascular disease; CVE, cardiovascular event; HS, healthy subjects; HM, healthy men; HW, healthy women.

while higher sICAM-1 levels predicted CAD progression and even the need for coronary bypass, they did not predict CVE.[73] Increased sICAM-1 levels predicted subsequent stroke risk in type 2 diabetes[74] but not in healthy white women.[75] Thus, the utility of elevated sICAM-1 levels may be context dependent. Interestingly, temporally increasing sICAM-1 levels were associated with all-cause mortality for men and women older than 64 years.[76] In systemic lupus erythematosus, sICAM-1 elevations predict future first cardiac events.[77]

In ACS setting, sICAM-1 levels are elevated and parallel the increased expression of the monocyte receptor CD11b[78] and higher CRP and titers of antibody to oxidized low-density lipoprotein.[79] After an acute MI, higher levels of sICAM-1 measured 7 days or 10 weeks later predicted subsequent coronary symptoms and events.[69,80,81] Similarly, for hypertensive type 2 diabetes patients, sICAM-1 level paralleled degree of atherosclerosis.[82] However, data are not uniform, as studies following up ACS[70] or coronary bypass grafting[83] observed no predictive value of sICAM-1 for subsequent CVE.

Perhaps most strongly supporting the value of sICAM-1 measurement, its elevation correlated with impairment of FMD in obese hypertensive children and adolescents,[84] and even in apparently healthy adolescents.[85] The elevated sICAM-1 level in smokers decreases upon smoking cessation, perhaps bolstering its value as a meaningful marker.[86]

Conclusions Regarding Endothelial Adhesion Molecules

Thus, taken together these data identify elevated sICAM-1 levels as having considerable potential as a useful prognostic marker for increased risk of CAD and future CVE, but broader application to clinical predictions in endothelial dysfunction generally is not known. So overall, more study of its value in the clinical setting is necessary. The other candidate markers (sVCAM-1, sE- and sP-selectin) can all be elevated in endothelial activation states (with sVCAM-1 being the most commonly used indictor), but they cannot be relied upon to distinguish between activation and dysfunction. Indeed, the usefulness of soluble adhesion molecules, either independently or in various combinations, for predicting or quantifying measured FMD has not been comprehensively studied.

Hemostasis and the Maintenance of Blood Fluidity

Endothelial integrity is fundamental for separation of blood components from thrombogenic subendothelial materials, and the luminal surface of the healthy endothelium itself is anticoagulant and antithrombotic[1,2,10] (see Chapter 42). For example, it displays thrombomodulin (TM) and the endothelial protein C receptor (EPCR), a glycocalyx carrying antithrombin (AT) and

tissue factor pathway inhibitor (TFPI); it elaborates tissue-type plasminogen activator, along with low levels of PAI-1; and it has an absence of TF. Further, the endothelium produces molecules that impede platelet reactivity, particularly NO. These prominent antithrombotic characteristics can be challenged during endothelial activation and are typically changed dramatically in damaged or dysfunctional endothelium.[10] However, few of the consequent blood alterations are sufficiently dominated by endothelial biology so as to offer utility in detecting endothelial status vis-à-vis activation and/or dysfunction.

Thrombomodulin

Although TM is also expressed by monocytes and other nonendothelial cells, its thrombin-stimulated conversion of protein C to activated protein C (APC) is understood to be predominantly endothelial in location, where this is robustly augmented by its companion molecule, EPCR. Inflammatory mediators (particularly tumor necrosis factor α [TNFα]) elevate blood sTM levels but conversely diminish endothelial TM expression, thereby lowering APC generation capability. The latter is observed in a number of inflammatory vascular syndromes associated with endothelial dysfunction, sepsis perhaps being the paradigmatic example,[9] but others include vasculopathies, diabetic angiopathy, and acute respiratory distress syndrome.[87,88] Yet no direct comparison to measured global endothelial dysfunction (e.g., by FMD) has been reported. Moreover, protein C biology is heavily influenced by endothelial-independent events and activities.

Nevertheless, low APC levels are independently associated with increased risk of CAD and some CVE,[89] and deficient conversion of protein C to APC characteristically identifies adverse prognosis in sepsis syndromes and reperfusion injury.[90,91] Conversely, increased plasma levels of sTM are associated with increased mortality from trauma and acute respiratory distress syndrome.[87] In sepsis patients, in whom APC generation is variably impaired,[9,91,92] higher sTM levels presage development of DIC (disseminated intravascular coagulation), multiorgan failure, delayed recovery, and mortality.[87,93] For apparently healthy subjects not having CAD, higher sTM levels identified diminished risk for future coronary and cerebrovascular events.[94] Yet in those having established CAD, higher sTM identified enhanced risk for future CVE.[95]

It has consequently been suggested that sTM level possibly has varying implications depending upon the specifics of the extant vascular biology in different clinical contexts. Possibly additionally contributing to biologic heterogeneity, multiple TM cleavage sites underlie sTM release. Some, but not all, of the resulting fragments retain anticoagulation activity.[96] The extent to which different TM fragments (retaining varying functional domains) impact differentially upon biology requires exploration. Whether elevated sTM levels can generally assist with the fine distinction between endothelial activation and dysfunction is debatable, yet their association with certain catastrophic endothelial failures is clear.

Von Willebrand Factor

Plasma vWF levels are mostly endothelial in origin, despite some contribution from platelets.[97] Release of stored high molecular weight (HMW) forms via Weibel-Palade body exocytosis[98,99] is an immediate-early endothelial response prompted by a spectrum of (patho)physiologic biologic mediators. Thereafter, its functional biology is dominated by anchorage to membrane P-selectin[100] and cleavage by liver-derived ADAMTS-13 (A Disintegrin And Metalloproteinase with a Thrombospondin type 1 motif)[101] to preempt the strong prothrombotic effect of HMW vWF.[102] Therefore, the largest forms of vWF are observed in plasma only under certain disease conditions,[103–106] and analysis of the vWF size distribution has not been found to be of value in assessment of endothelial function. On the other hand, high plasma vWF levels are associated with increased prospective risk for arterial thrombosis,[107] CAD,[108] and stroke[109] in the healthy population, and for future CVE in angina patients.[110] However, risk defined by vWF tracks closely with that defined by traditional risk factors and Framingham risk calculation, so it adds little value beyond classical risk assessment.[108,111] Interestingly, while vWF elevations in the atherosclerosis context are accompanied by inflammatory markers (e.g., sICAM-1, CRP), similar elevations in the apparently healthy that predict venous thromboembolism are not associated with these.[112] This may be an important clue vis-à-vis potential utility of vWF assessment more broadly in consideration of thrombotic risk.

Although measurement of plasma vWF has been used very widely to evaluate endothelial function, sometimes as the sole basis for assignment of "endothelial dysfunction," its utility is perhaps compromised by being too sensitive as a biomarker. Elevation of plasma vWF is probably a universal feature of endothelial dysfunction,[113] in part because the associated deficiency of endothelial NO production is the major endogenous inhibitor of Weibel-Palade body exocytosis.[98] Experimentally, pathologic vWF release and a consequent thrombotic renal microangiopathy occur in a relatively pure model of endothelial dysfunction, the eNOS knockout mouse.[114] Consistent with this illustrative association between vWF release and endothelial dysfunction, vWF elevations independently correlate with deficient brachial artery FMD in ACS,[115] hypertension,[116] and congestive heart failure[117] (FIGURE 48.3). However, elevated vWF levels are equally predictable in the disparate situations involving inflammatory activation since vWF behaves like an acute phase reactant.[113,118] It is further noteworthy that vWF levels vary with age, gender, race, and, in particular, genetics.[107] Overall, it seems improbable that vWF levels can distinguish between endothelial activation and dysfunction, although this issue certainly deserves more thorough clinical correlation and experimentation.

Other Biomarkers

Asymmetric Dimethylarginine

Plasma ADMA level, derived from multiple cell sources, is an emerging and unusual biomarker vis-à-vis endothelial status because it is a known inducer of endothelial dysfunction (as a competitive inhibitor of eNOS).[119] Elevated ADMA levels can be associated with deficient FMD,[120,121] and they seem to predict the future development of this even in healthy young subjects.[122] ADMA is proposed as an independent risk factor/indicator for CAD.[122] It predicts future adverse CVE in patients with established CAD[123] in high-risk CAD populations such as ACS,[124,125] hemodialysis and renal failure,[126–128] and in surgical[129] and critically ill[130] patients. In aggregate, these reports support the value of ADMA measurement, although much work is needed to examine its utility in additional clinical settings.

C-Reactive Protein

Derived from liver and blood monocytes, CRP is an acute phase protein that predictably is elevated in inflammatory states

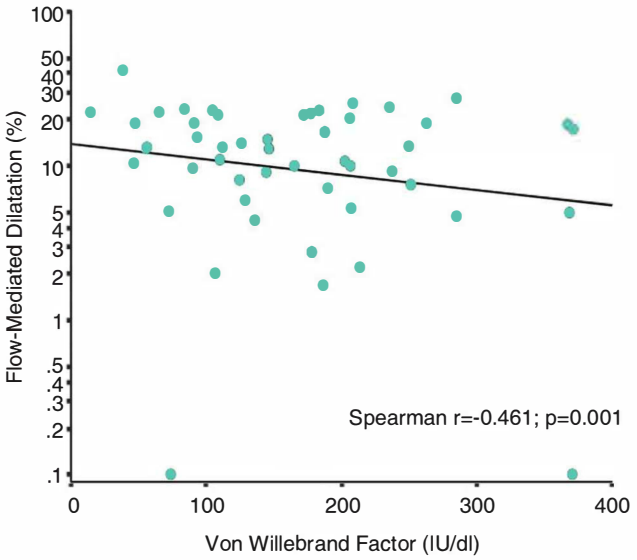

FIGURE 48.3 Flow mediated dilation is correlated with vWF level. These data are from patients with stable CAD. (Reproduced from Chong AY, et al. Endothelial dysfunction and damage in congestive heart failure: relation of low-mediated dilation to circulating endothelial cells, plasma indexes of endothelial damage, and brain natriuretic peptide. *Circulation* 2004;110:1794–1798, with permission from Wolters Kluwer Health.)

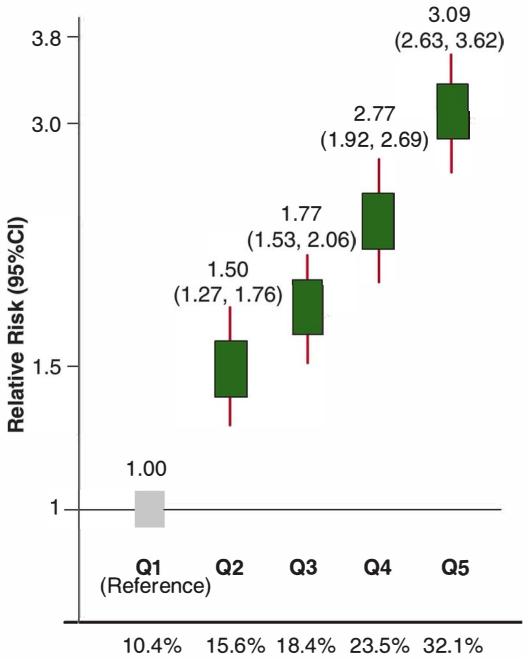

FIGURE 48.4 Relative prospective coronary risk increases in parallel with CRP concentration quintile. Numbers underneath the graph indicate what proportion of subjects falls into each of the quintiles of CRP level. However, enthusiasm regarding the utility of this relationship is mixed (see text). (Reproduced from Shah T, et al. Critical appraisal of CRP measurement for the prediction of coronary heart disease events: new data and systematic review of 31 prospective cohorts. *Int J Epidemiol* 2008;38:217–231, with permission of the International Journal of Epidemiology.)

generally, to approximately 500 mg/L in some cases, rendering it unusable as an endothelial biomarker in such contexts.[131] In the normal general population, baseline high sensitivity CRP (hsCRP) levels are divided roughly into thirds: <1, 1 to 3, and >3 mg/L. hsCRP measurement has emerged as a clinically useful biomarker in the subtle, subclinical inflammatory context of atherosclerosis natural history because of its amenability to precision measurement at low levels and its reportedly robust association with—and ability to predict—CAD.[131–134] Conversely, achievement of lower hsCRP levels using statin therapy is associated with lower incidence of recurrent events in CAD,[135] as well as diminished occurrence of venous thromboembolism[136] and CVE[137] in the apparently healthy. Many have argued that hsCRP level helps in cardiovascular risk stratification (FIGURE 48.4).

However, enthusiasm for this robustness of hsCRP is by no means uniform.[138] Levels of hsCRP are tightly associated with some classical CAD risk factors.[131] So while hsCRP elevation may be an independent indicator of endothelial dysfunction[139] and cardiovascular risk,[88,138] some have found that the hsCRP effect is actually less strong than that of traditional cardiovascular risk factors and, indeed, provides little if any incremental risk information.[138,140] Whether its measurement is truly of added clinical value in the patients who would benefit the most, those of intermediate cardiovascular risk, continues to be considered.

Further complicating interpretation, human CRP gene promoter polymorphisms that establish chronic elevations of plasma CRP are not associated with alteration of cardiovascular risk.[140,141] Thus, CRP by itself is probably not a biologic inducer of vessel wall disease, despite a variety of experimental observations finding that it induces predictably relevant adverse changes in monocytes and endothelium *in vitro*.[142–149] In this regard, three observations are most ominous. CRP triggers TF expression and procoagulant activity by monocytes *in vitro*,[143] eNOS uncoupling and impairment of endothelial-dependent vasodilation *in vivo*,[148] and *in vitro* degradation of the endothelial glycocalyx, a critical layer that normally preserves endothelial homeostasis.[149] Indeed, it has been argued that endothelial glycocalyx thinning is closely linked generally to endothelial dysfunction.[150] Among many effects, this results in release of proteins normally embedded in glycocalyx such as AT and TFPI.[151]

In summary, extant data argue against CRP being a pathogenic factor in and of itself, but hsCRP level does independently indicate future cardiovascular risk. How robust this association is, and whether it adds predictive value, remain to be settled. Clearly, hsCRP requires further study to examine any potential utility as a biomarker of endothelial dysfunction in nonatherosclerosis contexts.

Lipoprotein-Associated Phospholipase A2

Lipoprotein-associated phospholipase A2 (Lp-PLA_2) is derived from nonendothelial cells but is nevertheless an emergent biomarker that appears to be a strong, independent predictor of endothelial dysfunction in the atherosclerosis context.[152] Despite being an inflammatory responsive molecule, its association with endothelial dysfunction is reported to be independent of CRP, suggesting that Lp-PLA_2 deserves wider study as an endothelial biomarker.

ENDOTHELIAL MICROPARTICLES

The blood carries MP derived from platelets (PMP), monocytes (MMP) or other leukocytes, red cells (RMP), and endothelial cells (EMP). PMP are usually the most prevalent type, but the numbers of each type vary enormously depending upon clinical context and thereby exhibit some very interesting associations with vascular disease.[153] The various MP source types can be distinguished by their retention of some features of their cell of origin, enabling a focus upon EMP, which, are figuratively summarized in FIGURE 48.5.[154]

Most data merely emphasize EMP enumeration and its associations with pathophysiology. The extent to which EMP phenotype accurately reflects the extant phenotype and activation state of the source cell remains to be documented. Proteomic studies illustrate that EMP composition can vary depending upon the type of stimulus that promotes their formation,[155] but it is not known if phenotype acquisition precedes onset of the shedding process. Nonetheless, this approach may have the potential to offer more detailed and precise information about the functional status of the endothelium *in situ* than is presently obtainable outside of the research laboratory. At present, however, there are still substantial caveats regarding attainment and interpretation of such data, which are most properly regarded as identifying potential rewarding areas for clinical research development.

Definition and Formation of MP

Circulating MP are defined as small (0.1 to 1.0 μM in diameter), anucleate, phospholipid vesicles formed by exocytotic budding from activated/perturbed cells. They are distinguished from smaller exosomes (<100 nm) and from larger apoptotic bodies containing nuclear material (>1.5 μM). Shedding of MP is an orchestrated response to cell activation, one that involves multiple intracellular events.[156] Of total circulating MP, most bind annexin V, reflecting the externalization of procoagulant phosphatidylserine (PS) that typically accompanies their formation. On the other hand, the single study that examined it reported that even so, a substantial portion of MMP and EMP actually were annexin V negative.[157] The significance of this is unknown. The risk of misidentifying annexin V-positive apoptotic bodies or even small platelets as MP can be limited by application of rigorous methods. Although EMP derived from activation

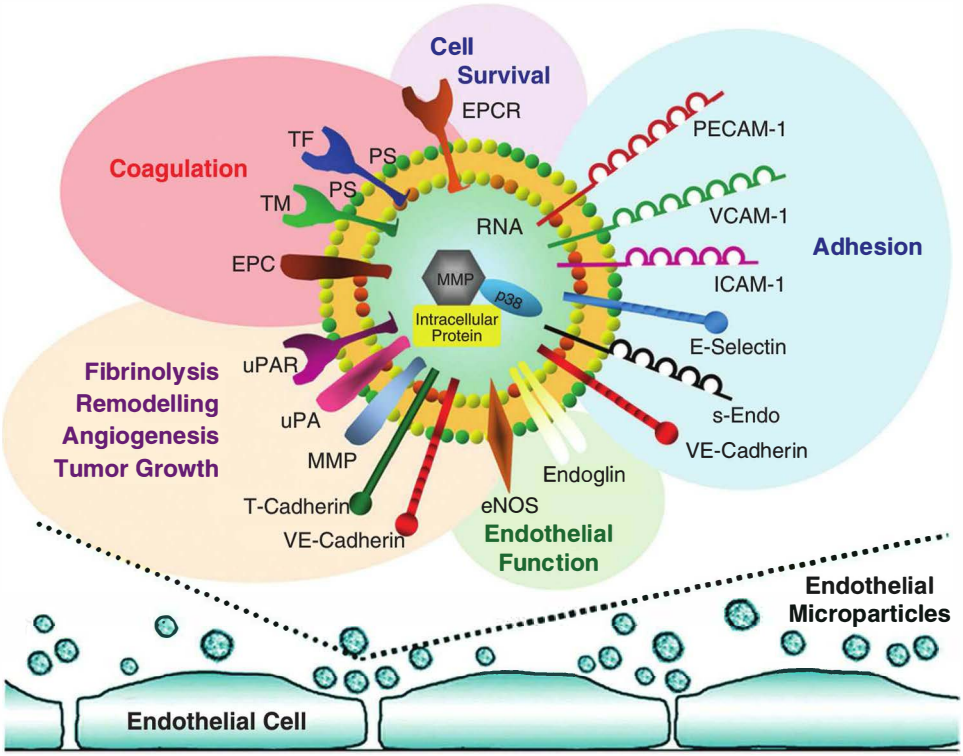

FIGURE 48.5 Depiction of a circulating endothelial-derived microparticle (EMP) showing many of the features reported for this type of MP. EMP features can include cytoplasmic components and surface features: endoglin (CD105); eNOS, endothelial NO synthase; EPC, endothelial protein C; EPCR, endothelial protein C receptor; E-selectin (CD62E); ICAM-1, intercellular cell adhesion molecule-1 (CD54); MMP, matrix metalloproteases; PECAM-1, platelet endothelial cell adhesion molecule-1 (CD31); PS, externalized phosphatidylserine; S-Endo/P1H12, melanoma cell adhesion molecule (CD146); TM, thrombomodulin; uPA, urokinase plasminogen activator; uPAR, urokinase plasminogen activator receptor; VCAM-1, VCAM-1 (CD106); VE-cadherin, vascular endothelial cadherin (CD144). Not shown on the EMP cartoon: α_v component of the vitronectin receptor (CD51); tissue factor (CD142). (Figure created and kindly provided by the laboratory of Dr. Francoise Dignat-George.) (Reproduced from Dignat-George F, et al. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* 2011;31:27–33, with permission from Wolters Kluwer Health).

versus apoptosis of cultured cells can be distinguished experimentally,[158] it is unclear that this distinction can be made in clinical samples. Shedding of EMP is initiated by thrombin, TNFα, endotoxin, CRP, aberrant shear stress, and other pathobiologic stimuli. Thus, elevations of circulating EMP are associated with a variety of vascular disorders (Table 48.2).

Structure and Phenotype of EMP

From the background total MP, identification of EMP is enabled by probing for surface proteins constitutively expressed by endothelial cells, for example, PECAM-1, endoglin, VE-cadherin, MelCAM (S-Endo–1, P1H12), and the vitronectin receptor[154,159]; P1H12/S-Endo–1 (CD146) or VE-cadherin are used most commonly. Additional rigor is provided by documentation of more than one endothelial marker, as well as negativity for markers of monocytes and of platelets. When EMP have been probed for phenotype in relevant clinical contexts (Table 48.2), they have displayed inducible endothelial markers, such as the CAMs and selectins. EMP expression of TF was observed in sickle cell anemia,[157] a disease that entails ongoing coagulation activation.[6] Interestingly, an unrelated proteomic evaluation of EMP obtained from cultured cells was interpreted to suggest that they comprise a circulating, storage pool of bioavailable TF.[160] In addition, EMP have been reported to carry/express oxidized lipids, transcription factors, mRNA, microRNA, matrix metalloproteinases, NADPH oxidase, CD39, protein C, EPCR, TM, plasminogen activator and its receptor, and other moieties.

An illustrative example of the potential robustness of circulating MP analysis is displayed in FIGURE 48.6.[157] These data from patients with sickle cell anemia (obtained using a rigorous method having outstanding specificity) demonstrate that a varying clinical context within a single disease can be associated with temporal changes in total MP number, proportionate contributions of the different source cells, and MP tissue factor antigen positivity.

Functions of EMP

Circulating MP can exhibit functionality. Therefore, it has been suggested that the EMP's surface composition of endothelial-derived molecules could function as a biologic address mechanism, resulting in targeting of EMP to specific locations defined by, for example, cognate receptor expression. Thus, it is relevant to consider which endothelial functions have been found for EMP.

Procoagulant

Like other MP types, EMP have procoagulant potential due to their externalized PS that facilitates prothrombinase assembly/activity. The paradigmatic example of this property is, of course, provided by the exuberant generation of PS-positive PMP during platelet activation. Likewise, MP bearing TF are understood to play a prominent role in thrombosis.[161,162] It has been proposed that the procoagulant activity of circulating MP varies with their phenotype: $PS^+/TF^+ > PS^-/TF^+ = PS^+/TF^- > PS^-/TF^-$.[163,164] Conversely, EMP can be positive for EPCR and APC,[165] for urokinase-type plasminogen activator (u-PA) and u-PA receptor (uPAR),[166] or CD39,[167] and exhibit corresponding antithrombotic and anti-inflammatory activities. The pro- or anticoagulant function of MP may well vary with context and cell of origin, something requiring much additional research attention.

Table 48.2

Circulating EMP (endothelial-derived microparticles) in human vascular disease

Condition	Endothelial MP
Atherosclerotic Risk and Disease	
Hypertension	↑ CD31+CD42-MP[186]
Diabetes	↑ CD144+ MP[188] ↑ CD51+ MP[189] ↑ CD31+CD42- MP[189]
Obesity	↑ CD31+CD42b- MP[187]
CAD	↑ CD51+ MP[186] ↑ CD31+CD42- MP[186] ↑ CD144+ MP[188] ↑ CD31+ MP[171]
LV dysfunction	↑ CD31+ MP[190]
Carotid disease	CD105+ MP[191]
ACS	↑ CD31+CD42- MP[191] ↔ C D51+ MP[191] ↑ CD54+ MP[192]
Acute stroke	↑ CD54+ MP[193] ↑ CD105+ MP[193] ↑ CD144+ MP[193] ↑ Annexin V+ MP[193] ↑ CD62E+ MP[194]
Obstructive sleep apnea	↑ CD31+CD42- MP[195] ↑ CD62E+ MP[195] ↑ CD31+AnxinV+ MP[195]
Nonatherosclerotic Vascular Diseases Having Increased Risk for Thrombosis	
Sickle cell anemia	↑ CD144+MP[157,196] ↑ CD144+CD142+MP[157] ↑ CD105+[196] ↑ CD105+ CD54+ MP[196]
Paroxysmal nocturnal hemoglobinuria	↑ CD105+ MP[196] ↑ CD105+CD 54+ MP[196] ↑ CD144+ MP[196]
Acute phase TTP	↑ CD31+CD62E+CD42b-, & ↑ CD54+CD62E+ & ↑ CD144+ & ↑ CD62E+vWF+ MP[158]
SLE +aPL ± thrombosis	↑ CD51+ MP[184]
Pre-eclampsia	↑ CD31+CD62E+ MP[199]

aPL, antiphhospholipid antibody; LV, left ventricle; MP, microparticles; SLE, lupus erythematosus; TTP, thrombotic thrombocytopenic purpura. For EMP features, CD designations are defined in legend to Figure 5. Features used to exclude PMP: CD41, GPIIb; CD42b, GPIba.

Endothelial NOS Impact

MP can impair eNOS function, as shown *ex vivo* by the adverse impact of EMP derived from cultures,[168] from renal failure patients,[169] and from CAD patients having myocardial

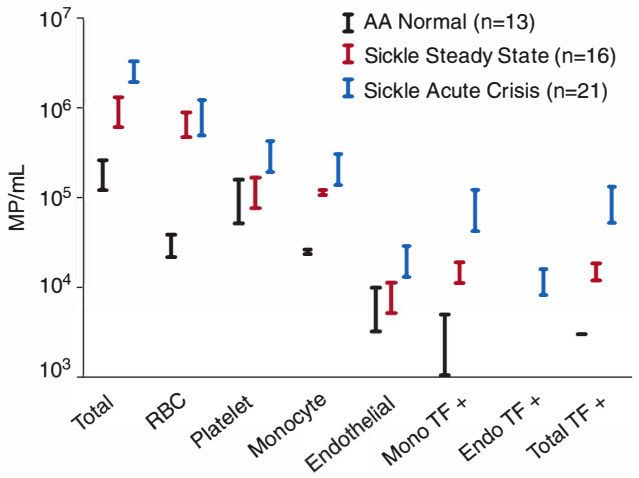

FIGURE 48.6 Example of circulating microparticle (MP) survey showing tissue factor (TF) positive MP. These data are from African American control subjects (*black bars*) and from sickle cell anemia patients either in "steady state" (*red bars*) or during an acute vaso-occlusive crisis (*blue bars*). These two sickle contexts are associated with chronic coagulation and platelet activation, which increase in the acute crisis state. Here, MP were defined rigorously: <1 μm in size plus positivity for annexin V binding plus ability to be pelleted out by ultracentrifugation. This had outstanding specificity but not optimal sensitivity (see text). Results indicate that (a) MP number varies widely in terms of cell-of-origin type, (b) MP type and number both vary with specific clinical context even within this single disease, and (c) additional instructive phenotype information can be identified and linked to cell-of-origin. (Data for this figure were derived from Shet AS, Aras O, Gupta K, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood* 2003;102(7):2678–2683.)

ischemia (but not those without ischemia)[170] upon endothelial-dependent dilation of rodent vascular rings. In the renal study, this adverse effect was not observed for EMP derived from healthy subjects.[169] Significantly, in patients with renal failure,[169] CAD,[171] and type 2 diabetes[172] brachial artery FMD correlated inversely with the elevated number of EMP[169,171,172] (FIGURE 48.7A,B). The renal failure study additionally noted that MP from nonendothelial sources (largely PMP outnumbering EMP by ~5:1) did not exhibit this correlation with FMD.[169]

In a low-disease burden model, enforced physical inactivity prompted EMP increases in normal volunteers,[173] interesting because lower levels of spontaneous physical activity induce a decrement in endothelial function (by FMD).[174] Thus, the data, albeit limited, suggest that elevated EMP levels not only result from endothelial dysfunction but also may promote it. The extent to which EMP induction of depressed FMD (an indicator of eNOS function) is accompanied by broader facets of endothelial dysfunction (e.g., inflammatory and prothrombotic phenotype) is not known and should be assessed. Whether this EMP response/effect is generally true in disease states is not known, although it does not escape attention that both EMP increases (Table 48.2) and depressed FMD seem to appear concurrently in multiple inflammatory states.

Other Signaling Effects and Biologic Roles

It is beginning to be apparent that the process of MP formation involves a degree of selective sorting of source cell molecules. This has been interpreted to raise the possibility that MP comprise a system of biologic cross-talk between cells, perhaps an orchestrated shuttling mechanism. Some studies have confirmed that MP can serve as messengers, transferring proteins and RNA/microRNA between cells.[175–178] Biologic roles for EMP in atherogenesis and atherothrombosis have been envisioned in terms of both pathogenic and protective mechanisms.[179,180] Likewise, participatory roles for MP in general have been proposed for sepsis,[181] angiogenesis,[182] thrombosis,[183] and the antiphospholipid syndrome in lupus.[184] Such proposals generally emphasize the roles of (higher numbers of) PMP and MMP, although sorting out which MP subpopulations (e.g., EMP vs. the others) contribute what to biology will be a formidable challenge.

Clinical Associations

Atherosclerosis Correlates

Although data are limited, and no comprehensive study has been done, studies in aggregate are beginning to indicate that EMP number may roughly parallel the natural history of atherosclerosis development and its characteristic deficient endothelial-dependent vasodilation. Elevated number of EMP is associated with abnormal arterial elasticity in apparent normals[185] and with severity of hypertension.[186] EMP count correlates inversely with FMD in various risk factor states such as obesity[187] and type 2 diabetes,[188,189] as well as in established CAD[171] (FIGURE 48.7B). Likewise, elevations of EMP count are associated with various cardiovascular complication events.[190–196] Finally, a study of 488 consecutive cardiology patients documented that EMP level roughly paralleled disease advancement in terms of risk/stage/acuity (FIGURE 48.7C), added further value to Framingham risk estimates, and predicted increased occurrence of subsequent CVE (FIGURE 48.7D).[197] Attainment of the latter observation specifically was perhaps predictably prejudiced by study design, but others similarly have argued that EMP count may assist in risk stratification.[198]

Other Disorders

Elevated EMP numbers also have been reported in thrombotic thrombocytopenic purpura (TTP), antiphospholipid syndrome, lupus anticoagulant, vasculitis, chronic venous insufficiency, venous thromboembolism, sickle cell anemia, paroxysmal nocturnal hemoglobinuria, eclampsia/preeclampsia but not gestational hypertension, and in a variety of inflammatory conditions including sepsis. A number of these are already known to be associated with abnormal FMD.

Conclusions Regarding MP

Methodology remains the greatest impediment to broad application of MP profiling by fluorescence-activated cell analyzer/sorter (FACS). The usable, gold-standard, but excessively cumbersome approach required a decade ago[157] (FIGURE 48.6) fortunately has been supplanted by multiple improvements.[154]

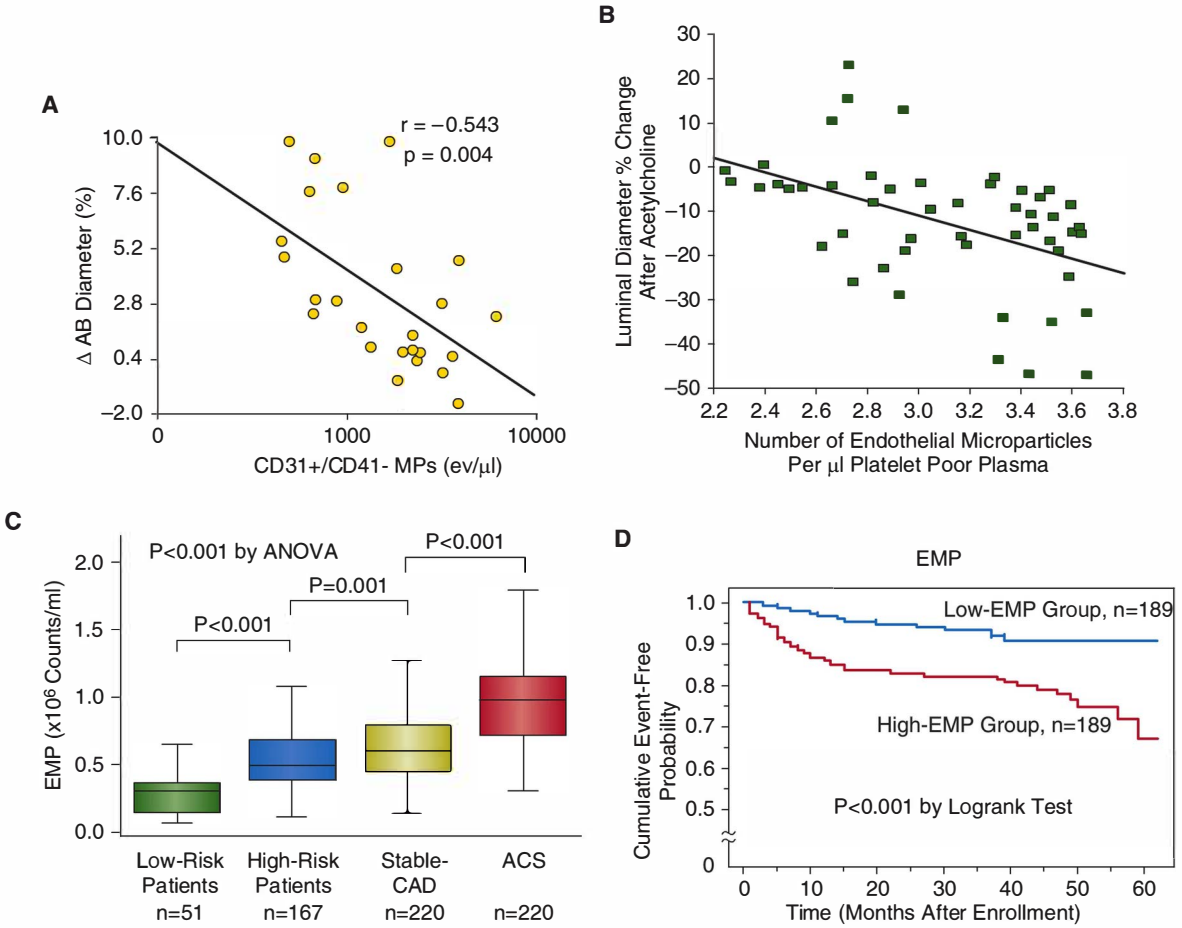

FIGURE 48.7 Relationship between EMP and endothelial function and coronary disease status. Flow mediated dilation (FMD) at the brachial artery (vertical axis) is inversely related to EMP count in end stage renal failure patients **(A)** (Reproduced from Amabile N, et al. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol* 2005;16:3381, with permission from the American Society of Nephrology.) and CAD patients **(B)** (Reproduced from Werner et al. Circulating CD31+annexin V+ apoptotic microparticles correlate with coronary endothelial function in patients with coronary artery disease *Arterioscler Thromb Vasc Biol* 2005;26:112–116, with permission from Wolters Kluwer Health.) **(C)** In consecutive patients, number of EMP increases in parallel with coronary disease status. (Reproduced from Nozaki et al. Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. *J Am Coll Cardiol* 2009;54:601–608, with permission from Elsevier.) **(D)** For the same CAD patients, those with higher EMP counts at presentation (*red*) had higher occurrence of subsequent CVE.

Nonetheless, ongoing attempts to achieve a consensus operational definition of MP and a standardized methodologic approach have been disappointing, as there are many issues that can impact results that still need to be standardized or solved.[154] The predominant challenge is presented by accurate assessment of the lower half of MP size spectrum (<500 nm), so many studies to date have focused only upon MP > 500 nm in size. Yet for optimal clinical utility, it seems probable that the full size range should be included (recognizing that MP of different sizes might have different phenotypes, functions, or disease associations), along with positivity/negativity for annexin V binding, at least two markers of parent cell of origin, and the desired markers of activation/functional status. Hopefully, the evolving availability of abundant fluorochrome choices, multiplex platforms, and latest generation of FACS hardware will allow such optimization to be achieved, in which case a wealth of clinically useful information may be obtainable.

A critical unsolved question is whether the phenotype of circulating EMP accurately reflects the extant features of the parent endothelium *in situ*, or whether it is established by the biologic process of MP formation itself. The available data (Table 48.2) are most consistent with the former. But if the latter is true, then EMP phenotyping will perhaps be disappointing as a "biopsy" of the endothelial surface *in situ*. Clearly, this critical issue requires research focus. Nonetheless, EMP enumeration plus phenotyping would seem to offer the greatest potential of all the blood biomarkers for eventual ability to glean multifaceted, clinically instructive information.

CIRCULATING ENDOTHELIAL CELLS

The CEC evident in fresh blood most likely have become detached from vascular walls, as evidenced by their recipient origin in subjects having previously undergone gender mismatched marrow transplantation.[200] They are identifiably endothelial by their positive staining for multiple endothelial markers and their inclusion of Weibel-Palade bodies.[154] The normal number of CEC (≤10/mL blood in virtually all normals, and <5 in most [Table 48.3]) comprises only approximately 10^{-11}% of the adult's total number of vessel wall endothelial cells. It is not known if their presence serves some biologic function, but suggestions that they may serve to "resurface" denuded vessel wall are perhaps whimsical. On the other hand, the worrisome implication of their appearance in blood lies in the presumed reciprocal existence of denuded vessel wall. The number of CEC in the normal total blood volume corresponds to an estimated <0.5 cm^2 of total denuded surface area, presumably an unconcerning amount. However, the CEC levels observed in pathologic states are sobering. The approximately 50 CEC/mL reached in sickle disease[201] and the approximately 200/mL reached in TTP[202] would correspond roughly to reciprocal denudation of approximately 7.5 cm^2 and approximately 30 cm^2, respectively—presumably not trivial. Whether this interpretation is actually realized in vascular biology is not known and deserves research attention.

CEC Detachment

The detachment mechanisms undoubtedly are complex and multifactorial but uniformly must involve impairment of endothelial cell anchorage to subendothelial matrix and adjacent cells. This event could be explained by various inciting processes including actual dislodgement by mechanical stress and a lifting response to biologic mediators such as TNFα,[203] inflammatory leukocyte proteases,[204] metalloproteases released from dysfunctional endothelial cells,[205] endothelial apoptosis that is hypothesized to be a prominent aspect of some vascular pathobiologies,[206] or the vanishingly slow turnover of the normal vascular wall endothelium. The latter may account for the tiny number of CEC found in normal individuals, but the inciting mechanism underlying abnormally increased CEC levels in disease undoubtedly varies amongst vascular disorders.

An increased number of CEC is usually assumed to reflect elevated generation, but in fact it is not known if CEC level is actually dominated by changes in clearance rate, a distinct possibility. It has been shown that infused autologous canine blood outgrowth endothelial cells are removed with probable single-pass kinetics,[207] which might imply that the CEC observable in fresh blood have very recently appeared. The balanced contributions of production and clearance also may well be different in different clinical disorders.

CEC Methodology

The rarity of CEC presents an enormous challenge to their enumeration and phenotyping.[154] Although prohibitively cumbersome, the gold standard method for CEC counting (direct manual immunofluorescent microscopic examination of an entire known amount of blood) has virtually 100% sensitivity and specificity.[201] Prior to this step, however, most studies have employed some form of centrifugation and/or enrichment using immunobeads carrying antibody to CD146 (either PIH12[201] or S-Endo-1[208]), although this results in loss of about 20% of CEC. Encouragingly, it may be possible to achieve acceptable specificity and reasonable CEC counts via direct FACS analysis without the immunobead step,[209] suggesting that FACS approaches to multiparameter phenotyping will enhance the potential utility of CEC assessment. A caveat is that depending upon the clinical context, serial observations should be considered. Longitudinally measured CEC counts in sickle patients, for example, varied significantly and rather chaotically depending upon clinical specifics.[201]

Information Derivable from CEC Analysis

While CEC enumeration and phenotypic profiling has by no means been done systematically or comprehensively, the accumulated reports focused upon single issues offer encouragement that this approach may have potential for obtaining clinically useful information.

Enumeration

CEC numbers are significantly increased in vasculotrophic infections, inflammatory vasculopathies, and atherosclerosis risk/disease. Such reports are summarized in Table 48.3 along with notations regarding ancillary findings of interest. Interestingly, elevations of CEC number have been observed to parallel increases of disease activity in systemic sclerosis,[210] systemic lupus erythematosus,[211] Mediterranean fever,[212] Kawasaki disease,[213] acute rejection of renal transplant,[214] and sickle cell anemia.[201] CEC level is reported to offer prognostic information in septic shock,[215] and it seems to predict risk for future CVE in hemodialysis[216] and ACS patients.[217]

Apoptotic Versus Viable CEC

CEC can be dead/necrotic cells (loss of permeability barrier), apoptotic cells (annexin V binding or TUNEL positivity), or still-viable cells[218] that exhibit very limited expansion potential if cultured[200]. These distinctions may well be indicative of the underlying disorder. Since endothelial cells are intolerant of loss of anchorage to survival-signaling matrix proteins, loss thereof triggers apoptosis pathways exhibiting (in cultured cells) a 10-hour half-time to death[219]. Thus, CEC should necessarily reside somewhere in the evolution of this process. Those dislodged most recently would have greater likelihood of being viable, so perhaps this could be an indicator of active dislodgement.

Yet even this is not so simple. For example, it was found that patients with sickle cell disease have an aberrantly higher proportion of CEC that are viable (64% vs. 30% in normals) rather than apoptotic,[220] seemingly implying that detachment is accelerated in this disease, especially since acute vaso-occlusive crises are associated with spikes in CEC count.[201] However, this unusually higher viability of CEC correlated with concurrent elevation of plasma levels of vascular endothelial growth factor (VEGF), a survival signaling growth factor,[220] so evaluation of CEC viability may not be a reliable indicator of blood residence time, at least in some cases. Despite this caveat, some enlightening results have been reported. For example, most CEC in antineutrophil cytoplasmic antibodies-associated small-vessel vasculitis are necrotic.[221] Apoptotic CEC dominate in many inflammatory disorders such as rheumatoid arthritis, whilst a

Table 48.3

Disorders with significantly elevated numbers of CECs*

Condition	CEC/mL nL	CEC/mL Cases	Noteworthy Special Observations
Atherosclerotic Disease and Risk-Related			
Coronary angioplasty [208]¶,§	<3	6–10	
ACS			↓ CD36 + CEC; some tissue factor + but generally
[222]¶,§	0	15	negative for end activation markers
[225]¶,§	<3	9	possible early marker non-ST elevation ACS
[226]¶,§	<3	11	Δ CEC ∝ Δ plasma vWF
			Δ CEC predicts future major events
			↑ eNOS + CD36 – CEC
[227]¶,§	11	52	CEC ∝ CRP
Childhood obesity [228]¶,§	<1	<1	↑ VCAM + CEC
PAD with rest pain [229]¶,§	1	3.5	∝ plasma vWF and TF levels
T2 diabetes [230]¶,§	10	69	
Pulmonary hypertension [231]†,§	<4	30	
Chronic heart failure [117]¶,§	<4	12	Strong correlation with ↓ FMD
Hemodialysis patients [216]¶,§	19	31	↑ CEC predicts future CVE
Nonatherosclerotic Vascular Diseases			
Sickle cell anemia [201,220,224,232]†,¶,§			↑ VCAM/ICAM/Psel/Esel + CEC
Steady state, nonacute	<3	13	↑ tissue factor + CEC
Acute vaso-occlusive crisis			↑ heme oxygenase-1 + CEC
		23	↑ CD36 + CEC
			↓ % apoptotic CEC ∝ ↑ [VEGF]
Thalassemias [233]#,§	4	47	↑ VCAM/ICAM/Esel + CEC &
			∝ TNF/VEGF/ProtC/ProtS [217]
Irreversible PAH [234]¶,§	3	57	
Sepsis syndromes [215]#,§			
- sepsis	<3	16	CEC # lower in survivors, higher in those who died
- septic shock		30	
Lupus erythematosus [209]#,‡	10	89	↑ annexin V + CEC ∝ ↓ FMD
Small vessel vasculitis [221]¶,§	5	136	↑ tissue factor and annexin V + CEC
Renal transplant rejection [214]¶,§	7	72	
Systemic sclerosis [210]‡	77	310	↑ Esel/CD106 + CEC & ∝ sol-Esel
Rickettsial disease [235]¶,§	1	162	
Kawasaki vasculitis [213]¶,§	6	15	
Aortoarteritis [236]†,§	16	58	Strong ∝ ESR
BMT conditioning [237]¶,§	8	16–44	

* Results shown as mean values, but greater information is derivable from the range of values within each disorder; citations are indicated by brackets. The symbol ∝ indicates "proportionate to". Methods vary: enrichment step utilized standard centrifugation (†) or density centrifugation (#) or immunobead enrichment (¶); counting/phenotyping utilized microscopy (§) or FACS (‡).
Abbreviations: ACS, acute coronary syndrome(s); BMT, bone marrow transplantation; CVE, cardiovascular events; ESR, sedimentation rate; FMD, flow mediated dilation as a direct indicator of endothelial dysfunction; PAH, pulmonary arterial hypertension.

temporal increase of the viable CEC was observed in patients with septic shock.[215]

Putative Vascular Origin of CEC

Actualization of identifying region of CEC detachment has been prevented by the unavailability of region-specific endothelial markers. It has been noted that CEC in sickle disease and in ACS are predominantly CD36 positive[201] and negative,[222] respectively, consistent with their respective natures as microvascular and macrovascular occlusive diseases.

Phenotype

An undetermined issue is whether CEC phenotype actually mimics that of the endothelium *in situ*. CEC activation phenotypes have been reported for a variety of disorders (Table 48.3). CEC examination also has been used to diagnose rickettsial disease[212] and to document therapeutic effect of attempted inhibition of endothelial inflammatory transcription.[223] Of present interest, TF (CD142) positive CEC have been detected in sickle disease[224] (FIGURE 48.9), in small vessel vasculitis,[221] and in acute myocardial infarction.[222]

CEC vis-à-vis Endothelial Dysfunction

CEC numbers are elevated in multiple clinical states known to involve endothelial dysfunction, including atherosclerosis risk/disease and sepsis (Table 48.3). In chronic heart failure[117] and in systemic lupus erythematosus[209] CEC levels were found to correlate inversely with FMD (FIGURE 48.8), but this correspondence has not been widely studied. Because of the seemingly dire implication of the presumed reciprocal denuded area indicated by CEC elevations, it seems reasonable to assume that these reveal existence of chronic endothelial dysfunction, but there is no specific proof of this. Whether this is true—and whether it is accompanied by untoward hemostatic activation—must be determined.

CONCLUSIONS

CEC can provide useful information if assessed manually (FIGURE 48.9), but if multiparameter profiling by FACS can be used accurately and reproducibly for this, much broader application will be enabled. However, CEC assessment needs to advance from essentially anecdotal descriptions of scantily profiled cells to a more systematic, rigorous, and critical assessment of the issues directly related to the believability of the information that their analysis yields. There are two fundamental issues that must be addressed if CEC evaluation can hope to advance from its current status as a boutique test of somewhat ambiguous meaning. To what extent does CEC phenotype mimic that of endothelium *in situ*, as opposed to being dominated by the (likely) temporally evolving state of their advancement toward apoptosis? If CEC phenotype is influenced by the evolution of apoptosis, can phenotyping still be informative, for example, by simultaneous, cell-by-cell documentation of viability status? Without answers to these questions, assessment of CEC will remain more of a curiosity than a biomarker of significance.

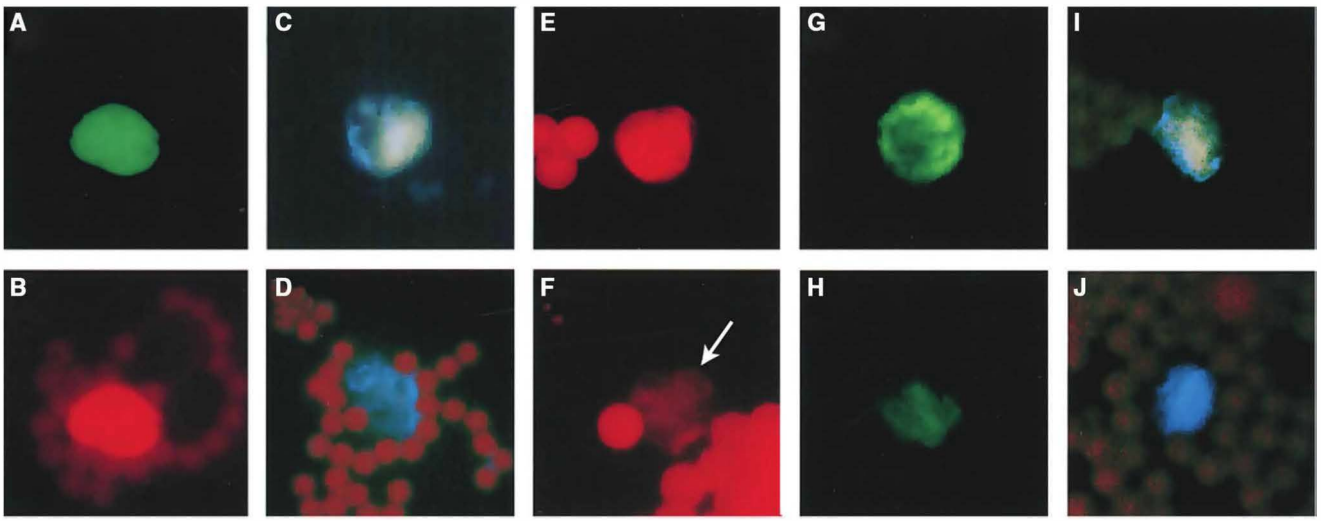

FIGURE 48.8 Assessment of circulating endothelial cell (CEC) tissue factor (TF) positivity. All panels show CEC obtained from sickle patients (smaller objects are the beads used for CEC enrichment). Panels are paired vertically to illustrate points. A single CEC is shown as positive for P1H12 **(A)** and TF antigen **(B)**. In **(C)**, a CEC that is P1H12 positive (*blue*) shows hybridization with antisense probe to TF mRNA (*yellow*), while panel **(D)** shows a control CEC having no hybridization using the control probe. **E:** A CEC stains positively with an antibody to factor VII/VIIa. **F:** That binding is abolished with a blocking antibody to TF (the faint CEC indicated by the arrow). **G:** A CEC incubated with factors VIIa and X exhibits factor X activation, as detected by a chromogenic substrate sensitive to factor Xa. **H:** That activity is abolished by a blocking antibody to TF. **I:** A CEC identified by P1H12 positivity (not shown) exhibits simultaneous expression of TF antigen (*blue*) and TF mRNA (*yellow-green*). **J:** The control CEC is positive for TF antigen (*blue*) but is negative for hybridization with the yellow control probe. Cells illustrated were representative of the sample slides from which they were selected. (Reproduced from Solovey A, et al. Tissue factor expression by endothelial cells in sickle cell anemia. *J Clin Invest* 1998;101:1899–1904] with permission of the American Society for Clinical Investigation.)

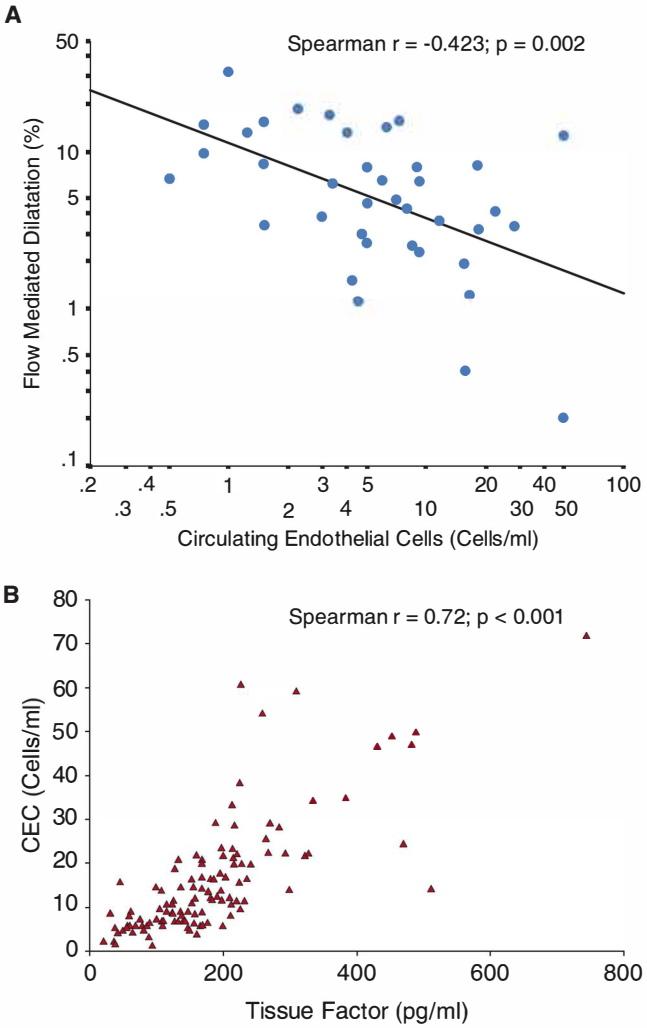

FIGURE 48.9 Relationship between circulating endothelial cell (CEC) count and other vascular parameters. **A:** In patients with congestive heart failure CEC count correlates inversely with FMD. (Reproduced from Chong AY, et al. Endothelial dysfunction and damage in congestive heart failure: relaxation of flow-mediated dilation to circulating endothelial cells, plasma indexes of endothelial damage, and brain natriuretic peptide. *Circulation* 2004;110:1794–1798, with permission from Wolters Kluwer Health.) **B:** In patients with ACS, CEC count correlates directly with plasma tissue factor content. (Reproduced from Lee KW, et al. Interrelationships of indices of endothelial damage/dysfunction (circulating endothelial cells, vWF and FMD) to tissue factor and interleukin-6 in acute coronary syndromes. *Int J Cardiol* 2006;111:302–308, with permission from Elsevier.)

ENDOTHELIAL PROGENITOR CELLS

Despite recent attempts to link varied levels of EPCs in blood with endothelial dysfunction, this cannot be recommended as a useful biomarker. Many "EPC" studies have utilized ambiguously defined and incompletely characterized cells and/or have assigned the term "progenitor" to what, in fact, are actually the outgrowth progeny of (possible) progenitors. Indeed, the universally cited seminal papers identifying "EPC" in blood[238] and using colony forming units ("CFU-Hill") to count them[239] both utilized a flawed methodology that actually detects a population of peripheral blood monocytes that undergo partial transdifferentiation in an environment rich in endothelial growth factors.[240,241] They thereby acquire some endothelial markers, but they retain myeloid markers and exhibit extraordinarily limited expansion potential; they are neither endothelial nor related to true endothelial progenitors. Fewer studies have actually directly or indirectly focused upon the circulating endothelial lineage cells that actually are capable of transplantation and high-fold expansion, their progeny usually referred to as blood outgrowth endothelial cells or late outgrowth endothelial cells.[200,240,241] Whether the parent cells of these are true progenitors, as is believed, has not been formally proven but is consistent with available evidence. Evidence is reasonably compelling for their residence and source being bone marrow,[200] but it alternatively has been suggested to be the blood vessel wall;[242] it could, of course, be both.

SUMMARY AND CONCLUSIONS

Despite the central role played by endothelial biology in physiologic homeostasis and its complex contributions to disease pathophysiology, there are no fully validated and universally applicable tests to document endothelial dysfunction generally in clinical medicine. The most specific available test is measurement of endothelial-dependent FMD at the brachial artery, an approach having clearly documented utility and unambiguous meaning in the setting of atherosclerosis and its risk factors. However, in endothelial dysfunctional states inferred from extant functional aberrancies causing clinical risk/harm, there are no comparable unambiguous biomarkers. The most generally useful biomarkers seem to be plasma levels of vWF and sICAM-1. Others, CRP and ADMA, are robust but probably have limited context specificity. Perhaps most intriguing are two emerging approaches, enumeration and phenotyping of CECs and blood MP. The latter would seem to offer the greatest promise for providing the richest (and real-time) insight into the functional status of endothelium *in situ*.

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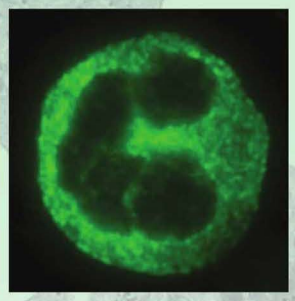
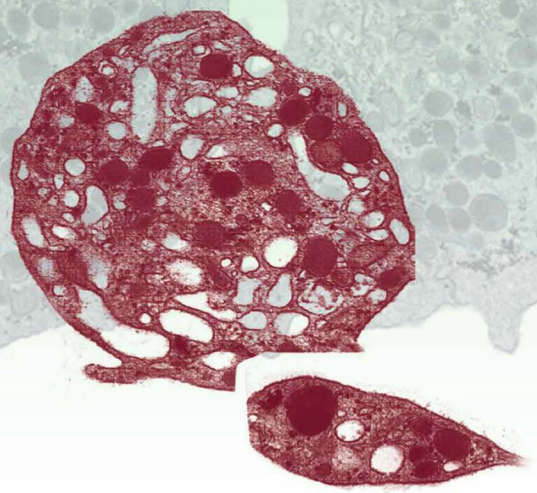
PART III





Disorders of Coagulation, Platelets, and Vessel Wall

SECTION A: BLEEDING DISORDERS OF COAGULATION AND FIBRINOLYSIS

CHAPTER 49

Overview of Inherited Hemorrhagic Disorders

GILBERT C. WHITE, II, HAROLD R. ROBERTS, AND VICTOR J. MARDER

The focus in Section A of Part III of this book is on inherited hemorrhagic disorders that result from deficiencies or abnormalities of the blood coagulation factors. Acquired hemorrhagic disorders, such as liver disease and diffuse intravascular coagulation (DIC), are presented in Part V of the text. Bleeding disorders from platelet defects are presented in the section on platelets (Section C, Part III) and bleeding disorders due to disorders that affect blood vessels are presented in the section on vascular defects (Section D, Part III). Altogether, worldwide, inherited hemorrhagic disorders due to deficiencies or abnormalities of blood coagulation factors number approximately 250,000. Thus, these are relatively rare but clinically important disorders.

Inherited disorders of blood coagulation have been known since antiquity. FIGURE 49.1 illustrates a passage from the Babylonian Talmud.[1] Rabbi Judah the Patriarch, redactor of the Mishnah, the second century compendium of Judaism's oral law, writes this passage which translates to: "If she circumcised her first child and he died, and a second one also died, she must not circumcise her third child." This demonstrates knowledge of the inherited nature of the bleeding disorder we now know as hemophilia and also demonstrates knowledge of the sex-linked nature of the disease as well as the clinical penetrance. General knowledge of inherited bleeding disorders such as hemophilia was further amplified when the daughters of Queen Victoria of England, a silent carrier for hemophilia A, married into a number of royal houses in Europe, including Russian, German, and Spanish royalty. The story of Princess Alexandra and her hemophilic son, Alexis, who was cared for by the mad monk Rasputin, is well known and was chronicled by Robert Massie, who was himself the father of a hemophilic son.[2]

Hemophilia in America goes back a long way too—relatively speaking—to the early colonial days in New England (FIGURE 49.2). John Oliver, the first known hemophiliac in the new world, was born in 1613 in London and came to Plymouth, MA, in 1639 on the ship, Jonathan, eventually settling in Ipswitch, MA. A daughter, Mary Oliver, was born in 1640. In 1656, Mary, an obligate carrier for hemophilia A, was married to Samuel Appleton and they proceeded to have eight children, three daughters and five sons. One son, Oliver Appleton, born in 1677, had hemophilia. Oliver lived to an advanced age and had three daughters. The oldest married Thomas Swain, Sr., of Reading, MA, and had seven children, two boys and five girls. The two sons both had hemophilia and bled frequently but became physicians, lived into their 30s, and had families. Dr. Thomas Swain, Jr., had a daughter who had three sons, two reported to be bleeders. Dr. Oliver Swain had three normal sons and a daughter who had three sons, all with hemophilia. A daughter of Thomas Swain, Sr., married John Bachilor and had three sons with hemophilia. Dr. Oliver Swain's daughter, Mary Swain, married Jeremiah Hartshorne in 1786 and gave birth to three sons with hemophilia. The Hartshorne family lived in Wakefield, MA, and, as it happened, next door to Dr. John Hay, a fellow of the Massachusetts Medical Society. Upon learning of the family history and after caring for the Hartshorne children, Dr. Hay published an *Account of a Remarkable Hemorrhagic Disposition Existing in Many Individuals of the Same Family* in the New England Journal of Medicine in 1813.[3,4] Coincidentally, a descendent of Thomas Swain, the New England landscape artist R. Swain Gifford, is an ancestor of the brother-in-law of one of the authors of this text.

Inherited disorders of blood coagulation range from the relatively common von Willebrand disease (vWD) to rare disorders such as factor XIII deficiency and multiple coagulation factor deficiencies (MCFD), from autosomal to X-linked in inheritance, and from severe to mild in clinical manifestations. Table 49.1 presents some of the distinguishing characteristics of each. The differential diagnosis of hemorrhagic disorders, both clinical and laboratory aspects, is discussed in Chapter 50. Specific coagulation tests are available for each factor, making unequivocal diagnosis of inherited deficiencies relatively easy, and industries to procure and sell specific clotting factor-deficient plasmas for specific assays exist, providing standardized substrate for the assays. Both immunologic and functional assays are available for most factors, enabling the diagnosis of both *quantitative*, defined as a proportional reduction in clotting factor protein and activity, and *qualitative*, defined as a reduction in clotting factor activity in excess of protein, disorders. The basis for clotting factor assays and how preanalytical variables can affect their accuracy is described in detail in Chapters 50 and 58. In general, the assays are sensitive to clotting factor activity, especially to levels of 20% of normal and lower, and are specific, making the diagnosis of most cases relatively straightforward. Where the diagnosis becomes difficult is with mild deficiencies, when clotting factor levels begin to blur with normal individuals, and in cases of general inhibitory substances, such as anti-phospholipid or so-called lupus-like antibodies, where multiple clotting factors may appear to be deficient. For example, the current difficulty with the diagnosis of mild type 1 vWD is well known and is discussed in detail in Chapter 52 by Sadler and Lillicrap on vWD.

While deficiency of procoagulants leads to bleeding, the clinical symptoms vary considerably (Table 49.1) and may provide a clue to the diagnosis. Hemophilia A and B are the most severe of the inherited bleeding disorders and have a curious predilection for bleeding into joints that is still poorly understood. As described in Chapter 51, this recurrent bleeding in joints leads to a chronic and progressive form of arthropathy that characterizes

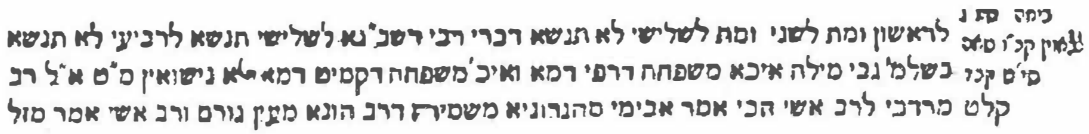


FIGURE 49.1 Hemophilia in Antiquity. A familial bleeding disorder, probably hemophilia, is described in the Babylonian Talmud, written by Rabbi Judah the Patriarch, redactor of the Mishnah, in the second century compilation of Jewish law. The translation is: "For it was taught: If she circumcised her first child and he died, and a second one who also died, she must not circumcise her third child. However, Rabbi R. Simeon b. Gamaliel said: She circumcises the third, but must not circumcise the fourth child."

the disorder and has some similarity to rheumatoid arthritis. The pathophysiology of the inflammatory changes that lead to the chronic arthropathy is reasonably well understood, but why bleeding occurs in the joints remains a mystery. It has been stated that factors VIII and IX must have some function supporting joint vessels. Bleeding from delicate mucous membranes is a feature of von Willebrand factor (vWF) deficiency (Chapter 52), suggesting that vWF-mediated platelet adhesion is particularly important in these tissues. Joint bleeding may occur in vWD but usually correlates with the level of factor VIII deficiency. Factor XIII deficiency has a high rate of intracranial bleeding, leading clinicians to emphasize the importance of prophylaxis in this disorder. Surprisingly, complete deficiency of fibrinogen (Chapter 54) produces a less serious bleeding disorder than might be expected from the deficiency of the protein that actually produces the clot. Why don't patients with afibrinogenemia bleed continuously? This is a question that is frequently asked by students and practitioners. In newborns with afibrinogenemia, bleeding from the umbilical stump is common. In children and adults, bleeding is typically in soft tissues and organs, but joint bleeding is not common. Other deficiencies (Chapter 53) are less common, and some, like factor XI deficiency, may be more variable in their bleeding but no less important if encountered. Despite the significant prolongation of the partial

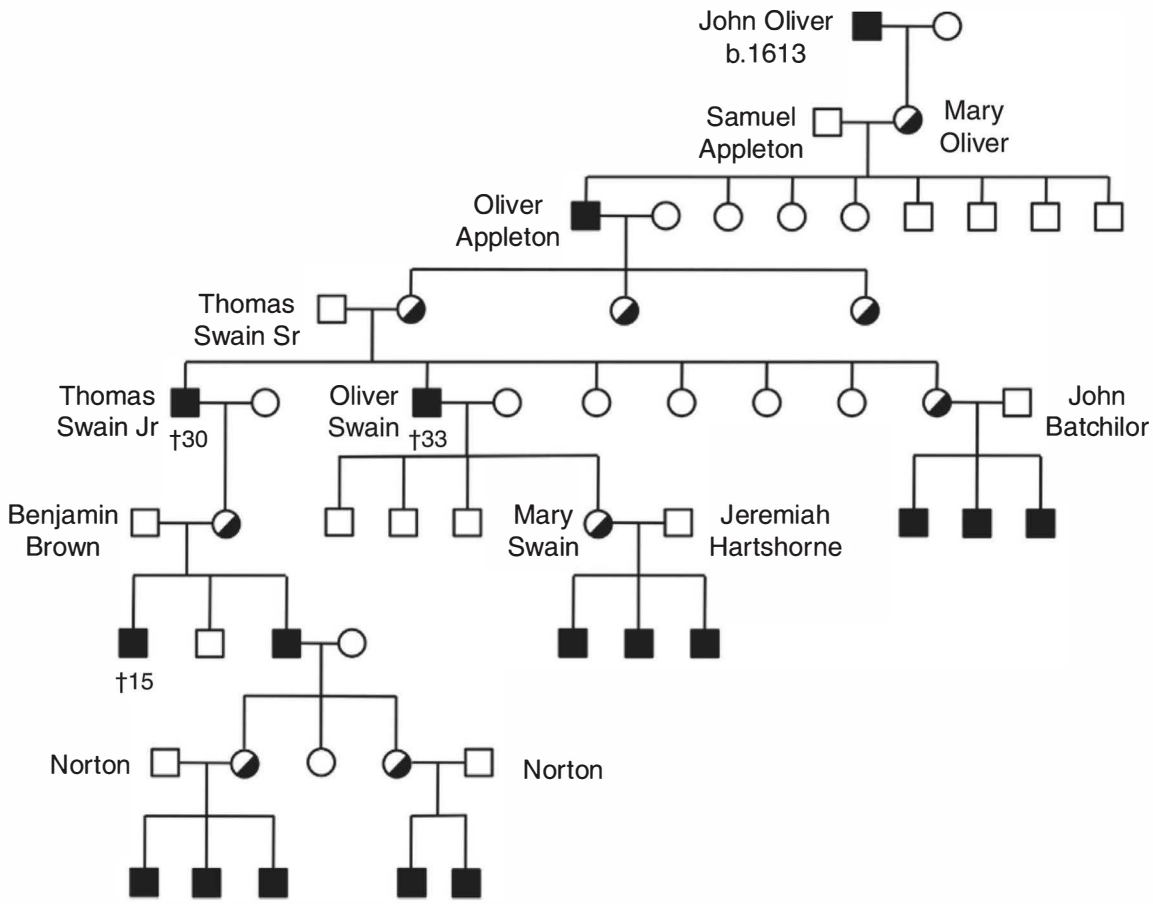


FIGURE 49.2 *Hemophilia in the New World*. Pedigree of first reported family with hemophilia in North America, as reported by Hay in 1813.[3] (The figure is reconstructed from descriptions in Hay's publication, in part.)

Table 49.1

Inherited hemorrhagic diseases

Defect		Frequency	Inheritance Pattern	Bleeding Manifestations	Diagnostic Testing			
					PT	PTT	TCT	BT
Fibrinogen abnormalities	Afibrinogenemia		Autosomal	Severe, but less so than severe hemophilia A and B	Infinite	Infinite	Infinite	Prolonged
	Dys-fibrinogenemia		Autosomal	Variable bleeding and/or clotting	Prolonged	Prolonged	Prolonged or shorten-ed	Normal
Prothrombin deficiency			Autosomal	Varies with prothrombin levels	Prolonged	Prolonged	Normal	Normal
Factor V deficiency			Autosomal	Mild-moderate	Prolonged	Prolonged	Normal	Prolonged
Factor VII deficiency			Autosomal	Moderate-severe	Prolonged	Normal	Normal	Normal
Hemophilia A			X-linked recessive	Variable, depending on factor VIII level	Normal	Prolonged	Normal	Normal
Hemophilia B			X-linked recessive	Variable, depending on factor IX level	Normal	Prolonged	Normal	Normal
Factor X deficiency			Autosomal	Variable, depending on factor X level	Prolonged	Prolonged	Normal	Normal
Factor XI deficiency			Autosomal	Variable, but not dependent on Factor XI levels	Normal	Prolonged	Normal	Normal
Deficiency of factor XII			Autosomal	None	Normal	Prolonged	Normal	Normal
Deficiency of prekallikrein			Autosomal	None	Normal	Prolonged	Normal	Normal
Deficiency of high molecular weight kininogen			Autosomal	None	Normal	Prolonged	Normal	Normal
Factor XIII deficiency			Autosomal	Severe	Normal	Normal	Normal	Normal
vWD		1:1,000,000	Autosomal; usually recessive; more rarely dominant	Moderate to mild, depending on level of deficiency and defect; predilection for mucous membranes	Normal	Normal or prolonged	Normal	Normal or prolonged
Deficiency of α_2 plasmin inhibitor			Autosomal	Moderate	Normal	Normal	Normal	Normal
Deficiency of PAI -1			Autosomal	Moderate	Normal	Normal	Normal	Normal

thromboplastin time (PTT) that occurs with deficiencies of factors XII, high molecular weight kininogen, and prekallikrein, there is no bleeding tendency. These proteins are important, though, providing, along with activated protein C, connections between the coagulation and inflammation pathways.

Deficiency of the regulators of fibrinolysis can also result in bleeding (Chapter 56). For example, in the absence of α_2-antiplasmin, the production of plasmin is less regulated, leading to enhanced fibrinolysis and a tendency to bleed. This is typically mild but can be severe. The diagnosis is suspected when a patient gives a lifelong history of bleeding, often after trauma, and when the usual screening tests of coagulation are normal and procoagulant levels are all normal. Similarly, deficiency of plasminogen activation inhibitor (PAI) can result in a bleeding tendency because plasminogen activation becomes unregulated, leading to increased plasmin levels. Symptoms and coagulation screening tests are similar to those seen in antiplasmin deficiency.

Bleeding tendency generally correlates with coagulation factor levels, but at a given level, there can be considerable variation in symptoms. For example, among patients with severe hemophilia A, the clinical symptoms can range from weekly bleeding to bleeding less than once a month; joint involvement can range from all joints to single joints. Even individuals with the same molecular defect can have varied symptoms. This has led to a search for so-called modifier genes, genes that can affect the frequency and severity of bleeding. Interestingly, prevalent gene defects associated with an increased risk of venous thrombosis do not appear to modify bleeding disorders, based on the evidence in hemophilia. Neither factor V Leiden nor the prothrombin 20210 polymorphism have been shown to consistently affect the annual bleed frequency, factor utilization, and/or arthropathy in hemophilia A,[5,6–15] even though hemophilic plasma with factor V Leiden generate more thrombin *in vitro* than hemophilic plasma with normal factor V.[16] This suggests that the balance between bleeding and clotting is not simple and may not reflect the sum of procoagulant and anticoagulant factors. Nevertheless, the strongest evidence that modifier genes do exist and do influence clotting are ABO blood group substances and their effect on vWF levels and on factor VIII levels, where vWF levels in individuals with type O blood (x = 74%) average 30% less than individuals with type A (x = 105%), B (x = 117%), or AB (x = 123%) blood.[17] The effect of blood group substances is believed to be through an effect on carbohydrate structure and vWF clearance. New genetic approaches such as genome wide association scanning (GWAS) and whole genome sequencing promise to make the search for meaningful modifiers more productive. This interest in identifying modifier genes in hemophilia stems in part from a desire to not only understand the difference in severity between individuals but also explore potentially new approaches to treatment. For example, there is currently strong interest in the pharmaceutical industry in the potential use of inhibitors of tissue factor pathway inhibitor as an approach to modify the bleeding risk in hemophilia, an approach that is supported by animal models.[18,19,20,21,22,23,24]

Multiple clotting factor deficiencies (Chapter 55) are among the most interesting deficiencies. Although clotting factor deficiencies may be coinherited, there is now evidence that these proteins may share common production pathways that create mechanisms for multiple deficiencies. One of the first of these to be described was the common deficiency of the vitamin K-dependent coagulation factors, factors II, VII, IX, X, protein C and protein S, initially called the Borgschulte-Grigsby syndrome.[25] Described clinically before molecular biology was understood, subsequent work has shown that two hepatic enzymes can produce this syndrome: γ-glutamyl carboxylase and vitamin K-epoxide reductase.[26,27] The former, cloned in 1991,[28] is responsible for posttranslationally converting glutamic acid to γ-carboxyglutamic acid at up to 12 sites in the carboxyterminal end of the vitamin K-dependent coagulation factors, thus enabling the factors to bind to phospholipid surfaces when the clotting reactions take place. The latter, cloned in 2004,[27,29] is responsible for an obligate oxidation and reduction of vitamin K to render it able to catalyze vitamin K carboxylase. Interestingly, the initial patient with combined vitamin K-dependent coagulation factor deficiencies has no detectable abnormality in either γ-glutamyl carboxylase or vitamin K epoxide reductase, spurring a search for a possible third gene that might lead to a Borgschulte-Grigsby-like syndrome. A second multiple clotting factor deficiency involves the deficiency of factors V and VIII. These are highly homologous cofactors in the process of blood coagulation. Both are transported from the endoplasmic reticulum to the Golgi by a specific calcium-dependent cargo receptor protein complex that includes LMAN1 (also called ERGIC53) and MCFD2. Abnormalities of either protein results in combined deficiency of both factors due to inefficient ER-to-Golgi transport.[30,31] The clue that combined V-VIII might be present is the finding of mild-moderate factor VIII deficiency that is inherited in an autosomal manner. Other multiple clotting factor deficiencies appear not to be coinherited and therefore represent other as yet undefined pathways that affect multiple factors and therefore represent common or shared mechanisms of synthesis or catabolism. Overall, multiple clotting factor deficiencies are uncommon causes of bleeding, but they emphasize the important point that defects outside the clotting factor genes can influence levels. Understanding the pathways in other forms of multiple clotting factor deficiencies will lead to a better understanding of the biology of coagulation.

Antibodies to clotting factors, called inhibitors because they inhibit the participation of the target clotting factor in the coagulation process, can occur. These may occur in individuals with inherited clotting factor deficiencies, or they may be spontaneous, in individuals with no underlying clotting defect. The former are considered alloimmune disorders; the latter are autoimmune disorders. In both situations, antibodies to coagulation factor VIII are most common. The development of an inhibitor is a serious occurrence and warrants considerable attention. Fortunately, effective treatments now exist that can either bypass the inhibitor to induce thrombin formation[32,33] or induce immunologic tolerance with disappearance of the antibody.[34,35,36] The ability to induce lasting tolerance is especially important as this is the only disorder known for which true tolerance can be induced. If the mechanism of tolerance in hemophilia can be understood mechanistically, it may be possible to apply the principle of tolerance to other immunologic disorders such as lupus and rheumatoid arthritis. In the 35 years since the discovery that prolonged

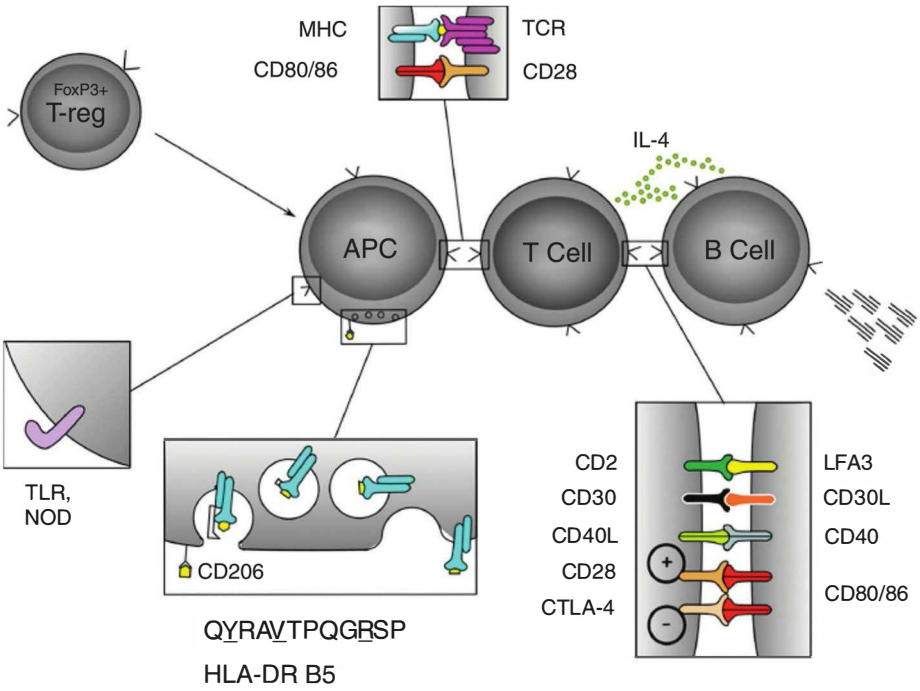


FIGURE 49.3 *Immunologic cellular cascade from APC to T-lymphocyte (T-cell) to antibody-producing B-lymphocyte (B-cell).* Peptide antigens such as FVIII are taken up by APCs. CD206, the macrophage mannose receptor, may be the endocytic receptor for factor VIII and mannose residues on VIII carbohydrates may mediate the binding to APCs (Dasgupta S Navrete A, Bayry J, et al. A role for exposed mannosylations in presentation of human therapeutic self-proteins to CD4+ T-lymphocytes. *PNAS* 104:8965, 2007.) After binding, VIII is internalized in endocytic vesicles where it is presented to MHC (major histocompatibility complex) class II molecules. The recognition of a given factor VIII peptide by an MHC molecule is specific and depends on the class II genotype of the individual. Once assembled, antigenic peptide-class II complexes are transported to the cell surface for presentation to helper T-lymphocytes. Antigenic peptide-class II complexes on the surface of APCs interact with the TCR on helper T-lymphocytes resulting in the generation of signals that result in T-cell activation and upregulation of surface molecules, including CD2, CD30, CD40L, and CD28. At the same time, synthesis of cytokines occurs. In Th1 helper cells, there is elaboration of cytokines such as interferon-γ and interleukin (IL)-2, which stimulate cell-mediated cytotoxicity. Th2 cells elaborate cytokines IL-4, IL-10, and IL-13, which stimulate antibody-producing B cells. Activated T-helper cells then activate B-cell clones through surface-mediated interactions and cytokines. These interactions stimulate B-cell growth, proliferation, and differentiation. TLRs and NOD-like receptors on APCs can enhance the process and increase the immune response. FoxP3 positive regulatory T-cells can down-regulate the process and decrease the immune response. (copyright G. C. White, from White et al.[40])

administration of factor VIII could induce tolerance in many individuals, significant advances in the immunology field have resulted in an increase in knowledge regarding the molecular events around antibody formation. The cellular cascade from antigen presenting cell (APC) to T-lymphocyte (T-cell) to antibody producing B-lymphocyte (B-cell) and the signals involved in these cellular interactions are known[37,38,39] (see FIGURE 49.3) and current efforts are aimed at understanding modulatory effects such as regulatory T-cells (Tregs).[41] and toll-like receptors (TLRs).[42] Knowledge gained over the past decade or two of the mechanisms involved in immunologic responses to peptide antigens such as the coagulation factors provides new directions for inducing tolerance. For example, the productive activation of T-cells by APCs requires an interaction between the MHC-peptide complex on APCs and the T-cell receptor (TCR) as well as a costimulation event, classically between CD80/86 on the APC and CD28 on T-cells. By blocking the costimulatory event, one can induce tolerance to antigen. Similarly, blocking interactions between T-cell and B-cell by, for example, blocking the CD4-L-CD40 interaction can inhibit the development of an antibody response. These various steps in the immune process represent novel targets for clinical therapy of inhibitors in hemophilia and in other disorders in which antibodies to coagulation factors arise. The characteristics of inhibitors and their diagnosis and treatment are covered in Chapter 57.

Overall, inherited hemorrhagic disorders are among the most important disorders in the field of thrombosis and hemostasis. Although even hemophilia is relatively uncommon, the proper diagnosis and treatment of these disorders requires knowledge

Table 49.2

Defects in soluble coagulation factors—clinical pearls

- The most common hereditary bleeding disorder is vWD with type 1 vWD as the most common subtype
- vWD is inherited as an autosomal dominant trait and is expressed in the heterozygous state
- Bleeding in this disorder resembles the type of bleeding seen with thrombocytopenia, with bruising and hemorrhage from mucous membranes
- Menorrhagia is common, and women with this common complaint should be screened for vWD
- The only two soluble clotting factors deficiencies that are inherited as sex-linked recessive characteristics are hemophilia A and B, resulting from defects in factors VIII and IX, respectively
- The occurrence of severe bleeding in men, especially associated with hemarthropathy, is strongly suggestive of one of these disorders
- Hemophilia A and B can be clinically indistinguishable, but classic hemophilia is much more common than hemophilia B
- Inhibitor antibodies occur more frequently in hemophilia A, and patients with such inhibitors are resistant to treatment and have increased morbidity and mortality
- The other deficiencies of soluble clotting factors are inherited in an autosomal recessive fashion, and bleeding occurs when the patient is homozygous or a combined heterozygote
- Deficiencies of factors XII, PK, and HK are not associated with bleeding, even after trauma or surgery
- A prolonged PT with a normal PTT and TCT suggests factor VII deficiency, whereas a prolonged PTT with a normal PT and TCT in a patient who is bleeding suggests a factor VIII or IX deficiency if the bleeding is confined to men or factor XI deficiency if the bleeding is mild and occurs in both sexes
- If all screening tests of coagulation are normal, deficiency of factor XIII, α_2-plasmin inhibitor or PAI-1 should be suspected, although these are very rare disorders
- When both the PT and PTT are prolonged, one should suspect deficiency of either factor V or factor X; bleeding from prothrombin deficiency and combined factor deficiencies can also present with these laboratory values, but these cases are more rare and can be easily diagnosed with assays for individual factors
- When the TCT as well as the PT and PTT are prolonged, dysfibrinogenemia should be suspected; when no clot is detected, afibrinogenemia is likely
- Acquired bleeding disorders can be confused with hereditary defects but are usually not lifelong and can be distinguished by a careful medical history

vWD, von Willebrand disease; PK, prekallikrein; HK, high molecular weight kininogen; PT, prothrombin time; PTT, partial thromboplastin time; TCT, thrombin clotting time; PAI-1, plasminogen activator inhibitor 1.

of the distinguishing clinical and laboratory features of each. In this section, the various inherited bleeding disorders involving blood coagulation factors are presented along with various approaches to treatment. Table 49.2 gives a salient summary and clinical pearls regarding defects in soluble clotting factors.

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CHAPTER

50 Differential Diagnosis of the Bleeding Patient

JILL M. JOHNSEN AND BARBARA A. KONKLE

The hematologist is often faced with the question of whether a patient has an underlying bleeding disorder. This may be in the setting of unexplained bleeding, either spontaneous or with minor or major trauma or procedures. Or, the hematologist may be asked to evaluate a person's risk of bleeding with an upcoming procedure, particularly in cases of a personal or family history of bleeding or screening laboratory results that suggest a bleeding risk. This chapter reviews key points to help determine whether an underlying bleeding disorder is present, first focusing on the history and physical examination. Basic screening laboratory tests usually available at the time of evaluation are reviewed, including the platelet count, prothrombin time (PT), also expressed as the international normalized ratio (INR), and activated partial thromboplastin time (aPTT). Laboratory tests useful in pinpointing specific hemostatic abnormalities are then discussed. Many of the disorders highlighted here are presented in more detail in other chapters in this text.

Bleeding disorders are classically divided into those of primary or secondary hemostasis. Disorders of primary hemostasis include those that affect initial platelet plug formation and typically result in mucosal bleeding, usually the result of a platelet or von Willebrand factor (VWF) disorder. Disorders of secondary hemostasis involve disorders of fibrin formation and often manifest as soft tissue or joint bleeding. [This is commonly due to specific coagulation factor deficiencies, notably factor VIII (FVIII) and factor IX (FIX) (hemophilia A and B, respectively)]. However, there is considerable overlap in symptomatology.[1] Thus, although the pattern of bleeding is an important clue to its etiology, a comprehensive approach is necessary to correctly identify the bleeding patient's underlying hemostatic defect.

THE BLEEDING HISTORY

From a detailed personal and family history, the clinician should have a good idea of whether (a) a bleeding disorder is present; (b) the disorder is inherited or acquired; (c) the pattern of bleeding suggests a primary or secondary hemostatic defect (or combination); and (d) a person's propensity to bleed has been enhanced by an underlying medical condition or exposure to medications or dietary supplements.

A history of bleeding is the most important predictor of future bleeding risk. In evaluating a patient for a bleeding disorder, a history of hemostatic challenges should be assessed. Does the patient have a history of spontaneous or trauma- or surgery-induced bleeding? What is the nature and timing of the bleeding? Was the bleeding severe enough to necessitate medical intervention or treatment for acute or chronic blood loss?

A negative bleeding history is reassuring, but should not be used alone as the basis for dismissing a bleeding disorder, particularly if the person has never experienced a significant hemostatic challenge. Assessing a positive bleeding history can also be challenging, particularly in milder cases, as normal individuals can respond positively when questioned about specific signs or symptoms, particularly easy bruising or menorrhagia. Bleeding score questionnaires have been developed by several groups in an attempt to quantify bleeding in inherited bleeding disorders.[2] These instruments may be useful as a guide to the type of questions to ask in a targeted bleeding history, but their utility in initial bleeding evaluations or scoring the severity of disease remains to be seen. Thus, a careful and thoughtful investigation of the bleeding history is critical in guiding any clinical evaluation of the bleeding patient.

Mucosal Bleeding

Epistaxis

Epistaxis is a common symptom, particularly in children and in dry climates, and may not reflect an underlying bleeding disorder. Clues that epistaxis is a symptom of an underlying bleeding disorder include lack of seasonal variation and bleeding of prolonged duration (>10 minutes), bleeding which requires medical attention, or frequent bleeding episodes.

Oral Mucosal Bleeding

Excessive bleeding or swelling after episodes of minor oral trauma may be a clue to an underlying bleeding disorder. Bleeding with eruption of primary teeth is seen in children with more severe bleeding disorders, such as moderate and severe hemophilia, but is uncommon in children with mild bleeding disorders. Spontaneous gum or cheek bleeding resulting in blood blisters, frankly bloody secretions, or profuse bleeding after tooth brushing in the absence of gum disease are also suggestive of an underlying bleeding disorder, especially disorders of primary hemostasis.

Dental extraction, particularly of molars or premolars, represents a significant hemostatic challenge. The limitations on wound closure imposed by the bony tooth socket and the presence of profibrinolytic enzymes in saliva and at the mucosal surface contribute to difficulties in achieving hemostasis. Excessive bleeding and/or recurrent bleeding after tooth extractions or other oral surgeries, or increased bleeding after dental cleaning, can be suggestive of an underlying bleeding disorder.

Gynecologic and Obstetrical Bleeding

Gynecologic and obstetrical bleeding are common presentations in women with underlying bleeding disorders (reviewed in Ref.[33]). Menorrhagia is a common symptom, but the history can be difficult as a complaint of heavy menses is subjective and

has a poor correlation with excessive blood loss. The diagnosis of menorrhagia is supported by anemia, soaking through a pad or tampon within 1 hour, or flooding at night.[4] Additionally, women with underlying bleeding disorders are more likely to have other bleeding symptoms and are much more likely to have menorrhagia beginning at menarche than are women with menorrhagia due to other causes (reviewed in Ref.[55]).

Postpartum hemorrhage is a common symptom in women with underlying bleeding disorders. This occurs most commonly in the first 48 hours after delivery but may also be manifest by prolonged, recurrent, or excessive bleeding after discharge from the hospital. Women with a history of postpartum hemorrhage are at an increased risk of recurrence in subsequent pregnancies. Women presenting with rupture of ovarian cysts along with intra-abdominal hemorrhage are also more likely to have an underlying bleeding disorder.[3]

Other Mucosal Bleeding

Gastrointestinal (GI) bleeding is usually due to underlying anatomic pathology and not a hemostasis defect. However, von Willebrand disease (VWD), particularly acquired VWD and types 2 and 3, can be associated with angiodysplasia of the bowel resulting in GI bleeding.[6] True hematuria is more likely to be due to an underlying urologic or renal problem and not a bleeding diathesis.[7] Rarely, recurrent hematuria of unclear etiology can occur in moderate and severe hemophilia.

Easy Bruising

The development of bruises (ecchymoses) after trauma is normal; however, an exaggerated response to trauma may be an indication of an underlying bleeding disorder. Ecchymoses presenting without known trauma, particularly on the trunk, and/or especially large (>3 cm in diameter) or palpable ecchymoses may be a sign of an underlying bleeding disorder. A lifelong history of significant ecchymoses suggests a congenital hemostatic defect, while the sudden development of new or multiple ecchymosis may reflect an acquired disorder. In pediatric and other vulnerable populations, ecchymoses can raise concerns for physical abuse, and the hematologist may be asked for an assessment of an underlying bleeding disorder (reviewed in Ref.[88]).

Minor Trauma

Bleeding after minor trauma is also normal. However a persistent history of excessive or prolonged bleeding after minor wounds, such as small sharp cuts from razors, paper, or knives, particularly bleeding which lasts >5 minutes or cannot be managed by the patient themselves, may suggest an underlying bleeding disorder. The hospitalized patient undergoes minor trauma with high frequency due to phlebotomy and the placement of intravascular catheters in addition to other procedures. The unexpected requirement for application of prolonged pressure or intervention at phlebotomy sites, vascular access sites, or after other minor procedures may be a sign of defective hemostasis.

Joint and Muscle Bleeds

Hemarthroses and spontaneous muscle or soft tissue hematomas are characteristic of moderate or severe congenital factor deficiencies, most commonly FVIII or FIX deficiency. It can also be seen in moderate and severe deficiencies of fibrinogen, prothrombin, and FV, FVII, and FX.[1] Spontaneous hemarthroses occur rarely in other bleeding disorders except for severe VWD, which can be associated with FVIII levels <5%. Bleeding into a joint results in severe pain and swelling, as well as loss of function, but is rarely associated with discoloration from bruising around the joint. The first bleeding episode into a joint is accompanied by considerable swelling, but as bleeding recurs and joint destruction progresses, swelling may be less prominent while pain and loss of movement may worsen. In patients without a diagnosis of hemophilia, joint bleeding may go unrecognized as such by the patient or family. The clinical history, therefore, should specifically include questions about symptoms of severe joint pain and swelling.

Excessive Traumatic or Surgical Bleeding

Hemorrhage in the setting of severe trauma can be due to a combination of the trauma injury itself, surgical bleeding, medications, underlying comorbidities, and acquired coagulopathies, which can have components of both transfusion-related coagulopathy (discussed below) and trauma-related coagulopathy. Trauma-related coagulopathy itself is multifactorial and can result from shock and resulting tissue hypoperfusion and organ dysfunction, hypothermia, acidemia, dilution, consumption of clotting factors, and injured tissue-mediated activation of anticoagulant and fibrinolytic factors. Not surprisingly, trauma patients with underlying coagulopathies have worse outcomes.[9]

Significant surgical bleeding can occur during surgery or in the postoperative period. A comprehensive assessment of sites of bleeding, procedures, and local measures to stop the bleeding is critical. Different procedures pose different hemostatic challenges that must be considered in the bleeding evaluation. Tonsillectomy is a significant hemostatic challenge from both the surgery and mucosal injury. Bleeding may occur early after surgery or more than a week postoperatively with loss of the eschar at the operative site.[10,11] Surgeries also inherently incur different degrees of blood loss in the normal patient. Some vascular or orthopedic procedures, such as spinal surgery, are bloody surgeries and can require transfusion support even in the hemostatically normal patient. On the other hand, other general surgeries, such as laparoscopic intra-abdominal procedures, can generally be performed with minimal blood loss. A number of major surgeries, such as coronary artery bypass grafting or aortic procedures, are often performed while the patient is exposed to one or more anticoagulant medications, which increases blood loss from the surgical sites and exacerbates bleeding from any other underlying anatomic defects.

Hemorrhagic events often occur in patients with comorbidities and/or multiple confounding factors. For example, the medical patient with liver failure (acquired factor deficiencies) being treated for infected ascites (risk for disseminated intravascular coagulation [DIC], antibiotic effects) may bleed briskly from esophageal varices (anatomic site) resulting in hypotension (tissue shock response) and a need for multiple transfusions (dilutional coagulopathy). However, even in cases instigated by a seemingly simple anatomic event, such as cleaning a penetrating wound with excessive bleeding requiring transfusion, factors can rapidly compound to a multifactorial coagulopathy. The approach to complex bleeding patient requires an open mind to reevaluate the history of the patient and periodic reassessments both for clinical and laboratory response to interventions and for new developments that may contribute to bleeding. Ongoing laboratory assessments in such patients should include at least a PT, aPTT, platelet count, and fibrinogen. Further laboratory

analyses will depend on laboratory results and underlying disorders. As patients with liver disease can have laboratory findings consistent with low-grade DIC, a diagnosis of DIC may require serial testing to show evidence of ongoing fibrinogen consumption.

Patients with underlying bleeding disorders are at higher risk to bleed in the setting of trauma or surgery. The hematologist should take into account the nature of the trauma or procedures performed and consider the opinion of the surgeon in assessing if the patient is bleeding excessively for the hemostatic challenge.

Transfusion-Related Coagulopathy

Transfusion-related coagulopathy occurs in the setting of large-volume transfusion replacing acute blood loss (reviewed in Ref.[109]). The primary cause is dilution and failure to adequately replace plasma coagulation factors or platelets in proportion with the number of red cell units transfused. Replacement can be guided by closely following the platelet count, PT/INR (for plasma), or fibrinogen levels (for fibrinogen concentrates or cryoprecipitate). In high-volume, rapid transfusion, the need for these blood components can generally be anticipated by the number of red cell units infused. Massive transfusion also results in infusion of significant doses of citrate with the blood products. Iatrogenic hypocalcemia, reflected by low ionized calcium levels, may also contribute to clinical bleeding. Citrate chelates calcium, an essential cofactor for clotting and for multiple other essential biologic processes, including nerve conduction and muscle contractility.

Underlying Systemic Disease and Bleeding

Acquired bleeding disorders are commonly secondary to, or associated with, systemic disease. The clinical evaluation of a patient with a bleeding tendency must therefore include a thorough assessment for evidence of underlying medical disorders. Bruising or mucosal bleeding may be the presenting complaint in liver disease, severe renal impairment, hypothyroidism, and conditions causing bone marrow failure. Less prevalent disorders, such as paraproteinemias or amyloidosis, occasionally also present in this fashion. In DIC, presentation may be with symptoms of bleeding and/or microvascular thrombosis, and the underlying trigger for the consumptive coagulopathy is not always immediately obvious,[12] such as in cases of occult malignancy.

Other Conditions Presenting with Signs or Symptoms of Bleeding

Medical conditions in which there is no coagulopathy or defect of primary hemostasis can present with signs or symptoms, which may suggest a bleeding disorder, but are instead caused by abnormalities of blood vessels or their supporting tissues. For example, in inherited collagen disorders, such as Ehlers-Danlos or Marfan syndrome, there is frequently a history of easy bruising or prolonged posttraumatic bleeding. However, other clues in the history and physical point to the underlying congenital defect, such as skin hyperelasticity or joint hyperextensibility and body habitus. Endocrine disorders (such as Cushing syndrome) or chronic exogenous corticosteroid exposure can result in changes in the skin and subcutaneous tissues that result in more pronounced subcutaneous bleeding in response to minor trauma. The bleeding is generally more superficial than in true ecchymoses. Senile purpura, common with advancing age, is also very superficial, usually occurs on sun-damaged skin, most commonly on the hands and forearms, and can also be mistaken for ecchymoses. Some autoimmune disorders can present with vasculitis reminiscent of petechiae and skin findings suggestive of bruising. Joint pain and swelling due to an acute arthritic flare, crystal arthropathy, or septic joint can mimic hemarthroses. These are often able to be differentiated from a true joint bleed by the history, an important distinction as suspicion for these other inflammatory conditions may lead to arthrocentesis, a procedure that generally should be avoided in patients with a hemarthroses related to an underlying bleeding disorder.

Medications and Dietary Supplements

A complete medication list, including both short-term and chronic medications and all herbal or supplement use, is essential and should be temporally correlated to the onset of bleeding. A common but sometimes overlooked contributor to bleeding is the use of aspirin or other nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs inhibit both cylooxygenase-1 (COX-1) and COX-2 pathways. Inhibition of COX-1 impairs primary hemostasis and can exacerbate bleeding from another cause or even unmask previously occult mild bleeding disorders. NSAIDs selective for the COX-2 enzyme have little platelet inhibitory effect and should not enhance or precipitate bleeding, but have instead been associated paradoxically with increased risk for thrombotic events.[13] However, all NSAIDs can precipitate GI bleeding by direct action at the GI mucosa, which may then be more severe in patients with underlying bleeding disorders. The aspirin effect on platelet function is permanent and can persist for up to 7 days as assessed by platelet aggregometry, although it frequently returns to normal by 3 days after the last dose.[14] The duration of the effect of other NSAIDs on platelets is shorter, because the inhibitor effect is reversible and depends on the half-life of the agent.

Other causes of drug-related bleeding range from the obvious, such as hemorrhage complicating thrombolytic therapy or bleeding in the setting of thrombocytopenia after chemotherapy, to the more diagnostically challenging, such as bleeding secondary to impaired platelet function in subjects on high-dose intravenous penicillin or caused by a combination of a qualitative platelet defect and moderate thrombocytopenia secondary to anticonvulsant therapy with sodium valproate (see Chapter XX for further discussion of drug-induced qualitative platelet defects). Furthermore, exposure to multiple medications contributes to the risk of adverse drug–drug or drug–supplement interactions, which can lead to altered drug levels and toxicity.

Use of alternative medicines and herbal supplements is commonly practiced and must be asked about specifically. Popular herbal supplements, such as gingko, ginseng, and garlic, are associated with altered hemostasis[15] (Table 50.1). Fish oil or concentrated omega 3 fatty acid supplements can also impair platelet function. Herbal supplements may also alter hemostasis indirectly by interacting with other drugs, particularly warfarin. In addition to these concerns, loose regulatory oversight of the supplement industry raises concerns for quality control of the products consumed. Herbal supplements may contain variable (or no) detectable amounts of the active agents claimed on the packaging, while other unlisted ingredients can be present due

Table 50.1

Herbal supplements associated with increased bleeding

Herbs with Potential Antiplatelet Activity
Ginkgo (*Ginkgo biloba*)
Garlic (*Allium sativum*)
Bilberry (*Vaccinium myrtillus*)
Ginger (*Zingiber officinale*)
Dong quai (*Angelica sinensis*)
Feverfew (*Tanacetum parthenium*)
Asian ginseng (*Panax ginseng*)
American ginseng (*Panax quinquefolius*)
Siberian ginseng/eleuthero (*Eleutherococcus senticosus*)
Turmeric (*Curcuma longa*)
Meadowsweet (*Filipendula ulmaria*)
Willow (*Salix alba*)
Coumarin-Containing Herbs
Motherwort (*Leonurus cardiaca*)
Chamomile (*Matricaria recutita, Chamaemelum nobile*)
Horse chestnut (*Aesculus hippocastanum*)
Red clover (*Trifolium pratense*)
Fenugreek (*Trigonella foenum-graecum*)

to poor manufacturing processes, misidentification, or substitution. NSAIDs are a common contaminant, particularly for those formulations claiming pain relief,[15] and warfarin was repeatedly identified in formulations of PC-SPES, a dietary supplement.[16]

Social History

The bleeding history must take into account the patient's exposure to hemostatic challenges in their daily lives. This includes assessing both common daily activities and the history of the individual's participation in sports or other vigorous physical activities. The lifestyle of an office worker or low-impact exerciser provides fewer opportunities for incidents of trauma compared to individuals who have physically demanding occupations or engage in contact sports. In the same vein, an infant who cannot walk would not be expected to exhibit the minor ecchymoses commonly found on the normal toddler. This history will help guide the clinician in assessing if bleeding symptoms, particularly ecchymoses or bleeding with minor trauma, seem excessive for the patient's circumstances.

FAMILY HISTORY

Young age at presentation, prolonged duration of symptoms, failure of previous hemostatic challenges, and positive family history can be indicators of an inherited disorder. The pattern of inheritance in families is also informative. VWD is generally inherited in an autosomal dominant pattern (except for types 2N and 3, which are recessive), but can have variable penetrance and expressivity (particularly type 1). Factor deficiencies in FVIII and FIX are sex linked, and as such affected males present with more severe disease. Thus, the presentation of a male newborn with umbilical stump bleeding and a family history of hemophilia in a maternal uncle are strongly suggestive of an inherited factor deficiency. On the other hand, an elderly patient with no previous history of excessive bleeding despite surgical challenges who presents with recent onset of massive bruising and forearm compartment syndrome is much more consistent with an acquired bleeding diathesis. Diagnostic difficulties arise, however, when a mild familial disorder is present in a patient who previously has not been challenged because of young age, previous good health, or when the bleeding symptoms are highly variable within families, as can occur with mild disorders.

The positive family history may be informative, but a negative family history does not necessarily rule out a genetic disorder. Up to one-third of cases of hemophilia A and B arise as a result of new mutations, and the family history will be negative for abnormal bleeding. Because most new mutations causing hemophilia will manifest first in the carrier female,[17,18] she may present with symptoms due to a decreased FVIII or FIX level without a diagnosis of hemophilia in the family. Alternatively, she may not have bleeding symptoms depending on her baseline factor level, which would vary by degree of random X chromosome inactivation (the Lyon hypothesis).[19] Bleeding in patients with FXI deficiency occurs most commonly in those with severe deficiency, although severely affected individuals may be asymptomatic. Most heterozygotes are asymptomatic. However, the relation between the FXI level and bleeding is not clear-cut,[20,21] and some heterozygotes bleed excessively after trauma. In patients where there is suspicion for an inherited bleeding disorder, gene mutation studies in family members may be helpful in confirming the diagnosis.

PHYSICAL EXAMINATION

The physical examination can provide helpful clues as to the etiology of the bleeding disorder, although many patients with mild bleeding disorders will have normal examinations. The skin, mucosa, and sites of skin compromise, such as vascular access sites or surgical or traumatic wounds, should be examined for ecchymoses, petechiae, oozing, and associated soft tissue hematomas. Palpable or larger ecchymoses (>3 cm), particularly in the absence of apparent trauma or on the trunk, are suggestive of an underlying disorder. Petechiae can be distinguished from small vasculitic, infectious, or embolic lesions in that they are never palpable, do not blanch, and tend to be distributed over the extremities, particularly the lower legs. Petechiae or mucosal purpura are seen in severe thrombocytopenia, but not usually in thrombocytopathies. On musculoskeletal examination, abnormal joint appearance and/or a loss of normal range of motion is suggestive of past hemarthroses, while active joint bleeding manifests with significant monoarticular arthralgia and effusion. Some rare disorders are associated with particular findings, such as abnormalities of the forearm in thrombocytopenia with absent radii, or defects in skin pigmentation in patients with syndromes associated with platelet granule or storage pool

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disorders. The physical examination of all systems should be thorough in search of signs associated with systemic conditions that may predispose to bleeding, such as liver or kidney disease, malignancy, infection, autoimmune disease, or collagen–vascular disorders.

LABORATORY EVALUATION

Careful history taking and clinical examination are essential components in the assessment of bleeding risk. The use of laboratory tests of coagulation and hemostasis cannot substitute for clinical assessment. Indeed, ample evidence exists that screening tests are unhelpful in the prediction of bleeding risk, especially when applied indiscriminately.[22–24] Although there is much interest in developing a global assay of hemostasis, to date there is no single assay that can predict bleeding or thrombosis. The clinician must frequently use the results of several assays combined with the clinical presentation to arrive at a diagnosis or an assessment of bleeding risk. The primary use of coagulation testing should be to confirm the presence and type of bleeding disorder in a patient with a suspicious clinical history. An exception may be the preoperative assessment in infants and young children, in whom the clinical history is less likely to be informative.

Because of the nature of coagulation assays, proper sample acquisition and handling is critical to obtaining valid results. In patients with abnormal coagulation assays and no bleeding history, properly performed repeat studies are often normal. Most coagulation assays are performed in sodium citrate–anticoagulated plasma that is recalcified for the assay. Because the anticoagulant is in liquid solution and needs to be added to blood in proportion to the plasma volume, incorrectly filled or inadequately mixed blood collection tubes will give erroneous results. Other patient factors can influence laboratory results. An elevated hematocrit (>55%) can result in an incorrect coagulation measurement because of a decreased plasma-to-anticoagulant ratio. An accurate result is obtained by adjusting the amount of sodium citrate solution added to the blood on the basis of the estimated plasma volume, using the formula:

$$\frac{(100 - \text{Hematocrit}) \times (\text{total tube volume})}{(595 - \text{Hematocrit})} = \text{Volume of Na Citrate (3.2\%) required}$$

Complete Blood Count and Peripheral Smear

The complete blood count (CBC) is a necessary component of the evaluation of the bleeding patient and is often instrumental in identifying an underlying platelet defect or evidence of chronic or ongoing blood loss. However, artifacts are common, and verification by repeat or manual testing is warranted in patients with a newly identified CBC abnormality. Thrombocytopenia is often first identified by automated cell counting. An assessment of reticulated platelets or estimation of average platelet size from the mean platelet volume can also be useful clues to the underlying cause of thrombocytopenia. New diagnoses of thrombocytopenia should be verified by examination of the peripheral smear to evaluate the quantity and morphology of the platelets, to look for abnormalities in other blood cells, and to exclude pseudothrombocytopenia. A common cause of pseudothrombocytopenia is platelet clumping in the blood collection tube as a result of either the anticoagulant ethylenediaminetetraacetic acid (EDTA)-induced platelet clumping or specimen mishandling. Pseudothrombocytopenia may also result from the failure of the automated cell counter to identify morphologically abnormal platelets, as in the case of hereditary platelet disorders or acquired bone marrow diseases.

Red cell indices and morphology may exhibit abnormalities relevant to the bleeding patient. Hypochromia and microcytosis may be evidence of iron deficiency and suggest chronic blood loss, while schistocytes reflect an underlying microangiopathy (such as DIC). Abnormal leukocyte counts and morphology can be clues to underlying bone marrow disorders or rare congenital syndromes.

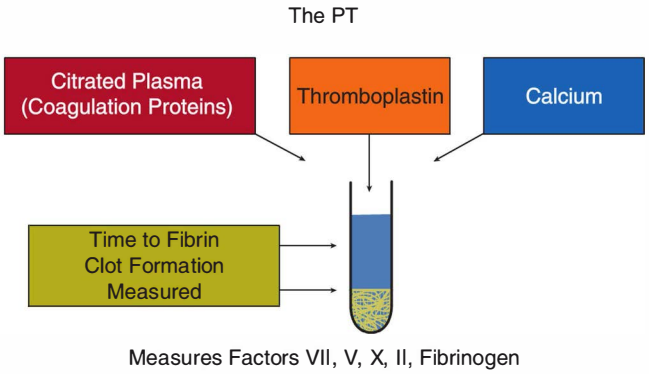

FIGURE 50.1 Elements of the PT are shown.

Prothrombin Time

The PT, developed by Quick et al.[25] in 1935, assesses the classic extrinsic coagulation system, including FI (fibrinogen), FII (prothrombin), FV, FVII, and FX (see FIGURE 50.1). The PT measures the time for clot formation of the citrated plasma after recalcification and addition of thromboplastin (reviewed in ref.[26]). Thromboplastin is a combination of tissue factor, the membrane receptor for FVII and FVIIa, and phospholipids. The speed of the PT reaction is influenced not only by the plasma concentrations of the extrinsic and common pathway clotting factors but also by the concentration and properties of the tissue factor.[27] The sensitivity to different factor deficiencies and coagulopathies varies by the source of thromboplastin. Human recombinant tissue factor, relipidated with natural or synthetic phospholipids, is now generally used.[28]

The degree of prolongation of the PT is dependent on the thromboplastin in the reaction as well as on the instrument used for the assay. In the past, the use of various thromboplastins and instruments resulted in varying levels of anticoagulation when the PT ratio was used to target warfarin therapy. To adjust for this variability, the overall sensitivity of different thromboplastins to reduction of the vitamin K–dependent clotting factors (FII, FVII, FIX, and FX) in anticoagulated patients is now expressed as the international sensitivity index (ISI).[29,30] This is determined by calibrating the thromboplastin against a standard thromboplastin, generally a well-characterized secondary WHO International Reference Preparation. An inverse

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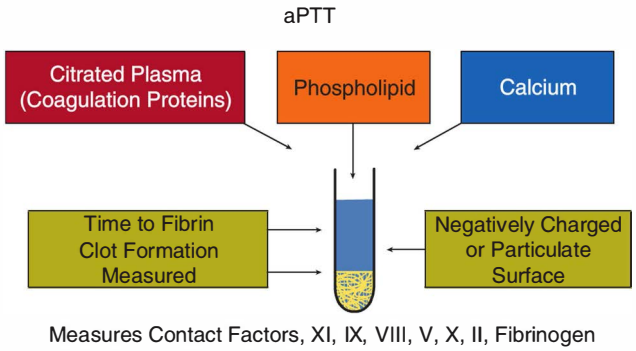

FIGURE 50.2 Elements of the activated partial thromboplastin time (aPTT) are shown. Contact factors are FXII, kininogen, and prekallikrein.

relation exists between ISI and thromboplastin sensitivity. The INR is then determined based on the formula:

$$\text{INR} = \left(\text{PT}_{\text{patient}} / \text{PT}_{\text{normal mean}}\right)^{\text{ISI}}$$

Although the INR was developed to assess anticoagulation due to reduction of vitamin K–dependent coagulation factors, it can be helpful in the evaluation of patients with liver disease, particularly when comparing values from testing performed at different laboratories. However, because progressive liver failure is associated with variable changes in coagulation factors, the degree of prolongation of either the PT or the INR is not necessarily associated with bleeding risk.[31] The INR is also generally not a reliable measure of warfarin anticoagulation in patients with an elevated baseline INR, such as in the case of antiphospholipid antibodies directed against prothrombin, which can prolong the PT at baseline.[32]

Activated Partial Thromboplastin Time

Like the PT/INR, the aPTT is performed on recalcified citrated plasma (see FIGURE 50.2, reviewed in Ref.[26]). It assesses the intrinsic and common coagulation pathways and was originally described as a "partial thromboplastin" time because the reagents did not induce hemophilic plasma to clot in a normal period of time as do "complete" thromboplastins.[33] The aPTT reagent contains phospholipids, derived from either animal or vegetable sources that function as a platelet substitute in the coagulation pathways. The aPTT test system also includes an activator of the intrinsic coagulation system, such as nonparticulate ellagic acid or the particulate activators kaolin, celite, or micronized silica. These provide a large surface area and increase the precision and reproducibility of the test.

The phospholipid composition of different aPTT reagents varies considerably and influences the sensitivity of individual reagents to clotting factor deficiencies and to inhibitors, such as heparin and antiphospholipid antibodies. Therefore, aPTT results will vary from one laboratory to another, and the normal range in the laboratory where the testing occurs should be used in the interpretation. The aPTT reagent will also vary in sensitivity to individual factor deficiencies. It usually becomes prolonged with individual factor deficiencies of 30% to 50%, but the coagulation laboratory should determine the sensitivity of the reagent it is using for each coagulation factor. The aPTT is commonly prolonged in patients with antiphospholipid antibodies, and reagents vary widely in their sensitivity to these antibodies. A clue that a prolonged aPTT is due to an antiphospholipid antibody can be variable aPTT results on samples between assays performed in different laboratories.[34] Heparin contamination is another common iatrogenic or artifactual cause of a prolonged aPTT, particular in hospitalized patients, where it also may be monitored by its anti-Xa activity (reviewed in Ref.[35]).

Thrombin Clotting Time

Thrombin clotting time (TCT), or simply thrombin time, is a simple test that measures the time for clot formation to occur in citrated plasma after the addition of thrombin.[36,37] It therefore reflects the action of thrombin on fibrinogen with the formation of fibrin. During this process, thrombin cleaves the α and β chains of fibrinogen at their amino-termini, releasing fibrinopeptides A and B. The residual fibrin monomer of one fibrinogen molecule interacts with others to form polymerized fibrin.

A prolonged TCT is caused by a deficiency or structural abnormality (dysfibrinogenemia) of fibrinogen or by the presence of an inhibitor of the thrombin–fibrinogen reaction. It is abnormal in patients with familial hypofibrinogenemia or dysfibrinogenemia and in patients with acquired deficiencies of fibrinogen (as in decreased production due to liver dysfunction, increased consumption due to DIC, or acquired dysfibrinogenemia). Fibrin-degradation products, common in DIC or acute thrombosis, can interfere with the assay. Monoclonal gammopathies, particularly IgM and IgA isotypes, also interfere with fibrin polymerization both in the TCT and *in vivo*. Clinically, the most important inhibitor causing prolongation of the TCT is unfractionated heparin.[38] Unexplained prolongations of the aPTT and TCT warrant evaluation for heparin exposure in the patient or contamination of the clinical test sample.

The TCT is also sensitive to direct thrombin inhibitors (DTIs) and correlates directly with plasma concentration. With modification, the TCT can be used to detect and monitor the IV thrombin inhibitors argatroban, lepirudin, and bivalirudin even in the setting of prolongation of the baseline aPTT, such as lupus anticoagulants and vitamin K deficiency.[39] TCT is very sensitive to the oral DTI dabigatran. At therapeutic dabigatran levels, the TCT displays a linear dose-response curve, while a normal TCT can be used to exclude the presence of dabigatran in the sample.[40] Low molecular weight heparins have a less potent effect on thrombin and exert relatively greater inhibition of FXa, with less, if any, effect on the TCT.

Ecarin Clotting Time

Ecarin is a metalloprotease isolated from the viper *Echis carinatus* that cleaves prothrombin to generate meizothrombin, a proteolytically active intermediate form of thrombin. DTIs inactivate meizothrombin, preventing the conversion of fibrinogen into fibrin and prolonging the ecarin clotting time (ECT). Although not widely available, the ECT can be used to detect and monitor all of the DTIs. However, the ECT is insensitive to heparin, which is unable to interact with meizothrombin due to steric interference by the heparin–antithrombin complex.[40,41]

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Reptilase Time

The snake venom reptilase (from *Bothrops atrox*) induces fibrin clot formation in plasma by a direct action on fibrinogen. In contrast to the action of thrombin, which cleaves fibrinopeptides A and B, reptilase cleaves only fibrinopeptide A.[42] Unlike the TCT, the reptilase time is unaffected by heparin and can be used as a quick way to rule out heparin contamination.[43] The reptilase time is generally more sensitive to dysfibrinogenemias than is the thrombin time.

Mixing Studies

Mixing studies are used to evaluate a prolonged aPTT, or less commonly PT, to distinguish between a factor deficiency and an inhibitor. In this assay, normal plasma and patient plasma are mixed in a 50:50 ratio, and the aPTT or PT is determined immediately after incubation at 37°C at varying time points, typically 30 minutes, 60 minutes, and/or 120 minutes. With isolated factor deficiencies, the aPTT will correct with mixing and stay corrected with incubation. With aPTT prolongation due to an antiphospholipid antibody, the mixing and incubation will show no correction.[34] In acquired neutralizing factor antibodies, such as an acquired FVIII inhibitor, the initial assay may or may not correct immediately after mixing but will prolong or remain prolonged with incubation at 37°C. Failure to correct with mixing can also be caused by the presence of other inhibitors or interfering substances, such as heparin, fibrin split products, and paraproteins.

Specific Clotting Factor Assays

Decisions to proceed with specific clotting factor assays will be influenced by the clinical situation and the results of coagulation screening tests. When bleeding is severe, specific assays are often urgently required to guide appropriate therapy. Following factor levels can permit a safe and logical approach to replacement therapy, preventing both undertreatment (which may result in adequate control of bleeding) and overtreatment (which may paradoxically increase risk of thrombosis).

One-stage assays are commonly used for the quantitation of specific clotting factors participating in both the extrinsic and intrinsic coagulation pathways. These tests are modifications of the aPTT or PT, in which the patient plasma is serially diluted in specific factor-deficient plasma. The concentration of the patient's clotting factor is calculated using a standard curve generated by re-reconstituting the deficient plasma with known quantities of the clotting factor.[37] An activity level can be assigned only if the values from the serial dilutions fall on a straight line and the line formed by the test plasma is parallel to that of standard normal plasma. Nonparallel plots usually result from an inhibitor in the patient plasma or, occasionally, from an unrecognized deficiency of another factor in the substrate-deficient plasma. A two-stage assay, based on the thromboplastin generation test, can also be employed and would be appropriate either as initial coagulation factor testing or for follow-up when the bleeding history is suspicious but disproportionate to the one-stage test result. Indeed, 5% to 10% of patients with mild hemophilia A have a normal one-stage FVIII measurement but a low two-stage FVIII measurement more consistent with the bleeding history.[44] Chromogenic substrate assays, which mimic the cleavage site on the natural substrate for the clotting factor being tested,[45] and less commonly protein quantification by enzyme-linked immunosorbent assay (ELISA), may be employed in the assessment of a factor deficiency.

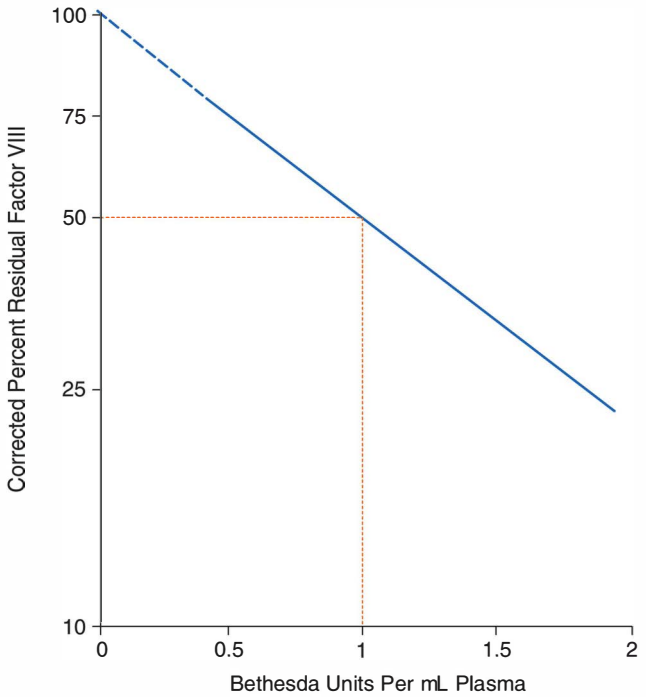

FIGURE 50.3 Bethesda assay for FVIII inhibitor quantitation. Relation between corrected residual FVIII activity and Bethesda titer is shown. If the result is <25%, serial dilutions of the patient's plasma are tested until the result is between 25% and 75%. The result is multiplied by the dilution to assign the Bethesda titer. One Bethesda unit is defined by a corrected residual FVIII of 50% in the assay (*dotted line*).

The Bethesda Assay for Factor VIII Inhibitor Quantitation

The Bethesda assay is used to detect and quantitate FVIII inhibitors[46] and is based on the ability of patient plasma to inactive FVIII in normal plasma. In this assay, patient plasma is serially diluted in normal plasma and residual FVIII activity measured after incubation for 2 hours at 37°C using a one-stage assay. The assay is controlled for normal FVIII decay using normal plasma diluted in buffer or FVIII-deficient plasma (Nijmegen modification). Percentage residual factor (corrected for control) is plotted on a logarithmic scale against units of inhibitor on a linear scale (see FIGURE 50.3). One unit is the amount of antibody that destroys 0.5 U of FVIII after incubation at 37°C for 2 hours. This assay can be adapted for inhibitors against other clotting factors, including porcine FVIII.

Factor XIII

FXIII deficiency cannot be detected by abnormalities in any of the routine coagulation screening tests. A high index of clinical suspicion is required for its recognition, and when it is suspected, a specific screening test for FXIII deficiency should be performed. The most commonly used procedure is a clot

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solubility test, which is based on the fact that non–cross-linked fibrin (due to FXIII deficiency or rarely, inhibitors to FXIII or fibrin) will be soluble in 5 M urea, 1% monochloroacetic acid, or 2% acetic acid. Functional and immunologic assays to quantitate FXIII are available.[47,48]

Fibrinogen

Fibrinogen is assessed in both quantitative and qualitative assays.[49,50] Clottable fibrinogen is determined similarly to the one-stage assay used for other clotting factors using either the TCT (Clauss method) or the PT as the basic assay method to determine fibrinogen activity. Immunologic methods are used to quantify the fibrinogen protein. A dysfibrinogenemia is diagnosed when the measurement of the total clottable fibrinogen is <80% of the immunologic fibrinogen in a sample.

Assessment of Fibrinolysis

There are a variety of immunologic, functional, and chromogenic tests for the determination of the components of the fibrinolytic system. The measurement of fibrin degradation is routine in patients suspected of having DIC or consumptive coagulopathies. D-dimers are the specific product of fibrin degradation formed through digestion of cross-linked fibrin by plasmin.

Enhanced fibrinolytic activity can be detected by the euglobulin clot lysis time (ECLT), a global test of fibrinolysis.[37] In this assay, plasma is treated by dilution to a low ionic strength and acidification to precipitate the euglobulin fraction, containing fibrinogen and plasminogen activators; fibrinolytic inhibitors such as plasminogen activator inhibitor (PAI-1) are partially precipitated. The euglobulin fraction is dissolved and the fibrinogen clotted; clot lysis is then timed. The ECLT is dependent on the activity of both tissue plasminogen activator (tPA) and PAI-1.[51] Short lysis times are indicative of increased fibrinolysis. Specific α_2-antiplasmin and PAI-1 assays are available, with the caveat that some assays are designed to detect elevations in these fibrinolytic factors and therefore limited in their sensitivity for detecting deficiencies.

Choice of testing for disorders of fibrinolysis is directed by the clinical suspicion of the underlying cause. Acquired states of accelerated fibrinolysis may contribute to bleeding that accompanies acute promyelocytic leukemia, carcinoma of the prostate or with prostatectomy, and end-stage liver disease. The latter is due to lack of clearance of fibrinolytic enzymes by the diseased liver. In these cases, measurement of the affected factors (fibrinogen, fibrin/fibrinogen degradation products, and D-dimers) to guide replacement is generally sufficient.

Familial bleeding disorders attributed to excessive fibrinolysis have been reported rarely, but include the autosomal recessive disorders of α_2-antiplasmin deficiency,[52] in which bleeding occurs because of the proteolytic digestion of procoagulants by plasmin, and deficiency of PAI-1,[53] the major inhibitor of tPA. High clinical suspicion would warrant specific factor testing.

von Willebrand Disease Testing

Both immunoassays and functional assays are available for the measurement of VWF in plasma. VWF protein antigen level (VWF:Ag) is measured by ELISA. VWF activity is measured by assays that test the interaction of VWF with its ligands, either platelets or collagen.[54,55] Ristocetin is an antibiotic that causes platelet agglutination by mediating an interaction between VWF and GPIb on the platelet surface. The ristocetin cofactor test (VWF:RCo) uses this interaction *in vitro* to assess the ability of VWF to bind platelets. Different concentrations of ristocetin are added to patient and control plasma in the presence of formalin-fixed platelets and platelet agglutination quantified. Decreased agglutination is indicative of low VWF activity, although a common polymorphism has also been described which decreases the affinity of VWF for ristocetin without perturbing its hemostatic function.[56]

The ristocetin-induced platelet aggregation (RIPA) also uses the platelet-agglutinating properties of ristocetin, but is less sensitive to mild deficiencies in VWF. In the RIPA, ristocetin is added to the patient's own platelets and plasma and platelet aggregation is measured. This test is useful to detect type 2B VWD and pseudo-VWD, in which the patients exhibit increased agglutination due to gain-of-function mutations in VWF or platelet GPIb respectively. The RIPA is decreased in Bernard-Soulier syndrome (GPIb deficiency) in addition to other forms of VWD. Alternative methods of quantifying the ristocetin-mediated interaction between VWF and GPIb have been developed, including ELISA and flow cytometry–based assays, although VWF:RCo remains the main benchmark of assessing VWF–platelet activity.

Collagen binding is assessed by ELISA and can vary depending on the type of collagen used to coat the plate. Collagen binding appears to be influenced by VWF multimer structure and has been previously suggested as a possible surrogate for disproportionate shifts in VWF multimer structure. However, genetic defects have been described in the VWF molecule that can affect collagen binding,[57] and therefore collagen binding assays are not always a surrogate for VWF multimer structure.

VWF structure should be assessed by a VWF multimer analysis in which VWF multimers are visualized on an unreduced low percentage (1% to 2%) agarose gel. Abnormalities in VWF multimer structure, such as changes in the abundance of high molecular weight multimers or alteration of the VWF triplet structure (a reflection of VWF proteolysis), are useful clues to the subtype of VWD.

Tests of Platelet Function

Assessment of platelet function should be performed in patients with a history suggestive of a disorder of primary hemostasis for which there is no obvious cause, such as VWD, thrombocytopenia, use of platelet inhibitory medications, or renal failure. Results of platelet function testing are highly dependent on sample handling and processing and are best performed in an experienced laboratory within 2 hours of sample collection. Ideally, the patient, rather than the blood sample, should go to the laboratory for on-site testing to attempt to optimize standardization of the procedure.

Platelet Aggregometry

Platelet function testing is commonly performed by platelet aggregometry, which is technically challenging and widely variable in execution. Despite these limitations, platelet aggregometry can be very useful when performed rigorously with validated standards in patients with a history of bleeding.[44] Although

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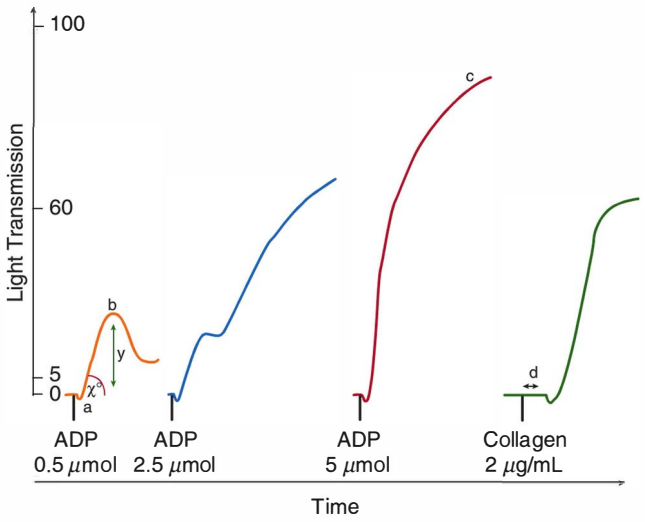

FIGURE 50.4 Typical traces obtained during platelet-rich plasma aggregation. *a*, shape change; *b*, primary wave aggregation; *c*, secondary wave aggregation; *d*, lag phase; χ°, angle of ascent of aggregation trace; *y*, height of aggregation trace; ADP, adenosine diphosphate.

other methods have been employed in the assessment of primary hemostasis, platelet aggregometry remains the gold standard in the diagnosis of platelet function defects.

Platelet aggregometry is performed *ex vivo* by light transmission aggregometry. Upon the addition of most aggregating agents, platelets change shape from discs to a more rounded form with pseudopods, resulting in a transient, small decrease in light transmission that is followed by increased light transmission as the platelets aggregate (see FIGURE 50.4).[58] Platelet aggregometry should always be performed with reference to a control sample and should take into account the patient's platelet count. The most commonly used agonists are ADP, epinephrine, collagen, arachidonic acid, and ristocetin (which is not a true platelet agonist, see VWF testing above), although other agents are sometimes used. ADP and epinephrine are weak agonists compared to collagen or thrombin. Aggregation with these agonists, particularly at low concentrations, will begin with a primary, reversible phase of aggregation, followed by a secondary irreversible phase. The secondary phase is dependent on thromboxane formation and release of granule content. Drugs that inhibit thromboxane formation, such as aspirin, will prevent the secondary phase of aggregation. Thus, the common agonists ADP, epinephrine, and collagen can all be helpful in identifying a platelet disorder.[59] Although platelet aggregometry is only modestly sensitive in identifying platelet disorders, bleeding disorders are likely if an aggregation defect is detected, particularly with two or more agonists.

As useful adjuncts to platelet aggregometry, the platelet release reaction and platelet granule content can be assessed using lumiaggregometry to measure the adenosine triphosphate (ATP) released after different agonists are added. The firefly enzymes luciferin and luciferase produces light proportional to the concentration of ATP, and the light is quantified in a luminometer.[60] Specific testing for platelet glycoproteins (such as the GPIb/V/IX complex or GpIIb/IIIa), analysis by electron microscopy to identify the ultrastructural defects associated with many inherited platelet disorders, and highly specialized testing of platelet granule components may be employed to further classify the nature of the defect in a suspected platelet disorder.

PFA-100

Different methods and instrumentation have been developed in an effort to globally assess platelet and VWF function. The PFA-100 (Siemens Diagnostics) measures the time to clot formation (and aperture closure) of citrated whole blood under flow in a membrane coated with collagen and either adenosine diphosphate (C/ADP) or epinephrine (C/EPI). The PFA-100 is rapid and easy to perform, but demonstrates wide variation between laboratories and is vulnerable to artifact during blood sampling and handling. The PFA-100 has varying sensitivity to VWD depending on the subtype and VWF levels, but it has low sensitivity for type 1 VWD and has limited sensitivity and specificity for common platelet disorders. Furthermore, the PFA-100 does not predict bleeding risk. Thus, the PFA-100 does not appear to be a useful general screening tool for bleeding disorders and is of limited utility in the evaluation of specific disorders of primary hemostasis.[61,62] Numerous other devices have been developed with the potential to measure primary hemostasis. However, most were developed to monitor antiplatelet therapies, are either investigational or of limited availability, and have not been validated in clinical practice.

Bleeding Time

The skin bleeding time is mentioned here for historical reference only. It previously was commonly employed in the bleeding evaluation in an effort to detect disorders of primary hemostasis. However, it is prone to operator error, insensitive, poorly reproducible, poorly predictive of bleeding, and invasive,[61,62] making it of limited clinical utility.

Genetic Testing

As the spectrum of mutations associated with defective hemostasis continues to expand, genetic testing may offer some assistance in the evaluation of a suspected inherited bleeding disorder. Gene mutations in many congenital coagulation factor deficiencies, such as FVIII, are well characterized, and the mutations almost always directly affect the clotting factor gene itself. Most subtypes of VWD have an identifiable mutation in the *VWF* gene, although about half of type 1 VWD patients do not.[63] Mutations in multiple genes have been associated with platelet defects, although many inherited platelet disorders remain molecularly unexplained.[64] Genetic testing is not widely available for many inherited bleeding disorders, but the field is rapidly evolving. Consideration of genetic testing should be based upon a high clinical index of suspicion and corroborating laboratory data, and only be performed in the setting of appropriate individualized genetic counseling before testing is performed and, if the patient is agreeable, again after the results are received.

CONCLUSION

Evaluation of the patient with bleeding requires careful attention to history and physical examination. Acquired perturbations of hemostasis resulting in coagulation factor deficiencies (such as

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Table 50.2

Inherited disorders of hemostasis, relative prevalence, and inheritance patterns

Disorders of Primary Hemostasis	Relative Prevalence[a]	Inheritance Pattern
VWD	More common	
Type 1 VWD		Autosomal dominant
Type 2 VWD		Autosomal dominant
Type 3 VWD	(Rare)	Autosomal recessive
Platelet disorders	Less Common[b]	
Glanzmann thrombasthenia	(Rare)	Autosomal recessive
Bernard-Soulier syndrome	(Rare)	Autosomal recessive
Platelet granule disorders (storage pool, gray platelet, etc.)		All patterns
Defects of secretion/signal transduction		All patterns
Defects in cytoskeletal proteins (MYH9-related, Wiskott-Aldrich, etc.)		All patterns
Disorders of secondary hemostasis		
FVIII deficiency (hemophilia A)	More common	X-linked recessive
FIX deficiency (hemophilia B)	More common	X-linked recessive
FI (fibrinogen) abnormalities	Less common	
Afibrinogenemia		Autosomal recessive
Hypofibrinogenemia		Autosomal dominant or recessive
Dysfibrinogenemia		Autosomal dominant or recessive
FII (prothrombin) deficiency	Rare	Autosomal recessive
FV deficiency	Less common	Autosomal recessive
FVII deficiency	Less common	Autosomal recessive
FX deficiency	Less common	Autosomal recessive
FXI deficiency	More common in Ashkenazi Jews, otherwise rare	Autosomal recessive
FXIII deficiency	Less common	Autosomal recessive
Combined factor deficiencies	Rare	
Combined FV and FVIII deficiency		Autosomal recessive
Combined FII, FVII, FIX, and FXI deficiency		Autosomal recessive
α_2-Antiplasmin deficiency	Rare	Autosomal recessive

[a]"More common" = prevalence of > 1:50,000; "less common" = prevalence < 1:50,000 but >100s; "Rare" = hundreds of families or less.
[b]Overall prevalence of platelet disorders has not been well characterized.

DIC, liver disease, warfarin, autoimmune disease) or platelet defects (aspirin, GPIIb/IIIa inhibitors, clopidogrel) are generally more common than congenital disorders of hemostasis, and thus must be considered in the differential diagnosis of the bleeding patient. However, inherited disorders, even when individually rare, are collectively common. Therefore, careful attention should be paid to clues of a congenital coagulation defect, such as early age of onset or a positive family history. Table 50.2 categorizes congenital bleeding disorders by their tendencies to present with a primary or secondary hemostatic defect, prevalence, and inheritance pattern. Logical use of screening and specialized coagulation testing will guide the diagnosis and lead to appropriate treatment to stop or prevent bleeding. Table 50.3 summarizes conditions known to contribute bleeding and their commonly associated PT and/or aPTT abnormalities, conditions that may result in abnormal PT and/or aPTT in patients without a bleeding history, and a list of conditions associated with thrombocytopenia. In conjunction with a thorough history and physical examination, these tables are a general guide to narrow the differential diagnosis of the bleeding patient. More specific information on the diagnosis and treatment of specific bleeding disorders can be found in other chapters of this book. Many of these conditions may present together or present with considerable heterogeneity, providing a seemingly daunting complex hemostatic picture at the outset. Through careful dissection of the data, the cause of the patient's hemostatic defect(s) and underlying bleeding risk should be able to be discovered and thoughtfully addressed.

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Table 50.3

Assembling the bleeding history, inheritance, and the aPTT and PT

Symptoms and Laboratory Findings	Inheritance Pattern	
Bleeding Patient	**Congenital**	**Acquired**
Normal PT, normal aPTT	VWD (with mild ↓ or normal FVIII), platelet disorders, FXIII deficiency, fibrinolytic factor defects (α_2-antiplasmin, PAI)	Acquired platelet dysfunction (medications, uremia), thrombolytics
Normal PT, prolonged aPTT ("extrinsic pathway")	Factor deficiencies (FVIII, FIX, FXI), VWD (due to lower FVIII)	Acquired inhibitor (e.g., FVIII), acquired VWD, heparin
Prolonged PT, normal aPTT ("intrinsic pathway")	Factor deficiency (FVII), fibrinogen defect	Vitamin K deficiency or warfarin, acquired inhibitor, antiphospholipid antibodies, liver disease (factor and fibrinogen defects)
Prolonged PT, prolonged aPTT (common pathway)	Factor deficiency (fibrinogen, prothrombin, FV and FX)	DIC, severe vitamin K deficiency/warfarin, severe liver disease, paraproteinemia, antiphospholipid antibodies (prothrombin), acquired FV inhibitor, amyloidosis (adsorption of factors, especially FX)
Asymptomatic Patient		
Normal PT, prolonged aPTT ("intrinsic pathway")	Factor deficiency (FXII, prekallikrein, kininogen, FXI)	Antiphospholipid antibodies
Prolonged PT, normal aPTT ("extrinsic pathway")	FVII deficiency, fibrinogen defects	Acquired fibrinogen defect, liver disease, warfarin/vitamin K deficiency, antiphospholipid antibodies
Prolonged PT, prolonged aPTT (common pathway)	Factor deficiency (fibrinogen, prothrombin, FV and FX)	Antiphospholipid antibodies, FV inhibitors
Thrombocytopenic Patient		
Thrombocytopenia	Congenital thrombocytopenia/syndrome, type 2B VWD	Platelet production defect (medications, sepsis/shock, bone marrow disease, autoimmune), consumption (hypersplenism, DIC, thrombosis, thrombotic thrombocytopenic purpura, HELLP [hemolysis, elevated liver enzymes, and low platelet] syndrome), dilutional (iatrogenic, pregnancy), immune mediated (underlying autoimmune disease, medications, immune thrombocytopenic purpura, antiphospholipid antibodies)

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CHAPTER

51

Clinical Manifestations and Therapy of Inherited and Acquired Hemophilia

EDWARD G.D. TUDDENHAM, PETER W. COLLINS, AND T.T. YEE

HISTORICAL INTRODUCTION

Knowledge of a congenital bleeding disorder affecting male infants can be inferred from rulings in the Babylonian Talmud[1] compiled about 500 CE. A dispensation was given to forgo circumcision in brothers and cousins of affected boys who had died after the ritual was carried out (presumably from bleeding), showing that the Rabbis recognized inheritance of a bleeding tendency through carrier females. Abu al-Qasim Khalaf ibn al-Abbas Al-Zahrawi, known as Albucasis (936–1013), the great Moorish surgical writer of 10th-century Spain, wrote of a certain village where boys and men suffered excessive bleeding after minor injury. He also gave the first practical advice for treating such bleeding by means of cautery, the success of which he observed. We can now interpret this report as referring to moderately severe hemophilia with a local founder effect.

The curious word for an abnormal inherited bleeding tendency which we use today—hemophilia (literally blood lover)—was introduced by Hopff in his doctoral thesis delivered at Wurzburg in 1828.

The medical literature of the 18th and 19th centuries on hemophilia was gathered in a monumental review published in 1911 by the Francis Galton Laboratory for National Eugenics of the University of London (now abbreviated to The Galton Laboratory). The review[2] described clearly all the clinical features that are seen today in untreated cases of hemophilia. However, they failed to recognize the key feature of X-linked inheritance—daughters of affected males are obligate carriers of the mutant gene. The correct theoretical explanation of chromosomal sex determination and of sex-linked inheritance in mammals had only just been proposed and had evidently not come to their notice. Notably absent from the nearly 1,000 pedigrees of hemophilic families illustrated in the review was that of the most famous hemophilic family in history, namely the descendants of Queen Victoria, who was an obligate carrier on pedigree evidence (FIGURE 51.1). In addition to her British descendants, she transmitted hemophilia to three other European Royal families through dynastic marriage alliances. No antecedents of Queen Victoria had a bleeding tendency, so it is likely that she inherited a new mutation in one or other of the parental gametes, as is the case for a third of carriers of hemophilia. Three of her daughters were carriers of the hemophilic gene and her youngest son Leopold had clinically severe hemophilia. The mutant gene then made its way into the German, the Spanish, and most famously the Russian Royal families. Leopold's daughter, the Princess Alice, was an obligate carrier and had two affected sons who died in childhood. The German affected boys also died young, but the Spanish princes with hemophilia survived into their 20s before succumbing to trauma-induced bleeding. Alexey Romanov, the Tsarevich (heir to the throne of Russia), was affected with severe hemophilia. Alexey was murdered on Lenin's orders with his family at Yekaterinburg during the civil war following the Bolshevik revolution. Hence by 1938, there were no surviving male descendants of Queen Victoria with hemophilia. In 1952, it emerged that there are two types of hemophilia—A and B. The question then arose, whether the "Royal disease" was type A or type B. It became a long-standing historical puzzle which was finally solved almost 60 years later, by a tour de force of forensic genetics, in favor of hemophilia B,[3] by genomic analysis of the Imperial family's bones, recovered from unmarked graves near Yekaterinburg (FIGURE 51.2).

GENETICS AND BIOCHEMISTRY OF HEMOPHILIA

The X-linked inherited bleeding disorders, hemophilia A and B, are due respectively to mutations at the factor VIII (FVIII) locus (*f8*) or the FIX locus (*f9*), both situated on the long arm of chromosome X at Xq2.8 and Xq2.6, respectively. The *f8*-linked condition, caused by deficient synthesis of coagulation FVIII, is known as hemophilia A, whilst the *f9*-linked condition, due to deficient synthesis of coagulation FIX, is called hemophilia B or Christmas disease (after one of the first described sufferers). The clinical features of the two conditions are indistinguishable, and they were not separated until complementation studies in 1952 uncovered the fact that blood from a majority of patients with X-linked hemophilia (type A) could correct the coagulation defect in blood from a minority (type B) and vice versa. FIX itself was discovered as a result of these studies, being called at first Christmas factor[4] to distinguish it from antihemophilic factor, the name already assigned to the putative entity lacking in the blood of type A patients. Biochemically, FVIII and FIX function together as a binary enzyme (FIXa) cofactor (FVIIIa) complex whose substrate is FX.

The incidence ratio of hemophilia A to B is about 5 to 1, and both conditions are rare: hemophilia A affects 1 in 5,000 males at birth whereas hemophilia B affects only 1 in 25,000 males at birth. Contrary to the commonly held belief that carriers are unaffected, about a third of females who are heterozygous for mutations at either locus manifest mild hemophilia due to skewed X inactivation.

Acquired hemophilia A is a rare autoimmune disorder caused by failure of self-tolerance toward FVIII occurring in later life, with production of antibodies neutralizing the activity of the patient's own FVIII. Hemophilia A and B are treated together in the following discussion of the pathology, clinical features, and treatment of the two disorders, only noting differences where relevant.

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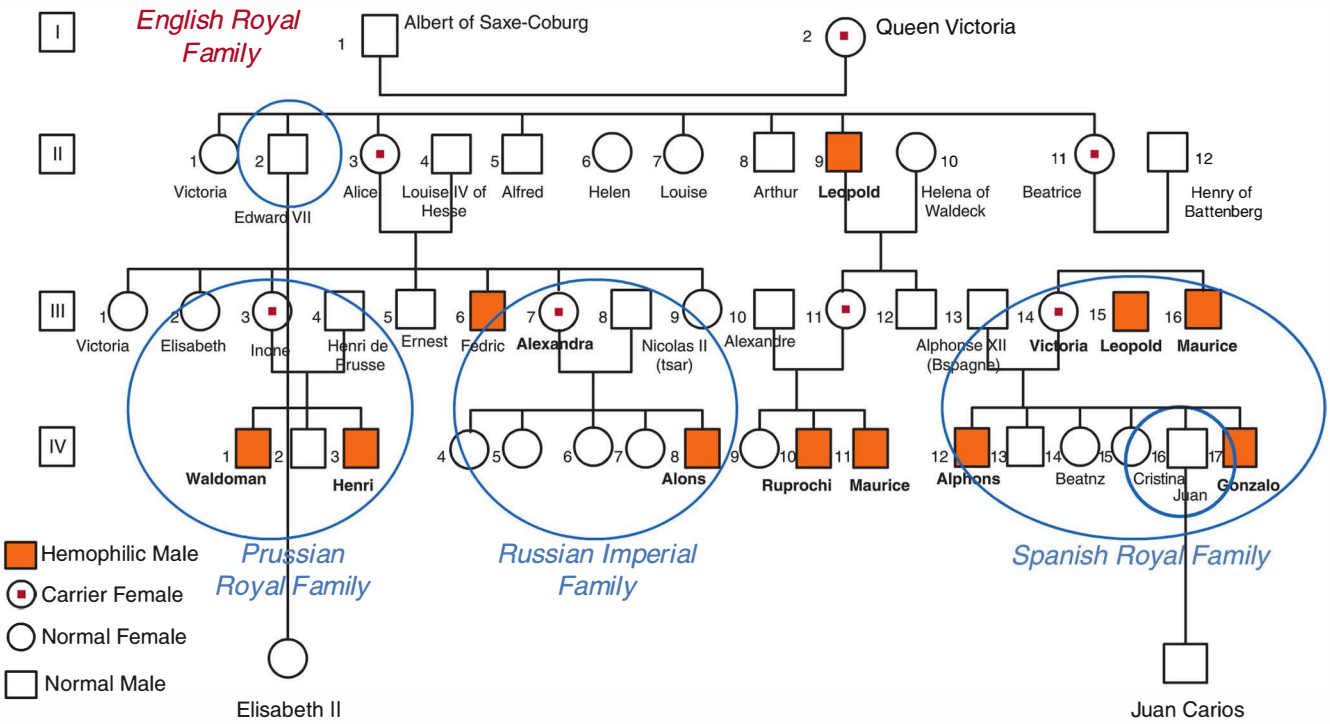

FIGURE 51.1 Queen Victoria's pedigree. The Royal disease is an X-linked recessive bleeding disorder that, until resolved by modern genetic analysis, could have been either hemophilia A or B as every affected male had died before blood tests could have decided the issue in 1950. Obligate carriers on pedigree are represented by a *circle* with a *red dot* in its centre. Affected males are shown as *red squares*. Of the four royal families afflicted by the disease (*blue circles*), the most tragic was the Russian. Alexei met his end by murder as did his sisters and parents. One of his sisters was a carrier according to the forensic analysis, probably Anastasia (FIGURE 51.2). (Reproduced from Lannoy N, Hermans C. The "royal disease"—haemophilia A or B? A haematological mystery is finally solved. *Haemophilia* 2010;16(6):843–847, with permission.)

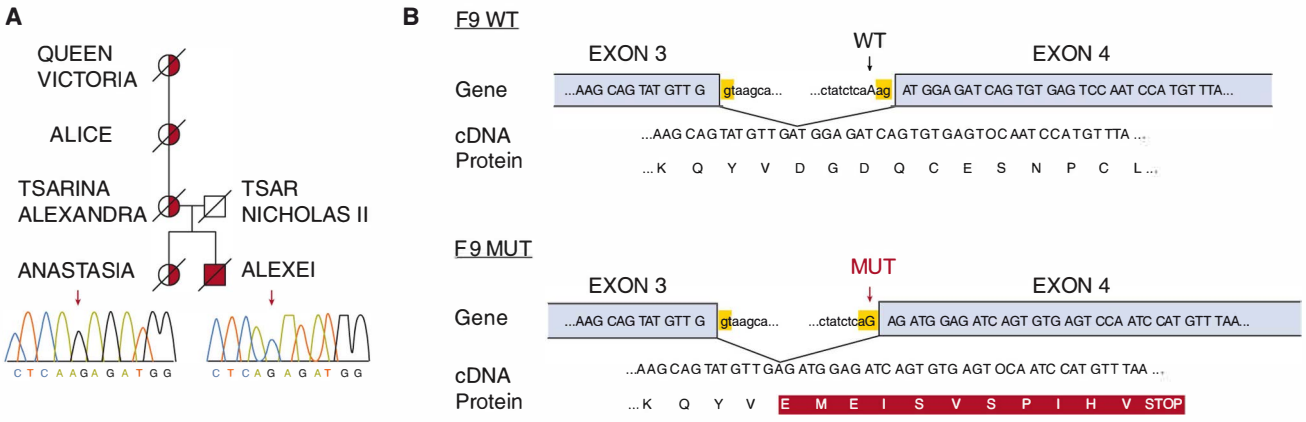

FIGURE 51.2 The Royal disease was caused by a point mutation in *F9*. **A**: Partial pedigree of the Royal family, showing transmission of the mutation from Queen Victoria to Empress Alexandra and from Alexandra to Prince Alexei, her haemophilic son. Alexei was hemizygous for the mutation, whereas Alexandra and one of her daughters, putative Grand Duchess Anastasia, were heterozygous carriers. **B**: The A-to-G mutation occurs just upstream of exon 4 in the F9 gene and is predicted to create a new splice acceptor site that could lead to production of a truncated factor IX protein. WT indicates wild type. (Reproduced from Rogaev EI, Grigorenko AP, Faskhutdinova G, et al. Genotype analysis identifies the cause of the "royal disease". *Science* 2009:326(5954):817, with permission.)

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PATHOPHYSIOLOGY AND PRINCIPLES OF TREATMENT

All clinical features of hemophilia A and B are due to deficiency of FVIII or FIX respectively, with consequent failure to generate adequate thrombin in response to injury and hence failure to form a stable hemostatic plug at sites of vascular damage. Correction of the defect by intravenous infusion of the relevant missing factor completely normalizes thrombin generation, since all other factors required for hemostasis are present in normal amounts. Several circumstances conspire to frustrate our efforts to normalize clotting in a hemophilic person's blood. FVIII and FIX are scarce proteins which are difficult to purify and difficult to make by genetic engineering. Hence, the cost of clinical factor concentrates is extremely high. Given a supply of factor concentrate, it still has to be introduced into the circulation by intravenous infusion. Vascular access is therefore critical for hemophilia care lifelong. Most problematic of all is the short half-life in the circulation of infused factor—8 to 12 hours for FVIII and 15 to 20 hours for FIX. In fact, four out of five hemophiliacs worldwide have little or no access to replacement therapy at all. Treatment regimens discussed below are a compromise designed to maximize the benefit of scarce resources and minimize the dire effects of bleeding, which if left unstaunched leads to progressive damage wherever bleeding is prone to occur.

If left without effective treatment the natural history of hemophilia is progressive loss of mobility from joint and muscle bleeding and early death due to exsanguination or uncontrolled internal bleeding, most often intracranial. Prior to the advent of factor concentrates in the 1970s, the most severely affected hemophiliacs died before the age of 20. In contrast to that dire prognosis, modern comprehensive hemophilia care, supported by the resources of an economically advanced state, can normalize life span and limit or eliminate tissue damage.[5] Adequate supplies of virus safe FVIII and FIX first became widely available in the 1990s; hence most hemophiliacs born before 1985, even in economically advanced countries, are living with the long-term effects of undertreatment in childhood and infection by hepatitis C and HIV, which were present in factor concentrates up to 1986.

GENETICS AND DIAGNOSIS OF HEMOPHILIA A AND B

Since there is no heterozygote advantage to carrying defects at the *f8* or *f9* loci and since the hemizygous male has severely limited reproductive fitness (absent treatment), the frequency of "hemophilic" gene defects in the population must be maintained by new mutations. Consequently and as predicted by Haldane (op. cit.), the mutations are highly diverse.

Usually genetic analysis is carried out following the measurement of blood factor level establishing the diagnosis in the affected male, but putative carriers of hemophilia A or B often have no living affected relative, due to early death. Alternatively they may be carriers of a recent mutation. To help such women with establishment of carrier status and genetic counseling, it is then necessary to sequence their *f8* and/or *f9* genes. Detection of a heterozygous mutation already noted in the databases associated with hemophilia, or (if not previously reported) plausibly causative of deficient/functionally defective FVIII or FIX, then confirms carrier status. The nature of the mutation can also predict the severity of the disorder. Severe gene lesions such as stop codons, large deletions, or the prevalent inversion in hemophilia A not only cause severe disease with no circulating factor but are also associated with high risk of neutralizing antibody (inhibitor) development after substitution therapy. Missense mutations on the other hand are usually associated with moderate (residual factor level 1% to <5%) or mild (residual factor > 5%) bleeding tendency.

In laboratory diagnosis of hemophilia A and B, the standard screening tests show a prolongation of activate partial thromboplastin time (APTT) with normal prothrombin time (PT). If this is the case, then specific factor assays to quantify FVIII and FIX are performed. Traditionally, residual FVIII or FIX <1% is defined as severe. However, the lower detection limit for both assays is about 1% and a residual level below 1% but above absolute zero can make some difference to bleeding frequency, that is to say—the clinical phenotype. This is notable when one compares the bleeding frequency of subjects with a null mutation against those with a missense mutation but factor level <1% who may even have no spontaneous bleeding.[6] Although uncommon, patients with multiple defects in their hemostatic system are also found.

An assay capable of detecting antibodies to FVIII or FIX is also a mandatory part of the laboratory workup. In hereditary hemophilia A and B antibodies only develop after treatment and may do so promptly thereafter. Inhibitors may develop after many years of treatment, as recent data from the UK national database show.[7] Thus, regular inhibitor testing is part of routine follow-up care in hemophilia.

There are two different ways of measuring FVIII activity, the one-stage clotting assay and the two-stage clotting assay. Ideally, both assays should be performed on all patients because many patients with mild hemophilia A have a much lower two-stage assay level than one-stage assay level. The clinical bleeding tendency is better predicted by the two-stage assay result. There are even a few patients who have normal one-stage assay result but clearly abnormal two-stage assay.[8]

THE CLINICAL PHENOTYPE IN HEMOPHILIA A AND B

A guide to bleeding frequency can be derived from the residual factor level in the patient's blood[9] (FIGURES 51.3A,B). Subjects whose residual level is <1% can attract clinical attention at birth, with bleeding complications of delivery or within a few months with spontaneous bruising, bleeding after circumcision, from primary dentition or hemarthrosis. Throughout life, such patients bleed apparently spontaneously, but also after minor trauma about once or twice a week in the most severe cases.

Patients whose residual level is 1% to <5% are said to have moderate hemophilia. Typically, they present at age 2 to 5 years with bleeding after significant trauma that has sufficiently intrigued a medical attendant to request a blood test including screening for coagulation disorders. If the residual level is over 5%, then the patient is said to have mild hemophilia. Bleeding only occurs in such cases after significant trauma or surgery. Patients with mild hemophilia are often first diagnosed in middle or late life and then only after severe trauma or major surgery brings the bleeding tendency to light. The diagnosis can

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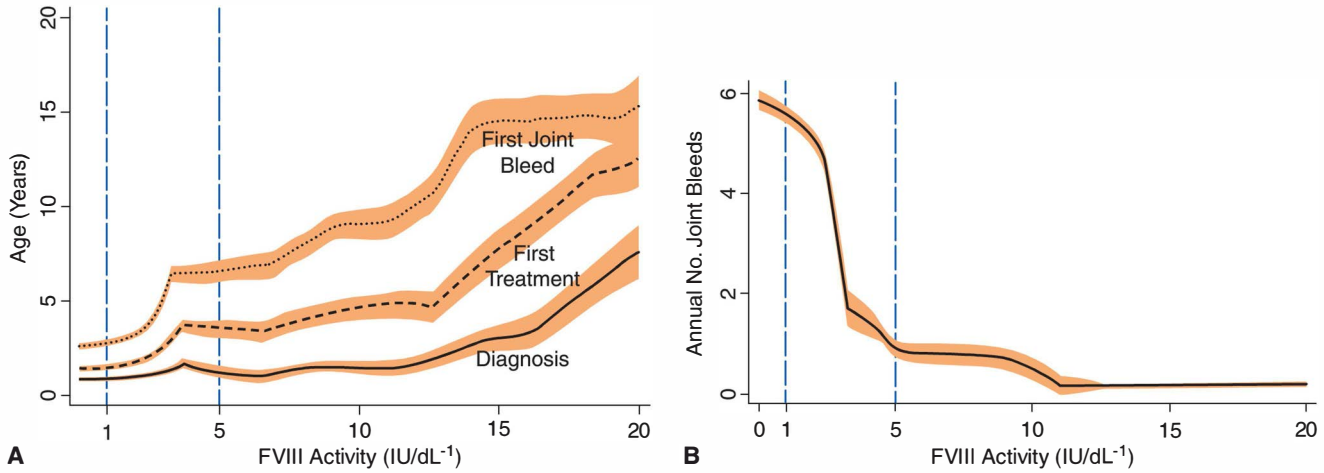

FIGURE 51.3 **A**: Age at diagnosis, onset of treatment, and joint bleeding according to factor VIII activity in a cohort of haemophilia A patients followed at one centre in the Netherlands (Ellis van Kreveld haemophilia centre). *Lines* are medians, *shaded areas* are interquartile range. (Reproduced from Den Uijl IE, Mauser Bunschoten EP, Roosendaal G, et al. Clinical severity of haemophilia A: does the classification of the 1950s still stand? *Haemophilia* 2011;17(6):849–853, with permission.) **B**:Annual number of joint bleeds according to factor VIII activity in the same cohort as in FIGURE 51.3A. Of note, there were no joint bleeds in patients with a baseline level above 12 IU/dL. (Reproduced from Den Uijl IE, Mauser Bunschoten EP, Roosendaal G, et al. Clinical severity of haemophilia A: does the classification of the 1950s still stand? *Haemophilia* 2011;17(6):849–853, with permission.)

go undetected for many years; as in one extreme example noted by one of us (ET), a 90-year-old gentleman was found to have 5% FIX that came to light because he bled around the site of catheter insertion for coronary angiography.

The anatomical structures most frequently affected by hemophilic bleeding are the major load-bearing joints in the order of ankles>knees>elbow>shoulders. The hips are often spared. A target joint is one where repeated bleeding is occurring, without fully recovering between bleeds. Such joints go on to develop progressive painful, deforming, and motion-restricting arthropathy until they become fixed by reactive fibrosis. Blood within a joint capsule is highly irritant and provokes proliferative synovitis with erosion of cartilage, formation of bone cysts and deformity, and progressive fibrosis.[10] The progress of such damage to joints is monitored for purposes of assessing response to treatment by standardized scales based on range of movement and function, radiology, or MRI.[11–14]

Muscles develop bleeding after direct trauma or exercise or spontaneously. If the blood is confined within a muscle capsule, pressure can build up until compartment syndrome develops. This is a surgical emergency since it can lead to ischemic necrosis and contracture with loss of all function. An incision in the fascia surrounding the affected muscle combined with adequate replacement therapy is muscle saving in such circumstances, though not without risk of continued bleeding.

Bleeding into deep muscles such as the iliofemoral muscles can occur in all severity levels of hemophilia. Hip flexion, abdominal pain, and femoral nerve entrapment symptoms should lead to suspicion of this site of bleeding.

Renal tract bleeding presenting with hematuria is common and often not related to any detectable renal tract disease.

Intracerebral bleeding has been the commonest cause of death in hemophilia, even to the present.

Bruising is commonly observed in hemophilic children on the legs, trunk, and arms but is not usually a cause of concern and does not need treatment if superficial and painless.

THE SEVEN AGES OF HEMOPHILIC MAN

Conception

The ethical choice not to have a hemophilic child is one that many carriers have made, in view of their first-hand observation of the suffering of their father or (N.B. not "and") brothers. Recently, preimplantation genetic diagnosis has made it possible to select embryos, obtained by in vitro fertilisation (IVF), for transfer according to their genetic status, and this technique has been successful for hemophilia.[15]

In Utero

Prenatal diagnosis, undertaken either to terminate an affected conceptus or to guide subsequent management of labor and delivery, is widely practiced by means of chorion villus biopsy. This allows direct access to tissue and therefore DNA of fetal origin, at 10 to 12 weeks, which can be tested for linkage to or directly for the specific mutation present in the family of the carrier.

Noninvasive testing for fetal DNA present in maternal blood has been developed using digital quantitative polymerase chain reaction (PCR).[16] From as early as the 11th week of gestation, it is now possible to determine by analysis of maternal blood not only the sex of the conceptus but also whether it is or is not affected. When the fetus is affected, the quantity of DNA bearing the mutation in the maternal plasma is increased above the level due to the maternal heterozygosity in a ratio that is high enough to enable perfect discrimination from the ratio when the male

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fetus is unaffected. Either the specific mutation or a linked informative single-nucleotide polymorphism may be used.

In utero bleeding is extremely rare in a hemophiliac fetus, suggesting that it is highly protected against trauma whilst in the womb.

Delivery of Affected Males

Passage through the birth canal of a term infant can only be achieved with considerable compression of the skull and deformation of the brain. At least 5% of hemophilic infants develop clear neurological signs of progressive bleeding requiring treatment. If the diagnosis of hemophilia has been made already, then the baby can be closely monitored and treated at the first sign of such bleeding. Tragedies occur when the diagnosis has not been expected, which is true of about one-third of such deliveries due to new mutations (see section on Genetics above). Current guidelines advise against prolonged labor, instrumental delivery, and use of scalp monitoring.[17] There should be a low threshold to proceed to cesarian section if labor is not progressing and difficulties in the birth canal are likely. A sample of cord blood should be taken to confirm or exclude hemophilia. FVIII may be boosted by the stress of birth such that an elevated level may be present in mild but not in severe hemophilia. FIX levels are reduced in normal term and more so in preterm cord blood. However, absent or very low levels are clearly indicative of hemophilia B.

The Infant

In neonates with severe hemophilia, the decision to infuse prophylactic concentrate at birth is controversial. Some authorities recommend replacement therapy without evidence of active bleeding whilst others wait for evidence of bleeding. The recommended practice in Europe and North America is to delay the start of regular prophylaxis until the infant starts to crawl and has his first joint or muscle bleed. This will vary from 12 to 18 months. To secure venous access, an indwelling port may need to be inserted and the parents trained in its use so that a regular program of prophylaxis can be maintained. This standard of treatment is now producing for the first time children and young men with severe hemophilia who have never had a serious bleed and who have normal joints. However, there is now also an increased incidence of inhibitory antibody development in children with severe hemophilia A, occurring within the first 50 infusions.

The School Years

Since the practice of early and continuous prophylaxis was instituted, hemophilic children have been able to attend mainstream schools and take part in all activities with the exception of violent contact sports. With maturity, the hemophilic child is encouraged to take responsibility for his condition and to self-administer concentrate. Most children with hemophilia can achieve this by the age of 10.

Adolescence and Transition to the Adult Center

There comes a time when the boy is reaching adolescence and should have his care transferred to an adult centre. When this time is reached varies, as does the timing of puberty. Special sensitivity to the patient's and parents' needs will ease this transfer, which should be managed formally according to a scheme that ensures none of the patient's needs are overlooked. Risk taking may manifest during adolescence, but if good habits of treatment and prophylaxis have been ingrained in childhood, then most young men with hemophilia can cope with the added stress of the teen years without losing control of their hemophilia.

Early Adulthood and Career

Hemophilia is no longer a bar to any level of educational attainment. Nor is it a bar to entry to any trade or profession, with the exception of front-line military and police work. No doubt, those bars too will be lifted one day, either by very long-acting preparations or by gene therapy (see section on Gene Therapy below).

Reproductive fitness of hemophilic males is now similar to that of other males. However, the question of inheritance does arise and is best dealt with in the context of a genetic counseling clinic with trained counselors, geneticist, and hematologist, roles that may be combined in one or two people. The obligate carrier daughter will herself in due course need genetic counseling. Numbers of carrier females and of affected male infants born to them are on the rise, thanks to improved therapy.

Middle Years

Men with severe hemophilia who have survived to the age of 35 and over have all been infected with hepatitis C, and most have also been infected with HIV in childhood. They may also have badly damaged joints with progressive arthropathy due to inadequate on-demand treatment of bleeding in their early years. Their medical needs contrast dramatically with those of the younger well-treated generations now reaching maturity. In those who have failed to clear hepatitis C, there is the potential for progression to cirrhosis with increasing risk of hepatocellular carcinoma and of terminal liver disease. These patients benefit from liver transplantation as the last resort, which also cures hemophilia A and B. Multiply disabled hemophiliacs (older men in developed countries and younger subjects from developing nations) depend for their mobility on the help of skilled orthopedic surgeons to replace joints and specialized physiotherapists to maintain function.

Later Life

For the first time in history, significant numbers of men with severe hemophilia are reaching their 6th, 7th and even 8th decades. A particular puzzle arises in relation to arterial degenerative disease and hemophilia. Although the rate of coronary thrombosis is reduced in hemophilia, due to the permanent state of anticoagulation that is characteristic of the condition, arterial disease itself continues unabated and often leads to the need for arterial stenting or bypass with subsequent requirement for antiplatelet agents. The compromise that may be recommended is to use lower doses of antiplatelet agent whilst maintaining prophylaxis of the hemophilia at a slightly higher level than usual for the patient.[18,19]

Old Age and Death

Whilst hemophilic life span, in those who are not subject to the dire effect of chronic viral infection, or of resistant inhibitors, is now approaching the norm for the general population, there still remains an excess of deaths due to intracerebral hemorrhage.[5] Prophylactic regimens do not deliver a normal level of FVIII or FIX all or even most of the time. Therefore, all patients spend most of their lives with subnormal thrombin generation.

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Perhaps, the aging arteries in the brain, which become more fragile with age, finally start to leak, and the absence of adequate hemostasis at that time and place is fatal.

One British hemophiliac has died who was found to have variant Creutzfeldt-Jakob disease (vCJD) prion in his spleen postmortem,[20] as a result of receiving concentrate made from blood products contaminated with the agent. All hemophiliacs who received concentrate made from UK plasma in the relevant period are deemed at risk to develop or transmit vCJD.

TREATMENT OF UNCOMPLICATED HEMOPHILIA A AND B

Mandatory normal hemostasis must be secured for treatment of head injury/intracranial hemorrhage, major trauma, impending compartment syndrome, exsanguination, and surgery. Normal hemostasis, that is, FVIII or FIX level maintained at 100% ± 5%, is most securely and economically achieved by continuous infusion. High-purity plasma-derived and recombinant factors may be used in continuous infusion; for this, purpose a suitable dose is made up and delivered by syringe pump at a rate of about 1 U/kg/h after a bolus dose has been delivered to raise the level rapidly to 100%. Interval assay levels, throughout the period where normal hemostasis is required, should be used to adjust the infusion rate.

Undertreatment is the commonest mistake made in hemophilia care. An example of the consequences of undertreatment may be seen in the recent analysis of outcomes of knee joint replacement.[21] Centers that covered surgery with normalization of factor level for 2 weeks followed by gradual reduction had a zero complication rate and centers using less factor had poor outcomes in up to 20% of patients. This is in keeping with laboratory studies in hemophilic mice.[22]

Formulae for Calculating Bolus Doses to Achieve 100%

FVIII dose in units = patients weight in kg × percent rise required to reach 100% × 0.5
FIX dose in units = patients weight in kg × percent rise required × 1.0
The same formulae are used to calculate any other desired target level as suggested in treatment plans below.

On-Demand Treatment of Joint and Other Bleeds

Treating a bleed at the earliest opportunity when it occurs is referred to as on-demand treatment. Provided the patient has his supply of treatment at home and can self-administer the drug intravenously, bleeding is minimized and the rate of joint damage progression is slowed. Due to lack of supply, on-demand treatment made available at a medical centre is the norm for severely affected patients in most of the world, although this is clearly a suboptimal approach to treating hemophilia.[23] Moderate and mildly affected patients are managed mainly with on-demand treatment. Self-therapy is always preferable as it enables the patient to take charge of his own treatment and eliminates delays. A general rule for hemophilia is "when in doubt treat with factor."

Dosage recommended for on-demand treatment is largely empirical with little in the way of trial data to support particular regimens. A common policy is to give a fairly small dose for an uncomplicated joint bleed if treated early and to follow with a second dose in 6 hours if the problem is not resolving rapidly. In hemophilia A, that would be a dose of 15 to 30 U/kg body weight. In hemophilia B with the somewhat lower recovery, 20 to 40 U/kg is suggested. For a more severe bleed induced by trauma or not responding to a lower dose of therapy, the recommended range is increased to 20 to 40 U/kg (hemophilia A) or 30 to 60 U/kg (hemophilia B).

Prophylactic Treatment for Hemophilia

The aim of prophylaxis is to convert the individual from a severe to a moderate bleeding phenotype and so decreasing or preventing musculoskeletal damage.[24] Prophylactic treatment has been shown to prevent joint damage and musculoskeletal disability in long-term retrospective cohort studies.[25,26,27] A prospective randomized clinical trial of prophylaxis versus enhanced on-demand therapy in young children showed reduced radiological joint damage in the prophylaxis arm.[23] On the basis of these studies, prophylaxis is now the recommended treatment for children with severe hemophilia and is being increasingly used in adults.[28] In addition to its proven role in reducing arthropathy, it is likely that prophylaxis reduces the risk of other severe bleeds, such as intracranial hemorrhage.

There is some controversy about the optimal dose and frequency of prophylactic infusions, and no studies have directly compared regimens. The regimen that was used in young children in the prospective randomized trial was 20 to 40 IU/kg on alternate days,[23] a regimen based on that pioneered in Sweden,[25] and many experts would now consider this to be the gold-standard prophylactic regimen in young children. However, similar long-term outcomes have been reported with lower dose regimens in the range of 10 to 15 IU/kg.[29] The use of 3 days a week regimen is no longer considered optimal therapy because of the 3-day gap that this entails and low FVIII levels during the third day before a dose[28,30] (FIGURE 51.4).

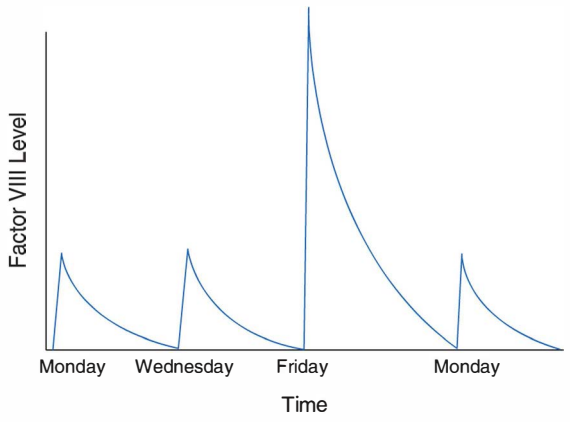

FIGURE 51.4 Implications of Monday, Wednesday, and Friday dosing. Illustration of the dose increment required to provide an equivalent trough level on Monday morning compared with the rest of the week for patients taking prophylaxis on Monday, Wednesday, and Friday. Assuming a 12-hour half-life, a fourfold dose increase on Friday morning compared to Monday and Wednesday would be required. The factor VIII scale is arbitrary. (Reproduced from Collins PW, Fischer K, Morfini M, et al. Implications of coagulation factor VIII and IX pharmacokinetics for clinical practice in the prophylactic treatment of haemophilia. *Haemophilia* 2011;17(1):2–10, with permission.)

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There is good evidence to show that prophylaxis should be started early in children with severe hemophilia.[23,26] In long-term follow-up studies, the best joint status is preserved if prophylaxis is started before recurrent bleeds have occurred,[26,31,32] and guidelines recommend starting prophylaxis at the latest after the first joint bleed.[28] Some hemophilia centers start prophylaxis before the first joint bleed, if venous access permits. There is no consensus on how prophylaxis should be initiated. Some experts recommend starting once-weekly prophylaxis with rapid escalation to full-dose regimens if bleeds occur and as the child becomes more active, whilst others prefer to introduce full-dose regimens as soon as prophylaxis is started. There are no convincing data to favor either view. The use of implanted central venous catheters may be required to allow prophylaxis in young children.

Prophylactic regimens should be tailored to the individual's bleeding pattern and activity and also take into account the patient's and their family's choices. Although the use of trough FVIII levels to adjust prophylaxis is often considered, the time spent per week with a low level may be more important than the trough level itself.[33] There are some data to suggest that dosing prophylaxis based on the patient's half-life to target a desired trough levels may lead to more cost-effective treatment, but this approach needs to be addressed in clinical trials before being widely adopted[34] (FIGURE 51.5).

Some centers are now using prophylaxis daily in very active patients or in those with problematic bleeds. The use of daily treatment results in much higher trough levels whilst using the same overall amount of FVIII or FIX as in less frequent regimens.[35] Daily treatment is a highly effective way to provide prophylaxis in the short or long term.

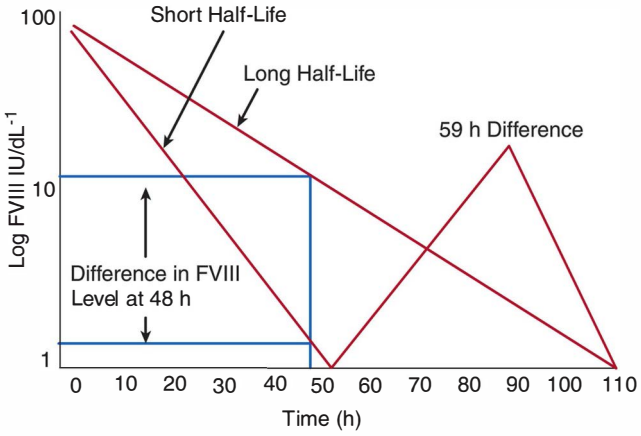

FIGURE 51.5 Effect of half-life on factor (FVIII) level following a bolus infusion. Illustration of the time taken for FVIII to reach 1 IU/dL in patients aged 10 to 65 years following an infusion of 30 IU/kg depending on variation in half-life within the normal range. Also illustrated is the variation in FVIII level at 48 hours depending on half-life. A short half-life is the 5th percentile and a long half-life is the 95th percentile of the normal range. This demonstrates that following a standard weight–based infusion, the time taken to reach 1 IU/dL varies by 59 hours depending on half-life. (Reproduced from Collins PW, Fischer K, Morfini M, et al. Implications of coagulation factor VIII and IX pharmacokinetics for clinical practice in the prophylactic treatment of haemophilia. *Haemophilia* 2011;17(1):2–10, with permission.)

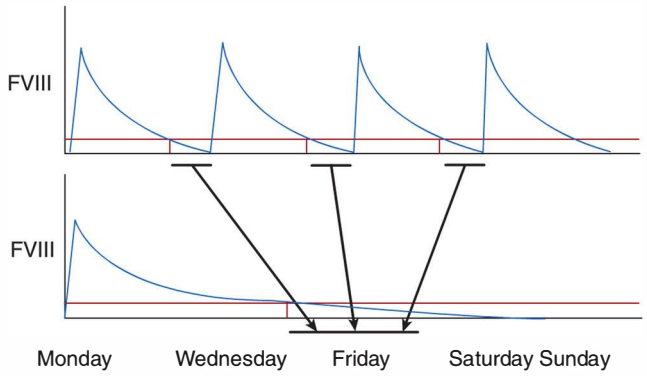

FIGURE 51.6 Potential implications of prolonged acting factor VIII (FVIII). Illustration of the length of time FVIII is below an arbitrary level assumed appropriate for an individual comparing standard FVIII infused on alternate days (*upper panel*) and a hypothetical prolonged acting FVIII infused once a week (*lower panel*). Patients on the prolonged acting FVIII spend more time per week below a specified level, and this is spread across both day and night as opposed to just during night as seen with alternate-day dosing. The FVIII scale is arbitrary. (Reproduced from Collins PW, Fischer K, Morfini M, et al. Implications of coagulation factor VIII and IX pharmacokinetics for clinical practice in the prophylactic treatment of haemophilia. *Haemophilia* 2011;17(1):2–10, with permission.)

Regimens for delivering prophylaxis will need to be revised if studies with longer acting FVIII and IX are successful. These products may allow less frequent infusions, but the effect of this strategy on bleeding needs to be closely studied because patients will spend longer with low levels than with conventional regimens (FIGURE 51.6).

There is debate about whether it is appropriate to stop prophylaxis when the patient has reached adulthood, but there are very limited data available. Observational studies have shown that, in the highly selected group of patients who decide to stop prophylaxis of their own accord, between 22% and 35% were able to continue with on-demand therapy without significant joint damage after 4 years follow-up.[36,37] The majority of patients, however, need to return to prophylaxis. In general, until new evidence is available, guidelines suggest that prophylaxis should be continued long term.[28]

Prophylaxis started in adulthood will reduce or even prevent bleeding, but the long-term effect on established musculoskeletal damage is unknown.[38,39] The cost of prophylaxis in adults is high; however, studies suggest that the use of pediatric regimens (e.g., 20 to 40 IU/kg) may not be appropriate for older patients because the coagulation factors have longer half-lives in adults. Hence, considerably lower infusion regimens will sustain a trough above 1 IU/dL and potentially allow much more cost-effective regimens to be used.[35]

TREATMENT OF INHIBITORS IN CONGENITAL HEMOPHILIA

With the introduction of concentrates that are safe from the risk of transfusion-transmitted infection, the development of an inhibitory antibody to infused FVIII or FIX is the most serious adverse event associated with replacement therapy.

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High-titer inhibitors are defined as >5 Bethesda units (BU)/mL and high-responding inhibitors are defined as an increase in titer to above 5 BU/mL after exposure to concentrate. Patients with a Bethesda titer above 5 BU/mL do not, in general, respond to factor concentrate and bypassing agents are usually required. (1 BU is the amount of inhibitor capable of neutralizing 0.5 units of FVIII after 2 hours incubation.)

Most inhibitors develop early during a patient's treatment history after a median of about 10 exposure days; emerging evidence suggests that the incidence of inhibitors increases again in later life.[7] Risk factors for the development of inhibitors include severe hemophilia, especially caused by null mutations and a positive family history of inhibitors. Peak treatment moments defined at 5 or more consecutive days of treatment are also associated with inhibitor development.[40] The relative risk of inhibitor formation with plasma-derived and recombinant FVIII is debated, but systematic reviews suggest that the incidence of inhibitor formation is similar with both types of concentrate.[41,42]

Inhibitor Eradication

Treatment to eradicate an inhibitor by immune tolerance induction (ITI) should be undertaken unless the inhibitor is low titer and transient.[43] Based on registry data, it is common practice to delay the start of immune tolerance until the inhibitor titer has declined to below 10 BU/mL because this is associated with a higher chance of eradication.[44] It is also common practice to use rFVIIa to treat bleeds whilst waiting for the inhibitor to decline to reduce the risk of an anamnestic response. Other features associated with successful immune tolerance are less well established but a high historic peak titer and a short time between inhibitor development and ITI have been suggested as being important. Complete remission after ITI is reported to be between 60% and 80%, dependent on the risk factors of the patients studied.

ITI for patients with hemophilia A, an inhibitor titer below 10 BU/mL and a historical peak titer <200 BU/mL, has been the subject of a prospective randomized trial.[7] The regimens compared were 200 IU/kg daily versus 50 IU/kg on alternate days, and results show that the two arms were similar in terms of the proportion of patients who achieve a complete remission, but the patients treated on the low-dose arm took longer to achieve remission and had significantly more bleeds.

The type of FVIII that should be used for ITI is controversial. Retrospective, uncontrolled data suggest that plasma-derived concentrate with a high von Willebrand factor (vWF) content may be more efficacious than recombinant FVIII.[45] However, as yet, this has not been confirmed in well-designed clinical trials, and many clinicians prefer to use recombinant FVIII for first-line ITI.

Patients who not achieve inhibitor eradication with first-line ITI are a difficult group to treat. Anecdotal reports and uncontrolled case series suggest that a proportion will respond to changing to a plasma-derived concentrate with a high vWF content, and many clinicians will attempt a second ITI with these products.[45] A prospective trial is underway to investigate this question.[46] There has been recent interest in the use of immune suppression with the anti-CD20 agent rituximab in this group. Although a proportion of patients appear to respond sufficiently well for some to be treated with FVIII rather than bypassing agents, a durable complete remission is uncommon.[47]

Hemostatic Treatment of Bleeds

The management of bleeds in patients with hemophilia A and inhibitors depends on the bleed site and severity and the characteristics of the inhibitor.[42] Low-titer inhibitors may be overwhelmed with high and frequent doses of FVIII and, if adequate FVIII levels are achieved, good hemostatic effects can be expected. Patients with low-titer, low-responding inhibitors can be treated with FVIII for most bleeds, but if the inhibitor is high responding then many clinicians reserve FVIII for major bleeds.

High-titer inhibitors (>5 BU/mL) do not usually respond to FVIII and a bypassing agent is the treatment of choice. At the time of writing, the two available bypassing agents are recombinant FVIIa (rFVIIa, Novo Nordisk) and the plasma-derived activated prothrombin complex concentrate FEIBA (Factor Eight Inhibitor Bypassing Activator, Baxter Inc.). These agents have been shown to have similar haemostatic efficacy when used to treat hemarthroses at home.[48,49] Both agents appear to have a low risk of thrombosis when used in patients with congenital hemophilia.

The standard dose of rFVIIa is 90 μg/kg every 2 hours initially, with decreased frequency as the bleed is controlled. Most hemarthoses can be controlled with 1 to 3 doses if treated early. The use of 270 μg/kg as a single dose has been shown to be as efficacious as 3 doses of 90 μg/kg when treating hemarthroses, and this is an option for some patients.[49,50] This higher dose of rFVIIa has not been studied in other types of bleeding, and caution is required in patients with risk factors for arterial thrombosis.[51] FEIBA is generally used at a dose of 25 to 100 U/kg 8 to 12 hourly to a maximum dose of 200 U/kg in 24 hours. The length of treatment with either agent depends on the site and severity of the bleed, and some situations require a period of prophylactic treatment after the initial bleed has been controlled to prevent recurrence of bleeding. Some centers have reported successful treatment with immunoadsorption and infusion of FVIII.[52] This treatment modality is not widely available but can be useful in unresponsive patients.

In the past, porcine FVIII has been used to treat bleeds associated with inhibitors because the cross-reactivity of antihuman FVIII antibodies with porcine FVIII is low. Good hemostatic response was reported in most patients, but this product is no longer available. Clinical trials with a recombinant B-domain delete porcine product are underway (Inspiration Biotech Inc. company sponsored trial), and safety and efficacy data are awaited.

Surgery

Surgery in hemophilia patients with high-titer inhibitors is a high-risk undertaking since neither bypassing agent has consistent and predictable hemostasis efficacy. All surgery should be performed in centers with experience in treating this patient group. Both FEIBA and rFVIIa have been used successfully for surgery, and both seem to offer effective hemostasis in 80% to 90% of patients.[53,54] The optimal regimen for rFVIIa and FEIBA in surgery remains to be established, and a number of treatment schedules have been published. rFVIIa has been successfully used as a continuous infusion.[55] Protocols using rFVIIa in the initial perioperative period with switch to FEIBA for extended postoperative rehabilitation have been reported.[56]

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Prophylaxis

Patients with severe hemophilia and inhibitors are at high risk of developing disabling arthropathy, and as a result interest has been increasing in the role of prophylaxis in this patient group. In most inhibitor patients, FVIII cannot be used for prophylaxis because the half-life of circulating FVIII is too short or an anamnestic response may be provoked.

There are a number of published case reports and cohort studies that suggest bypassing agents reduce the number of bleeds in patients with inhibitors, but there is a high chance of a positive reporting bias and the studies need to be interpreted cautiously. Regimens tend to be either rFVIIa at 90 μg/kg daily or FEIBA 50 to 100 IU/kg alternate day. Some reports describe daily FEIBA prophylaxis.[57–61]

A prospective study has investigated rFVIIa given at 90 μg/kg daily for 3 months in patients who were experiencing very high numbers of hemarthroses. Although the number of bleeds during prophylaxis fell by 45% to 59% compared with on demand, the study design lacked a control arm and so it is not clear whether the reduced number of bleeds was the effect of the rFVIIa or partly due to the natural variation in bleeding over time seen in many patients with hemophilia. Furthermore, despite the statistically significant reduction in bleeds, the actual number of bleeds (mean 2 to 3 a month) remained high[59] and the clinical benefit of this is unclear.

A prospective randomized crossover study of FEIBA prophylaxis given at 85 U/kg 3 days a week also enrolled patients with significant numbers of bleeds.[62] The bleed rate on prophylaxis fell, but again the actual number of bleeds (median 3 hemarthroses in 6 months) remained high. At the present time, there is more published experience with FEIBA and the longer half-life of this product may make it a more practical option for many patients. It is, however, associated with anamnesis and should be avoided in those where ITI is planned. Patients who are proceeding to ITI should be managed with rFVIIa. A common initial dose is 90 μg/kg once daily. If bleeds continue despite this treatment, dose escalation has been shown not to be effective[59] and increasing frequency to twice daily is a more logical option.

Inhibitors in Mild Hemophilia

Patients with mild hemophilia A have a lower prevalence of inhibitors than those severely affected. Inhibitors in patients with mild hemophilia B are extremely rare. Inhibitors developing in mild hemophilia have been associated with intensive treatment especially at the time of surgery, and close surveillance of patients at this time is required.[63]

Some inhibitors cross-react with the patient's native FVIII and reduce their baseline FVIII level, resulting in a more severe bleeding phenotype. In these patients, bypassing agents are often required to manage bleeds. In patients in whom the antiFVIII antibody does not cross-react and whose baseline FVIII level is stable, a clinically useful response may be achieved with an infusion of Desmopressin, leading to the release of endogenous FVIII.[64]

There are limited data on inhibitor eradication in patients with mild hemophilia.

Factor IX

Treatment of patients with FIX inhibitors is complicated because allergic reactions to infused FIX are reported, and in some cases these can be very severe.[65] Furthermore, infused FIX is associated with the development of nephrotic syndrome.[66] FIX can be used in patients with a low-titer FIX inhibitor who do not experience allergic reactions. In patients with high-titer inhibitors, rFVIIa or FEIBA can be used. In patients with a history of allergic reactions to FIX, rFVIIa is the treatment of choice because FEIBA contains FIX. In general, patients with inhibitors to FIX respond less well to ITI than those with FVIII inhibitors, and the risk of nephritic syndrome is high.

CLINICALLY SIGNIFICANT INFECTIOUS COMPLICATIONS OF HEMOPHILIA TREATMENT

The availability of clotting factor concentrates (CFCs) made from large plasma pools from the mid-1970s changed the lives of hemophilia patients and the health care professionals involved in their care. There was tremendous mood of optimism as the modern replacement therapies alleviated the many of the worst problems of hemophilia, and the life expectancy for a man with hemophilia in the developed world had increased from 40 years in the 1960s to 69 years by 1980.[67] But effective treatment is rarely free from adverse effects, as the emergence of infectious complications of blood products and development of FVIII/FIX inhibitory antibodies became the next tragedy and clinical challenge for the hemophilia community.

HIV Infection

Hemophilia individuals have a significant risk of acquiring an infection from plasma even if the agent has a low prevalence rate as CFCs are produced from large plasma pools. Prior to 1985, there was no blood screening programs and no viral inactivation methods applied to the production of CFCs, and the true impact of transfusion-transmitted viral infections was not appreciated until the magnitude and consequences of the HIV epidemic in hemophiliacs became evident in the mid-1980s.

The first reports of *Pneumocystis carinii* pneumonia among patients with hemophilia appeared in the Morbidity and Mortality Report in 1982,[68] and only in April 1984 the major breakthrough for the discovery of HIV came about with the isolation of a human T lymphotropic retrovirus from cultured T lymphocytes of two brothers with hemophilia B.

The first HIV seroconversion in the United Kingdom in a man with hereditary bleeding disorder was in 1979,[69] and since 1986 no HIV infection has occurred through infusion of plasma-derived CFCs. The percentage of patients infected with HIV differs widely from country to country, and approximately 80% of severe hemophilia A patients were infected with HIV in the United States,[70] whereas in the United Kingdom during 1969 to 1985, 4,865 hemophilia individuals of all severity received nonheat-treated blood products and approximately 25% were infected with HIV.[71]

Initial treatment for HIV with azidothymidine monotherapy for patients with AIDS and AIDS-related complex[72] became available in 1987, but management of HIV was revolutionized with the advent of combination called highly active antiretroviral therapy (HAART) in 1996. Many hemophilic men died before HAART, and in the United Kingdom the death rate rose steeply from 8/1,000 in 1985 to 1992 in seronegative individuals to 81/1,000 in 1991 to 1992 in seropositive individuals.[73] The increased survival

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conferred by HAART has led to an increased risk of noninfectious comorbidities such as cardiovascular disease, diabetes mellitus, bone fractures, and renal failure seen as part of the metabolic toxicities of antiretroviral medications.[74]

HCV Infection

Hemophilia individuals who received CFCs during the 1970s and early 1980s before heat treatment was applied became infected with HCV on the first exposure, and these small cohort studies have shown that over 90% of individuals treated with nonvirus inactivated large pool concentrates became infected with HCV.[76,75,77]

HCV infection is self-limited in about 15% of cases where HCV PCR is negative in the setting of anti-HCV antibody positivity. The remaining 85% develop chronic hepatitis with viremia and abnormal liver function. Majority of hemophilia patients are infected with HCV genotype 1 infection, and current standard therapy achieves 50% sustained virological response for genotype 1 and 80% for genotype 2 and 3. There is encouraging evidence from phase 2/3 trials, indicating that the addition of a protease inhibitor (e.g., telaprevir and boceprevir) to pegylated interferon-α/ribavirin substantially improves sustained virologic response rates in both treatment-naïve and treatment-experienced patients with HCV genotype 1.

Yee et al.[78] have shown HIV-HCV coinfected patients have a progression of 19% to liver-related death after 25 years and HCV monoinfection has a slow progression rate of 3% to a liver-related death. As hemophilia patients continue to grow older, clinicians will be challenged with end-stage liver disease, hepatocellular carcinoma, and the need for liver transplantation. Consequently, eradication of these hepatitis viruses with antiviral agents and chemoprevention methods may decrease the risk of end-stage liver disease and hepatocellular carcinoma.

HIV-HCV coinfection is not a contraindication for liver transplant, and recent data from a London liver transplant unit revealed the median age for liver transplant in 18 HCV infected hemophilia patients was 52 years and the median survival of patients with and without HIV coinfection was 26 and 118 months, respectively.[79]

HBV Infection

Most individuals treated before the introduction of donor screening for hepatitis B virus (HBV) in 1972 were exposed to HBV (MRC report BJH 1974), and about 2% of HBV-exposed hemophilia patients developed persistent HBs Ag-positive infection.[80] Routine HBV and HAV vaccines are given to all patients diagnosed with a bleeding disorder.

Parvovirus Infection

Parvovirus B19 is a ubiquitous DNA virus infecting up to 50% of people by the age of 15 years. This prevalence, combined with its small size, which makes filtration difficult, and its resistance to inactivation by available virucidal processes, has led to the transmission of Parvovirus B19 by plasma-derived factor replacement products used to treat hemophilia.[81] Parvovirus B19 has been found in recombinant (non–plasma-derived) FVIII products as well, most likely introduced by the albumin used as a stabilizer.[82] Continued surveillance and studies are warranted to reduce the risk of Parvovirus B19 being spread by blood products.

Variant Creutzfeldt-Jakob Disease

vCJD is caused by a prion protein (PrP) and manifests as the human counterpart of bovine spongiform encephalopathy (BSE). The BSE incidence peaked in 1993, and its rapid spread throughout United Kingdom resulted in clinical cases of vCJD attributable to dietary exposure. It is from a group of individuals harboring asymptomatic subclinical vCJD infection donating blood that the risk of secondary transmission arises for the individuals with bleeding disorders receiving blood and blood products. To date, there have been no cases of transmission of vCJD by blood products in the United States and no case of clinical vCJD has been identified in any hemophilia patients who have received implicated batches of UK plasma products. Recent detection of PrP in the spleen of a patient with hemophilia who had received implicated UK sourced FVIII concentrates but showing no evidence of a neurological disorder has raised anxiety in the hemophilia cohort. As there is no validated screening test for vCJD and the prevalence of subclinical vCJD infection in the general population remains unknown, continued surveillance of individuals "at risk" and widespread public health measures need to continue.

ORGANIZATION OF HEMOPHILIA CARE

By localizing hemophilia care at treatment centers, a sufficient critical mass of patients is gathered to attract and justify the specialized attention of a range of health care professionals. The first and minimal requirement for such a center is an interested physician with access to a laboratory capable of making accurate diagnosis with specific factor assays. Once a location for a hemophilia centre has been established and the patient register started, it is time to support the physician with specialist nursing and physiotherapy professionals and therapeutic factors. Other professionals whose services to the hemophiliac and his family are needed include orthopedic surgery, infectious disease, hepatology, psychology, and genetics. In the United Kingdom, such facilities are designated "comprehensive care centres" if they have registered a target number of severely affected patients (at least 40) and offer a full range of staff and services. A hub and spoke model of centre organization serves to facilitate access for patients living across a geographically dispersed region. Central collection of national statistics of hemophilia treatment enables planning at government level of service delivery and enables monitoring of progress in hemophilia care.

NEW THERAPEUTIC AGENTS IN DEVELOPMENT

The golden era of cloning the genes for the coagulation factors spanned the 1980s with FVIII cloned in 1984, FIX in 1983, and FVII in 1986. There is now a ferment of activity in the area of hemostatic drug development with biosimilar and modified versions of all three factors on trial. These products include versions of FVIII and FIX with prolonged half-life obtained by addition of polyethylene glycol to the protein, or fusion of the clotting factor with albumin or with Fcγ. The best prolongation of half-life has been reported for glycopegylated FIX with a half-life of over 90 hours. If such a product can be used safely for prophylaxis then regimes of dosing every 3 weeks become possible[83,84] (FIGURE 51.6).

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Another route to hemostasis in hemophilic blood would be to reduce the strength of natural coagulation inhibitors that shut down the extrinsic pathway, in particular tissue factor pathway inhibitor, since this pathway is normal in hemophilia A and B. Proof of concept for this approach has been obtained in *ex vivo* studies,[85] but human trials have not been started at the date of this writing.

GENE THERAPY FOR HEMOPHILIA

Hemophilia was seen as early as the 1980s to be a suitable target for gene therapy, because the missing protein only has to be produced in low amounts that do not need tight regulation to achieve a beneficial effect. Mice and dogs with both hemophilia A and B have been successfully cured of hemophilia using a variety of gene vectors.[86] Clinical studies of gene therapy in humans with hemophilia have been safe but not efficacious until this year when an Anglo-American group reported on a study in hemophilia B using an adeno-associated virus vector.[87] Of the first six subjects so treated, who each had severe hemophilia B, all have been converted to moderate or mild hemophilia B phenotype. Correction of bleeding tendency has been sustained for up to 18 months due to a rise in steady-state level of FIX in the range of 2% to 6%, achieved without serous toxic side effects.

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CHAPTER

52

von Willebrand Disease: Diagnosis, Classification, and Treatment

J. EVAN SADLER AND DAVID LILLICRAP

von Willebrand disease (vWD) was discovered by Erik von Willebrand,[1,2] a Finnish internist who investigated a family living in Föglö, one of the Åland Islands in the Gulf of Bothnia, separating Sweden and Finland. The proband was a 5-year-old girl with a severe bleeding tendency. Four of her affected sisters died of hemorrhage before the age of 4 years, and the proband herself died at the age of 13 years with her 4th menstrual period.[3] The parents and several other relatives of both sexes had mild bleeding symptoms, suggesting autosomal inheritance. The severity of bleeding was variable throughout this large pedigree, and some obligate heterozygous individuals were asymptomatic. The most common sites of bleeding were skin, uterus, and mucous membranes rather than deep tissues. Patients had a prolonged bleeding time, but had normal coagulation time, clot retraction, and platelet count. von Willebrand distinguished this condition from hemophilia and Glanzmann thrombasthenia but was unable to determine whether the defect lay in the blood, the vasculature, or the platelets.

Considerable progress has been made in understanding the pathophysiology of vWD. In the 1950s, patients with vWD were reported to have low levels of blood clotting factor VIII (FVIII), and transfusions with plasma fractions from healthy individuals or patients with hemophilia were shown to correct the bleeding tendency in vWD.[4–8] These observations suggested that vWD was caused by abnormalities in a plasma protein, now known as von Willebrand factor (vWF). In 1972, vWF was purified,[9] and genetic variants of vWD were identified and correlated with structural differences in vWF.[10] The development of multimer gel electrophoresis[11,12,13] uncovered additional heterogeneity in vWD. In 1985, the structure of vWF was determined by protein sequencing and cDNA cloning.[14–17] This was followed almost immediately by the characterization of mutations that cause severe vWD.[18,19] Today, hundreds of mutations in many subtypes of vWD have been described.[20,21] An online database of vWD mutations is maintained by the International Society on Thrombosis and Haemostasis at the University of Sheffield and is accessible at http://www.vwf.group.shel.ac.uk/index.html.

We now understand many aspects of vWF biosynthesis, structure, and function, as discussed in Chapter 13. vWF is a multimeric blood protein that performs two major roles in hemostasis; it mediates the adhesion of platelets to sites of vascular injury and it is a carrier protein for FVIII. These activities require the assembly of vWF into large multimers and interactions with several ligands. Inherited defects in vWF may interfere with biosynthetic processing, or disrupt specific ligand-binding sites, thereby causing bleeding by impairing either platelet adhesion or blood clotting. Depending on the disease mechanism, vWF mutations may cause autosomal dominant or recessive vWD. This knowledge provides a framework to understand the pathogenesis and classify the many variants of vWD.

DIAGNOSIS AND CLASSIFICATION OF VON WILLEBRAND DISEASE

Three major categories of vWD are distinguished: partial quantitative deficiency (type 1), qualitative deficiency (type 2), and total deficiency (type 3) (see Table 52.1).[22,23] Qualitative type 2 vWD is divided further into four variants (2A, 2B, 2M, and 2N) on the basis of details of the clinical phenotype. These six categories correspond to distinct pathophysiologic mechanisms and they correlate with distinct clinical features and therapeutic requirements, as discussed in a subsequent section. The laboratory features of vWD subtypes are summarized in Table 52.2.

von Willebrand Disease Type 1

vWD type 1 includes partial quantitative deficiency of vWF and is commonly inherited as an autosomal dominant trait. vWD type 1 accounts for more than 70% of all vWD, although the prevalence of vWD type 1 is not known precisely. Symptomatic vWD that leads to medical treatment affects approximately 35 to 100 per million individuals, which is comparable to the prevalence of hemophilia A.[24] Screening programs often identify many more individuals with low vWF and mild symptoms and suggest that vWD could have a prevalence of approximately 1%,[25] but very few patients identified by population screening subsequently have significant bleeding.[26] Finally, screening patients attending the offices of primary care physicians has indicated a prevalence of symptomatic vWD of approximately 1 in 1,000.[45] vWD type 1 includes variants previously designated as type IA[27]; type I-1, I-2, and I-3[28]; type I-platelet normal and type I-platelet low[29]; and vWD Vicenza. vWD Vicenza was formerly classified as vWD type 2M, but the consensus now favors classifying it as vWD type 1. Most studies indicate that vWF Vicenza has a normal vWF function/antigen ratio (vWF:RCo/vWF:Ag),[30,31] but some suggest the protein is dysfunctional.[32]

Many factors make vWD type 1 difficult to diagnose with confidence. Mild bleeding symptoms are sufficiently common that coincidental association with low vWF levels also is frequent, even when patients have a positive family history of bleeding.[33] Furthermore, bleeding symptoms are not specific for vWD, and apparent transmitters of low vWF or a bleeding phenotype may themselves be phenotypically normal. Therefore, the criteria used for diagnosis are important determinants of the apparent prevalence of vWD type 1.

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Table 52.1

Classification of vWD

Type	Description
1	Partial quantitative deficiency of vWF
2	Qualitative vWF defects
2A	Decreased vWF-dependent platelet adhesion and a selective deficiency of high molecular weight vWF multimers
2B	Increased affinity for platelet GPIb
2M	Decreased vWF-dependent platelet adhesion without a selective deficiency of high molecular weight vWF multimers
2N	Markedly decreased binding affinity for FVIII
3	Virtually complete deficiency of vWF

vWF, von Willebrand factor.
From Sadler JE, Budde U, Eikenboom JC, et al. Update on the pathophysiology and classification of von Willebrand disease: a report of the Subcommittee on von Willebrand factor. *J Thromb Haemost* 2006;4(10):2103–2114.

Bleeding Symptoms

Few large studies of symptoms in vWD have been published, and they rarely distinguish among subtypes of vWD. For example, the extensive compilations of Silwer[34] and of Buchanan and Leavell[35] include patients with all variants of vWD. Because other variants are relatively uncommon, these surveys remain useful guides to the clinical features of vWD type 1. The most common symptoms (see Table 52.3) are epistaxis, skin bruises, hematomas, prolonged bleeding from trivial wounds, oral cavity bleeding, and excessive menstrual bleeding. Gastrointestinal bleeding appears to be relatively rare, but may be serious when it occurs.

Epistaxis may be severe and prolonged. Easy bruising is common in vWD. Prolonged bleeding after minor trauma to skin or mucous membranes is characteristic of vWD. Severe hemorrhage after major surgery may continue for several weeks. Prolonged oral bleeding is common from gums, or with biting of the tongue or lips. Heavy bleeding is also common after tooth extraction or other oral surgery such as tonsillectomy and adenoidectomy. Menorrhagia is a typical presenting symptom in women with vWD, but is also frequent in otherwise healthy women.[34]

Coexistent conditions or drug interactions can strongly influence the severity of symptoms in vWD. Bleeding may be exacerbated by the use of aspirin or corticosteroids and decreased by the use of oral contraceptives. Treatment with sodium valproate for epilepsy can lower the level of vWF and induce bleeding.[36] The risk of hemorrhage may be increased by liver disease, uremia, defects in connective tissue such as Ehlers-Danlos syndrome,[37] hypothyroidism,[38] and focal lesions such as gastric or duodenal ulcers, or gastrointestinal angiodysplasia.[39]

Standardized questionnaires have been developed to describe and quantitate bleeding symptoms.[40,41] These assessment tools are being refined and validated for prospective use with pediatric and adult patients to diagnose vWD.[42,43–45] These tools may help direct the intensity of laboratory investigation and also facilitate communication of the bleeding severity between health care professionals.

Laboratory Testing

The goal of laboratory testing in vWD type 1 is to exclude other causes of bleeding, document vWF deficiency, and exclude qualitative vWF defects. As a complete laboratory evaluation for vWD is quite elaborate, the efficiency of testing can be increased by using initial screening tests for vWD. vWF levels vary with physiologic

Table 52.2

Selected laboratory features of vWD subtypes

vWD	FVIII	vWF:Ag	vWF:RCo or vWF:CB	RIPA	Multimer Structure
Type 1	Decreased	Decreased	Decreased	Decreased	Normal in plasma and platelets
Type 2A	Decreased or normal	Decreased or normal	Decreased relative to vWF:Ag	Decreased relative to vWF:Ag	Large and intermediate multimers absent from plasma; variable in platelets
Type 2B	Decreased or normal	Decreased or normal	Decreased or normal	Increased at low ristocetin concentration	Large and intermediate multimers absent from plasma; normal in platelets
Type 2M	Decreased or normal	Decreased or normal	Decreased relative to vWF:Ag	Decreased relative to vWF:Ag	Large and intermediate multimers present in plasma and platelets
Type 2N	Moderately decreased (<20 IU/dL)	Normal	Normal	Normal	Normal in plasma and platelets
Type 3	Moderately decreased (<20 IU/dL)	Absent or trace	Absent	Absent	None or trace in plasma or platelets

vWD, von Willebrand disease; vWF, von Willebrand factor; RIPA, ristocetin-induced platelet aggregation.

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Table 52.3

Frequency (%) of bleeding symptoms in vWD

		Buchanan and Leavell[a]		
Symptoms	Swedish Series[b] (264 Cases)	Men (95 Cases)	Women (104 Cases)	Normal[b] (500 Cases)
Nose bleeding	62.5	73	64.5	4.6
Menorrhagia	60[c]	—	51	25.3[c]
Bleeding after tooth extraction	51.5	28.5	38	4.8
Ecchymoses and hematomas	49.2	—	—	11.8
Ecchymoses	—	55.7	67	—
Hematomas	—	8.5	7.5	—
Bleeding from minor cuts or abrasions	36	37	33.5	0.2
Gingival bleeding	34.8	33	36	7.4
Postoperative bleeding	28	19	20	1.4
Bleeding at delivery	23.3[c]	—	10.5	19.5[c]
Gastrointestinal bleeding	14	24.5	9	0.6
Traumatic oral and lip bleeding	11.7	—	—	0.6
Petechiae	11.5	16	18.5	1.2
Joint bleeding	8.3	12.5	5	0
Hematuria	6.8	7.5	2	0.6
Ovarian bleeding	6.8[c]	—	—	—
Bleeding after tonsillectomy	6.1	—	—	—
Bleeding during shedding of teeth	4.9	—	—	—
Bleeding at abortion	3.8[c]	—	—	—
Intramuscular, deep subcutaneous, or submucosal bleeding	2.7	—	—	—

[a]Data of Buchanan JC, Leavell BS. Pseudohemophilia: report of 13 new cases and statistical review of previously reported cases. *Ann Intern Med* 1956;44:241–256.
[b]Data of Silwer J. von Willebrand's disease in Sweden. *Acta Paediatr Scand Suppl* 1973;238:1–159.
[c]For women older than 15 years.

stress, and where possible, tests should not be performed in proximity to hemorrhagic events, pregnancy, acute infection, or strenuous exercise. Optimal testing strategies have not yet been validated experimentally, and the approach described here is an attempt to synthesize various expert opinions.

Initial tests for any hemostatic disorder include a complete blood count with platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT). Additional tests specific for vWD include ristocetin cofactor activity (vWF:RCo), collagen-binding activity (vWF:CB), vWF antigen (vWF:Ag), and FVIII level.

vWF:RCo is a useful initial assay for vWF concentration and platelet-binding function,[25] but the test is difficult to standardize and is not very precise. Therefore, vWF:Ag may be used to confirm low values for vWF:RCo. Like the vWF:RCo test, vWF:CB also depends on vWF concentration but measures the interaction of vWF with immobilized collagen. vWF:CB tests are not as widely available as vWF:RCo, but are useful for a comprehensive evaluation of vWF functional defects.[46,47]

Discrepancies between vWF:RCo and vWF:Ag, or between vWF:CB and vWF:Ag, suggest a qualitative vWF defect that should be investigated along with other tests, as discussed in the section, *von Willebrand Disease Type 2*. However, some common vWF sequence variations affect the vWF:RCo assay. For example, African Americans have relatively decreased vWF:RCo, and this variation is explained by differences in the frequency of the vWF Asp1472His polymorphism. vWF 1472His has normal hemostatic function but decreased vWF:RCo compared to vWF 1472Asp. vWF 1472His occurs in 19% of whites and 62% of African Americans, which accounts for the observed differences in vWF:RCo/vWF:Ag between these populations and can lead to the overdiagnosis of vWD type 2M.[48] The vWF mutation Pro1467Ser also profoundly decreases vWF:RCo/vWF:Ag (<0.1) with no impairment of hemostasis.[49] Conversely, the polymorphism Ala1381Thr modestly increases vWF:RCo.[50] Mutations associated with vWD type 2B Malmö/New York may also belong to this category.

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vWF levels may be normal intermittently in vWD, and abnormal results should be confirmed.[51] For example, the PT, aPTT, FVIII, and vWF tests may be repeated after an interval of 2 weeks. If the results are inconsistent, they may be performed again. Unfortunately, such repeated testing is impractical or not feasible for many patients. With the limitations discussed in the subsequent text, vWD is excluded by arriving at repeated normal values of FVIII and concordant vWF:RCo, vWF:CB, and vWF:Ag on serial testing. If these initial tests are abnormal, other tests are indicated.

An abnormal aPTT is evaluated in concert with the FVIII level. An FVIII that is disproportionately lower than the vWF:Ag would suggest either hemophilia A, a defect that impairs vWF ability to bind FVIII (vWF:FVIIIB) as occurs in vWD type 2N, or another cause of FVIII deficiency such as an antibody against FVIII. If the FVIII is normal, then other causes of a prolonged aPTT should be considered.

Low values for vWF by any test can be hard to evaluate because the range of normal values for plasma vWF concentration is very broad. vWF levels also depend on ABO blood type (see Table 52.4), and the mean vWF:Ag level is approximately 25% lower for individuals of blood type O compared to other blood types.[52] Some additional variation has been attributed to the secretor locus that specifies some oligosaccharide antigens of the Lewis blood group system.[53,54] However, most of the variation in vWF levels is due to unknown factors other than either vWD or blood type. The problem is illustrated in FIGURE 52.1, using data for blood type O. For this population of normal type O blood donors, the mean level of vWF:Ag was 86 IU/dL, with a range (±2 SD) of 41 to 179 IU/dL.[52] Therefore, blood type O alone can cause vWF levels low enough to suggest the diagnosis of vWD. By halving the values for this normal population, the distribution for individuals of blood type O and only one functional vWF allele can be estimated to have a mean vWF:Ag of 43 IU/dL (see FIGURE 52.1), but there is considerable overlap with the normal distribution. Because individuals heterozygous for vWD mutations constitute much <1% of the population,[24]

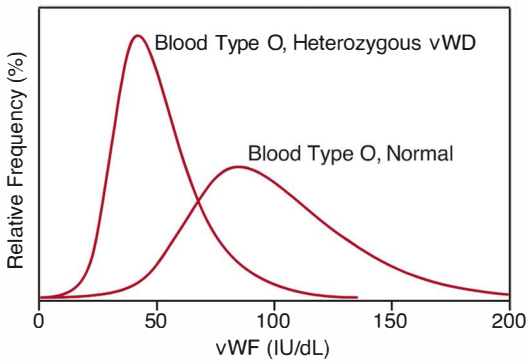

FIGURE 52.1 Distribution of vWF:Ag levels in normal blood donors of blood type O and hypothetical distribution of vWF:Ag levels in individuals of blood type O who are heterozygous for vWF null alleles. The reference normal value of 100 IU/dL is based on the World Health Organization standard pooled plasma. (Adapted from tabulated data of Gill JC, Endres-Brooks J, Bauer PJ, et al. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood* 1987;69:1691–1695.)

Table 52.4

Influence of ABO blood groups on vWF:Ag in volunteer blood donors

		vWF:Ag (Percentage of Pooled Normal Plasma Standard)	
ABO Type	n	Geometric Mean IU/dL	Geometric Mean ± 2 SD IU/dL
O	456	86	41–179
A	340	121	55–267
B	196	134	65–275
AB	109	141	73–271

The groups were statistically different from each other as follows: O versus A, B, and AB, $P < 0.01$; A versus AB, $P < 0.01$; B versus A, $P < 0.05$.
vWF, von Willebrand factor.
Modified from Gill JC, Endres-Brooks J, Bauer PJ, et al. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood* 1987;69:1691–1695. The modified table includes vWF:Ag values converted to WHO international units per deciliter (IU/dL).[23]

moderately low vWF levels cannot reliably identify patients with vWD type 1. Whether blood type–adjusted means should be used routinely remains controversial, but it seems increasingly likely that the ABO blood type is one of perhaps many genetic modifiers that influence vWF plasma levels. In these initial tests, comparison of functional assays with the level of vWF:Ag must not suggest a qualitative abnormality of vWF, as discussed in the section on vWD type 2. If available, ristocetin-induced platelet aggregation (RIPA) should not indicate abnormal sensitivity to ristocetin, and the plasma vWF multimer distribution should have a normal complement of large multimers.

The template bleeding time has been used to diagnose vWD, but its value for this purpose is difficult to demonstrate. A prolonged bleeding time is not specific for vWD, and does not predict bleeding at surgery.[55–57] The bleeding time is subject to even greater normal variation than vWF levels, has poor reproducibility, and often is normal in vWD.[51] The test can distinguish populations that are defined independently, but generally it is not useful when applied to individuals. The bleeding time may be helpful for assessing the efficacy of therapeutic interventions in selected patients with vWD, but it does not appear to have sufficient sensitivity or reproducibility to contribute strongly to the diagnosis or exclusion of vWD. With all of these considerations, performance of the bleeding time has now been discontinued in many institutions.

A platelet function analyzer (PFA)-100 has been developed to test platelet plug formation *in vitro* by forcing a sample of citrated whole blood at high shear through a porous membrane coated with collagen plus adenosine 5′-diphosphate or collagen plus epinephrine. Normal platelets become activated upon contact with the membrane and form occlusive aggregates. The time required for blood flow to stop is termed the closure time, which is prolonged by defects in vWF or platelet function. The PFA-100 has been proposed as a sensitive and reproducible alternative to the bleeding time, but lack of specificity remains a concern and the clinical utility of PFA-100 testing remains uncertain.[58–60] As previously reported for the bleeding

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time,[55–57] a prolonged PFA-100 closure time was not found to predict increased surgical bleeding.[61]

Other vWF tests may be performed in specialized diagnostic or research laboratories. Approximately 15% of vWF in the blood is located within the α-granules of platelets and is secreted upon platelet activation.[62] Platelet vWF can be assayed by methods similar to those used for plasma vWF, and the results can help identify some variants of vWD type 1.[29] Botrocetin is a snake venom protein that binds vWF.[63,64] Like ristocetin, botrocetin induces high-affinity binding to platelet glycoprotein (GP) Ibα. Tests of botrocetin-induced platelet aggregation or "botrocetin cofactor activity" have been developed that are analogous to vWF assays employing ristocetin, although botrocetin and ristocetin affect vWF function by distinct mechanisms.[65] A variant of the vWF:RCo assay has been described that replaces platelets with recombinant, gain-of-function GPIbα immobilized on microtiter plates. This enzyme-linked immunosorbent assay style test is very precise and sensitive compared to conventional vWF:RCo assays[66] but is not yet available commercially.

Criteria for Diagnosis of von Willebrand Disease Type 1

No matter what criteria are used, the extreme range of normal vWF levels complicates the diagnosis of vWD type 1, and a satisfactory general answer to this dilemma has not yet been devised. Because of this diagnostic uncertainty, the risk and benefit of specifically diagnosing vWD type 1 should be balanced for each patient.

The diagnosis of vWD type 1 is easy to make when the patient has significant mucocutaneous bleeding, the vWF level is clearly low (e.g., <30 IU/dL), and the family history is strongly positive with high penetrance. Such families usually show clear coinheritance of low vWF with bleeding symptoms, and usually, mutations in the vWF gene can be found.[29,67–69,70–74] For these patients, a diagnosis of vWD type 1 seems appropriate because they often can benefit from changes in lifestyle, or from specific treatment to prevent or control bleeding. Also, genetic counseling is straightforward when the inheritance of the disorder can be predicted with confidence. Unfortunately, this combination of features is relatively rare.

More commonly, patients have a medical history of only menorrhagia or other infrequent mild bleeding, their vWF levels are borderline low (e.g., 30 to 50 IU/dL), and their family members do not consistently have either low vWF or bleeding symptoms. In such cases, low vWF level and bleeding are likely to associate coincidentally rather than causally.[33] Family studies usually show that bleeding symptoms and low vWF levels are not coinherited, and linkage to the *vWF* gene cannot be demonstrated.[26,41,70–74,75,76] vWF levels that are slightly more than 2 SD below the population mean confer a relatively small relative risk of menorrhagia or other bleeding[33,77,78] and do not correlate with increased bleeding at surgery.[79] To label these patients as vWD type 1 has little clinical utility. It may, in fact, harm them by affecting their relationship with employers and insurance providers, by causing unnecessary changes in lifestyle, and by inducing unjustified fear of transmitting a genetic disease. A modestly low vWF level may be treated instead, as a biomarker for modestly increased bleeding risk. Empiric therapy to raise vWF may be considered for these patients with low vWF if they should bleed.

Molecular Defects in von Willebrand Disease Type 1

Recent studies have indicated that vWD type 1 is associated with candidate mutations at the *vWF* locus in approximately 65% of cases.[71,73,74] There is significant allelic heterogeneity, with >100 different mutations being documented to date. Missense mutations predominate, with the Tyr1584Cys substitution being the most frequent variant reported, showing a prevalence of 5% to 15% in different populations.[71,73,74] This mutation is associated with a mild phenotype (vWF:Ag ~40 IU/dL in heterozygotes), usually accompanies blood type O, and has a penetrance of approximately 70%.[80]

vWD type 1 can occasionally be caused by frameshifts, nonsense mutations, or deletions that are similar or identical to mutations found in patients with vWD type 3.[81–83] As first shown by Erik von Willebrand, some heterozygous members of such families may not have bleeding symptoms, so that possession of a single such mutant allele does not consistently cause bleeding symptoms. Variation in symptoms may be due to chance coinheritance of a second mutated vWF allele. For example, in two families in the Netherlands, compound heterozygosity for vWD type 1 and vWD type 2N was found in patients with substantial bleeding, whereas relatives with either single mutant allele were asymptomatic.[84]

vWD type 1 sometimes is inherited as an autosomal dominant trait with very high penetrance, and this strongly dominant phenotype is usually associated with missense mutations.[70–74] Many of these missense mutations cause intracellular retention of mutant vWF subunits.[85] In one family, a Cys1149Arg missense mutation was identified that caused the retention of mutant subunits in the endoplasmic reticulum and also inhibited the secretion of normal vWF subunits, possibly by causing the intracellular retention of heterodimers between mutant and normal subunits.[86,87] Another missense mutation at a cysteine residue, Cys1130Arg, was found in two other families with a similar dominant phenotype, although the effect on vWF biosynthesis was not reported.[86] Two independent mutations that cause the heterozygous deletion of vWF exons 4 to 5 were shown to dominantly inhibit the secretion of normal vWF.[88] This dominant mechanism provides a plausible biochemical explanation for the transmission of exceptionally low vWF levels and bleeding symptoms with high penetrance.

Quantitative vWF deficiency also can be caused by mutations that lead to the increased clearance of vWF from the blood, provided that the clearance mechanism does not exhibit a strong preference for large multimers.[89,90,91] For example, the mutation Arg1205His causes vWD Vicenza, which is characterized by vWF:Ag < 15 IU/dL and plasma vWF multimers that are sometimes even larger than those found in normal plasma. vWF Vicenza appears to be cleared from the blood approximately fivefold more rapidly than normal vWF.[31] It has been proposed that these accelerated clearance variants, which comprise approximately 15% of type 1 vWD cases, be subclassified as vWD type 1c.[89]

Other mutations resulting in vWD type 1 may be located in *vWF* introns or in distant regulatory elements. Furthermore, the results of a large genome-wide association study meta-analysis indicate that several genes involved in storage organelle formation and protein clearance functions are strongly associated with plasma vWF levels.[92] Variation at these novel loci may act as primary causative mutations or genetic modifiers in vWD type 1.

von Willebrand Disease Type 2

For vWF to perform its hemostatic functions, it must be assembled into large multimers, and functional binding sites must be constructed for FVIII, for platelet receptors, and for ligands in

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connective tissue. Mutations in vWD type 2 can disrupt any of these properties, and vWD type 2 accounts for between 7% and 30% of all vWD in various reports. Such qualitative abnormalities of vWF often are associated with discrepancies between the level of vWF:Ag and functional assays such as vWF:RCo, vWF:CB, or vWF:FVIIIB. Four subtypes are currently recognized (Tables 52.1 and 52.2). The prevalence of the subtypes appears to be increasing as awareness of them increases, although the distribution of subtypes differs considerably among treatment centers. A survey in France found that patients with vWD type 2 were divided as 30% type 2A, 28% type 2B, 8% type 2M (or unclassified), and 34% type 2N.[93] A similar study in Germany found 74% type 2A, 10% type 2B, 13% type 2M, and 3.5% type 2N.[94]

Discrimination between qualitatively normal vWF and abnormal vWF can be difficult by laboratory testing, especially because vWF:RCo assays become somewhat unreliable when the vWF:Ag level is <20 IU/dL. A common strategy is to compare values obtained in functional assays (vWF:RCo, vWF:CB, vWF:FVIIIB) with vWF:Ag; a ratio <0.6[95] or <0.7[96,97] is inferred to be abnormal, and a ratio larger than the selected value is interpreted as normal. This approach seems reasonable, although the sensitivity and specificity of a particular ratio would depend on the performance characteristics of the individual assays. The discriminant power of vWF multimer analysis is also a significant determinant of vWD type 2 diagnosis.

von Willebrand Disease Type 2A

A disproportionately low vWF:RCo or vWF:CB, relative to vWF:Ag, may reflect decreased affinity of vWF for platelets or collagen, respectively. The most common cause of this defect is the absence of large vWF multimers (see FIGURE 52.2), which is characteristic of vWD type 2A. This subtype can be diagnosed by the combination of markedly reduced vWF:RCo or vWF:CB and compatible multimer gel analysis (FIGURE 52.2). vWD type 2A appears to account for most of vWD type 2.[98] vWD type 2A includes variants previously designated as type IIA[12]; type IIA-1, IIA-2, and IIA-3[28]; type I-platelet discordant[29]; type IB[27]; type IIC[99]; type IID[100]; type IIE[101]; type IIF[102]; type IIG[103]; type IIH[104]; and type II-I.[105]

vWD type 2A usually is a dominant disorder and is caused in approximately 80% of cases by single amino acid substitutions in or near domain A2 (residues 1,480 to 1,672) of the mature vWF subunit[20] (see FIGURE 52.3). These mutations lead to the absence of large vWF multimers either by impairing intracellular multimer assembly or by promoting intravascular proteolysis of vWF.[106] Some mutations may cause vWD type 2A by a combination of these mechanisms. Defects in multimer assembly produce an absence of large multimers in both plasma and platelet vWF. In contrast, increased susceptibility to proteolysis selectively depletes the large multimers of plasma vWF because

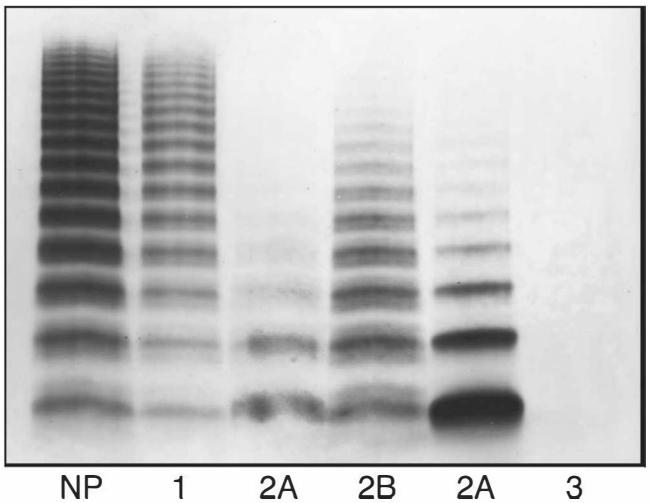

FIGURE 52.2 Multimer patterns in vWD. Samples of plasma from a normal individual (*N*) and from patients with vWD type 1, type 2A (two unrelated patients), type 2B, and type 3 were electrophoresed through a 1.3% agarose/sodium dodecyl sulfate (SDS) gel and von Willebrand factor (vWF) was detected by Western blotting. (Reprinted from Sadler JE. von Willebrand disease. In: Bloom AL, Forbes CD, Thomas DP, et al., eds. *Haemostasis and thrombosis*, 3rd ed. Edinburgh, UK: Churchill-Livingstone, 1994:843–857, with permission.)

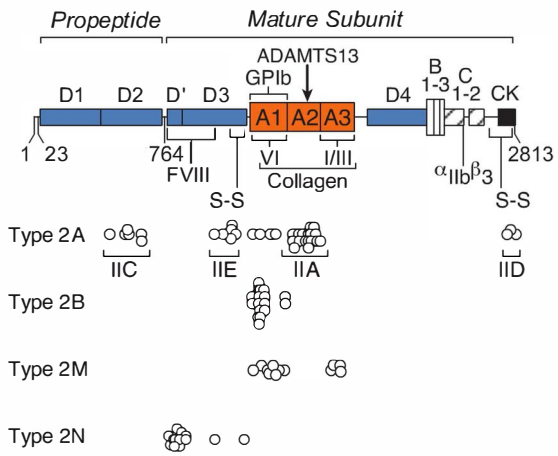

FIGURE 52.3 Mutations in vWD type 2. Amino acid residues are numbered by codon number 1 to 2,813 for prepro-vWF. The signal peptide contains residues 1 to 22, the propeptide contains residues 23 to 763, and the mature subunit contains residues 764 to 2,813. The locations of conserved structural domains (A, B, C, D, and CK) are indicated. Intersubunit disulfide bonds (not shown) connect CK to CK domains and D3 to D3 domains. The locations of binding sites are indicated for FVIII, platelet glycoprotein (GP)Ib, collagen, and platelet $\alpha_{IIb}\beta_3$. The metalloprotease ADAMTS13 cleaves a Tyr–Met bond in domain A2 (*arrow*). *Circles* mark the positions of mutations that cause the vWD phenotypes noted at the left. For vWD type 2A, the *labeled brackets* show the location of mutations that correspond to variants with characteristic multimer patterns discussed in the text. These include variants with increased sensitivity to proteolysis (*IIA*) or defective multimer assembly due to mutations in the propeptide (*IIC*), the D3 domain (*IIE*), or the CK domain (*IID*). References to the individual mutations can be found in the database of vWD mutations[20] maintained by the International Society on Thrombosis and Haemostasis at the University of Sheffield and accessible at http://www.shef.ac.uk/vwfvwf.group.shel.ac.uk/index.html. (Adapted from White GC II, Sadler JE. Von Willebrand disease: clinical aspects and therapy. In: Hoffman R, Benz EJ, Shattil SJ, eds. *Hematology: basic principles and practice*, 4th ed. Philadelphia, PA: Elsevier Churchill Livingstone, 2005:2121–2136, with permission.)

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platelet vWF is relatively protected from proteolysis. The plasma metalloprotease, a disintegrin and metalloprotease with thrombospondin type 1 motifs member 13 (ADAMTS13), cleaves the Tyr1605–Met1606 bond within domain A2 of vWF,[107,108] and this cleavage accounts for the major proteolytic fragments of the vWF subunit that are found in plasma vWF.[109] Mutations that increase the susceptibility of vWF to ADAMTS13 cause the loss of large, hemostatically effective multimers from plasma and result in bleeding. Conversely, deficiency of ADAMTS13 causes thrombotic thrombocytopenic purpura associated with unusually large vWF multimers, as discussed in Chapter 99.

A similar dominant phenotype is caused by mutations within the cystine knot motif at the carboxyl terminus of the vWF subunit. These mutations disrupt the initial dimerization of pro-vWF subunits in the endoplasmic reticulum. The mutant subunits remain capable of forming disulfide bonds in the Golgi apparatus, and the vWF found in the blood consists of small multimers comprised mainly of an even number of subunits, with prominent abnormal intermediate bands composed of an odd number of subunits.[110–113] vWD type 2A caused by mutations in the cystine knot domain corresponds to the variant originally named "type IID" (FIGURE 52.3).[100,114]

vWD type 2A also may result from defective multimerization within the Golgi. Mutations in the D3 region can cause a dominant phenotype characterized by small multimers that are indistinct or "smeary," suggesting heterogeneity of the disulfide bond arrangements that probably is caused by disruption of normal intersubunit disulfide bond formation.[94,111,113] These mutations correspond to the variant, originally named "type IIE" (FIGURE 52.3).[101] A recessive form of vWD type 2A is caused by mutations within the vWF propeptide that impair the assembly of disulfide bond–linked multimers (FIGURE 52.3).[113,115–118] The small plasma vWF multimers in these patients are characterized by an exceptionally clean appearance, lacking the usual intermediate or "satellite" bands that are associated with proteolysis *in vivo*; these mutations correspond to the variant originally named "type IIC."[99,101]

These studies indicate that vWD type 2A can be caused by several distinct defects in vWF biosynthesis or proteolytic susceptibility, and some can be distinguished by multimer gel electrophoresis. These various defects are united, however, by a common mechanism of impaired hemostasis resulting from the absence of large vWF multimers, and currently, they cannot be distinguished clinically by differences in severity of bleeding or response to therapy.

von Willebrand Disease Type 2B

The mutant vWF in vWD type 2B has increased affinity for binding to GPIbα on platelets.[12] This defect causes spontaneous binding of large vWF multimers to platelets *in vivo*, followed by removal of the large vWF multimers and a variable decrease in the platelet count. The remaining small multimers are not hemostatically effective, and patients with vWD type 2B have a bleeding diathesis that may seem disproportionately severe for their vWF level. Thrombocytopenia in vWD type 2B can be intermittent and is frequently exacerbated by stress factors, such as infection or pregnancy.[119–124] vWD type 2B has been misdiagnosed and treated as autoimmune thrombocytopenia.[120–122,124–127] vWD type 2B includes variants previously designated as vWD type IIB,[12] type I New York,[128] and Malmö.[129]

The results of initial laboratory tests may be difficult to interpret in vWD type 2B. vWF levels occasionally are in the normal range, but vWF:Ag usually is decreased.[12] vWF:RCo is usually decreased more than vWF:Ag, suggesting a diagnosis of vWD type 2A. Less commonly, vWF:Ag and vWF:RCo are decreased proportionately, which suggests a diagnosis of vWD type 1. In most cases of vWD type 2B, the plasma vWF lacks the largest multimers, and multimer gels do not reliably distinguish vWD type 2B from vWD type 2A (FIGURE 52.2).

The diagnosis of vWD type 2B depends on the results of RIPA. In platelet-rich plasma (PRP) from normal controls, RIPA requires vWF and exhibits a characteristic dose response to ristocetin (see FIGURE 52.4). In vWD type 2B, brisk platelet aggregation occurs at low concentrations of ristocetin that has little or no effect on PRP from normal controls.[12] Positive results in this test are found in one other rare disease, platelet-type or pseudo-vWD, in which mutations in platelet GPIbα cause a gain-of-function phenotype very similar to vWD type 2B. Increased RIPA at low ristocetin concentrations is sensitive and fairly specific for vWD type 2B. The RIPA test is not sensitive to moderate decreases in vWF concentration[130] and therefore is not an effective screening assay for other subtypes of vWD.

In the absence of RIPA data, the only sign pointing to vWD type 2B may be thrombocytopenia. For patients misdiagnosed as vWD type 1, the correct diagnosis of vWD type 2B may be discovered accidentally by finding that thrombocytopenia develops or is markedly worsened by desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP).[119] For this reason, the platelet count should be followed in all patients who receive their first diagnostic or therapeutic trial of desmopressin.

The binding site on vWF for platelet GPIbα is within vWF domain A1, between amino acid residues 1,226 and 1,479. At least 25 mutations are known to cause vWD type 2B, and all of them are within domain A1 (FIGURE 52.3). The crystal structure of a complex between vWF domain A1 and a fragment of GPIbα suggests that large conformational changes occur in both proteins when they bind.[131] The locations of vWD type 2B mutations indicate that they are likely to stabilize the bound conformation of domain A1, which could allow binding to platelet GPIbα in the absence of vascular injury and thereby cause the observed dominant, gain-of-function phenotype.[131]

vWD type 2B Malmö/New York merits special consideration. This variant of vWD type 2B is characterized by abnormal RIPA—consistent with a diagnosis of vWD type 2B—but a normal vWF multimer pattern and normal platelet count, even after administration of DDAVP.[128,129] Patients with this phenotype rarely have significant bleeding,[132] suggesting that vWD type 2B Malmö/New York is either an extremely mild form of vWD type 2B or a normal vWF variant in which specific sequence changes cause increased RIPA *in vitro* without affecting hemostasis *in vivo*. The mutations associated with vWD type 2B Malmö/New York are Pro1266Leu, Pro1266Gln, and Arg1308Leu. These mutations also occur in the vWF pseudogene that is located on chromosome 22, and their occurrence in the vWF gene on chromosome 12 appears to be a consequence of recombination between these loci.[133]

von Willebrand Disease Type 2M

vWD type 2M ("M" for "multimer") includes variants in which platelet adhesion is impaired but the vWF multimer distribution is normal. This phenotype may be produced by mutations that inactivate specific binding sites for ligands on platelets or in connective tissue.[22] For example, many patients

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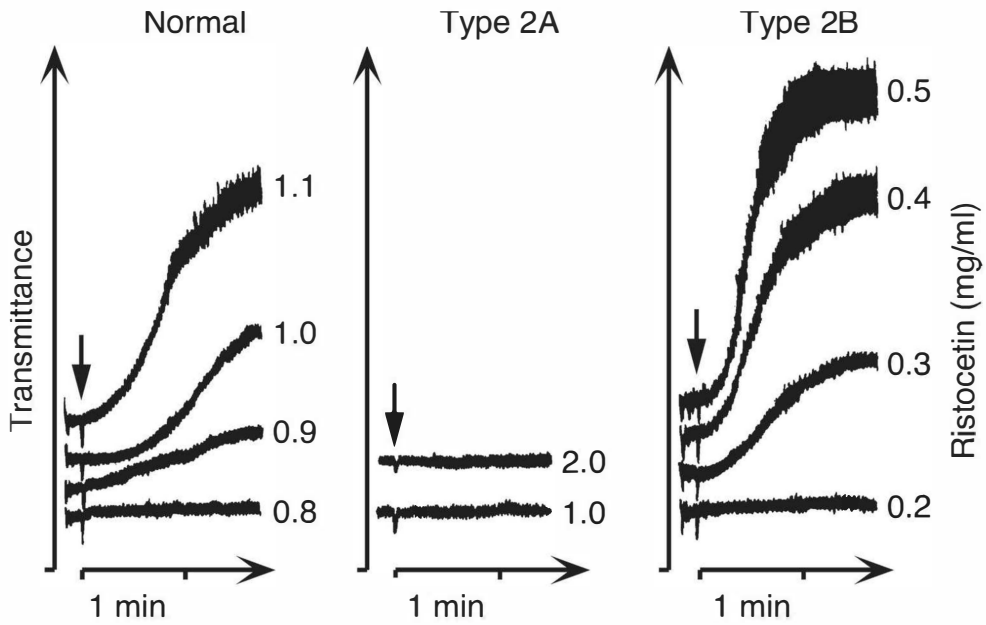

FIGURE 52.4 RIPA in a healthy control and patients with vWD type 2A and type 2B. Stirred samples of PRP were supplemented at the time marked with the *arrow* (↓) with the indicated concentrations of ristocetin, and light transmission was recorded as a function of time. Normal samples always required more ristocetin than did vWD type 2B samples to achieve comparable rates of platelet agglutination. (Adapted from Ruggeri ZM, Pareti FI, Mannucci PM, et al. Heightened interaction between platelets and factor VIII/von Willebrand factor in a new subtype of von Willebrand disease. *N Engl J Med* 1980;302:1047–1051.)

with vWD type 2M have been described with mutations in vWF domain A1 that impair binding to platelet GPIbα (FIGURE 52.3).[93,113,134–136] Only four mutations that impair binding to collagen have been reported in vWF domain A3,[137–139] although the apparent low prevalence of domain A3 mutations might reflect the relatively limited use of vWF:CB assays compared to vWF:RCo assays. Screening laboratory results generally are similar to those in vWD type 2A (Table 52.2), but multimer gel analysis shows that large multimers are present. vWD type 2M includes variants previously designated as type B,[140] type IC,[141] and type ID.[142]

von Willebrand Disease Type 2N

The FVIII-binding site on vWF is near the amino-terminus of the mature subunit. Missense mutations that selectively inactivate this binding site (FIGURE 52.3) produce an interesting autosomal recessive vWD phenotype in which the platelet-dependent functions of vWF are preserved and the multimer pattern appears normal, but FVIII levels are low, usually between 5 and 25 IU/dL.[143,144] This autosomal mimic of hemophilia A is named vWD "type 2N" after "Normandy," the birth province of one index case. The correct diagnosis is made either by demonstrating that the patient's vWF has markedly decreased binding affinity for FVIII in vWF:FVIIIB assays or through the demonstration of *vWF* mutations known to result in this phenotype.

The prevalence of vWD type 2N is not known, but it might be higher than currently appreciated. For example, 31 affected families were identified between 1990 and 1995 in France, where only two centers perform vWF:FVIIIB assays.[145] Some of these patients with vWD type 2N had been misdiagnosed with hemophilia A or as carriers of hemophilia A. Screening of patients with hemophilia A has shown that a small percentage of those with apparent mild hemophilia A may instead have vWD type 2N.[146]

Although vWD type 2N superficially resembles mild hemophilia A, or a hemophilia carrier state with low FVIII levels due to extreme lyonization, the therapeutic requirements in vWD type 2N are quite distinct. In particular, highly purified or recombinant FVIII preparations give poor FVIII recovery and survival *in vivo* because the patient's endogenous vWF cannot stabilize it normally.[147,148] For the same reason, treatment with desmopressin often does not effectively increase plasma FVIII levels.[148] Desmopressin may produce acceptable results in milder forms of vWD type 2N (e.g., Arg854Gln) but is unlikely to be beneficial in cases with more severe 2N mutations (e.g., Arg816Trp). Importantly, genetic counseling for an autosomal recessive disease is very different from that for an X chromosome–linked disease. Because of these issues, the differential diagnosis of vWD type 2N versus hemophilia A should be considered in any patient with low FVIII levels in whom the evidence for X-linked inheritance is incomplete, or in whom initial therapy with desmopressin or pure FVIII gives unexpectedly poor results.

von Willebrand Disease Type 3

vWD type 3 is a recessive disorder in which vWF protein is essentially absent. Total deficiency of vWF causes a secondary deficiency of FVIII, and such patients have a severe combined defect in platelet adhesion and blood clotting. Laboratory testing shows undetectable vWF:RCo and vWF:Ag, as well as a prolonged aPTT that is explained by a low FVIII level. If the

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condition is congenital, other testing is not usually necessary to make the diagnosis.

Heterozygous relatives of patients with vWD type 3 usually are phenotypically normal, but may sometimes have mild bleeding symptoms and meet criteria for vWD type 1.[149–152] Review of published data for 117 obligate heterozygotes indicated that they had a mean plasma vWF level of 45 IU/dL with a range of 5 to 130 IU/dL; some had mild bruising, epistaxis, menorrhagia, or bleeding after tooth extraction.[33] Such variability was evident in the first report by von Willebrand—the proband was one of five siblings with vWD type 3 and both parents had mild symptoms, but several relatives who could transmit the disease appeared to be unaffected.[1] Similar variability in symptoms is evident in many other families in whom mutations have been characterized. vWD type 3 appears to be caused mainly by deletion, frameshift, and nonsense mutations,[20,81–83,152] but approximately 25% of mutations are missense. The mutation in the original Åland Islands families studied by von Willebrand was shown to be a single-nucleotide deletion in exon 18, causing a frameshift and premature termination within the propeptide.[153]

Questions regarding the "expressivity" or "penetrance" of vWD in the relatives of type 3 patients are related to questions concerning the pathophysiology of vWD type 1. For example, the prevalence of vWD type 3 is reported to be 2.5 to 3 per million in Scandinavian countries and is 0.1 to 1.6 per million in many counties of Europe and the Middle East.[154] An intermediate prevalence of approximately 1.5 per million was reported for the United States and Canada.[155] These figures provide approximate values for q^2, where q is the gene frequency for type 3 alleles; therefore, the prevalence of heterozygous individuals ($2pq$) is 660 to 3,400 per million. The prevalence of heterozygotes could be even higher because of the shortened life expectancy in vWD type 3. However, the prevalence of symptomatic vWD type 1 appears to be <10% of these values. Therefore, a single nonfunctional vWF allele may sometimes be associated with vWD type 1, but rarely is sufficient to cause disease.

DIFFERENTIAL DIAGNOSIS OF VON WILLEBRAND DISEASE

vWD type 2 and type 3 are relatively easy to diagnose and appear at present to be genetic diseases of a single locus, the *vWF* gene. The most common variant, vWD type 1, often is difficult to diagnose and appears to involve factors other than mutations in the *vWF* gene. vWD must be distinguished from other inherited or acquired disorders of platelet function or FVIII deficiency. A few conditions are characterized by laboratory features that are remarkably like certain vWD subtypes and therefore pose special diagnostic challenges.

Acquired von Willebrand Syndrome

Acquired von Willebrand syndrome (AVWS) refers to vWF deficiency that is not inherited but is secondary to another cause. AVWS is usually a chronic condition in which patients may have mucocutaneous bleeding symptoms and laboratory parameters consistent with the diagnosis of vWD. Although the incidence of AVWS is unclear, a survey of 260 patients with myeloproliferative or lymphoproliferative disorders identified 25 patients with a ristocetin cofactor activity of <40 IU/dL, eight of whom had evidence of a vWF inhibitor.[156] In many cases, a multimer pattern consistent with type 2A vWD was identified, suggesting an increased effect on high molecular weight multimers. AVWS typically is caused by one of three mechanisms: autoimmune clearance, proteolysis induced by increased fluid shear stress, or binding to cell surfaces.[157,158]

Autoimmune clearance probably accounts for the common association of AVWS with monoclonal gammopathy of unknown significance (MGUS). Other conditions thought to induce immune clearance of vWF include lymphoproliferative diseases such as non-Hodgkin lymphoma and multiple myeloma and autoimmune diseases such as systemic lupus erythematosus and autoimmune thyroiditis. Antibodies to vWF have been demonstrated in a few patients tested, suggesting the assays are insensitive or the mechanism of disease may not always involve circulating antibodies.[157,158]

Cardiovascular conditions with pathologically increased fluid shear stress will promote the cleavage of circulating large vWF multimers by ADAMTS13, thereby causing AVWS. A classic presentation of AVWS is associated with aortic stenosis and bleeding from intestinal angiodysplasia. The AVWS and bleeding resolve upon surgical replacement of the stenotic valve.[159] AVWS also has been described in patients with mitral valve prolapse, pulmonary hypertension, ventricular septal defect, and patent ductus arteriosus.[157,158,160–162]

AVWS caused by increased vWF binding to platelets has been described in reactive thrombocytosis and in thrombocytosis associated with myeloproliferative diseases including essential thrombocythemia, polycythemia vera, and chronic myelogenous leukemia. The platelet count correlates inversely with the level of large vWF multimers, and reducing the platelet count can restore a normal vWF multimer distribution.[163,164] vWF binding to malignant lymphocytes and other cancer cells has also been described, sometimes associated with the expression of platelet GPIbα or other vWF-binding proteins on the tumor cell surface.[157,158]

Hypothyroidism may sometimes cause decreased vWF levels attributed to decreased vWF synthesis, and the levels return to normal with thyroid replacement therapy.[38] Certain drugs including sodium valproate[36] and ciprofloxacin[165] are reported to be associated with AVWS.

Platelet-Type Pseudo–von Willebrand Disease

vWD type 2B is characterized by exaggerated affinity of plasma vWF for GPIbα on platelets. The vWD type 2B phenotype is mimicked closely by an analogous but relatively rare gain-of-function defect in platelet GPIbα that is referred to as "platelet-type" or "pseudo" vWD.[166–168] Both vWD type 2B and platelet-type pseudo-vWD are associated with decreased plasma vWF and FVIII, increased sensitivity to ristocetin in the RIPA assay, the absence of the largest vWF multimers from plasma, and the presence of all sizes of multimers within platelets. Furthermore, administration of desmopressin to patients with either disorder can cause transient thrombocytopenia and spontaneous platelet aggregation.[119,169] Platelet-type pseudo-vWD can be distinguished from vWD type 2B by suitable mixing experiments: addition of normal vWF (e.g., in normal plasma) to patient platelets causes platelet aggregation in platelet-type pseudo-vWD, but not in vWD type 2B.[167,169,170] Four mutations in the platelet GPIbα subunit have been described in patients with platelet-type pseudo-vWD: Gly233Val,[171] Gly233Ser,[172] Met239Val,[173] and a 27 bp deletion

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in the macroglycopeptide-coding region of the gene.[174] The affected residues in the missense mutants are located in a small region that changes structure and adopts a β-sheet conformation upon binding to vWF domain A1. The mutations that cause platelet-type pseudo-vWD appear to enhance the affinity of binding, by stabilizing this β-sheet conformation.[131]

Hemophilia A

As discussed in the preceding text, vWD type 2N may be misdiagnosed as hemophilia A, particularly if the family history does not provide clear evidence for X chromosome–linked inheritance of FVIII deficiency. Conversely, patients with hemophilia A who also have platelet dysfunction, commonly caused by aspirin use, can have mucocutaneous bleeding similar to that of patients with vWD.[175]

Other Disorders

Because patients with vWD type 2B may present with thrombocytopenia, this subtype of vWD has been misdiagnosed as autoimmune thrombocytopenia in adults and infants.[120–122,124–127]

TREATMENT OF VON WILLEBRAND DISEASE AND ACQUIRED VON WILLEBRAND SYNDROME

Although the bleeding in vWD is usually mild, particular care to achieve adequate hemostasis should always be taken in patients undergoing invasive procedures and in those with trauma. In addition to the severity of bleeding (or potential surgical bleeding), treatment is guided by the vWD subtype, so that whenever possible the correct subtype should be established prior to therapy. Because the bleeding time is a poor reflection of bleeding risk and other laboratory testing such as the vWF:Ag and vWF:RCo levels may be difficult to obtain in a timely fashion, intervention may be guided by clinical criteria. In general, with appropriate therapy, adequate hemostasis should be obtained so that surgical procedures may be safely undertaken in all forms of vWD.

Agents Used in the Treatment of von Willebrand Disease

Desmopressin

Desmopressin (DDAVP) is a synthetic analog of vasopressin that has <1% of the pressor effect of the natural hormone. Originally used as replacement therapy for central diabetes insipidus, this agent was shown to successfully treat bleeding in hemophilia A and vWD.[176,177,178] Administration of desmopressin results in a rapid increase in plasma levels of FVIII and vWF in normal individuals and those with type 1 vWD. The rapid rise suggests an effect on preformed vWF that is released from the Weibel-Palade bodies of the endothelium, but the mechanism of this effect is uncertain.

Surgical prophylaxis and treatment of minor bleeding can usually be accomplished with desmopressin. Desmopressin may be administered intravenously, subcutaneously, or as an intranasal spray. When given intravenously, the usual dose is 0.3 μg/kg diluted in 50 mL of 0.9% sodium chloride and infused over 30 minutes. The maximal response in the vWF levels occurs in 30 to 60 minutes and generally persists for at least 6 hours. When indicated, repeat doses are usually given every 12 to 24 hours.[177,179,180] The response is consistent in any one individual and usually results in a twofold to fourfold increase in vWF. Some vWD type 1 variants, such as vWD Vicenza (Arg1205His), are associated with a much greater initial response (up to 10-fold increment in vWF), but by 4 hours the vWF levels have returned to baseline. This reflects the accelerated clearance of these variants and emphasizes the importance of obtaining a vWF level at >2 hours after desmopressin administration. Several early reports suggested that patients developed tachyphylaxis (decreased response) to repeated doses of desmopressin, and this is thought to be caused by depletion of vWF from its storage sites. On an average, the increment in vWF is approximately 30% less with a second dose given 24 hours subsequent to the initial dose, but the response was not reduced further after one or two additional doses.[181] Repeated dosing at intervals of <24 hours is also more likely to cause tachyphylaxis. Three to four days after desmopressin is stopped, a full response can again be anticipated with readministration of the drug. If suitably concentrated preparations of injectable desmopressin are available, a subcutaneous dose is approximately as effective as the same intravenous dose and suitable for home therapy.[182]

In some countries, a highly concentrated form of desmopressin containing 1.5 mg/mL (Stimate, CSL Behring) is available as an intranasal spray. The recommended adult dose is 300 μg given in divided doses, 150 μg in each nostril. In children and adults weighing <50 kg, the recommended dose is 150 μg. Note that preparations of intranasal desmopressin used for diabetes insipidus or enuresis are too dilute as intranasal therapy for bleeding in vWD. The therapeutic levels achieved with intranasal desmopressin are similar to a dose of approximately 0.2 μg/kg intravenous desmopressin.[177] For patients with type 1 vWD, peak responses in vWF activity occur in 1 to 2 hours and the plasma concentrations of vWF generally are not as high as with intravenous desmopressin. The responses are also less predictable than with intravenous therapy.[183] Nevertheless, this approach is useful for patients who are in remote settings or who require treatment of minor recurrent bleeding (e.g., menorrhagia). The medication should be stored under refrigeration but appears stable for up to 3 weeks when stored at room temperature for use during travel.

Because the response to desmopressin varies among individuals, a trial dose should be evaluated before any therapeutic or prophylactic use of desmopressin. Blood samples are obtained for baseline measurement of vWF:Ag, vWF:RCo (or vWF:CB), and FVIII. Then a single dose of desmopressin, 0.3 μg/kg in 50 mL of normal saline, is given intravenously over 30 minutes. The subject should be monitored for vital signs, flushing, and headache. Additional samples for measurement of vWF:Ag, vWF:RCo, and FVIII should be obtained at 1 hour and 4 hours after the start of the infusion. Values at 1 hour represent the maximum response. Values at 4 hours allow calculation of the circulatory half-life of the released factors.

Target plasma levels of vWF and FVIII depend on the hemostatic challenge. Minor bleeding and minor surgical procedures may be covered satisfactorily with increases of vWF and FVIII to at least 30 IU/dL at 1 hour after desmopressin, provided the half-life is normal. Major bleeding and major surgery may require levels >100 IU/dL that remain >50 IU/dL before the next dose.

Useful responses to desmopressin are common in vWD type 1 and rare in vWD type 2 variants.[178] Almost all patients with type 3 vWD are insensitive to desmopressin and do not have

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a therapeutically useful response. The percentage of patients with vWD type 1 that responds to intravenous desmopressin varies from 90%[179] to approximately 30%.[32] Some of this variation reflects different definitions of response. Some patients with vWD type 2A, type 2M, or type 2N have a useful response to desmopressin, and these patients may be identified by their response to a test dose.[184,185] Administration of desmopressin to patients with vWD type 2N is associated with the expected twofold to threefold increase in vWF:Ag and vWF:RCo, but patients with severe FVIII-binding defects often have a small and transient response in FVIII level with a half-life of only 2 to 3 hours.[143,186] In contrast, patients with the relatively common mutation Arg854Gln have a mild FVIII-binding defect with higher baseline FVIII levels of 21.8 ± 9.8 IU/dL[145] and frequently have a useful FVIII response to desmopressin.[32,186]

When given to patients with vWD type 2B, desmopressin increases the levels of vWF and FVIII, but also causes or exacerbates thrombocytopenia. For this reason, plasma-derived FVIII/vWF products are generally recommended for use in vWD type 2B (Table 52.5). Nevertheless, a few patients have received desmopressin with good hemostatic responses and no unexpected bleeding or thrombosis.[187,188] Therefore, if plasma product therapy must be avoided, desmopressin could be considered.

It is generally recommended that a test dose of intranasal desmopressin also be evaluated before the clinical use of this form of the drug, even in patients who have previously responded to intravenous desmopressin.

The most common adverse reaction to intravenous desmopressin, occurring in up to 10% of patients, is vasodilatation with facial flushing and, less commonly, headache and mild hypotension. Extending the period of infusion from 30 to 60 minutes generally lessens these side effects as does administering the drug by the subcutaneous route. Intranasal desmopressin is associated with similar reactions and may also be associated with local reactions including nasal congestion, rhinitis, and upper respiratory symptoms. Hyponatremia, the result of the antidiuretic effect of desmopressin, tends to occur in children younger than 2 years, and in elderly patients who have received several doses. Hyponatremia has been described with either intravenous or intranasal desmopressin and, when severe, may cause seizures.[180,183] Monitoring of the serum sodium level and fluid restriction should be instituted for patients receiving more than two to three doses. Hyponatremia resolves rapidly after desmopressin is discontinued. There have been several isolated reports of stroke and myocardial infarction among patients receiving desmopressin. However, a retrospective review of patients receiving desmopressin for major surgery found no difference in thrombotic events between patients who did and did not receive the drug.[189] Most cases of thrombosis have been described in older men who have other thrombotic risk factors so that a concern remains in these patients requiring therapy.

Unlike vasopressin, desmopressin does not appear to cause uterine contractions. Nevertheless, because of the concern over a possible oxytocin-like effect, desmopressin is not often used during pregnancy, but may be administered as soon as the baby is delivered. Safety in nursing mothers has not been defined.

Plasma-Derived von Willebrand Factor

Cryoprecipitate is prepared from plasma and is enriched in FVIII, vWF, and fibrinogen. Blood collected for the preparation of cryoprecipitate is screened for a number of infectious agents including human immunodeficiency virus (HIV), hepatitis B, and hepatitis C, but at present is not virucidally treated. Therefore, a small risk of viral contamination remains, similar to that of red cell transfusions. One unit of blood yields approximately 8 to 10 mL of cryoprecipitate containing 100 to 150 IU of vWF. When prepared from single donors and processed individually, cryoprecipitate has a vWF multimeric structure similar to that found in plasma.[190] Cryoprecipitate is given as an intravenous infusion, and a typical dose is 1 to 2 "bags" per 10 kg of body weight. Matching for ABO type is not necessary, and the product does not need to be given through a leukocyte-depleting filter. Cryoprecipitate was once the preferred plasma-derived product for the treatment of bleeding in vWD. However, concern over viral transmission has led to the abandonment of cryoprecipitate in favor of virucidally treated FVIII/vWF concentrates in almost all circumstances.

FVIII/vWF-containing products are either derived from plasma or prepared as a recombinant product from cultured cells. Plasma-derived products are virucidally treated, and available data suggest that the risks of transmitting HIV, hepatitis B, and hepatitis C have been essentially eliminated. Highly purified or "monoclonal" plasma FVIII concentrates and recombinant FVIII products lack sufficient vWF to be useful in the treatment of any form of vWD. In contrast, plasma-derived FVIII/vWF concentrates that contain 15 to 200 IU FVIII per mg protein (commonly described as "intermediate purity") also contain substantial amounts of vWF. Because these products are virucidally treated, they have become the preferred products for the treatment of vWD.

For patients who do not tolerate or respond adequately to desmopressin, treatment with a plasma-derived FVIII/vWF concentrate such as Humate-P may be necessary. When examined by gel electrophoresis, the vWF present in plasma-derived FVIII concentrates shows some evidence of limited degradation compared to fresh plasma vWF. Nevertheless, these products can correct the hemostatic defect in patients with vWD.[191,192] Humate-P (CSL Behring) is one of the most extensively utilized FVIII/vWF concentrates. This concentrate appears effective for the prevention of bleeding perioperatively and for the treatment of other bleeding episodes. Each vial of Humate-P is labeled with both FVIII units and vWF units (ristocetin cofactor units) so that particular attention is paid to this information to ensure correct dosing. Examples of treatment guidelines are summarized in Table 52.5. Monitoring of vWF activity may be helpful to reduce the hemostatic risk of underdosing and the extra cost of overdosing.[193] Humate-P is generally well tolerated with few adverse events.[193] Continuous infusion of Humate-P may be considered because this appears effective and consumes approximately one-half of the total dose required when given by intermittent bolus injection.[194]

Data with Alphanate (Alpha Therapeutics) and other plasma-derived FVIII/vWF concentrates suggest that these products are also effective in correcting the vWF activity level and therefore may be considered for use in vWD.[191,192] Because the concentration of vWF in these products is not yet standardized in the United States, dosing is determined in units of FVIII activity. Initial treatment with 40 FVIII IU/kg intravenously every 12 hours is usually sufficient to maintain the vWF activity level >50 IU/dL.

Concentrates that contain both vWF and FVIII have the potential to cause disproportionately high plasma FVIII

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Table 52.5

Usage of plasma-derived FVIII/vWF concentrates in the treatment of vWD

Hemostatic Challenge	Therapy
Major surgery or bleeding	50 IU FVIII or vWF:RCo/kg body weight, followed by 25 to 40 IU/kg every 12 or 24 h, adjusted to keep the nadir level of FVIII or vWF:RCo >50 IU/dL until healing is progressing, typically for 5 to 10 d
Minor surgery	40 IU FVIII or vWF:RCo/kg body weight every 24 or 48 h, adjusted to keep the nadir level of FVIII or vWF:RCo >30 IU/dL until healing is progressing, typically for 2 to 4 d
Dental extraction	30 IU FVIII or vWF:RCo/kg body weight as a single dose, to maintain the level of FVIII or vWF:RCo >50 IU/dL for 12 h
Spontaneous minor bleeding	25 IU FVIII or vWF:RCo/kg body weight every 24 h to keep the nadir level of FVIII or vWF:RCo >30 IU/dL until bleeding stops, usually for 2 to 4 d
Labor and delivery	40 IU FVIII or vWF:RCo/kg body weight daily, adjusted to maintain the FVIII or vWF:RCo >50 IU/dL h on the day of delivery and for 3 to 4 d postpartum

Doses may be increased 20% for subjects younger than 18 years, to compensate for their increased plasma volume.
FVIII, factor VIII; vWF, von Willebrand factor.
Adapted from Mannucci PM, Chediak J, Hanna W, et al. Treatment of von Willebrand disease with a high-purity factor VIII/von Willebrand factor concentrate: a prospective, multicenter study. *Blood* 2002;99:450–456.

levels when administered to patients with vWD. Both the transfused FVIII and endogenously synthesized FVIII are stabilized by binding to the transfused vWF, and after several days of therapy the vWF level may be normal but the FVIII level may exceed 400 IU/dL.[178] These levels of FVIII may be associated with venous thrombosis and pulmonary embolism in patients with vWD exposed to other thrombotic risk situations,[178,195,196] and standard measures for thromboprophylaxis should be considered.

A virucidally treated, highly purified vWF concentrate that contains little FVIII has been used successfully to increase the levels of vWF in patients with vWD.[191,192,197,198] This product is not yet available in the United States. Because the FVIII content is so low, the normalization of FVIII levels depends on endogenous FVIII production and may require 12 hours in patients with severe vWD. Therefore, prophylactic treatment with purified vWF should be given 12 to 24 hours before any elective procedure, and acute correction of both FVIII and vWF levels requires concomitant treatment with a source of FVIII.

A recombinant human vWF concentrate is currently undergoing clinical trial evaluation (Baxter Healthcare). This concentrate also contains recombinant FVIII, and thus immediate replacement of FVIII is also provided by this product. This concentrate has previously been evaluated in dog models of vWD, and it appears to be safe and effective.[199]

Alloantibody inhibitors of vWF are rare complications of transfusion therapy in vWD type 3, reportedly occurring in 3% to 10% of patients.[200,201] Large vWF gene deletions appear to correlate with the risk of developing inhibitors.[201] With one possible exception, the few inhibitors that have been characterized were polyclonal Ig (immunoglobulin) G antibodies.[201] Patients with vWF inhibitors often have severe allergic reactions to transfused vWF that can include back pain, abdominal pain, hypotension, and anaphylaxis.[200,202]

Antifibrinolytic Agents

Tranexamic acid and ε-aminocaproic acid (Amicar) inhibit fibrinolysis and may be used alone or in combination with other hemostatic agents. These agents are most useful in oropharyngeal bleeding and menorrhagia. They are generally contraindicated in urinary tract hemorrhage because of the risk of a fibrin clot obstructing the urinary outflow tract. Tranexamic acid (10 to 15 mg/kg every 8 to 12 hours) or ε-aminocaproic acid (50 to 60 mg/kg every 4 to 6 hours) may be given orally or by intravenous infusion.[203] These agents may also be used as a mouthwash for oral bleeds or dental procedures. Tranexamic acid mouthwash, 10 mL of a 4.8% solution given as a "swish and spit," may be better tolerated than ε-aminocaproic acid in some patients.[180,204]

Platelet Transfusions

Some patients with vWD type 3 have had a rapid improvement in the bleeding time after platelet transfusion despite an incomplete response to a prior infusion of plasma-derived vWF.[205] Both platelet and plasma vWF contribute to hemostasis,[206] and platelet transfusion (e.g., a plateletpheresis collection of 4 to 5×10^{11} platelets) may be considered as adjunctive therapy for bleeding that is refractory to treatment with FVIII concentrate.[207]

Platelet transfusions are not likely to be helpful in the treatment of thrombocytopenia associated with type 2B vWD.[208]

Other Therapy

Short courses of conjugated estrogens have been used in surgical prophylaxis and for treatment of bleeding associated with angiodysplasia.[209] A short course of corticosteroids may be effective for hematuria.

Patients with vWD type 3 and alloantibodies to vWF have been treated with purified plasma or recombinant FVIII that contains no vWF. The infused FVIII is cleared rapidly, but therapeutic

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levels (40 to 60 IU/dL) can be maintained by administering FVIII at a total dose of 700 to 900 IU/kg/d intravenously by continuous infusion or bolus infusions every 4 hours.[210,211] Recombinant FVIIa (NovoSeven) also has been used to treat surgical or postpartum bleeding in these patients by continuous infusion (approximately 20 μg/kg/h) or bolus infusion (~90 μg/kg every 4 hours).[211,212]

Therapy for Problems Specific to Women with von Willebrand Disease

The incidence of symptomatic vWD in women is disproportionate as compared to that in men, representing approximately two-thirds of all patients,[34,75] although autosomal inheritance requires an equal frequency of mutant gene transmission to both sexes. The increased impact of vWD on women of childbearing age is a consequence of the repeated hemostatic challenges of ovulation, menstruation, pregnancy, and delivery. Bleeding into the corpus luteum may occur after ovulation, and midcycle pain may be severe enough to prompt surgical exploration.[34,213] The prevalence of menorrhagia in vWD is not known precisely because few studies have used quantitative measures of blood loss, and bias in patient selection is possible. With these limitations in mind, approximately 65% of women with vWD appear to have menorrhagia (>80 mL blood loss per menstrual cycle), compared to 9% to 14% of controls, and menorrhagia for women with vWD often begins at menarche.[34,214,215]

Conversely, approximately 6% to 13% of women with unexplained menorrhagia have vWF levels more than 2 SD below the mean.[214,216] Independent of whether subjects meet criteria for diagnosing vWD, a low vWF level appears to be a modest risk factor for menorrhagia, with a relative risk of approximately 2 to 4 for vWF levels below approximately 50 IU/dL.

Excluding patients with vWD type 3, intranasal desmopressin every 12 to 24 hours for up to 3 days may be effective for menorrhagia in vWD, if the side effects can be tolerated.[217] Two randomized placebo-controlled trials in a total of 44 women, approximately half with vWD, reported a trend in favor of desmopressin for menstrual bleeding, but the differences between the two arms were not statistically significant.[214,218] A larger open-label study in 90 women, many with vWD, reported shortened or decreased bleeding in 88% of treated menstrual cycles.[219]

Menorrhagia in all types of vWD may be treated successfully with hormonal therapy (danazol, progesterone, or combined oral contraceptive pills).[215,220] In appropriate patients, desmopressin and hormonal therapy can be combined.

Antifibrinolytic agents can be very effective for menorrhagia,[221,222] and, although experience is limited in vWD, case reports suggest these agents could be useful alone or as adjuncts to other treatments.[223] Tranexamic acid has been used at 1 g every 6 hours for 5 days,[221] or 4 g in a single daily dose for 3 to 5 days.[223] ε-Aminocaproic acid has been used at dosages of 5 g three times daily for 5 days,[224] or 3 g every 6 hours for 3 to 5 days.[225] ε-Aminocaproic acid is thought to reduce fertility by inhibiting embryo implantation although the data are scanty. For this reason, antifibrinolytics generally are not recommended during the first trimester of pregnancy or for patients considering pregnancy.

Pregnancy usually is tolerated well by patients with vWD.[226] In most patients, except for those with vWD type 3, vWF levels increase during the third trimester and protect against hemorrhage late in pregnancy. However, miscarriage or therapeutic abortion early in pregnancy, while vWF and FVIII levels are low, carries an increased risk of maternal hemorrhage.[227,228] Provided the levels of both vWF and FVIII are >50 IU/dL, the risk of hemorrhage during labor and delivery does not appear to be increased. Exceptions to this generalization may occur for patients with vWD type 2. Retrospective series that include both vWD type 1 and type 2 suggest that primary postpartum hemorrhage (>500 mL blood loss in the first 24 hours) occurs in approximately 16%[229] to approximately 18.5%[228] of patients. After delivery, the levels of FVIII and vWF fall rapidly over several days, and secondary postpartum bleeding (bleeding after 24 hours in excess of "normal" lochial loss) may occur with an incidence of 20%[228] to 25%.[229] Therefore, patients should be monitored for at least 1 week postpartum, and they should be advised that bleeding may occur up to 3 weeks after delivery.

Progressive pregnancy-associated thrombocytopenia is common and may be the presenting manifestation of vWD type 2B.[122] Increased bleeding has not been reported to accompany pregnancy-associated thrombocytopenia in vWD type 2B. Patients with vWD type 2B Malmö/New York do not develop thrombocytopenia when given DDAVP or during pregnancy.[129,132]

Maternal hemorrhagic complications can be minimized by prophylactic therapy to raise factor levels to >50 IU/dL at delivery and to maintain them at that level for 3 to 4 days postpartum.[200,228,230] Factor levels >50 IU/dL appear to be sufficient to administer epidural anesthesia safely.[228,231] In general, FVIII/vWF concentrates are used prior to delivery, switching to desmopressin if appropriate after delivery. FVIII/vWF concentrate may reverse the thrombocytopenia and correct the bleeding tendency of vWD type 2B for delivery.[124] In all patients, complete uterine evacuation and contraction after delivery are important to prevent bleeding.[230]

Treatment of Acquired von Willebrand Syndrome

Treatment of AVWS involves consideration of both the underlying disease and the bleeding disorder. Because the mechanism of AVWS is variable, identifying effective therapy can be difficult.[157]

The optimal treatment of AVWS associated with autoimmune disease is yet to be defined. Immunosuppressive therapy with oral prednisone, 1 mg/kg/d, or intravenous cyclophosphamide, 700 mg/M² monthly has been used successfully.[232,233] For patients with malignant lymphoproliferative disorders such as non-Hodgkin lymphoma or multiple myeloma, successful treatment of the underlying disease with cytotoxic chemotherapy or surgical removal of the tumor typically results in resolution of the AVWS. In a few cases, the return of AVWS heralded a relapse.[234–237] For patients with MGUS, treatment of the associated clonal lymphoid disorder with cytotoxic or immunosuppressive chemotherapy usually does not alter the course so that prevention and treatment of bleeding represents the mainstay of therapy.

Intravenous immunoglobulin (IVIG) was first proposed in 1976 for the treatment of AVWS,[238] and many reports describe the success of this approach for patients with AVWS caused by immune-mediated clearance of vWF.[157,234,239–243] Active bleeding may be treated with IVIG 1 g/kg/d for 1 to 2 days with resolution of the hemorrhage within several days of the infusion. Repeated prophylactic infusions of IVIG 1 g/kg every 3 to 4 weeks have been used successfully in the prevention of bleeding.[242,243] IVIG

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appears to be more effective for patients with IgG than IgM MGUS.[242] Patients in whom no underlying disease is identified may respond to IVIG, suggesting that an underlying immune-mediated disorder may be responsible.

Desmopressin or FVIII/vWF concentrate is effective in up to one-third of patients with AVWS associated with MGUS or malignant lymphoproliferative diseases, although the primary mechanism of disease is increased clearance. A test dose of desmopressin can be helpful to identify responsive patients.[157]

For patients with myeloproliferative disease or solid malignancies, treatment of the underlying disease with surgery or chemoradiation therapy appears to be effective. Control of the platelet count in myeloproliferative disease seems to be effective in increasing the plasma concentration of large vWF multimers.[244,245] An unusual association of AVWS with Wilms tumor has been described, and the bleeding disorder resolved with the successful treatment of the malignancy.[246] Cases of AVWS associated with medications resolve when the drug has been discontinued, and those associated with hypothyroidism resolve with thyroid hormone replacement therapy.[38]

Patients with AVWS caused by increased fluid shear stress generally do not respond to desmopressin, FVIII concentrates, or IVIG, but require correction of the underlying cardiovascular abnormality to normalize vWF function.[157] For example, AVWS associated with aortic stenosis typically resolves upon successful repair of the defective valve.[159]

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CHAPTER

53

Rare Inherited Coagulation Disorders Other than Hemophilia

PIER MANNUCCIO MANNUCCI, FLORA PEYVANDI, AND MASSIMO FRANCHINI

The rare inherited coagulation disorders (RICDs), usually transmitted in an autosomal recessive fashion, represent 3% to 5% of all the inherited deficiencies of coagulation factors and include fibrinogen, factor (F)II, FV, FV+FVIII, FVII, FX, FXI, FXIII, and the multiple deficiency of vitamin K–dependent factors.[1,2] Their distribution is variable in the world, with a prevalence in the general population of the presumably homozygous forms ranging from approximately 1 in 2 million for prothrombin (FII) to 1 in 500,000 for FVII deficiency (the most common) (Table 53.1).[3] In countries with large Jewish communities, FXI deficiency is more prevalent. In Middle Eastern countries and Southern India, where consanguineous marriages are customary, autosomal recessive traits occur more frequently in the homozygous state.[4]

Data on the distribution of patients affected by RICDs are limited. Given their rarity, few centers have the possibility to follow and manage a consistent number of patients, so that scientific reports are usually limited to small numbers. Two recent large registries help to derive the worldwide distribution of RICDs: one by the World Federation of Haemophilia (WFH, www.wfh.org) and the other by the International Rare Bleeding Disorders Database (RBDD, www.rbdd.org).[5] The WFH database, which started in 1998 but collected information since 2004, includes information from 108 countries (2008 update), while the RBDD, developed in 2004, reports data from 68 countries. According to these registries, FXI and FVII deficiencies are the most prevalent RICD worldwide, with frequencies of 29% and 33% of the total RICDs, followed by FX (9%), fibrinogen (8%), FV (8%), FXIII (6%), and combined FV+FVIII (5%) and FII deficiencies (2%) (FIGURE 53.1).[6] These results were also confirmed by smaller national registries, such as those of Central and South America, Egypt, France, India, Iran, and the United Kingdom.

There are considerable variations in bleeding patterns among patients with RICDs, but on the whole, bleeding manifestations affecting critical organs such as the central nervous and musculoskeletal systems appear to be less frequent than in hemophilia A and B.[7] The laboratory diagnosis of RICDs is carried out by means of screening tests such as the activated partial thromboplastin time (APTT), the prothrombin time (PT), and the thrombin time (TT) in subjects reporting a clinical and family history of bleeding (Table 53.1).[4] Abnormal results of screening tests should be followed by coagulation factor assays, in order to specify the diagnosis. Molecular diagnosis is based on the search of mutations in the genes encoding the corresponding coagulation factors. Exceptions are the combined deficiency of FV and FVIII, caused by mutations in genes encoding proteins involved in the intracellular transport of FV and FVIII (multiple coagulation factor deficiency 2 [MCFD2] and lectin mannose binding protein 1 [LMAN1]), and the combined deficiency of vitamin K–dependent proteins (FII, FVII, FIX, and FX), caused by mutations in genes that encode enzymes involved in posttranslational modifications and in vitamin K metabolism (γ-glutamyl carboxylase [GGCX] and vitamin K epoxide reductase [VKOR]). Genetic analysis is important mainly for prenatal and preimplantation genetic diagnosis, especially in countries with a high rate of RICDs and consanguinity.[8] Because it is not always clear whether or not the identified mutation is the cause of the deficiency of a clotting factor, family history associated with allele frequency in the general population is important to rule out that the identified mutations are common polymorphisms.[7]

Treatment of RICDs is a difficult task, because information on the clinical management of RICDs is often scarce and replacement therapy of coagulation factors may require unlicensed products that are not readily available.[9,10] The patients' personal and family history of bleeding are important guides for management.[9] Dosages and frequency of replacement therapy with products containing the deficient factor depend on the minimal hemostatic level of the factor, its plasma half-life, and the type of bleeding episode. Unfortunately, information on the safety and efficacy of the few available products is scarce and experiences from their use are significantly more limited than those available for congenital hemophilia. Other treatments in RICDs are nontransfusional adjuvant drugs (i.e., antifibrinolytic amino acids, estrogen–progestogen).[10]

After these general statements on RICD, the next section will deal specifically with each factor deficiency.

FIBRINOGEN DEFICIENCY

Fibrinogen is a 340 kDa protein synthesized in the liver, with a plasma concentration of approximately 1.5 to 3.5 g/L and a half-life of approximately 4 days (Table 53.1). The fibrinogen molecule is a homodimer, each half consisting of the three nonidentical polypeptide chains Aα, Bβ, and γ. The genes encoding fibrinogen Bβ (*FGB*), Aα (*FGA*), and γ (*FGG*), ordered from centromere to telomere, are clustered on chromosome 4 in a region of approximately 50 kb (Table 53.1). Fibrin is produced by proteolytic cleavage of fibrinogen by thrombin, with the release of fibrinopeptides A and B and generation of insoluble fibrin monomer followed by polymerization. Fibrinogen is also important in primary hemostasis through its ability to support normal platelet aggregation.[9]

Fibrinogen deficiency is heterogeneous and two main phenotypes can be distinguished. In some cases, plasma and platelet levels of the protein are not measurable or very low, leading to afibrinogenemia and hypofibrinogenemia. In other cases,

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Table 53.1

Epidemiologic, molecular, and laboratory characteristics of rare inherited bleeding disorders

Deficiency	Estimated Prevalence	Gene on Chromosome	Minimum Hemostatic Levels	Plasma Half-life	Coagulation Assays		
					PT	PTT	TT
Fibrinogen	1:1,000,000	4	50 mg/dL	2–4 d	↑	↑	↑
Prothrombin	1:2,000,000	11	20%–30%	3–4 d	↑	↑	N
Factor V	1:1,000,000	1	15%–20%	36 h	↑	↑	N
Combined factor V+VIII	1:1,000,000	18 (*LMAN1*) 2 (*MCFD2*)	15%–20%	FV 36 h FVIII 12–14 h	↑	↑	N
Factor VII	1:500,000	13	15%–20%	4–6 h	↑	N	N
Factor X	1:1,000,000	13	15%–20%	40–60 h	↑	↑	N
Factor XI	1:1,000,000	4	15%–20%	40–70 h	N	↑	N
Factor XIII	1:2,000,000	6 (*FXIII-A*) 1 (*FXIII-B*)	2%–5%	11–14 d	N	N	N
Vitamin K–dependent coagulation factors	Reported in about 30 families	2 (*GGCX*) 16 (*VKORC1*)	15%–20%	See corresponding factors	↑	↑	N

PT, prothrombin time; APTT, activated partial thromboplastin time; TT, thrombin time.

low clottable fibrinogen contrasts with normal or moderately reduced fibrinogen antigen, causing dysfibrinogenemia and hypodysfibrinogenemia.[3]

Mutation Analysis

A homozygous deletion of approximately 11 kb of the *FGA* as a cause of inherited afibrinogenemia was for the first time identified by Neerman-Arbez et al.[11] in four members of a Swiss family. Since then many other mutations have been identified in afibrinogenemia (in homozygosity or in compound heterozygosity) or hypofibrinogenemia.[8] While the majority of mutations are in the *FGA* gene, missense mutations leading to complete fibrinogen deficiency appear to be clustered in the highly conserved *C*-terminal globular domains of the Bβ and γ chains. Recent functional studies of these mutations in transfected cells have demonstrated either impaired assembly or secretion of the fibrinogen hexamer. To date, only two missense mutations located at the start of the coiled coil in *FGA* (Cys64Phe and Met70Arg) were found in fibrinogen deficiency.[12,13]

Besides the large number of rare, individual mutations identified in congenital afibrinogenemia, two common mutations were found in individuals of European origin, both in *FGA*: the Asp153SerfsX4 mutation and the *FGA* 11-kb deletion, both on multiple haplotypes. Two further residues of interest in screening for dysfibrinogenemia are residue Arg16 in exon 2 on *FGA*, which is part of the thrombin cleavage site in the fibrinogen Aα chain, and residue Arg275 in exon 8 on *FGG*, which is important for fibrin polymerization.[14]

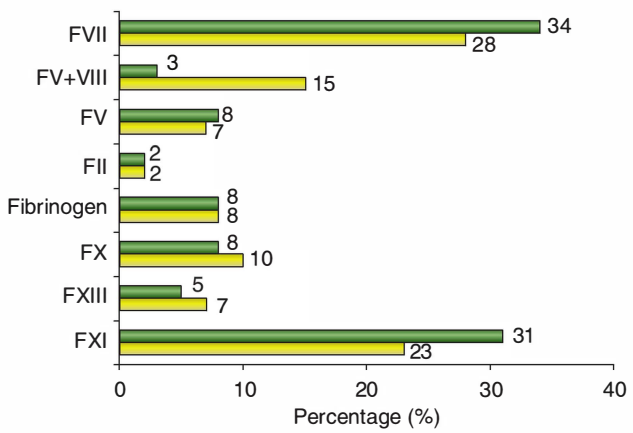

FIGURE 53.1 Prevalence of RICDs derived from RBDD (*green-filled bars*) and WFH (*yellow-filled bars*) registries.

Clinical Phenotype and Treatment

Whereas the majority of patients with dysfibrinogenemia do not bleed at all, afibrinogenemic patients have a bleeding tendency that may manifest in the neonatal period, with 85% of cases presenting with umbilical cord bleeding.[15] Bleeding may also occur in the skin, gastrointestinal tract, or central nervous system, while recurrent joint and muscle bleeding leading to persistent damage to the musculoskeletal system is less frequent. Clinically milder symptoms such as epistaxis and menorrhagia are also frequent. First trimester abortion is common in afibrinogenemic women, less common in dysfibrinogenemia, and not reported so far in hypofibrinogenemia.[16,17] Postpartum bleeding is relatively frequent when no prophylactic replacement therapy is given. On the whole, bleeding problems are often not dramatic in patients with afibrinogenemia, despite the fact that screening coagulation tests (PT, APTT) usually result in no clot formation and that the skin bleeding time is prolonged.[2]

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Both arterial and venous thromboembolic complications have been reported in afibrinogenemic patients, in spite of the fact that in the majority of patients no traditional thrombotic risk factors were present. One possible but as yet unproven explanation for this predisposition is that even in the absence of fibrinogen platelet aggregation does occur due to the action of von Willebrand factor. Moreover, in contrast to hemophiliacs, afibrinogenemic patients are able to generate thrombin, both in the initial phase of limited production and in the secondary burst of greater thrombin generation.[2]

Pertaining to replacement therapy, four products are available: fresh-frozen plasma (FFP), cryoprecipitate, virus-inactivated FFP, and fibrinogen concentrate. Safety commands avoidance of the first two if possible, because they are not virus inactivated. The latter is the treatment of choice, because it is virally inactivated and thus safer than cryoprecipitate or FFP and does not cause volume overload.[18,19] The conventional mode of treatment delivery is episodic and on demand, with fibrinogen administered as soon as possible after onset of bleeding. Long-term secondary prophylaxis (made possible thanks to the relatively long half-life of fibrinogen) with administration of fibrinogen every 7 to 14 days (particularly after central nervous system hemorrhages) has been proposed (Table 53.2). Prophylactic therapy also appears to be necessary for successful completion of pregnancy: fibrinogen replacement must begin at least at 5 weeks gestation to prevent abortion and to maintain fibrinogen

Table 53.2

Treatment of inherited coagulation disorders

Deficient Factor	On Demand	Surgery	Long-term Prophylaxis
Fibrinogen	SD-treated plasma (15–20 mL/kg) Fibrinogen concentrate (50–100 mg/kg) Target level: 50–100 mg/dL	Fibrinogen concentrate (50–100 mg/kg) Minimum level: 100 mg/dL	Fibrinogen concentrate (30–100 mg/kg every week) Target level: 50–100 mg/dL
Prothrombin	SD-treated plasma (15–20 mL/kg) PCC (20–30 U/kg) Target level: 20%–30%	SD-treated plasma (15–20 mL/kg) PCC (20–30 U/kg) Minimum level: 20%–40%	PCC (25–40 U/kg every week) Target level: 20%–30%
Factor V Factor V+VIII	SD-treated plasma (15–20 mL/kg) Target level: 10%–20%	SD-treated plasma (15–20 mL/kg) Minimum level: 25%	No data available
Factor VII	FVII concentrate (30–40 mL/kg) PCC (20–30 U/kg) rFVIIa (15–30 μg/kg every 4–6 h) Target level: 10%–15%	FVII concentrate (30–40 mL/kg) PCC (20–30 U/kg) rFVIIa (15–30 μg/kg every 4–6 h) Minimum level: 10%–15%	FVII concentrate (30–40 mL/kg, three times per week) rFVIIa (10–20 μg/kg, two to three times per week) Target level: 10%–15%
Factor X	SD-treated plasma (10–20 mL/kg) PCC (20–30 U/kg) FX/FIX concentrate (10–20 U/kg) Target level: 10%–20%	SD-treated plasma (10–20 mL/kg) PCC (20–30 U/kg) FX/FIX concentrate (10–20 U/kg) Minimum level: 10%–20%	PCC (30–40 U/kg, two to three times per week) FX/FIX concentrate (20–40 U/kg, one to two times per week) Target level: 10%–20%
Factor XI	SD-treated plasma (15–20 mL/kg a.d.) FXI concentrate (15–20 U/kg a.d.) Target level: 15%–20%	Antifibrinolytic agents (250 mg/d) SD-treated plasma (15–20 mL/kg a.d.) FXI concentrate (15–20 U/kg a.d.) Minimum level: 15%–20%	No data available
Factor XIII	SD-treated plasma (3 mL/kg) FXIII concentrate (10–20 U/kg) Target level: 2%–5%	SD-treated plasma (3 mL/kg) FXIII concentrate (10–20 U/kg) Minimum level: 5%–10%	SD-treated plasma (15–20 mL/kg every 4–6 wk) FXIII concentrate (10 U/kg every 4–6 wk) Target level: 2%–5%
VKCFD	Vitamin K (10 mg i.v. or s.c.) SD-treated plasma (15–20 mL/kg) Target level: 20%–30%	Vitamin K (10 mg i.v. or s.c.) SD-treated plasma (15–20 mL/kg) Minimum level: 20%–30%	Vitamin K (10 mg orally every week)

SD, solvent/detergent; PCC, prothrombin complex concentrate; rFVIIa, recombinant activated factor VII; VKCFD, vitamin K–dependent coagulation factors deficiency; i.v., intravenously; s.c., subcutaneously; a.d., alternate days..

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levels at >1.0 g/L throughout pregnancy (a level of >1.5 g/L is recommended for delivery).[17]

PROTHROMBIN DEFICIENCY

Prothrombin (FII) is a 72 kDa single-chain glycoprotein synthesized by the liver. It is one of the vitamin K–dependent coagulation factors and requires posttranslational carboxylation to become functionally active. Prothrombin consists of four domains—the Gla domain, kringle 1 and kringle 2 domains, and a serine protease domain—and is encoded by a gene of approximately 21 kb located on chromosome 11. FXa activates prothrombin on the surface of platelets in the presence of FV and calcium. During prothrombin cleavage, the activation peptide fragment 1 + 2 is released.[20]

Prothrombin deficiency is probably the rarest inherited bleeding disorder, with an estimated prevalence of 1:2,000,000 in the general population.[4] Based on the measurement of prothrombin (FII) activity and antigen level in plasma, two main phenotypes are distinguished: hypoprothrombinemia (both levels are concomitantly low) and dysprothrombinemia (normal or near normal synthesis of a dysfunctional protein).[20] No living patient with undetectable plasma prothrombin has been reported so far, consistent with the demonstration that in mice complete prothrombin deficiency by gene knockout is incompatible with life, resulting in embryonic or neonatal lethality.[21]

Mutation Analysis

Genetic variants were identified all along the prothrombin gene, although frequently they involve the catalytic site, consistent with the fact that they affect the enzymatic activity of the protein.[22] Mutations in different exons may cause similar phenotypic expression of prothrombin. Most of the mutant prothrombins derive from missense mutations (80%), although insertion/deletions (10%), nonsense (6%), and splice-site (4%) mutations have also been described. The abnormalities may be classified according to the site of the defects: (a) in the activation mechanism or (b) in the protease activity of thrombin. The latter can in turn be subdivided into two subtypes: one in which there is defective amidolytic activity and another in which there is a defective interaction of thrombin with substrates because of mutations in the molecular recognition domains.[23] Other mutations indirectly affect the catalytic activity of thrombin, by perturbating of the Na^+ binding site of the enzyme. However, while *in vitro* Na^+ binding seems to play a major role in thrombin activity, the natural mutant prothrombins with altered Na^+ binding described so far (Arg517Gln and Lys556Thr, in exon 13 and 14, respectively) had only a mild hemorrhagic phenotype.[24] Recent biochemical characterization of the mutants Des-Lys9/10 and Phe299(7)Val revealed an important role of the A-chain for a correct function of the catalytic B-chain of thrombin. Perhaps these natural mutations affect the interaction of divalent metals with thrombin, recently localized at the interface between the A- and B-chain that seem to stabilize the active conformation of thrombin.[25,26]

Clinical Phenotype and Treatment

Clinically, heterozygotes with prothrombin deficiency are usually asymptomatic, while homozygotes with activity levels <10% of normal experience severe bleeding manifestations, such as spontaneous hematomas and hemarthroses, intracerebral bleeding, and gastrointestinal hemorrhages. In homozygous women, menorrhagia is frequent.[24,27]

Patients with a prothrombin coagulant activity above 20% usually do not require replacement therapy, but antifibrinolytic agents may be considered.[28] Replacement therapy is needed only in homozygous patients, in case of bleeding or to ensure adequate prophylaxis of bleeding before surgical procedures. Since no prothrombin concentrate exists, prothrombin complex concentrates (PCCs) are used to treat patients (Table 53.2),[20] because in several clinical settings, higher levels of prothrombin may be achieved with PCCs without the harm of potential volume overload induced by FFP. However, all PCCs contain other vitamin K–dependent coagulation factors, which increase unnecessarily in plasma of patients with normal levels and could potentially cause thrombotic complications.

FACTOR V DEFICIENCY

FV is a large glycoprotein of 249 kDa, encoded by a gene on chromosome 1, synthesized by hepatocytes and megakaryocytes (Table 53.1). FV and FVIII have approximately 40% amino acid homology in their respective A and C domains and their overall protein domain structures are identical.[29] Platelets contain approximately 20% of total circulating FV.[30] FV is activated by thrombin and the resulting heterodimer FVa acts as a cofactor for FXa in the conversion of prothrombin to thrombin.[29] FV also contributes to the anticoagulant pathway by downregulating FVIII activity, so that FV deficiency also may rarely result in a thrombotic tendency.[31,32] The majority of FV deficient cases are characterized by the concomitant deficiency of FV activity and antigen levels (type I deficiency), but approximately 25% of them have normal antigen levels, indicating the presence of a dysfunctional protein (type II deficiency).

Mutation Analysis

Studies of the molecular basis of severe FV deficiency started in 1998 with the identification of the first causative mutation.[33,34] So far, a total of 56 genetic defects have been identified.[29] Of them, 48 seem to be responsible for the type I deficiency, only one being recurrent (Tyr1702Cys, repeatedly found in Italians), confirming the remarkable allelic heterogeneity of the disease. A mild type I deficiency has also been associated with two FV variants: the functional Met2120Thr polymorphism, which causes about 25% reduction in FV levels, and the HR2 haplotype defined by a group of more than 10 polymorphisms. Among them, the dominant contribution of the Asp2194Gly variant was demonstrated by expression experiments in eukaryotic cells.[35] Only one genetic defect associated with type II deficiency Ala221Val has been reported so far.[29]

A genotype/phenotype discrepancy can be observed in FV deficiency because, despite carriership of the same mutations, some patients often present a more severe bleeding pattern than others. The possibility that severe FV-deficient phenotype may be modulated by a concomitant procoagulant defect was investigated by Duckers et al.[36] showing that congenital FV deficiency associated with reduced plasma levels of tissue factor pathway inhibitor—which decrease the FV requirement for minimal thrombin generation to <1%—perhaps protects patients from severe bleeding.

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Clinical Phenotype and Treatment

Frequent symptoms in patients with FV deficiency are epistaxis and menorrhagia, which occur in approximately 50% of cases. Other less common symptoms include hemarthroses and hematomas, which occur in about 25% of patients, whereas life-threatening episodes in the gastrointestinal tract and in the central nervous system are rare (6%).[37]

There is no FV concentrate available and FV is not present in significant amount in cryoprecipitate nor in PCCs.[38] Thus, replacement therapy of FV is only achieved through FFP, preferably virus-inactivated (Table 53.2).[2] Platelets contain FV, so that platelet transfusions have been sometime used therapeutically, particularly in patients with FV deficiency owing to the development of a specific inhibitor. In FV deficiency, this limited availability of therapeutic weapons should prompt pharmaceutical companies to develop and make available plasma-derived or recombinant FV products.

COMBINED DEFICIENCY OF FACTOR V AND FACTOR VIII

Combined FV and FVIII deficiency is an autosomal recessive bleeding disorder with an incidence of about 1 in million, and it is a clinical entity distinct from FV deficiency and FVIII deficiency[8] that are transmitted with different patterns of inheritance (autosomal recessive for FV, X-linked for FVIII) and involve proteins encoded by two different genes. The molecular mechanism of the combined deficiency (F5F8D) was not understood until 1998,[39] when it was causally associated with mutations in the *LMAN1* (previously named as *ERGIC-53*), encoding a 53 kDa type-1 transmembrane protein chaperoning the intracellular transport of both factors.[40,41] In 2003, another locus associated with F5F8D was identified in about 15% of affected families with no mutations in *LMAN1*: the *MCFD2* that encodes the MCFD2 protein acting as a cofactor for LMAN1 through the recruitment of correctly folded FV and FVIII in the endoplasmatic reticulum.[42,43]

Mutation Analysis

LMAN1 is encoded by a gene of approximately 29 kb located on chromosome 18 and containing 13 exons, while MCFD2 is encoded by a shorter gene of approximately 19 kb located on chromosome 2 and containing 4 exons. To date, a total of 48 mutations are described (32 in *LMAN1* and 16 in *MCFD2*). All the *LMAN1* mutations reported so far are null mutations, with the exception of a cysteine-to-arginine mutation that disrupts a disulfide bond required for oligomerization and destabilizes the protein. In contrast, both null and missense mutations have been identified in *MCFD2*. In particular five of six reported missense mutations (Asp81Tyr, Asp89Ala, Asp122Val, Asp129Glu, and Ile136Thr) disrupt LMAN1 binding, indicating that LMAN1 and MCFD2 must function as a unit to transport FV and FVIII.[43] Zhang et al.[44] have performed a genotype–phenotype analysis to evaluate if mutations in the two different genes are associated with differences in FV and FVIII plasma levels. Mean plasma levels in patients with MCFD2 mutations were significantly lower than those in patients with LMAN1 mutations, suggesting that MCFD2 may play a primary role in the export of FV and FVIII from the endoplasmatic reticulum. The *MCFD2* IVS2+5G>A appears to be one of the most common mutation causing F5F8D, being identified in at least 13 unrelated families from different geographic regions (India, Italy, United States, Serbia, and Germany).[7]

Clinical Phenotype and Treatment

Combined F5F8D is characterized by concomitantly low but measurable levels (usually 5% to 20%) of the two coagulation factors, both as coagulant activity and antigen. The concomitant presence of two coagulation defects does not appear to enhance the hemorrhagic tendency observed in each defect separately. Thus, symptoms are usually mild, with a predominance of easy bruising, epistaxis and bleeding after dental extractions.[45] Menorrhagia and postpartum bleeding are also reported in some affected women. More severe symptoms (such as hemarthroses, gastrointestinal, central nervous system, and umbilical cord bleeding) occur very seldom. Soft tissue hematomas are also unusual.[45]

As there are no FV concentrates available, replacement of FV and FVIII can currently be achieved only through the use of FFP, preferably virus-inactivated,[38] which contains both. For FVIII replacement, a large number of products are available: the synthetic hormone desmopressin (DDAVP) can be successfully used for minor bleeding episodes to raise FVIII only.[10]

FACTOR VII DEFICIENCY

Factor VII is a vitamin K–dependent glycoprotein of approximately 50 kDa that circulates in plasma in two forms, the majority as a single-chain inactive form (with a concentration of 0.5 μg/mL) and in a much smaller amount as the active two-chain form. The FVII gene maps to chromosome 13, spans approximately 12 kb of DNA, and consists of nine exons encoding a mature protein of 406 amino acids. Through its interaction with tissue factor, FVII is fundamental for the initiation of coagulation.[46]

FVII deficiency is the most common RICD (1:500,000).[47] Plasma levels of FVII coagulant activity (FVII:C) and FVII antigen (FVII:Ag) are influenced by a number of genetic and environmental factors (sex, age, cholesterol, and triglyceride levels) and by *FVII* polymorphisms.[48] The majority of patients have concomitantly low levels of FVII:C (measured with functional assays) and FVII:Ag (measured with immunologic assays), but several cases are characterized by normal or low borderline levels of FVII:Ag contrasting with lower levels of FVII:C.[49]

Mutation Analysis

The molecular basis of FVII deficiency is more extensively characterized than those of other defects, owing to the relatively high frequency of this defect and small size of the gene.[50] Numerous mutations (available at online database http://europium.csc.mrc.ac.uk) are described. Missense mutations are the most frequent (70% to 80%), and splice-site changes are also well represented, while nonsense mutations and small deletions are rare, particularly in the homozygous condition.[50] Mutations are located throughout the gene, suggesting that all domains are important in maintaining the structure and function of FVII. The severely deficient cases are either homozygous or double heterozygous for mutations that disrupt the expression of the protein (e.g., deletions, insertions, splice-site and promoter mutations), resulting

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in FVII:C levels typically <1% of normal. Individuals with a mild/moderate clinical phenotype are homozygous or doubly heterozygous for missense mutations; however, some missense mutations are also associated with a severe phenotype.[51,52]

The most frequently reported mutations (Ala294Val, Ala294Val, 11125delC, Arg304Gln, and Cys310Phe) are associated with all ranges of clinical severity. Neither FVII:C nor gender provides an explanation for this heterogeneity, suggesting that other modifying factors may influence the different clinical manifestations in patients carrying the same mutation. FVII levels are modulated by *FVII* polymorphisms that are associated with up to fivefold differences in mean FVII levels. Tightly linked polymorphisms in the 5′ regulatory region regulate FVII levels and differential promoter activity *in vitro*. The Arg353Gln substitution in the catalytic domain, tightly associated with the decanucleotide insertion (g.–323–324insCCTATATCCT) and with the −401T allele in the promoter region, decreases FVII secretion, thereby reducing plasma levels. The same effect is given by the g.11293–11294insAA polymorphism in the 3′ untranslated region of a *FVII*, which negatively affects the steady state of mRNA levels and leads to a reduction of FVII mRNA by 40%, which in turn reduces FVII protein levels.[53] An additional polymorphism, located in intron 7 of *FVII* and characterized by the presence of a variable number of 37–base pair (bp) repeats, is associated with variable rates of FVII transcriptional activity.[50]

Clinical Phenotype and Treatment

The severity of symptoms of FVII deficiency is quite variable. Some patients with severe deficiency do not bleed at all after major challenges of hemostasis. Life- or limb-endangering bleeding manifestations are relatively rare, the most frequent symptoms being epistaxis and menorrhagia.[54] The high incidence of central nervous system bleeding (16%) reported by Ragni et al.[47] in a series of 75 patients affected by FVII deficiency was not confirmed by two subsequent large series of Italian and Iranian patients.[51,55] Thrombotic episodes (particularly deep vein thrombosis), have also been reported in 3% to 4% of patients with FVII deficiency, particularly at the time of surgery and replacement treatment, but spontaneous thrombosis may also occur.[56] The mechanism of these complications is obscure.

Current therapeutic options include, PCCs, plasma-derived FVII concentrates, and recombinant FVIIa (Table 53.2),[46] because, the very short half-life of FVII makes it difficult to use FFP without causing volume overload. Recombinant FVIIa is the optimal replacement therapy, since it can be used at a very low dosage (10 to 20 μg/kg).[57] Pregnancy does not require special precautions and uncomplicated delivery is possible with no prophylaxis. However, all postpartum bleedings reported so far occurred in women with relatively low FVII coagulant activity (<15 IU/dL) not on replacement therapy. Therefore, delivery should take place under the coverage of short-term replacement.[46] It must be noticed that rFVIIa given for inherited FVII deficiency has sometime caused formation of a specific inhibitor.

FACTOR X DEFICIENCY

Factor X occupies a key position in the coagulation cascade, as its activated form is the first enzyme in the common pathway of thrombin formation.[58] The gene for FX is 22 kb long, is located on chromosome 13 and encodes a protein of 59 kDa. FX synthesis occurs in the liver and circulates in plasma at a concentration of 10 μg/mL as a two-chain molecule (Table 53.1). Activation of FX occurs by means of a tenase complex consisting of tissue factor, FVIIa, calcium ions, and a suitable phospholipid surface. FXa is the most important activator of prothrombin and the presence of the prothrombinase complex (FXa, FVa, calcium ions, and a suitable negatively charged phospholipids membrane) accelerates 280,000-fold the conversion of prothrombin to thrombin.[59] Severe (homozygous) FX deficiency is inherited as an autosomal recessive disorder with an incidence of 1:1,000,000 in the general population. The clinical phenotypes of FX deficiency are characterized by concomitantly low levels of coagulant activity and antigen but also by low coagulant activity contrasting with normal or low borderline antigen values.[60]

Mutation Analysis

To date, 105 mutations including 85 missense, 14 insertion/deletions (3 gross deletion + 11 micro insertions/deletions), 6 splice sites, 2 nonsense, and 1 in the 5′ flanking region, are reported. Although most mutations are in exon 8, the number of mutations located in each exon is proportional to the length of the exon itself, indicating the absence of a hotspot region on *FX*. Among the reported mutations, only one (C61X) was identified in the homozygous state in three unrelated patients. Moreover, only a few of the entire group of missense mutations were found in more than one family. In particular the Arg-1Thr, associated with a severe phenotype (FX:C <1%), was identified in homozygous state in four unrelated patients from Iran, while the Gly380Arg, associated with intracranial hemorrhages, was identified in six homozygous patients from Costa Rica. The Pro343Ser (FX Friuli), associated with reduced FX:C (4% to 9%), was identified in more than 10 patients coming from Northern Italy. Other mutations such as Gly-20Arg, Gly94Arg, and Gly222Asp, all associated with a severe laboratory phenotype, were reported from different countries.[58] No association was found between polymorphisms located in the promoter region and FX plasma levels.[61]

Clinical Phenotype and Treatment

Severely affected individuals (FX:C <1 IU/dL) sometimes present in the neonatal period with umbilical-stump bleeding. The most frequent symptom in FX deficiency is epistaxis, seen with all severities of deficiency. Menorrhagia occurs in half the women of reproductive age. Hemarthroses, severe postoperative hemorrhage, and central nervous system hemorrhage have also been reported.[62–64]

The treatment of FX deficiency patients is based upon the administration of FFP and PCCs (Table 53.2).[58] However, PCCs may be associated with thrombotic complication owing to the attainment of high concentrations of FII, FVII, and FIX after PCCs preparations. To overcome this problem, a freeze-dried human coagulation FIX and FX concentrate (Factor X P Behring, CSL Behring) with specified amounts of FX (and FIX) has been developed. Even though pregnancy is accompanied by increased concentrations of FX, women with severe FX deficiency and a history of adverse pregnancy outcomes (such as abortions, placental abruption, or premature births) may benefit from prophylactic replacement therapy.[65]

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FACTOR XI DEFICIENCY

FXI is a homodimeric serine protease mainly synthesized in the liver and encoded by the 23 kb long *FXI* located on chromosome 4.[66] The disease is characterized by a decrease of the functional activity of this plasma protein, usually accompanied by correspondingly low FXI:Ag. More rarely, normal or borderline level of plasma FXI:Ag is associated with a dysfunctional form of the protein. The estimated prevalence of the severe FXI deficiency in most populations is about 1:1,000,000, but prevalence is much higher in Ashkenazi Jews, heterozygosity for FXI gene mutations and the corresponding mild FXI deficiency being as high as 8%.[67]

Mutation Analysis

Up to date, more than 180 mutations affecting *FXI* have been identified (available online: http://www.isth.org/default/index.cfm/publications/registries-databases/mutations-rare-bleeding-disorders/factor-xi/). The high allelic heterogeneity of FXI deficiency is highlighted by the distribution of genetic defects throughout the whole gene. Missense mutations represent more than 60% of all variants, only seven of them leading to the dysfunctional form of the deficiency.[68]

Only two mutations are responsible for most cases of FXI deficiency in Ashkenazi Jews, while mutations are more individual and private in non-Jews.[68,69] The type II Jewish mutation is a stop codon in exon 5, compatible with the observation that patients homozygous for this defect usually have undetectable levels of FXI. The type III Jewish mutation is a missense mutation in exon 9, leading to the substitution of phenylalanine at position 283 with leucine.[70,71] The mutation causes defective secretion of the protein from cells, but some FXI is ultimately produced so that these patients have measurable levels of FXI (at ~10%). Type II/III compound heterozygosity is the commonest cause of severe to moderate FXI deficiency in Ashkenazi Jews.[71]

Clinical Phenotype and Treatment

In comparison with hemophilia A or B, bleeding manifestations in FXI deficiency are much less predictable, even in severe deficiency, because bleeding is poorly related to FXI:C levels but rather to the site of injury. Thus, when a site of injury with local high fibrinolytic activity is involved (e.g., urogenital tract, oral cavity after dental extraction, or tonsillectomy), the risk of bleeding is increased (49% to 67%) in comparison to sites with less local fibrinolytic activity (1.5% to 40%).[72] Usually patients with severe FXI deficiency (1% or less) are mildly affected and most bleeding manifestations are injury related. Surprisingly patients with low but measurable levels of FXI are also mild bleeders, so that clinical phenotypes are not strikingly different in these two groups.[66,73] Women with FXI deficiency are prone to excessive bleeding during menstruation, but case series of women affected by severe FXI deficiency showed that 70% of pregnancies were uneventful despite no prophylactic treatment.[74–76] Also patients with alloantibodies that inhibit FXI do not bleed spontaneously, but only during surgery or trauma.[66] The Glu117stop mutation predisposes to the development of inhibitors.[77]

Treatment is based upon the use of antifibrinolytic agents, FFP, FXI concentrate, and low-dose recombinant FVIIa, but attention should be paid in order to reduce the risk of thrombotic complications (especially when FXI concentrates are employed), volume overload, allergic reactions, and development of anti-FXI inhibitors.[66] As surgery or trauma can be associated with excessive and prolonged bleeding unless adequately treated, a careful evaluation of patients with severe FXI deficiency is recommended prior to surgery, as well as meticulous planning of the procedure and the postsurgical course.[78] FXI has a relatively long half-life (40 to 70 hours), so that the infusion of 15 to 20 mL/kg virus-inactivated FFP on alternate days should be sufficient to keep FIX at trough hemostatic levels of 15% to 20% (Table 53.2).

FACTOR XIII DEFICIENCY

FXIII, also called fibrin-stabilizing factor, is the final enzyme in the blood coagulation pathway and functions to cross-link α- and γ-fibrin chains, resulting in a stronger clot with an increased resistance to fibrinolysis. The plasma factor consists of two catalytic A subunits (FXIII-A) and two carrier B subunits (FXIII-B). FXIII-A is synthesized in cells of bone marrow origin, while FXIII-B is produced in the liver. The corresponding genes are located on chromosomes 6 and 1.[79,80]

Inherited FXIII deficiency is an autosomal recessive disorder with a frequency of approximately 1:2,000,000 in the general population. In FXIII deficiency, plasma levels of FXIII-A, measured as functional activity or immunoreactive protein, are usually extremely reduced, whereas the FXIII-B subunit is reduced but always at measurable levels.[79] In 1999, the Scientific and Standardization Committee of the ISTH approved a new classification at the DNA level: FXIII-A deficiency (former type II deficiency), FXIII-B deficiency (former type I deficiency), and a possible combined deficiency FXIII-A and FXIII-B.[80]

The diagnosis of FXIII deficiency is made after the demonstration of increased clot solubility in 5 M urea, monochloroacetic acid, or acetic acid. FXIII activity may also be determined quantitatively by measuring the incorporation of fluorescent or radioactive amines into proteins.[81] Specific ELISA tests have been developed to measure FXIII-A and FXIII-B antigen levels.[82] It must be emphasized that the accuracy of functional FXIII assay is at the moment rather limited and hence it is not easy to diagnose partial factor deficiencies and assess their clinical significance.

Mutation Analysis

More than 70 mutations within *FXIII-A* have been published to date and most of them are missense/nonsense point mutations. An updated listing of mutations is available (http://www.f13-database.de). Multiple missense/nonsense mutations were also reported for a number of codons (77, 260, 326, 413 and 541) with codon changes AAC-AAA and AAC-AAG.[79] In the review by Ivaskevicius et al.[83] the two most frequent mutations affecting *FXIII-A* were the IVS5-1 G>A splice-site and the Arg661stop mutations. Mutations are scattered throughout the *FXIII-A* and in a few studies the mutant proteins were expressed in cultured mammalian cell lines, showing that the introduction of the mutation often caused destabilization of the protein structure and intracellular degradation.

On *FXIII-A* several polymorphisms have been identified. The most common are Val34Leu, Tyr204Phe, Pro564Leu,

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Val650Ile, and Glu651Gln.[83] The Val34Leu variant is the most studied, with the amino acid substitution occurring in the activation peptide sequence, three amino acids upstream from the thrombin cleavage site. The Leu34 allele variant promotes accelerated cleavage of the activation peptide once activated by thrombin and has been associated with a decreased risk of myocardial infarction, although its role in coronary artery disease is still debated.[84] In *FXIII-B*, only four mutations are identified to date: a single-nucleotide deletion at the acceptor splice junction of intron A/exon 2 (IVS1(-2)delA), a Cys430Phe missense mutation, a G deletion in exon 9, and an AAC triplet insertion within codon Tyr-80 in exon 3.[83]

Clinical Phenotype and Treatment

Individuals with FXIII deficiency have an increased tendency to bleed.[85] Animal models of factor XIII deficiency (FXIII-A knockout mice) present with intrathoracic, intraperitoneal, and subcutaneous hemorrhage.[86] In humans, bleeding from the umbilical stump in the first few days of life is common. Intracranial hemorrhage, hematomas, and prolonged bleeding following trauma are also characteristic.[87] Delayed wound healing is reported in approximately 30% of FXIII-deficient patients, while hemarthroses are less common than in hemophiliacs.[83] In a recent study made on the largest group of patients with severe FXIII deficiency (93 Iranians), the most frequent mucosal tract bleeding symptom was bleeding in the oral cavity (lips, tongue, gum) followed by menorrhagia and epistaxis.[87] Twenty percent of patients in the reproductive age had intraperitoneal bleeding that occurred at the time of ovulation, while 50% of pregnant women had at least one miscarriage.[87] The same patterns of symptoms, including the threat of intracranial bleeding in untreated patients, was also recently shown by Ivaskevicius et al.[83] in the frame of an international registry based mostly upon European data.

The clinical severity of FXIII deficiency, and particularly the high risk of intracerebral bleeding prompts regular prophylaxis.[79] This mode of treatment is facilitated by hemostatic levels of FXIII as low as 2% to 5% and the very long plasma half-life of infused FXIII (11 to 14 days), so that replacement material can be infused at longer intervals (20 to 30 days) (Table 53.2).[88] During pregnancies, monthly prophylactic infusions of FXIII concentrate are necessary to prevent abortions. There are three types of FXIII-containing products: FFP (preferably virus-inactivated), a plasma-derived, virus-inactivated FXIII concentrate, and a recombinant FXIII-A2 concentrate that contains no human or mammalian products and is manufactured in *Saccharomyces cerevisiae* (ZymoGenetics, Inc. Seattle, Washington, USA).[89] Recombinant FXIII-A2 homodimers associate in plasma with the endogenous FXIII-B to form the stable heterotetramer FXIII-A2B2. In a phase I clinical trial, recombinant FXIII-A2 was found to have a half-life similar to that of native FXIII and appeared to be safe.[89]

VITAMIN K–DEPENDENT COAGULATION FACTORS DEFICIENCY

Coagulation FII, FVII, FIX, and FX require γ-carboxylation of critical glutamic acid residues within their Gla domains to enable binding of calcium and attachment to phospholipid membranes.[90,91] The γ-carboxylation reaction is catalyzed by the hepatic enzyme GGCX that requires reduced vitamin K (KH2) as a cofactor. During the γ-carboxylation reaction, KH2 is converted to vitamin K epoxide, which is recycled to KH2 by the VKOR enzyme complex.[92,93] Inherited dysfunction of GGCX or the VKOR complex results in the secretion of poorly carboxylated FII, FVII, FIX, and FX that have poor hemostatic function. The γ-carboxylation of glutamic acid residues is also required for the activities of the anticoagulant proteins C, S, and Z.[90] The GGCX and VKOR proteins are encoded by two genes: the *GGCX* (13 kb—15 exons) located on chromosome 2 and the *VKORC1* (5,126 bases—3 exons) located on chromosome 16.

Mutation Analysis

The vitamin K–dependent coagulation factor deficiency (VKCFD) is a rare autosomal recessive bleeding disorder that arises from point mutations in either the *GGCX* (type I) or *VKOR* (type II) genes, the only exception being a kindred with a 14 bp deletion in intron 1 of *GGCX*.[92] To date a few mutations have been described for the type I disorder: Asp31Asn, Trp157Arg, Leu394Arg, His404Pro, Arg485Pro, Trp501Ser, and Thr591Lys, and two recently identified splice-site mutations: a G to T transversion of the first nucleotide of intron 2 (c. 1358+1G>T) and an A to G transversion of the third nucleotide of intron 11 (c. 10363+3A>G). The only mutation identified so far leading to type II is the homozygous Arg98Trp mutation.[94–96]

Clinical Phenotype and Treatment

Patients with severe VKCFD (i.e., factors levels below 5U/dL) are often symptomatic in the neonatal period, with umbilical stump bleeding or even intracranial hemorrhage.[97,98] Severely affected children may also have skeletal abnormalities such as nasal hypoplasia, distal digital hypoplasia, epiphyseal stippling, and mild conductive hearing loss. Older patients are usually less clinically severe and may show easy bruising and mucocutaneous or postsurgical bleeding.[99]

Treatment with oral or parenteral vitamin K1 (phytomenadione) should be started as soon as possible in all patients at diagnosis. However, large parenteral doses of vitamin K do not always correct FII, FVII, FIX, and FX activities, and there is clear biochemical evidence that the molecules are not fully carboxylated by such treatment.[90] Thus, in the group of patients with insufficient response following vitamin K1 administration, virally inactivated FFP could also be used in case of acute bleeding episodes or before surgery (Table 53.2). PCC is the alternative choice when a rapid increase of clotting factor levels is necessary. However, PCCs have been associated with thrombosis and disseminated intravascular coagulation (DIC), and therefore should be used with caution.[91]

GENERAL CONCLUSIONS

The clinical expression of the RICDs is more variable than that of hemophilia and may present challenges in both diagnosis and management. The unique proprietary nature of these mutations complicates the approach to the control of these diseases through prenatal diagnosis in families with affected members, because it renders necessary the actual identification of the underlying mutation in each kindred.

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The relation between genotype and phenotype is not always clear cut. Even though in general severe mutations predicting no protein production (stop codons, deletions, insertions, splicing abnormalities) are associated with more severe factor deficiencies and clinical phenotypes, there are a number of cases with severe factor deficiencies and phenotypes that are associated with missense mutations. However, missense mutations are usually associated with milder phenotypes in spite of undetectable factor levels, probably because some trace of protein is produced in these cases that cannot be detected with the currently available assays. The most severe RICDs are afibrinogenemia and severe FXIII and FX deficiencies, which present with the highest rate of life-threatening bleeding symptoms, whereas the least severe disease is FXI deficiency, with at least one third of patients remaining asymptomatic.

Treatment of RICDs consists in the use of the most purified blood product available that contains the deficient factor. Due to the rarity of each deficiency, specific factor concentrates are not often available as they are for hemophilia A and B. Dosages and frequency of treatment depend on the minimal hemostatic levels of the deficient factor, plasma half-life, and type of bleeding episode. General recommendations on treatment on demand, prophylaxis, and on surgery in patients with RICDs are summarized in Table 53.2. The avoidance of transmission of blood-borne infectious agents is the primary requisite in the choice of replacement material. Costs are also another important determinant. Virally inactivated factor concentrates are available for several deficiencies and should be preferred when virally inactivated plasma is not available or repeated infusions causing fluid overload are needed, as may occur at surgery or in cases of bleeding in the central nervous system. An updated registry of the available clotting factor concentrates, published by the WFH can be downloaded at the web address http://www.wfh.org/index.asp?lang=EN.

In conclusion, even though in the last few years substantially more knowledge on the prevalence, clinical manifestations, and genotype analysis of RICDs has been achieved, additional efforts are required to ameliorate the global care of RICD patients. Particular attention should be devoted to the health care status of women affected by RICDs. It is necessary to conduct appropriately designed studies for prompt diagnosis, prophylaxis, or on demand treatment that may significantly improve the usually poor quality of life of affected women. The pharmaceutical industries should devote more efforts to improve the availability of factor concentrates. An FV product and a prothrombin product are particularly needed. Finally, registries on RICDs should continue and should be funded by research and regulatory agencies, because they are the only weapons that can answer the many unsolved questions that pertain to the management of these very rare disorders[100].

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CHAPTER

54 Afibrinogenemias and Dysfibrinogenemias

DENNIS K. GALANAKIS

Congenital fibrinogen disorders have provided novel insights into structure/function relationships and into the central role of fibrinogen in hemostasis. They are generally classified as quantitative (type I, a- or hypofibrinogenemia) and qualitative (type II, dysfibrinogenemia) groups. Afibrinogenemia was first reported in 1920,[1] hypofibrinogenemia in 1935,[2] and dysfibrinogenemia in 1958.[3] The first fibrinogen mutation (Detroit) was described in 1969[4] using protein sequencing, and use of DNA sequencing was first applied in 1989 to identify fibrinogens Hershey II and Leogan.[5] Subsequent application of mass spectrometry remains the preferred method to confirm results from genetic techniques. Aside from a 1974 compilation[6] and an updated listing in *http://www.geht.org/databaseang/fibrinogen/*, recent reviews provided detailed updates of this steadily expanding area of investigation.[7–10] To date, afibrinogenemia accounts for 23%, hypofibrinogenemia for 26%, and dysfibrinogenemia for 51% of all three. Estimated from the present selection of mutations, dysfibrinogenemia accounts for 51%, afibrinogenemia for 23%, and hypofibrinogenemia for 26%. This relative frequency and and the ~0.6% prevalence of apparent congenital hypofibrinogenemia among in a 5,746 Japanese patient population[11] suggest an over a prevalence of ~2% for all three disorders, and a possibly similar prevalence in other populations. Also, with the present selection of mutations, afibrinogenemia and dysfibrinogenemia are most frequently caused by FGA and FGG mutations, 38% and 35%, respectively as compared to 27% by FGB. Apart from its hemostatic functions, fibrinogen binds to vascular endothelial and other cells, interacts with many extracellular matrix components (see Chapter 16), and enables placentation from the middle of first trimester to full term.[12–14] Moreover, fibrin provides nonsubstrate binding for thrombin, serves as platform for assembly of fibrinolytic proteins, and is the preferred substrate for the protease plasmin.

AFIBRINOGENEMIA

Causative Mutations

This is an autosomal recessive disorder that often results from consanguinity. Mutations that cause afibrinogenemia (Table 54.1) are typically homozygous causing deficiency at the DNA or at the RNA level by affecting mRNA splicing or stability, or by affecting protein synthesis, assembly, or secretion.[10,15] Since the first mutation was identified in 1999,[16] more than 80 novel mutations have been described. Null mutations are most common and include frameshift or large deletions, and early truncating nonsense, or splice site mutations. Less frequently, missense or late-truncating nonsense mutations allow synthesis of the corresponding chain but impair intracellular fibrinogen assembly and/or secretion.

Clinical and Laboratory Manifestations—Hemorrhage

Hemorrhagic diathesis commences during the neonatal period and in some cases at a later age.[17] Umbilical cord bleeding occurs in up to 85%, and other common sites are gastrointestinal tract, skin, and genitourinary tract. There is an increased incidence of splenic rupture,[18] and intracranial bleeding is the major cause of death. Hemarthrosis and/or muscle hematomas are reported in about half. In menstruating women, menometrorrhagia is common and spontaneous first-trimester abortion is typical.[19] Predictably, clotting time is infinite, bleeding time is prolonged, and fibrinogen-dependent platelet aggregation is impaired.[20] Also, erythrocyte sedimentation rate is decreased,[21] and delayed hypersensitivity skin test reaction sites do not develop the normal induration.[22]

Thrombosis

Paradoxically, venous or arterial thrombosis occurs in some probands.[23] Thrombosis risks such as antithrombin III, protein C deficiency, or others, present in few probands,[24] are absent in most.[25] Among six unrelated probands,[25] arterial thrombosis sites included iliac, hypogastric, and other peripheral arteries. A prevailing explanation is that in the absence of fibrin, unsequestered thrombin induces vaso-occlussive platelet aggregate formation. This is based on evidence that fibrin sequesters thrombin,[26,27] and that in a fibrinogen knockout mouse model[28] spontaneously formed platelet aggregates embolized six times more frequently than those of wild-type controls and were often vaso-occlusive. Also, plasma levels of prothrombin activation peptides and of thrombin–antithrombin complexes are elevated in such probands, and both normalize following fibrinogen replacement.[29,30] Among eight unrelated probands with venous thromboembolism (VTE), the affected veins included portal vein, mesenteric vein, cerebral sinus, and others.[25] An intriguing feature is the association with fibrinogen infusion in five probands with venous and two with arterial thrombosis, suggesting a causative role by infused fibrinogen. Among possible explanations is substantial enrichment of plasma cryoprecipitate, and possibly other fibrinogen preparations, with soluble fibrin/fibrin(ogen) complexes[31,32] known to shorten fibrinogen clotting times.[33] Also, active thrombin was demonstrated in such complexes from a dysfibrinogenemic proband with recurring thrombosis.[34] Such thrombin may be released by proteolytic degradation of the complexes at the wound or other sites. Suggesting another possible mechanism, fibrinogen excess decreases plasminogen binding to fibrin polymers.[35]

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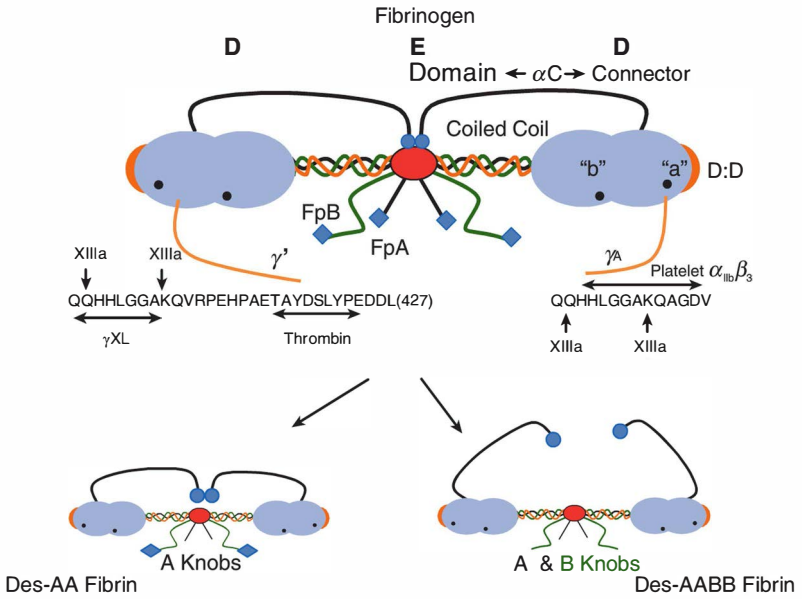

FIGURE 54.1 Schematic representation of functional regions of fibrinogen and fibrin. The C-terminal sequences of the γ_A and γ' chains are amplified for clarity, showing the high affinity thrombin binding, platelet interaction, and factor XIIIa catalyzed cross-linking (XL) sites. Elsewhere on the D region are the 'a' and 'b' pockets, and the D:D interface indicating the sites between D s of two molecules that associate during fibril elongation. The lower left panel indicates the slight weakening of αC tethering to the E region by FpA release, reflecting the contributing role to the tether by intact FpA.[131] The lower right panel illustrates the untethering and dissociation of αC s following FpB release.[128,132]

HYPOFIBRINOGNEMIA

Mutations that cause afibrinogenemia are usually homozygous and in a few cases compound heterozygous. They cause decreased plasma concentrations of normal fibrinogen when they are heterozygous (Table 54.2), often with decreased assembly and/or secretion of the variant fibrinogen. Substitutions Aα C36G or R[36] disrupt formation of Aα36C-Bβ65C and Aα36C-γ23C bonds,[37] while AαC45Y[36] and C45F[38] disrupt formation of the AαC45-γ23C bond.[39] As detailed elsewhere,[36] these confirmed experimental data showing that these bonds are required for hexamer assembly and/or secretion. Among mutations in the FGB gene two missense, R255H[40] and D346Y,[41] and one nonsense mutation, W470X,[42] result in changes in the β-domain of the distal D nodule of fibrinogen, suggesting impaired secretion. A second nonsense mutation, Y71X,[7] likely results in impaired assembly since the encoded chain lacks the β-domain of the distal D nodule. A single splice-site mutation at the 5′ site of FGB intron 6 (IVS6 + 1G > A) results in a frameshift after P319, adding 18 novel residues prior to a stop codon.[43] Like other mutations in this region (Table 54.2), variant fibrinogen may be assembled but is not secreted. Also not secreted is fibrinogen with γC153R.[44] In two unrelated probands with hemorrhagic diathesis,[45,46] substitution A82G is accompanied by a BβP265L in one and by an FGG splice-site mutation (IVS2 + 1G > A) in the other. Considered together, these γ chain variants are likely synthesized but not incorporated into hexamers.

Four mutations result in hypofibrinogenemia, *hepatocellular intracytoplasmic* inclusions, and progressive liver disease in the same proband (Table 54.2). Three include missense mutations, γG284R (Brescia),[47] γR375W (Aguadilla),[48] and γT314P (Al DuPont),[49] all occurring within or near the "a" pocket. Defective polymerization of fibrinogen Brescia was attributed to its hypersialylation, a characteristic of fibrinogen in liver disease.[50] The fourth, a γ346-350 del (Angers)[51] occurring within the five-stranded beta sheet of the γC, results from deletion of FGG nucleotides 7690A-7704G which deletes the splice site at position 7703. This generates a new (donor) splice site at position 7688, resulting in the in-frame deletion of five amino acids (GVYYQ) 346 to 350. Interestingly, other heterozygous mutations in these fibrinogen regions result in either assembly and secretion (dysfibrinogenemia) or selective secretion of only normal molecules (hypofibrinogenemia) but no intracellular accumulation.[52]

Clinical Manifestations

Hemorrhagic manifestations are common when plasma fibrinogen levels are in the range of 0.1 to 0.5 g/L[1] (normal 1.50 to 3.50 g/L[1]) by clotting assays. Symptoms include bruising, epistaxis, menometrorrhagia, miscarriage, and hemorrhage after trauma, surgery, or during pregnancy and parturition. By contrast, probands with plasma fibrinogen at diagnosis as low as 0.60 to 0.70 g L^{-1} are usually asymptomatic. The moderately prolonged thrombin (TT) and reptilase (RT) times are corrected by equal mixtures with normal plasma. Similar results may be obtained when a variant fibrinogen comprises a minor fraction of plasma fibrinogen (hypodysfibrinogenemia).

DYSFIBRINOGENEMIA

Dysfibrinogenemia is an autosomal dominant disorder that comprises mostly heterozygous mutations that result in both variant and normal fibrinogen in circulation. At least 500 families are known to date. Predictably,[53] plasma fibrinogen consists of normal and abnormal homodimers and of heterodimers, and its level by clotting assays is typically decreased, less than half that by antigen assay which is usually in the normal range. In a minor proportion of mutations (Table 54.2), fibrinogen is decreased by both assays and it is known as hypodysfibrinogenemia.

Defects that Impair Conversion of Fibrinogen to Fibrin

Following fibrinopeptide cleavage, fibrin monomers self-assemble to form protofibrils that polymerize by lateral contact to form fibers and networks (FIGURE 54.2). The specific interactions resulting in normal fiber formation have been elucidated in x-ray crystallographic structures of fibrinogen fragments D and factor XIIIa-cross-linked

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Table 54.1

Mutations resulting in afibrinogenemia

FGA

cDNA	Mature Chain	Type	Reference
4.1 kb del		Large del of exon 1	209
c.3-4insC	F17Lfs	Fs, exon 1	210
c.54+1G>A		Spl. site mutation, intron 1	211
c.54+3A>G		Spl. site mutation, intron 1	210
11 kb del		Large del, exons 2–6, recurrent	210
c.94G>T	G13X	Nonsense mutation, exon 2, escapes nonsense-mediated decay (NMD)	212
c.117Tdel	V21Wfs	Fs mutation, exon2, escapes NMD	212
C196-197InsT	S47Ffs	Fs mutation, exon 3	211
c.209T>G	M51R	Missense mutation, exon 3, impairs fibrinogen secretion	213
c.229-231del3ins12	V58insPLMX	Del and ins exon 3, premature stop codon	214
c.285T>A	Y76X	Nonsense mutation, exon 3	215
c.356C>G	S100X	Nonsense mutation, exon 3 escapes NMD	213
c.364+1-+4del(GTAA)		Spl. site mutation, intron 3, causes intron skipping	215, 216
c.385C>T	R110X	Nonsense mutation, exon 4, escapes NMD	212
c.431-432del(AA)	K125Sfs	Fs mutation, exon 4	210
c.448C>T	Q131X	Nonsense mutation, exon 4	217
c.502C>T	R149X	Nonsense mutation, exon 4, escapes NMD	212, 218
c.510+1G>T		Spl. site mutation, intron 4, causes cryptic spl. site use	215,216
15 kb del		Large deletion exons 5 and 6	219
c.541C>T	R162X	Nonsense mutation, exon 5	220
c.563-564insT	L169Ffs	Fs mutation, exon 5	220
c.607C>T	Q184X	Nonsense mutation, exon 5	221
c.609-610insTGA	L185X	Insertion, stop codon, exon 5	211
c.635T>G	L166X	Nonsense mutation, exon 5	220
c.711-712insT	K219X	Fs mutation, exon 5	222
c.743G>A	W229X	Nonsense mutation, exon 5	220
c.786-789delGAGA	E243Dfs	Fs mutation, exon 5 predicted to encode 157 aberrant aa	223
c.835delA	T260Pfs	Fs mutation, exon 5, predicted to encode 141 aberrant aa	224
c.885G>A	W276X	Nonsense mutation, exon 5	220
c.934delA	S293Afs	Fs mutation, exon 5 predicted to encode108 aberrant aa	215
c.945delT	G297Efs	Fs mutation, exon 5, predicted to encode 103 aberrant aa	215
c.946G>T	G297X	Nonsense mutation exon 5	210
c. 1001G>A	W315X	Nonsense mutation exon 5	210
c.1025delG	G323Efs	Fs mutation exon 5, predicted to encode 78 aberrant aa	220
c.1037delA	N327Tfs	Fs exon 5, predicted to encode 74 aberrant aa	220
c.1055delC	P333Lfs	Fs mutation exon 5 predicted to encode 68 aberrant aa	210
FGB			
c.114+ 2076A>G		Mutation in intron 2 causes expression of a 50 bp cryptic exon	226
c.139C>T	R17X	Nonsense exon 2	227
c.248-249delAGinsT	K53Ifs	Fs exon 2	220

(Continued)

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Table 54.1

Mutations resulting in afibrinogenemia *(Continued)*

c.605T>A	L172N	Missense mutation exon 4 leads to aberrant splicing	228
c.887G>A	W266X	Nonsense mutation exon 6	214
c.958+13C>T		Splice site mutation intron 6	229
c.1148T>G	L353R	Missense exon 7 impairs secretion	230
c.1244+1G>T		Splice site mutation intron 7	229
c.1289G>A	D400D	Missense exon 8 impairs secretion	230
c.1330G>C	G414S	Missense exon 8 impairs secretion	231
c.1399T>G	W437G	Missense exon 8 impairs secretion	232
c.1400G>A	W437X	Nonsense exon 8 impairs secretion	233
FGG			
c.78+5G>A		Spl. site mutation	234
c.98delA	N7Tfs	Fs in exon 2	215
c.124-3C>G		Spl. site mutation intron 2	215
c.307+5G>A		Spl. site mutation intron 3	220
c.400C>T	R108X	Nonsense, split between exons 4 and 5, escapes NMD	9
c.448delC	L124X	Fs exon 5 creates stop codon	5
c.666+660A>T		Intron 6 causes 75bp cryptic exon inclusion	9
c.667A>T	R197X	Nonsense exon 7	225
c.759G>T	E231X	Nonsense exon 7	9
2395G>A (5th nt of IVS-3)	77Stop,	impaired hexamer assembly	250

Del, deletion; Ins, insertion; Spl, splice; Fs, frame shift, bp, base pairs.

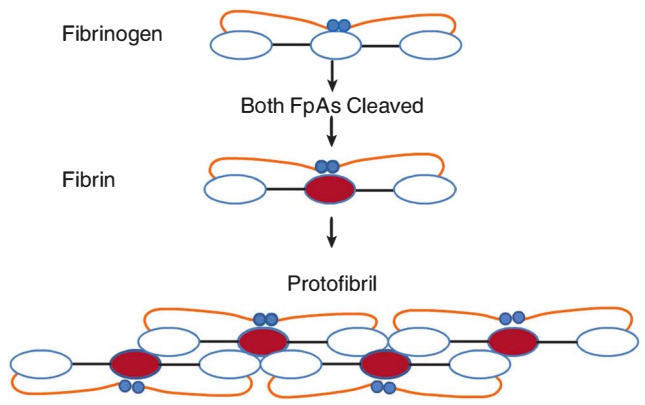

FIGURE 54.2 Schematic representation of fibrinogen and its conversion to fibrin. Release of FpA initiates polymerization. Each central (or E shown in red) fibrin region associates with the outer (or D) regions of two other molecules initiating a double stranded, half staggered, two molecule thick fibril, known as protofibril. As elongation progresses FpB release, significantly slower than that of FpA, is accelerated. Following FpA release, illustrated in Fig. 54.1, the αCs become untethered and dissociate. Each free αC associates with one or more counterpart(s) on neighboring fibrils (not shown) and in this manner promotes lateral assembly.

fragment D-D in the presence of peptides GlyProArgPro-amine (GPRPam) and GlyHisArgPro-amine (GHRPam), which mimic the *N*-terminal α and β sites of fibrin, respectively.[54–57] Substitutions occurring within these crystal structures have enabled correlations of clinical and biochemical data with the three-dimensional fibrinogen structure. Mutations in the E region (Table 54.2) impair one or more of the following functions: thrombin binding to fibrinogen, fibrinopeptide cleavage, and fibrin polymerization.

Defects in the "A" Knob

Variants include G17V (Bremen), P18L (Kanazawa and Kyoto II), R19S (Detroit), R19G (Oslo IV, Aarhus, Mannheim I, Matsumoto V, and Milano XIII), R19N (Munich I), and V20D (Canterbury). Structural data indicate that the free amino group of the "A" mimic (GPRPam) interacts directly with the side chain of residue γD364 in the "a" pocket, suggesting that the side chain is not critical.[54] Apparently, substitution of a Val for Gly17 impairs both thrombin-catalyzed fibrinopeptide A (FpA) release and polymerization since the peptide Val-Pro-Arg-Val partially inhibits fibrin polymerization.[58] Structure data also show that the positively charged side chain of R19 interacts with residues in the "a" site, such as γD330. Consequently, substitutions with Gly, Ser, or Asn will abolish these interactions. The substitution of Leu for Pro 18 likely alters the structure of the "A" knob and thereby impairs the strength of the "A":"a" interaction.

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Table 54.2
Mutations resulting in hypofibrinogenemia

FGA

Name, Reported Nucleotide Defect	Peptide Defect	Site	Clinical Feature(s)	Type Reference
–1138C>T, upstream of FGA	(decreases enhancer activity)	Promoter		MS[235]
c.180+2T>C, spl. site		FGA intron 2		Spl[236]
Le Seyne, 6292T>G	Aα C36G	E		MS[36]
Marseille, 6292T>C	Aα C36R	E		MS[36]
Marseille II, 6780G>A	Aα C45Y	E		MS[36]
Cremona, 1749G>T	Aα C45F	E		MS[38]
Caracas VI	Aα N80del	CC	Hem. Thr.	Del[119]
Montreal, c.1543G>A	Aα D496N	αC-dom.		MS[237]
Grand Lyon, 5011-5012 replaced by TTGGAAATTT>Fs	A α last 62 residues replaced by 72 new residues	αC-dom.	Hem.	Fs[209]

FGB

c.958+1G>A		Spl. site		Spl.[42]
Tottori II, 3356T>G	Bβ Y41Stop	E	Hem.	Stop[238]
3404A del	Bβ 59PFs	E		Fs[239]
	Bβ Y71X	E		NS[7]
Lyon, 4075T>A	Bβ M118K	CC	Hem. Misc'ge	MS[162]
	Bβ L202Q/R47X	D/CC		MS/NS[238]
Paris IX, 5909A>G/IVS7-1G>C	Bβ Y236C	D	Hem. Thr. Misc'ge	MS[163]
Merivale, 6654G>A, Hannover XII, homoz.	Bβ R255H	D	Hem.	MS[40,240]
Hannover XV	Bβ R255C	D		MS[240]
Nottingham II	Bβ R294G	D		MS[241]
	Bβ D316Y	D		MS[242]
IVS6+1G>A, Fs	Bβ P319+ 18 residues	D		Stop[229]
7611 A>G	Bβ N320G	D		MS[41]
Villeurbanne II, c.1067A>G	Bβ Y326C	D	Hem.	MS[243]
	Bβ D346Y	D		MS[42]
Patient A, 7893 CAG>TAG	Bβ G393X	D	Hem.	NS[41]
	Bβ Q396X	D		NS[41]
	Bβ W402X	"b"		NS[162]
c.1328A>G	Bβ A413S	"b"	Hem.	MS[244]
c.1331G>C	Bβ G414A	"b"	Hem.	MS[244]
Hannover XVIII/XX and Unicov	Bβ G414S	"b"		MS[240]
7969>7972, GGGG>GGG	Bβ G419V, Fs 419>434, 435Stop	"b"	Hem.	Stop[245]
Lyon II, 7919A>G	Bβ W432X	"b"		NS[162]
Mumbai, homoph.	Bβ G434D	D		MS[246]
	Bβ G444S/W47X	D		MS/NS[247]
Spl. site	Bβ 440-461del	D		Del[248]
	Bβ W470X	D	Hem.	NS[249]
	Bβ D496N	D		MS[243]

(Continued)

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Table 54.2

Mutations resulting in hypofibrinogenemia *(Continued)*

FGG				
2525C>G, spl. site G>A	γA82G, homoz.	CC	Severe Hem. Misc'ges	MS[164,251]
Poznan, c.331A>T	γ85KStop	CC	Hem.	Stop[252]
Matsumoto IV	γC153R	D		MS[44]
Colombus. c.677G>T	γG200V	D		MS[153]
Bratislava, Imp'd secr'n	γW227C	D	Hem.	MS[253]
Lyon IV, 5857A>C	γN230H	D		MS[162]
Middlemore	γN230D	D		MS[254]
Lyon IV	γN256H	D		MS[162]
Brescia	γG284R	DD	Hep. Storage	MS[47]
Dorfen	γA289V	DD	Hem.	MS[255]
Hillsborough	γG309D	D		MS[256]
Manheim II	γH307Y	D		MS[257]
7590G>A	γS313N	D		MS[258]
Al du Pont, c.1018C>A	γT314P	D	Hep. storage	MS[49]
	γD316Y	D		MS[242]
Patient B, 7611A>G	γD320G	D		MS[41]
Lyon III, c.1099G>A	γA341T	"a"		MS[243]
Tolaga Bay, c.1100C>T	γA341V	"a"		MS[96]
Impaired secretion	γN345S	"a"	Hem.	MS[38]
Angers, 7690A-7704G del	γ346-350del	"a"	Hep. Storage	Del[51]
Muncie, C>T	γT371I	"a"	Hem.	MS[259]
Aguadilla, C>T	γR375W	"a"	Hep. Storage	MS[48]
Matsumoto VII, 7651G del	γ387Ifs>25 res>Stop	D		Fs[260]

Misc'ge, miscarriage; Spl., splice; Fs, frameshift; MS, missense; NS, nonsense; Hem., hemorrhagic; E, E region; "a", "a" pocket; "b", "b" pocket; D, unspecified D region site(s); Dom.: domain, CC, coiled coil.

Unexpectedly, the heterozygous Aα V20D substitution[59] results in absence of residues Aα1-20, thus explaining the approximately half of normal amount of released FpA. This apparently reflects a new furin-susceptible Aα20-21 bond, which enables intracellular removal of the mutant peptide.

Defects in FpA Release

Substitutions at AαR16 are most frequent with over 80 unrelated families known to date. Most are heterophenotypic and asymptomatic. They include either AαR16H or AαR16C and one AαR16S family.[60] Unique to AαR16H is the slower FpA than fibrinopeptide B (FpB) release.[32] In contrast, thrombin does not cleave FpA from AαR16C fibrinogen, although it does from its modified (e.g., S-aminoethylated) isolated chains.[61,62] In the rare homophenotypic probands (i.e., all fibrinogen consists of abnormal homodimers) with either AαR16H[32,63] or AαR16C,[64–66] clotting times are prolonged by the former and infinite by the latter. In such AαR16C probands, this reflects fibrinogen incoagulability, since FpB is releasable, but no clot forms under physiologic conditions.[67] In the case of AαR16H, the reversed FpA/FpB release sequence results in clots formed when most if not all FpB and minor amounts of FpA have been released.

Substitutions of AαD7N, AαL9P, AαP11G, and AαG12V all display delayed FpA release, underscoring the importance of this region on FpA cleavage. Studies including nuclear magnetic resonance and crystallography demonstrate extensive interactions between this region and thrombin, including disruption of a critical electrostatic interaction by the AαD7N (Lille) substitution.[68] Introduction of Pro into the α-helical stretch between residues Aα7 and 10 (AαL9P, Magdeburg I) is believed to inhibit interaction with thrombin by altering conformation.[69] Similarly, the AαG12V (Rouen and Saint-Germain) substitution is predicted to disrupt the Aα1-12 β turn and slightly displace Gly13 and Gly14.[70] The Saint-Germain report also describes delayed FpB release.[71] In addition, AαE11G (Mitaka II) destabilizes the β turn and disrupts the salt bridge between fibrinogen Aα E11 and R173 of thrombin.[72]

Defects in FpB Release and B Knob

Only heterophenotypic Cys substitutions were identified at or near the Bβ14 thrombin cleavage site (Table 54.3). Cleavage of FpB exposes the *N*-terminal β chain site, "B" knob, which binds noncovalently to a complementary polymerization "b" pocket

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in the *C*-terminal region in the β chain (β397-432) of another fibrin(ogen) molecule. This interaction is modeled in the crystal structures obtained in the presence of GHRPam (see previous reviews[7,8]). Only one substitution has been found in the "B" knob: heterophenotypic Bβ G15C (Ise, Fukuoka II, Kosai, Ogasa, and Hamamatsu II). Functional studies showed normal FpA release, approximately 50% FpB release, impaired thrombin- and reptilase-catalyzed polymerization, and impaired desA- and desAB-fibrin monomer repolymerization.[73,74] In addition to impairing the function of "B" knob, these results suggest they impair that of the "A" knob whose second part[8] resides within β.15-42 The sulfhydryl group of each varied. Some formed complexes with albumin attributable to disulfide links via the albumin's solitary-free C34 sulfhydryl,[75] some formed fibrinogen–fibrinogen dimers, and some were free sulfhydryls. Three were asymptomatic and two were associated with thrombosis.

Among eight Bβ R14C unrelated probands (including those in the online registry, vide supra), six were associated with thrombotic events,[76,77] occurring at a young age in at least four, and in several probands in one family (Table 54.3). This association, however, has not occurred in every affected family and remains unclear. There was one proband with hemorrhage and one asymptomatic.[78] Release of FpA was normal and release of FpB was half-normal. Reptilase-induced polymerization (Seattle) was also impaired, suggesting that the mutation itself impairs polymerization.[78] Studies on fibrinogen Ijmuiden identified molecular species linked to albumin (40%), in free sulfhydryl form (36%), and as fibrinogen–fibrinogen dimers (30%).[77] Bβ R44C (Nijmegen, heterophenotypic) impaired fibrin polymerization. Its association with thrombosis contrasts with the same mutation in two unrelated and asymptomatic families (author's unpublished data).

Defects in Thrombin Binding

Three different Bβ chain substitutions resulted in delayed fibrinopeptide release linked to impaired thrombin binding. One was the homophenotypic Bβ A68T (Naples) substitution that impaired thrombin binding (<10%) to fibrin and markedly delayed FpA and FpB release.[79] Crystallographic data suggest that this substitution impairs interactions between fibrinogen and thrombin exosite I.[80] Other studies indicate that binding of thrombin to low-affinity sites (located in the E region) is abolished and high-affinity binding to the γ′ chain is impaired.[81] The second is a heterophenotypic fibrinogen (New York I) with a large deletion of residues Bβ9-72, encoded by FGB exon 2.[82] Its delayed FpA and FpB release is also attributable to diminished thrombin binding. A third substitution, a heterophenotypic Bβ A61K (Miami),[83] is associated with repeated spontaneous abortions and impaired polymerization. A reptilase-incoagulable subfraction (60% of plasma fibrinogen) displayed thrombin binding <10% of normal controls (author's unpublished data).

Defects that Impair Fibrin Polymerization

The intermolecular D:E interaction results in half-staggered, double-stranded fibrils (FIGURE 54.2) and lateral association of fibrils with fibers. More specific interactions have been elucidated in x-ray crystallographic structures of fibrinogen fragments D and FXIIIa–cross-linked fragment D-D in the presence of the peptides GPRPam and GHRPam, which mimic the *N*-terminal regions of the α and β chains of fibrin, respectively.[54,56,57,84] Identification of specific amino acids in these structures and their substituted counterparts caused by mutations enabled a correlation of corresponding biochemical data with the three-dimensional structure. Also, binding of calcium to high-affinity sites γAsp318, Asp320, Phe322, and Gly324[84] is impaired by mutations in this stretch.

Defects within the "a" Pocket

This pocket encompasses γ chain residues 337 to 379. Crystallographic studies showed that Q329 shifts on GPRPam binding; the substitution γQ329R (Nagoya) may disrupt this shift, thereby impairing polymerization.[85] Substitutions γD330V, Ales[86] and Milano I,[87] and D330Y, Kyoto III[88] disrupt ionic interactions with Aα R19. Biochemical analyses of these variants demonstrate that the Aα R19 residue is critical for polymerization.[86,88] Similarly, substitutions γD364 to His and Val, Matsumoto I[89] and Melun[90] respectively, disrupt ionic interactions with the free amino group of the "A" knob mimic, and both substitutions impair polymerization. A γ-chain insertion of 15 residues between γQ350 and γG351, with a concomitant G351S change (Paris I), was first noted in the protein[91,92] and later defined by genetic analyses.[93] The inserted segment has two Cys residues that likely form a disulfide bond, which alter conformation around residues γ350 and γ351. These alterations can account for the impaired polymerization, factor XIIIa cross-linking, and platelet binding.[94] A study of three γ364 substitutions, Ala, His, or Val, disclosed coagulability with normal FpA release but absence of "A": "a" interactions, implying that "B": "b" interactions enabled polymerization.[95] This is in general agreement with the polymerization behavior of homophenotypic Aα R16H (Kingsport).[32] Two polymerization-impairing mutations near the "a" pocket resulted in novel glycosylation γA327T (Tokyo V) and γK380N (Kaiserslautern I). Impaired polymerization by the latter with a new glycosylation site at N380 was corrected by desialylation, suggesting the extra sialylation impaired polymerization. Substitutions γA341D and γA341V[96,97] resulted in impaired "A":"a" interactions and impaired calcium binding,[98] implying that this residue is required for both functions. Two novel Cys substitutions within the "a" pocket were covalently linked to albumin, γY354C[99] (Homburg VII), and γS358C[100] (Milano VII). Both impaired polymerization, and this was not corrected by removal of albumin from Milano VII, suggesting it was attributable to γ358C *per se*.

Defects within the D:D Interface

This interface, first seen in the crystal structure of D:D,[84] encompasses γ275R-300S, near or within which many mutations with variable clinical presentation were reported. Among them are γG268E (Kurashiki), γR275C (numerous unrelated families), γR275H (numerous), γR275S (Kamogawa), γA279D (Auckland), γY280C (Banks Peninsula), γN308I (Baltimore III), γN308K (several), γ309D (Hillsboro), and γM310T (several). By scanning electron microscopy, γR275C (Tokyo II) clots displayed altered network structure,[101,102] which along with other studies underscores the importance of the D:D interface in polymerization. Similarly, impaired polymerization by γA279D and γY280C most likely reflects altered D:D interface, as suggested by evidence of a direct intermolecular γ280:γ275 interaction.[84] The crystal structure also shows γ308 and γ310 lying on the D surface juxtaposed to γ275, intimating altered D:D interface by each mutation. Consistent with this, electron microscopic analysis showed that the abnormal clot structure of γM310T is virtually normalized by enzymatic removal of the novel γ308N-linked carbohydrate cluster.[103]

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Table 54.3

Mutations resulting in dysfibrinogenemia

Mature Peptide Change (Name)	Dysfunction	Site	Clinical	Reference
Defects in the E Region				
AαD7N (Lille)	FpA & Polym	Thr. bdg.		68,261
AαL9P (Magdeburg I)	FpA & Polym	Thr. bdg.		69
AαE11P (Mitaka II)	FpA & Polym	Thr. bdg.	Hem.	262
AαE12V, (Rouen, St. Germain) homoz.	FpA & Polym	Thr. bdg.	Hem.	70,71
AαR16C (Many)	FpA & Polym	Aα16-17		6,263
AαR16C, homoz. (Metz, Frankfurt XIII)	FpA & Polym	Aα16-17	Hem.	64
AαR16H (Many)	FpA & Polym	Aα16-17		263
AαR16H, homoz. (Giessen + two others)	FpA & Polym	Aα16-17	Hem.	32,63,264
Aα R16S (Frankfurt XI)	FpA & Polym	Aα16-17		60
AαG17V(Bremen I)	FpA & Polym	"A" knob	Hem.	58
AαP18L (Kyoto II, Kanazawa)	Polym	"A" knob	Thr.	265,266
AαR19S, homoz. (Detroit)	FpA & Polym	"A" knob	Hem.	4
AαR19N(Munich I)	FpA & Polym	"A" knob	Hem.	267
AαR19G (Aarhus, + 4 others)	FpA & Polym	"A" knob	Hem.	268,269
AαV20D (Canterbury)	FpA & Polym	"A" knob	Hem.	59
BβR14C (Ijmuiden, + 3 others)	FpB Polym	Bβ14-15	Hem./Thr.	77,270,271
BβG15C, homoz. (Hamamatsu II,+ 4 others)	FpB Polym	"B" knob		272
BβR44C (Nijmegen)	Polym	"B" knob		77
BβA61K (Miami)	Polym	Thr. bdg.		83
BβH67L (Sumperk) homoz.	Polym	Thr. bdg.		273
Defects in the Coiled-Coil Region				
BβS111Del (Kyoto IV)	Polym (Augmented)	CC		120
BβM118K (Lyon)		CC	Hem. Thr.	162
Aα Chain				
AαN80 del (Caracas VI)	Polym	CC	Hem./Thr.	119
AαN106D (Plzen)	Polym	CC	Thr.	274
AαR141S, homoz. (Lima)[a]	Polym	CC	Hem.	124
Bβ111S del (Kyoto IV)	Polym	CC		120
BβK148D/BβR255H[4] (Merivale)	Polym	CC/D	Hem.	40
BβN160S (Niigata)[a]	Polym	CC		125
BβR166C (Longmont)	Polym	CC		275,276
Bβ137N-141E del (Epsom). Hypod.	Polym	CC	Hem. Misc'ge	122
Defects in the αC Region				
AαR268Qfs, homoz. (Otago), Hypod.	Polym	αC-conn.	Hem.	137
AαR289G		αC-conn.		277
AαQ328P (Seoul II)	Polym XL	αC-conn.	Thr.	278

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Table 54.3

Mutations resulting in dysfibrinogenemia *(Continued)*

Aα452 GPD–>WS-Stop) (Milano III)	Polym	αC-conn.		143
AαS434N (Caracas II)[a]	Polym	αC-conn.		133,279
AαN325I (Krakow) Hypod.	Polym	αC-conn.		280
AαD454Stop, homoz. (Nieuwegein) Endo.	Polym	αC-conn.		144
AαR454C (Bordeaux)		αC-conn.	Thr.	281
AαK461Stop, homo. (Marburg)[a], hypod. Endoth.	Polym	αC-conn.	Hem.	140
AαS466Pfs, homoz. (Wilmington)	Polym	αC-conn.	Hem.	282
AαS466X (Caracas I)	Polym	αC-conn.	Hem.	283
AαE467Stop (Guarenas)	Polym	αC-conn.	Hem.	284
AαA475+HCLAStop (Lincoln)	Polym	αC-dom.	Hem.	141
Aα494fs +23 residue truncation at 517 (Perth)	Polym	αC-dom.	Hem.	142
AαH494P+23 res>517Stop (Mannheim V)	Polym	αC-dom.	3 Misc'ges	165
AαA499SfsStop (San Giovanni Rotondo)	Polym	αC-dom.		138
AαV522Afs, Aα524fs-548Stop, AαV522E (Indianapolis IV, III), V526E, AαR554L (Indianapolis)		αC-dom.	Amyloid	171,285,286,168
AαS532C-albumin (Caracas V)	Polym	αC-dom.	Thr.	135,157
FGA nucleotides 5011A-5012C replaced by TTGGAAATTTT > fs: Aα C-terminal 66 residues replaced by 72 novel residues (Grand Lyon)		αC-dom.	Hem.	162
Defects in Other Sites in the D Region				
BβL172Q/R17Stop, 5157T>A/3282C>T. 5157T>A, activates splicing site, deletes ~70% of Bβ chain length. Hypod.	Polym	CC	Hem.	123
BβY236C/BβIVS7-1G>C, spl. site (Paris IX), Hypod.	Polym	D	Hem. Thr.	163
BβR237S (Znojmo)	Polym, Plt	D	Thr.	287
BβA335T (Pontoise)[a]	Polym	D		104
BβN351K (Rokycany)	Polym, Plt	D		287
BβG414S (Unicov)		"b"		273
Bβ462Stop >W +12 residues (Osaka VI) stopSPMRRFLLFCM	Polym	D	Hem.	105
γG165R/AαR16C (Milano XII) γG165R (Hannover XIV)	Polym Polym	D/E D		106
γQ239H (Vicenza)	Polym	D		288
W253G homoz. (Darlinghurst)	Polym	D	Hem. Thr.	289
γY262C (Liberec)	Polym	D		290
γG268E, homoz. (Kurashiki)	Polym	D		291
γR275C (Baltimore IV), 18 other families	Polym	D:D		292
γR275H (Barcelona IV), 17 other families	Polym	D:D		293
γR275S (Kamogawa I)	Polym	D:D		294
γA279D (Auckland)	Polym	D:D		295
γY280C (Banks Peninsula)	Polym	D:D	Hem.	296

(Continued)

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Table 54.3

Mutations resulting in dysfibrinogenemia *(Continued)*

γG292V (St. Gallen, Baltimore I)	Polym	D:D	Hem.	107,297
γN308I (Baltimore III)	Polym	D		298
γN308K (Kyoto I), 3 other families	Polym	D	Variable	299
γM310T[c] (Asahi I)[a]	Polym Plts	D	Hem.	300
γS313R, hypod. (Detroit II)	Polym	D		301
γT314I (Kagoshima)	Polym	D	Thr.	302
γD318Y (Bastia)[b]	Polym	D		303
γ319-320 del (Vlissingen, Otsu I)	Polym Plts	D	Hem. Thr.	304,305
γ320D del (Des Moines) Hypod.	Polym	D	Thr.	306
γC326S (Cordoba & Hannover XIII) Hypod.	Polym	D		108
γC326Y[b] (Suhl & Frostburg) Hypod.	Polym	D	Thr.	99,307
(γA327T[b] (Tokyo V)[a]	Polym XL	D	Thr.	308
γQ329R (Nagoya I)	Polym	D		85
γD330Y (Kyoto III)	Polym	D		88
γD330V (Milano I)	Polym	D		87
γD330V, homoz. (Ales & Milano)	Polym	D		86
γT335R (Fremantle)	Polym	D		309
γM336I[b] (Hanover VI & XXIII)[b] Hypod.	Polym	D	Thr.	99
γN337K (Bern I)	Polym	"a"		109
γA341D (Seoul)[a] Hypod.	Polym	"a"	Thr.	97
γG352C and T353P (Ilam)	Polym	"a"		310
γ350-351ins (Paris I)	Polym XL Plts	"a"	Hem.	93
γN345D (Stuttgart) Hypod.	Polym	"a"	Thr.	99
γG351S/γA82G (Leipzig II) Hypod.	Polym	"a"/Coil	Hem.	311
γG351S (Praha III)	Polym	"a"	Hem. Thr.	274
γY354C[b] (Homburg VII) Hypod.	Polym	"a"	Hem.	99
γA357T (Frankfurt I)	Polym	"a"		60
γS358C (Milano VII)	Polym	"a"		100
γN361K (Poissy II)	Polym	"a"	Hem.	110
γY363N (Praha III)	Polym	"a"	Thr.	274
γD364H (Matsumoto I)	Polym	"a"		89
γD364V (Mellun I)	Polym	"a"	Thr.	312
γR375G[b] (Osaka V)	Polym	"a"		112
γS378P (Philadelphia) Hypod.	Polym	D	Hem.	313
γK380N, homoz. (Kaiserslautern)[a]	Polym	D	Thr.	314
γV411I (Hershey IV)	Plts	D	Hem.	315

Homoz., homozygous; XL, impaired fibrin crosslinking; Hypod., hypodysfibringenemia; Misc'ge, miscarriage; Plts, platelets; FpA or FpB, impaired FpA or FpB release; Polym polymerization; Thr. bdg., thrombin binding; Hem., Hemorrhage; "a", "a" pocket; "b", "b" pocket; D, unspecified D region site(s); conn.: connector. CC, coiled coil.

[a]Accompanied by extra glycosylation detailed in the text.

[b]Decreased calcium binding and/or impaired protective effect of calcium on plasmic lysis.

[c]Of two Bβ mutations in different alleles in the family, the R255H segregated with hypofibrinogenemia, while the K148D segregated with normal fibrinogen (1.4 g L^{-1}) levels. Standard capital letter labels are abbreviations used in DNA and RNA sequences to denote nucleotides and in protein to denote amino acids.

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Defects in Other D Region Sites

The Bβ A335T[104] (Pontoise) substitution results in extra glycosylation in Bβ333N and a markedly prolonged clotting time. Causing impaired polymerization, an insertion of 12 residues at the *C*-terminus of the Bβ chain resulted when the normal stop codon at 462 was changed to encode Trp (Osaka VI).[105] The resulting single new cysteine formed disulfide-linked dimers of variant molecules. Clots of this variant had thinner fibers in a lacy meshwork with large pores and were more fragile and compressible than controls. The γG165R (Milano XII) mutation was copresent in heterozygosity with Aα R16C.[106] Circular dichroism analysis disclosed altered folding of the *C*-terminal part of the γ chain, likely because γG165R falls between disulfide-bound residues γ153 and γ182. Molecular modeling of γG292V (St. Gallen I)[107] suggested that (a) the Val substitution alters conformation so fibrin(ogen) γ302-γ303 is more accessible to plasmin and (b) it impairs polymerization by disrupting hydrogen bonds between γ297 and γ375, thus destabilizing "A": "a" interactions. The γC326S substitution displayed albumin bound to its unpaired Cys.[108] Both γM336I[99] (Hanover VI) and γN337K[109] (Bern I) are near the calcium-binding site and the "a" pocket and likely impair polymerization by altering both structures. Impaired polymerization by γN361K[110] (Poissy II) implies that residue 361 contributes to the "A": "a" interaction. Normalized polymerization by increased calcium concentrations also suggests that this mutation affects calcium binding. The γR375G (Osaka V) substitution was similar,[111,112] with impaired polymerization normalized by calcium, impaired protective effect of calcium on plasmin degradation, and reduced high-affinity calcium binding. These results indicated a role for γ375 in calcium binding and the "a" pocket functions.[112] Some γchain mutations impaired fibrinogen-dependent platelet aggregation.[113–115] Since platelets interact with the *C*-terminal γ400-411site and an auxiliary γ370-383 site,[116] the impaired platelet aggregation by these mutations possibly reflects allosteric effects.

Defects within the Coiled-Coil Region

Identified in wide-angle x-ray diagrams of fibrinogen fibers,[117] the D and E regions are joined by α-helical coiled-coil rods that encompass amino acids Aα50-166, Bβ81-191, and γ24-134. In the coiled-coil, most nonpolar residues point inward to form the core and the polar residues are exposed to the solvent on the outside. Evidence indicates that most of the elasticity of the molecule resides in its coiled coil.[118] Two single deletions, Aα80N del[119] (Caracas IV) and Bβ111S del[120] (Kyoto IV), occurred within the all-polar region near the carbohydrate attachment point at γ52N and are thought to disrupt α-helicity. The former decreased polymerization and clot stiffness and the latter enhanced lateral polymerization. This region is a touch point where the coiled coils from different molecules associate in an antiparallel fashion in the fibrinogen crystal and possibly in polymerized fibrin.[121] A Bβ N167-E171 deletion is associated with severe hypodysfibrinogenemia and undue bleeding.[122] Severe hypodysfibrinogenemia is also associated with a compound heterozygosity consisting of a missense Bβ L172Q, with a truncation resulting from exposure of a cryptic acceptor splice site in exon 4, and R17Stop.[123] Each of two other mutations, Aα R141S[124] (Lima) and a Bβ N160S (Niigata),[125] results in a consensus sequence enabling novel glycosylations. Impaired polymerization is not corrected by deglycosylation at residue 158, indicating it was attributable to the Ser substitution (Niigata). In contrast, deglycosylation of Aα N139 (Lima)[126] corrects the polymerization defect, suggesting the novel glycosylation impairs polymerization. Substitution of Bβ R166C (Longmont) resulted in disulfide-linked fibrinogen dimers[127] whose removal did not correct the impaired polymerization. Taken together, these data suggest steric hindrance or blocking of proper alignment of monomers into protofibrils, of protofibrils into fibers, or both. Lastly, afibrinogenemia was found in a compound heterozygote with Aα M51R in one allele and a stop Aα51 codon in the other,[127] intimating failure of intracellular assembly or secretion by each mutation. Normal/mutant heterozygosity of either mutation likely results in normal or decreased plasma fibrinogen (hypofibrinogenemia).

Defects in the αC Region

The αC region (residues Aα221-610) extends from the D region and folds back toward the center (E) region.[128] It includes a flexible segment (Aα221-391), the "αC-connector," and an independently folded compact segment (Aα392-610), the "αC-domain."[129,130] In fibrinogen, the two αC-domains interact with each other and with the E region (FIGURE 54.1) while in fibrin they switch to intermolecular interactions. FpA release weakens their E region tethering.[131] Following FpB release, αCs are untethered and dissociate from one another facilitating lateral assembly of protofibrils.[128,132] Most mutations within αC generally result in impaired polymerization. Aα S434N substitution[133,134] creates a consensus sequence enabling novel glycosylation. In two other single substitutions, Aα S532C (Caracas V) and Aα R554C (Dusart, Chapel Hill III, Nashville, and another unnamed), the novel Cys residue is covalently linked to albumin. Electron microscopy (Caracas V[135] and Dusart[136]) disclosed abnormal fibrin clot structures.

Seven mutations result in truncations in the αC region, ranging from a 56% truncation terminating at residue 271, Otago[137] to a 15% truncation terminating at residue 518, San Giovanni Rotondo.[138] The Otago truncation reflects a frameshift mutation encoding Aα R268QEP, is homozygous with severe hypodysfibrinogenemia, and suggests an αC role in fibrinogen assembly and secretion. Also suggesting such a role are truncations with similarly low levels of fibrinogen variants Keokuk (10% of normal, Aα Q328X in the simple heterozygote),[139] Marburg (10% to 20%, homozygous Aα K461X),[140] Lincoln (20%), a heterozygous frameshift mutation Aα A475+HCLAStop,[141] and Perth (15%), a heterozygous frameshift with addition of 23 novel residues after Aα494.[142] In sharp contrast, two homozygous truncations at Aα452 (GPD–>WS-Stop, Milano III)[143] and Aα454 (Nieuwegein)[144] circulate at normal levels (2.6 g and 1.7 g L^{-1}, respectively). These two dysfibrinogens differ from one another in their terminating residues. Milano III[143,145] is a frameshift change in which two novel residues, W453 and S454, follow the Aα G452 mutation before a stop codon. In the Nieuwegein[144] defect, a nucleotide insertion at P453 codon results in a premature stop codon at 454. Turbidity studies indicate that each of these truncations decreases lateral aggregation, consistent with the long-known αC enhancement of this process.[132] Studies with fibrinogen Lincoln[141] (frameshift at Aα475A which translates as four new residues before terminating at a new stop codon, 476HCLAStop) showed that despite the new Cys residue, the variant chain was not found in albumin-bound fibrinogen. Also unique, the Aα Q328X truncation (Keokuk) occurred in two compound heterozygotes, with an FGA (IVS1 + 4A > G)

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splice-site mutation that results in no viable Aα chains from the second allele, a known cause of afibrinogenemia in homozygotes.[10] Only the truncated Aα chain was found in circulating fibrinogen whose lateral fibrin aggregation was markedly decreased. In general, mutations with albumin-bound complexes indicate that absence of the specified αC segment, rather than the presence of albumin, leads to impaired polymerization.

Clinical manifestations. The plasma prothrombin time (PT) is more often prolonged than the activated partial thromboplastin time (aPTT), possibly because PT is performed without a preincubation step and is thereby more sensitive to time lag prolongation. Such prolongation may be masked by the preincubation, with activating agents during the aPTT test. TT and RT are characteristically prolonged. For plasma fibrinogen measurements, TT-based[146] assays are clearly more sensitive than those that are PT based,[147] particularly when fibrinogen levels by either assay are decreased. Abnormal thromboelastograms may also reflect hypo- or dysfibrinogenemia[148] and are particularly sensitive when the test is performed in the absence of platelets. That is, owing to platelet retraction the typically small proportion of normal molecules may be sufficient to result in a normal or near-normal signal graph, while without platelets this is insufficient to correct the altered signal graph caused by the preponderance of abnormal molecules. Most dysfibrinogenemia mutations are asymptomatic (Table 54.2). Among symptomatic probands, a common feature is that other affected family members are asymptomatic. For example, a massive single hemorrhagic event in the Fibrinogen Louisville, AaR16H, proband (Table 54.3) did not occur in other affected family members, suggesting other possible contributing factors. In a retrospective study of clinical manifestations among >260 dysfibrinogenemic probands,[149] 55% were asymptomatic, 20% presented with a thrombotic history or event, and 25% with a hemorrhagic history or event, and among 2,376 patients with deep vein thrombosis 0.8% had dysfibrinogenemia.

Association with thrombosis. Among probands with a thrombosis history or presenting event, other genetic/acquired risks of thrombosis were present in a relatively small number. Some examples[150–153] include heterozygous factor V_{Leiden} with γR375C (Cedar Rapids), γR275H (Crete) or γG200V (Columbus), and AαT312A on contraceptive medication. Mutations associated with thrombosis include a small number with both thrombosis and hemorrhage. In roughly half of the thrombosis group (Thrombosis U, FIGURE 54.3), the nature of the copresence or association is unclear, while in the other half (Thrombosis R) clinical and other data strongly suggest a relationship to the mutation (Table 54.4). There is a variety of mutation types (i.e., deletion, insertion, missense, frameshift, etc.) and they occur in many molecular sites (FIGURE 54.4). Two mutations (Champagne au Mont d'Or and Lozanne) associated with thrombosis were identified using electrophoretic screening of fibrinogen from 217 individuals with recurrent or early age thrombosis and normal clotting times.[154] Five others were identified by liquid chromatography and mass spectrometry analyses of fibrinogen from 33 patients with thromboembolism-related pulmonary hypertension.[155] Among these, finer fibrin network and hypofibrinolysis (in three) were shown by retrospective analyses. An additional two, fibrinogens New York I,[82] heterophenotypic, and Naples,[79] homophenotypic, were associated with recurring thrombosis in more than one family member with the same mutation. Each mutation resulted in markedly impaired thrombin binding (vide supra), implying a causative mechanism for thrombosis. The heterophenotypic substitution AαR554C was found in four unrelated families with recurring thrombosis

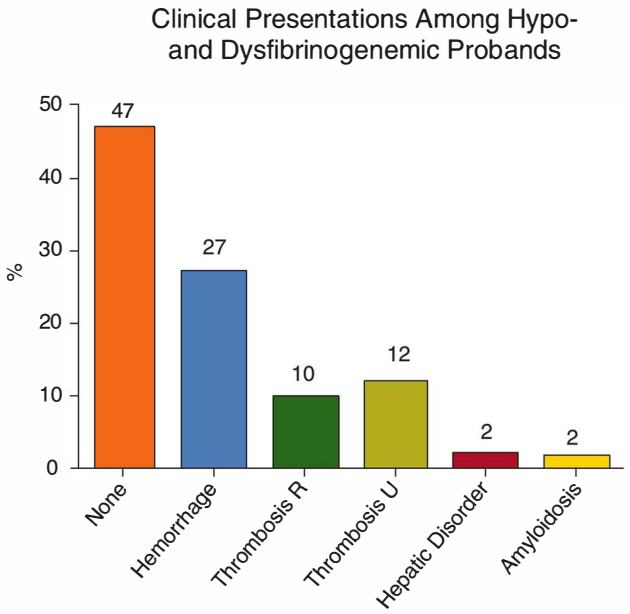

FIGURE 54.3 Clinical presentations among 194 probands with hypofibrinogenemia or dysfibrinogenemia. Numbers on top of each column reflect the % of the total in all columns.

in multiple affected members (Dusart, Chapel Hill III, Nashville, and one unnamed other).[156] The fine clot network (Dusart) was resistant to hypofibrinolysis.[8] This raises the possibility that the covalently linked albumin, relatively resistant to plasmin,[75] masks or blocks access to critical plasmin-susceptible bonds. Recurring thrombosis was also described in multiple probands in each family with heterophenotypic AαS532C,[157] AαQ328X (with Aα IVS4+1G >T),[139] and γD364V.[90]

Association with hemorrhagic diathesis. Including 4% with both thrombotic and hemorrhagic events, 27% of 194 mutations (FIGURE 54.3) are associated with hemorrhage, which is common among probands with fibrinogen levels of approximately 0.1 to 0.5 g L^{-1} by clotting assays. Frequent hemorrhagic events include recurring skin bruising with or without epistaxis. Unexplained and recurring miscarriage and/or hemorrhage during pregnancy including parturition are common.[158,60] However, undue hemorrhage during or following surgery, trauma, or minor injury is highly variable, suggesting that for a given mutation the threshold for undue hemorrhage in pregnancy is lower than in other clinical settings. Hemorrhagic diathesis from E region mutations is typical among homophenotypic AαR16H (two unrelated families, reversed FpA/FpB release sequence) and AαR16C[64,65] (three unrelated families, incoagulable fibrinogen at 37 degrees). In one AαR16H proposita (fibrinogen 0.61g L^{-1}), clot lysis by plasmin was accelerated[159] and likely contributed to the delayed FpA release-associated hemorrhagic diathesis. A similar presentation is associated with some heterophenotypic mutations within or near the "A" knob (vide supra). Most mutations within FpA are associated with hemorrhagic symptoms (Table 54.3), possibly reflecting both diminished thrombin binding and FpA release. Most polymerization defects are asymptomatic (Table 54.3). For those with a hemorrhagic history, the mechanism(s) is unclear, save fibrinogen Paris I with defective cross-linking of γchains and platelet interaction, those with severe hypodysfibrinogenemia, and those associated with defective α chain cross-linking.

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Table 54.4

Mutations associated with thrombosis

Peptide Phenotype (Site)	Name[Reference], Mutation(s)	Presentation	Affected Family Prob.	Other Comments
Aα L69H (CC)	San Diego IV[155]	One of 5 in 33 patients with VTE-pulmonary hypertension	None	Detected by special screening (see text)
AαN106D (CC)	Plzen[274]	VTE	None	26-year-old proband
Aα272 in frame 39-residue duplication (αC-conn.)	Champagne au Mont d'Or[154], 117 bp ins	Recurring VTE, 42 y.o., plasma clotting tests normal	None	Detected by special screening (see text)
Homophenotypic AαQ328Stop both siblings (αC-conn.)	Keokuk[139], FGA codon 328 C>T (Stop)	Both siblings with severe hypod. and severe hem. diathesis. Male: Recurrent VT after cryoprecipitate infusion	Female: 6 Misc'ge	Both compound heterozygotes with IVS-4 + 1g > t (silent allele)
AαR454C (αC-dom.)	Bordeaux[281]	VTE at age 25	None	Normal plasma clotting times
AαI451Wfs, homoz. (αC-dom.)	Milano III[143,145]	VT at age 13	None	Impaired polym.
AαS532C (αC-dom.)	Caracas V[135]	VTE	Many	Hypofibrinolysis
Aα R554H (αC-dom.)	San Diego V[155]	One of 5 among 33 patients with VTE-pulmonary hypertension	None	Detected by special screening (see text)
AαR554C-alb (αC-dom.)	Dusart[156] (4 unrelated families)	Recurring VTE in young adults and children	VTE in many probands	Impaired polym. hypofibrinolysis
Heterophenotypic Bβ9-72 del (B knob, thr. bdg.)	New York I,[82] exon 2 del	Recurring VT	VT in others	Impaired thr. bdg., polym.
BβA59Pfs (Thr. bdg.)	3404del[239]	Thr. in infancy		Fs
Homophenotypic BβA68T (Thr. bdg.)	Naples[316], codon 68G>A	Recurring arterial and VT	VT in three homozygotic probands	Impaired thr. bdg. and polym.
BβP235L/γR375W(D:D) BβP235L/γY114H(CC), BβP235L (D)	San Diego I[155] San Diego II[155] San Diego III[155]	Three of 5 in 33 patients with VTE-pulmonary hypertension	None	All detected by special screening (see text)
Bβ236YStop (D)	Lozanne[154], 5909a>g	Recurrent VTE, clotting tests normal, age 31 y	Mutation in family members	Detected by special screening
γT314I (D)	Kagoshima[302]	VT, age 36 y		Postpartum hemorrhage
γD318V (D)	Caen[317], c.1031A>T	VT, age 5 y	None	
γD318G (D)	Giessen IV[317]	VT, child	None	
γA327T (D)	Tokyo V[308] hypod.	Recurrent VTE, age 43 y	None	Clot resists lysis
γD330V (D)	Ales[86]	Strokes, before age 36 y		Thrombotic strokes
γD364V ("a")	Melun[90]	VT and arterial thrombosis, miscarriage	VT in many siblings	Normal fibrin polym.

Homophenotypic: indicates that either genotypes were not reported (e.g., Detroit, Metz), were homozygotic, or were compound heterozygotic by allele analysis (e.g., Kingsport, Frankfurt XIII); Homoz., homozygous; Hypod., hypodysfibringenemia; Misc'ge, miscarriage; Ins, insertion; Fs, frameshift; Polym, polymerization; Thr. bdg., thrombin binding; Hem., hemorrhagic; VT, venous thrombosis; D, unspecified D region site(s); Dom.: domain, conn.: connector. CC, coiled coil; y, year old.
Criteria for inclusion: thrombosis at young age (<40 y), in multiple related and unrelated probands, and probands randomly discovered in a thrombosis population.

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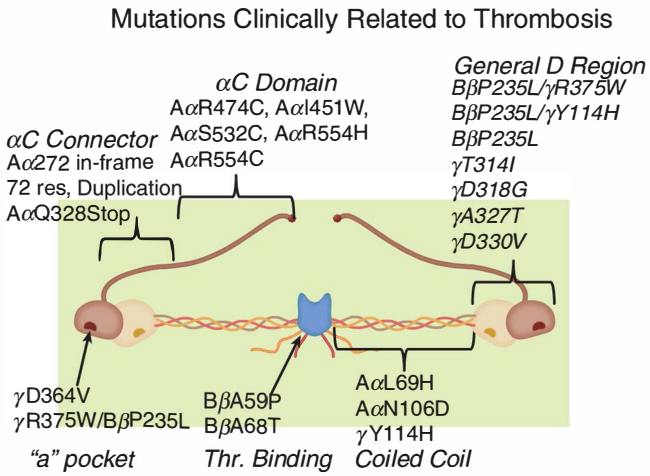

FIGURE 54.4 Schematic representation of molecular sites of fibrin(ogen) mutations whose clinical features strongly suggest a relationship to thrombosis as detailed in the text and Table 54.4.

Pregnancy complications. Although the precise frequency of miscarriage among afibrinogenemic fertile women is unknown, case reports indicate it is typical,[9,10,17,160] in general agreement with data from animal studies showing fibrinogen is required to maintain gestation to term.[12,13,14] Miscarriage is also frequent in pronounced hypo- or hypodysfibrinogenemia. Examples of mutations with dysfibrinogenemia and associated miscarriage include homophenotypic AαR16C (Metz,[161] Frankfurt XIII[64]), BβM118K (Lyon[162]), BβY236C (Paris IX[163]), γA82G (Hannover VII[164]), BβAsn137-Glu141del (Epsom),[122] AαH494P+23 res>517Stop (Manheim V),[165] and homophenotypic AαQ328Stop (Keokuk).[139] Many other reports in which the mutations were not identified[19,166] described miscarriage, bleeding during second or third trimester including the intra- and peripartal period, and abruption placenta. An unique example is perivillous placental thrombosis and recurring postpartum VTE along with intrapartal hemorrhage associated with the single pregnancy of the homophenotypic AαR16H proposita.[32]

Hereditary amyloidosis. A few mutations within the fibrinogen αC-domain are linked to hereditary amyloidosis, a protein misfolding disorder resulting in tissue deposition of insoluble fibrils containing peptides with the abnormal sequence. There are 25 known causative mutations among various proteins.[167] Renal involvement is typical, the most frequent lesion consisting of glomerular deposits. The typical presentation is nephrotic syndrome and hypertension with a median age of 55 years. The first report of amyloid identified fibrinogen peptides encompassing Aα499-580 with an AαR554L substitution.[168] Other mutations include AαE526V[169] and two frameshift mutations that encode truncations: a mutation at codon 524, resulting in termination at Aα548,[170] and one at codon 522, also resulting in termination at Aα548.[171] A possible explanation is intimated by the minor catabolic fraction of circulating fibrinogen that lacks intact αC.[172] The resulting free mutant αC peptides may form insoluble aggregates that are deposited in tissues and lead to amyloidosis. A retrospective DNA and tissue analysis of 350 cases of systemic amyloidosis disclosed 18 cases with renal amyloidosis due to the AαE526V mutation.[173] In a study providing new insights, among 22 patients with fibrinogen amyloid (21 with AαE526V and one R554L), all of whom received both renal and hepatic transplants,[167] coronary and systemic atheromas were found in 67% and 55%, respectively. Vascular atheromas and endocardial biopsies from these contained purely fibrinogen amyloid. Also, the combined liver/renal transplantation dramatically improved renal allograft survival.

Therapy

There are no controlled studies on fibrinogen replacement, but there is general agreement from case reports that at a mean weakly dose of 0.06 g/kg body weight it prevents/stops bleeding from fibrinogen anomalies,[10] notwithstanding that in one report frequencies of bleeding episodes between groups with and without prophylactic replacement were similar.[174] Cryoprecipitate (~0.25 g fibrinogen/container) is commonly used. Fresh-frozen plasma (0.5 to 0.75 g fibrinogen/container) is less frequently used owing to undesirable hemodynamic effects of its large volume. A fibrinogen concentrate[175] (that lacks the viral disease transmission risks) has also become available in United States. Numerous case reports clearly indicate that with a history of one or more miscarriages further miscarriage is prevented by fibrinogen replacement.[158] Similarly, undue bleeding or miscarriage after the first trimester can also be successfully treated/prevented. Suggested regimens (Table 54.5) reflect those in various reports.

Hyperfibrinogenemia

Circulating fibrinogen levels are determined partly (30% to 59%) by genetic[176–178] and partly by environmental factors.[23] The latter include the acute-phase response[179,180] during which the fibrinogen level is regulated by cytokines,[181–184] mainly IL-1 and IL-6. Underscoring genetic determinants, overexpression of all three fibrinogen genes was required to achieve fibrinogen levels of 1.4 to 1.7 times the normal level in an experimental mouse model.[185] Levels above the normal range are commonly associated with pregnancy, venous thrombosis, malignancies, and inflammation. Hyperfibrinogenemia is found in approximately 26% of patients with a large array of different malignancies.[186] Fibrinogen levels of >5g L^{-1} carry a nearly fourfold higher venous thrombosis risk than those of <3gL^{-1}.[187] Of considerable interest are levels within the upper part of normal range that are associated with FGB promoter region polymorphisms such as −854G>A, −455G>A, −148C>T, R/K448, and BclI.[188] Among these, −148T and −455A are associated with cardiovascular disease.[189] That is to say, fibrinogen levels in the upper tertile, relative to those in the lower tertile, are associated with a 2.3-fold greater risk of cardiovascular disease.[190,191] Cross-sectional prospective studies imply that such elevations are independent predictors of coronary artery disease, stroke, and peripheral artery disease.[191] Moreover, the magnitude of the increased fibrinogen levels correlates with the severity of peripheral arterial disease, with the extent of myocardial necrosis following infarct, and with cerebrovascular occlusions. Additionally, the disease risk is disproportionately increased in individuals with the highest fibrinogen levels indicating a threshold effect. Fibrinogen is a major determinant of blood viscosity whose increased level is also a strong predictor of cardiovascular events in hypertension, stroke, and diabetes.[192] Also, circulating soluble fibrinogen/fibrin complexes,[193] which are inherently viscid and procoagulant,[33] are likely to increase with increased fibrinogen and contribute

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Table 54.5

Recommended fibrinogen replacement during pregnancy

First Trimester	Third and Fourth Trimesters	During Labor or Caesarian Section
Afibrinogenemia		
As soon as gestation is established begin replacement and maintain plasma fibrinogen at ~1 g/L[1].	At least weakly infusions required, the amount required will increase two to three times by late third trimester.[318]	Infuse 5-g fibrinogen bolus; initiate a slow infusion to maintain 1.5 g L^{-1} for 24 h (will need ≥ 5g).
Dysfibrinogenemia		
Fibrinogen >0.6 and <1.5 g L^{-1} similar to pregestation levels, and no history of bleeding	Obtain levels every trimester.	Replacement need is unlikely, unless bleeding occurs before delivery.
Fibrinogen <0.6 g L^{-1} (bleeding risk is increased)	Follow replacement regimen as in afibrinogenemia.	Follow replacement regimen as in afibrinogenemia.
Hypofibrinogenemia		
Fibrinogen >0.6 and <1.5 g L^{-1} (bleeding risk is low)	Follow the dysfibrinogenemia procedure, if needed	Replacement as in afibrinogenemia may be needed.
Fibrinogen <0.6 g L^{-1} (bleeding risk is increased)	Follow replacement regimen as in afibrinogenemia.	Follow replacement regimen as in afibrinogenemia.

to the increased blood viscosity. Among the effects of elevated viscosity is increased blood flow shear stress[192] and thereby activation of endothelial cells[194,195] that leads to expression of various adhesion molecules such as intercellular adhesion molecule-1, $\alpha_v\beta_3$, and $\alpha_5\beta_1$.[196,197] All three are receptors for fibrinogen whose binding to each receptor results in multiple alterations of vascular function.[198] In other studies, histopathologic data indicate the presence of fibrinogen and its degradation products during the progression of atheroma,[199,200] and elevated fibrinogen levels are closely related to the enhanced rate of coronary calcification and increased media–intima thickness, both measures of premature atherosclerosis.[201] At the cellular level, fibrinogen favors permeability of endothelial cells, extracellular accumulation of low-density lipoprotein cholesterol, and formation of foam cells. Fibrinogen also induces migration of monocytes and vascular smooth muscle cells, increases platelet reactivity or aggregation, and enhances inflammation.[23,202] Also, elevated fibrinogen level is a major determinant of amount of formed thrombin.[203] A study using a transgenic mouse model[204] demonstrated that elevated fibrinogen augments fibrin deposition into specific organs, interacts with an unidentified and independent modifier of hemostatic activity that regulates fibrin turnover and deposition, and exacerbates neointimal hyperplasia under stasis-induced vascular remodeling conditions. In a study of a normal variant known as γ', (8%–15% of total fibrinogen), it is increased in stroke and coronary disease and this is independent of increased plasma fibrinogen.[205] This is a splicing variant with a longer γ chain[206] (FIGURE 54.1). Its last four C-terminal γ residues are replaced by a unique 20-residue extension[207] within which residues 414 to 427 possess the high affinity, noncatalytic thrombin-binding site.[208] Mediated by thrombin exosite 2, binding to this variant sequesters free thrombin and by limiting its catalytic potential regulates thrombin generation via the intrinsic pathway. Considered together, the foregoing observations intimate that elevated plasma fibrinogen contributes substantially to the development of the atheroma lesion.

Definitions

FpA: fibrinopeptide A. "A" knob: site exposed by FpA release, encompassing residues α17-19 (GlyProArg). "a" pocket: site interacting with "A" knob

FpB: Fibrinopeptide B. "B" knob: site exposed by FpB release encompassing Bβ15-19 (GlyHisProArg)

"b" pocket: Bβ397-432

αC: Aα chain region encompassing Aα 220-610

αC-domain: region encompassing Aα 372-610

αC-connector: region encompassing Aα 220-371

Heterozygous: mutation or polymorphism affecting one allele

Homozygous: mutation of polymorphism affecting both alleles

Heterophenotypic: expression of both alleles of a heterozygous proband

Homophenotypic: expression of both variant alleles of a homozygous proband or of only one of two variant alleles of a heterozygous proband (i.e. the second allele is not expressed).

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CHAPTER

55 Familial Multiple Coagulation Factor Deficiencies

BIN ZHANG AND DAVID GINSBURG

The familial multiple coagulation factor deficiencies (FMFDs) are a group of rare inherited disorders characterized by a simultaneous decrease in the levels of two or more coagulation factors. Earlier reports of deficiencies in various combinations of coagulation factors prompted Soff and Levin[1] in 1981 to classify these disorders into six types (FMFD I to VI). Combined deficiency of FV and FVIII (F5F8D, or FMFD I) and combined deficiency of vitamin K–dependent clotting factors (VKCFD, or FMFD III) are the best known and comprise the vast majority of reported cases of FMFD. This chapter focuses on these two disorders. Little is known about other types of multiple coagulation factor deficiencies, as few new cases have been reported since 1981. As a consequence, this old classification is of limited relevance today and not generally used in practice.[2]

COMBINED DEFICIENCY OF FACTOR V AND FACTOR VIII

History

Combined deficiency of factors V and VIII (F5F8D) was first described in a pair of Swiss siblings in 1954.[3] Since then, more than 80 families with F5F8D have been identified around the world. As is the case for rare autosomal recessive disorders, F5F8D is often associated with consanguineous marriages. However, it is likely that F5F8D is underdiagnosed, in part due to its often mild bleeding manifestations. Some F5F8D patients may be misdiagnosed as having mild hemophilia A or parahemophilia (FV deficiency). F5F8D appears to be particularly prevalent among Middle Eastern Jews and non-Jewish Iranians, estimated at approximately 1:100,000 in these populations.[4,5,6] This high frequency is probably due, at least in part, to the prevalence of consanguineous marriages in these populations. The molecular basis of F5F8D was a mystery until 1998.[7] Initial speculation centered on a defect in a precursor protein for both factors V and VIII,[3] but no such precursor could be identified. A low level of protein C inhibitor activity in patient plasma was reported in 1980,[8] but was later found to be caused by an artifactual loss of protein C inhibitor in frozen samples.[9–12] Subsequent genetic studies identified the cause of this disorder as deficiencies in a unique secretory pathway shared by FV and FVIII.[13,14,15]

Genetics and Biology

Inheritance of F5F8D is autosomal recessive and distinct from the coinheritance of both FV deficiency and FVIII deficiency. Heterozygotes are asymptomatic and their plasma levels of FV and FVIII are indistinguishable from normal. Using homozygosity mapping and positional cloning approaches, the first gene for F5F8D was identified in 1998[13] as *LMAN1* (lectin, mannose-specific 1). The encoded protein (LMAN1, also referred to as ERGIC-53), though of previously unknown function, was known to localize to the endoplasmic reticulum (ER)-Golgi intermediate compartment (ERGIC).[16,17] Mutations in *LMAN1* account for approximately 70% of F5F8D patients. No *LMAN1* mutations could be identified in approximately 30% of affected families.[18,19] In 2003, the second cause of F5F8D was identified in the latter subset of families as mutations in *MCFD2* (multiple coagulation factor deficiency gene 2).[14] To date, at least 33 different *LMAN1* mutations[13,18–20,21] and 17 different *MCFD2* mutations[14,21,22,23] have been identified (FIGURE 55.1). The diverse nature of the mutations indicates multiple independent genetic origins. Haplotype analyses performed on two *MCFD2* mutations (c.149+5G>A and c.407T>C) that are identified in multiple distinct populations demonstrated multiple independent genetic origins for each mutation.[24] However, founder mutations may account for all or the majority of F5F8D in some isolated populations. For example, two mutations in *LMAN1* account for nearly all Jewish F5F8D patients. One of these mutations is prevalent in Jews originating from the island of Djerba in Tunisia, but not found among North African Jews.[25] The second *LMAN1* mutation is unique to Middle Eastern Jews.[13] Another notable mutation is the missense mutation abolishing the initiation codon (c.2T>C) of *LMAN1*, which has only been reported in families of Italian origin.[21,26]

Mean FV and FVIII levels are slightly but significantly lower in *MCFD2* patients compared to *LMAN1* patients. However, the levels in these two groups overlap extensively, and individual patients with *MCFD2* or *LMAN1* mutations are clinically indistinguishable.[21] Plasma FV and FVIII levels for a given patient are also significantly correlated, suggesting that deficiencies in either LMAN1 (ERGIC-53) or MCFD2 exert a similar impact on the secretion of both FV and FVIII.[21] Circulating FV exists in both plasma and the α-granules of platelets as a 330-kDa single-chain polypeptide. In humans, the platelet FV pool is thought to originate from endocytosis of plasma FV,[27,28] whereas in mice, platelet FV is derived exclusively from biosynthesis within the megakaryocytes.[29,30] Comparison of platelet FV levels in F5F8D patients and normal controls indicates that platelet FV and plasma FV are reduced to the same extent for both the *LMAN1* group and the *MCFD2* group.[21] Therefore, mutations in *LMAN1* or *MCFD2* similarly affect the steady-state levels of FV in both pools.

LMAN1 is a 53-kD homohexameric transmembrane protein that belongs to a conserved class of L-type animal lectins with homology to leguminous mannose-binding lectins.[31,32] The

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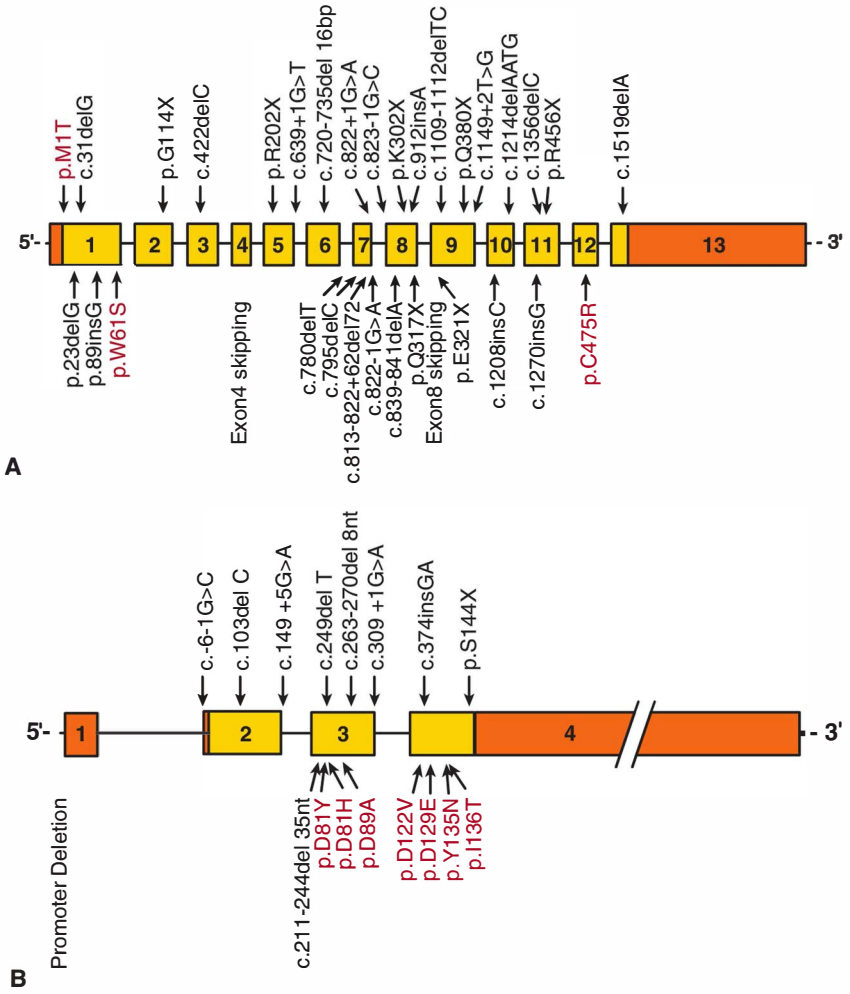

FIGURE 55.1 Structures of *LMAN1* and *MCFD2* genes and mutations identified in combined deficiency of factor V and factor VIII. **A:** Most mutations in *LMAN1* are predicted to result in complete loss of protein function. Three missense mutations, shown in *red*, also lead to loss of function. **B**: In addition to insertion, deletion, and splice site mutations in *MCFD2*, multiple missense mutations also occur in both EF-hand domains and were shown to abolish binding to LMAN1(14;21;37). Exons are numbered and drawn to scale. *Shaded rectangles* represent the coding regions of the genes, with the open portions of exons 1 and 13 in *LMAN1* and exons 1, 2, and 4 in *MCFD2* representing the 5′ and 3′ untranslated regions.

LMAN1 *C*-terminus is exposed to the cytoplasm and contains the ER exit and retrieval motifs. The ER luminal side of the protein contains a carbohydrate recognition domain (CRD) and a coiled-coil domain. MCFD2 is a 16-kDa soluble protein with two Ca^{2+}-binding motifs, known as EF-hand domains, at the *C*-terminus with a structure similar to that of calmodulin.[33] MCFD2 interacts with LMAN1 to form a stable Ca^{2+}-dependent complex with a 1:1 stoichiometry, and the two proteins colocalize in the ERGIC.[34] Crystal structure and cell biology studies indicate that the *N*-terminus of the LMAN1 CRD interacts with the EF-hand domains of MCFD2.[35,36] Only three missense mutations have been identified in *LMAN1*. The first missense mutation destroys the initiation codon of the gene. The other two mutations either destabilize the protein[24] or abolish MCFD2 binding.[20] Seven *MCFD2* missense mutations have been identified within the EF-hand domains, which disrupt the LMAN1-MCFD2 interaction, resulting in F5F8D.[14,21,37]

One of the central problems in cell biology is how newly synthesized proteins are sorted for transport to their final destinations. An increasingly accepted model envisions that "cargo receptors," localized to the ER membrane, selectively package secreted proteins into "vesicles" for transport to the Golgi body.[38,39] The finding of *LMAN1* gene mutations as a cause of F5F8D provided the first direct evidence for a mammalian ER cargo receptor, suggesting that LMAN1 mediates export of FV and FVIII from the ER to the Golgi (FIGURE 55.2). To date, the LMAN1-MCFD2 complex remains the only cargo receptor identified in higher eukaryotes,[38,39] though two other potential cargo receptors have been described in yeast.[40,41] LMAN1 and MCFD2 should act in concert to facilitate transport of FV and FVIII, since mutations in either gene lead to impaired secretion of FV and FVIII. However, the precise role of MCFD2 in cargo receptor function is still not clear. Both LMAN1 and MCFD2 have been shown to bind FV and FVIII directly,[14,34,37,42] with the sugar-binding site of LMAN1 and the EF-hand domains of MCFD2 important for these interactions.[37,42] The heavily glycosylated B domains of FV and FVIII may be important in mediating secretion through the LMAN1-MCFD2 pathway.[34,43,44] An interesting question is whether the ER to Golgi transport of other proteins is also altered in F5F8D patients. This seems likely, given the ubiquitous expression patterns of both LMAN1 and MCFD2,[14] as well as the presence of LMAN1 and MCFD2 orthologs in lower eukaryotes without a blood clotting system. Indeed, a number of other proteins have been identified to either interact with LMAN1 or exhibit delayed secretion in cultured cells with a

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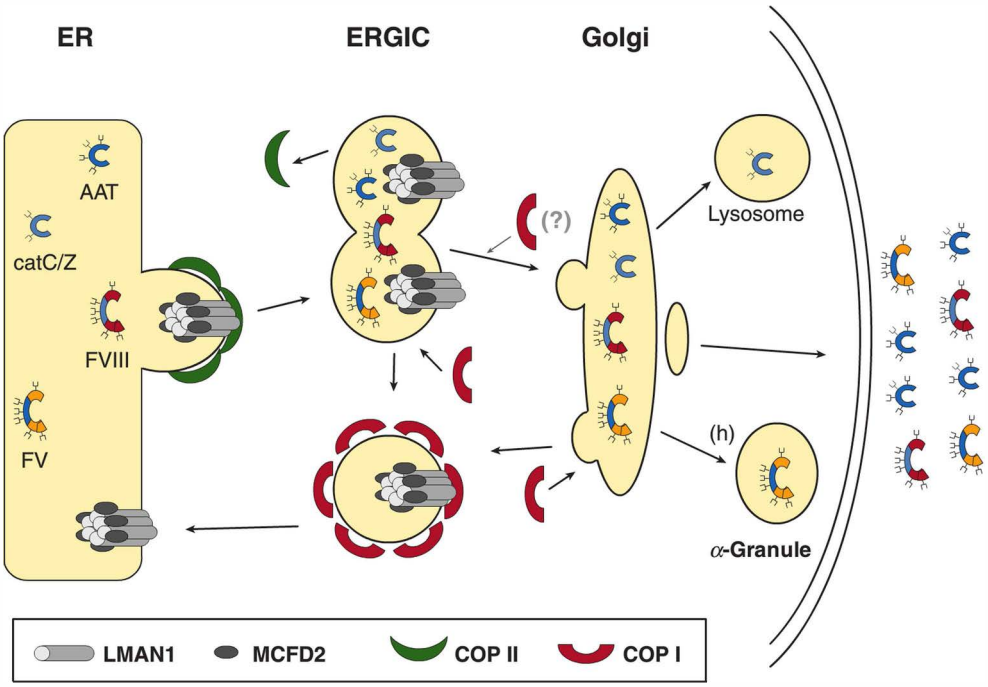

FIGURE 55.2 Model of receptor-mediated ER to Golgi transport of FV and FVIII and other potential cargo. Correctly folded FV/FVIII molecules are recruited to the COPII-coated vesicle budding from the ER by binding to the LMAN1-MCFD2 complex. Upon budding, these vesicles uncoat and fuse with each other to form the ERGIC. Release of FV/FVIII from LMAN1-MCFD2 occurs in the ERGIC. The LMAN1-MCFD2 complex is recycled back to the ER in COPI-coated retrograde vesicles as FV and FVIII are transported to the Golgi. Cargo proteins undergo further sorting and modifications in the Golgi. CatC and catZ are targeted to the lysosome. FV, FVIII, and α_1-antitrypsin (AAT) are secreted from cells, with the exception of megakaryocytes, in which FV is packaged into α-granules for storage. (Modified by permission from Baines AC, Zhang B. Receptor-mediated protein transport in the early secretory pathway. *Trends Biochem Sci* 2007;32:381–388, copyright (2007) Elsevier Ltd.)

dysfunctional LMAN1 (FIGURE 55.2).[45–48] However, transport deficiency in other proteins is likely to be at a level insufficient to produce a clinical phenotype, as no other abnormalities, aside from the deficiencies of FV and FVIII, have been identified in F5F8D patients.

Clinical Manifestations

Seligsohn et al.[4] studied 14 patients in 7 families of Middle Eastern Jews. Peyvandi et al.[5] and Mansouritorgabeh et al.[6] studied a total of 46 patients from 23 families of non-Jewish Iranians. In addition to these relatively large studies, a number of case reports have also been published.[23,26,49–76] Patients with this disorder exhibit plasma FV and FVIII activity level at approximately 10% of normal, with a range of 5% to 30%, though factor levels as low as 1% have been reported.[5,49] FV and FVIII clotting activity and antigenic level are both concordantly decreased.[1,67,77] Bleeding symptoms appear to be comparable to those observed in patients with single deficiencies of FV or FVIII.[4,5] No abnormalities outside of these hemostatic changes have been identified in F5F8D. Spontaneous bleeding symptoms are common, including easy bruising, epistaxis, and menorrhagia. Hemarthroses are less frequent, though reported in up to one-third of patients in some series.[5,6,49] In contrast, gastrointestinal bleeding, hematuria, and intracranial bleeding are only rarely observed. Excessive bleeding is particularly common during or after trauma, tooth extraction, surgery, or labor. Postcircumcision bleeding was reported to be common among Iranian patients,[5] but rare among Jewish patients,[4] possibly due to the age at which circumcision is performed (at birth among Jews and usually at 5 to 7 years of age among Muslims). In contrast to hemophilia A, the levels of FV and FVIII correlate poorly with bleeding severity.

Diagnosis and Differential Diagnosis

The diagnosis of F5F8D should be suspected in patients with a prolonged prothrombin time (PT) and partial thromboplastin time (PTT) and a history of mild to moderate bleeding beginning in childhood. Diagnosis is confirmed by the demonstration of FV and FVIII levels in the 5% to 30% range. A positive family history, if present, should be consistent with an autosomal recessive inheritance. All other routine laboratory results would be expected to be normal. Differential diagnosis includes coinheritance of hemophilia and parahemophilia, though this is extremely rare. Coinheritance of FV deficiency and type 1 von Willebrand disease (vWD) may be more common,[50,57] given the higher frequency of vWD.[78] von Willebrand factor levels could be determined to exclude this possibility.

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Treatment

Due to the small number of patients, only limited experience is available to guide therapy, though severe or life-threatening bleeding is fortunately rare. Fresh-frozen plasma has been effective in F5F8D in raising both FV and FVIII levels and should be used for the treatment of life-threatening hemorrhage, preparation for major surgery, and child birth.[79,80] Regular prophylaxis is generally not required.[81] DDAVP (1-deamino-8-D-arginine-vasopressin) has been used for mild to moderate bleeding, and results in an up to threefold increase in FVIII, but does not alter the FV level. DDAVP has also been used in combination with plasma infusion to compensate for the shorter half-life of plasma FVIII. Recombinant FVIII may also be combined with plasma infusion to raise FVIII level. Overall, treatment must be individualized to the specific patient and the severity of clinical bleeding.

COMBINED DEFICIENCY OF VITAMIN K–DEPENDENT CLOTTING FACTORS

History

The first case of VKCFD was described in 1966 in an infant girl who exhibited significant bleeding from the first week of life.[82] The proband was found to have low or undetectable levels of factors II, VII, IX, and X, with no evidence of hepatic disease or malabsorption. High doses of vitamin K partially restored clotting factor levels with clinical improvement.[83] Over 20 additional cases of VKCFD have been reported since that time.[84,85,86,87–103,104]

Genetics and Biology

γ-glutamyl carboxylase (GGCX) catalyzes a posttranslational modification of glutamate (Glu) residues into γ-carboxyglutamate (Gla) residues.[105–107] This carboxylation is required for the activities of coagulation factors II, VII, IX, and X, as well as the anticoagulant factors protein C, protein S, and protein Z.[108,109] Carboxylation occurs at Glu residues located in a homologous approximately 45–amino acid "Gla domain." The presence of Gla residues is thought to enable these proteins to adopt a calcium-dependent conformation that allows binding to phospholipids. Other proteins known to undergo γ-carboxylation include osteocalcin,[110] matrix Gla protein (MGP),[110] Gas6,[111] nephrocalcin A-D,[112] as well as several putative carboxylated proteins identified through nucleotide database screening (PRGP1, PRGP2, TMG3, and TMG4).[113,114] Vitamin K is an essential cofactor for γ-carboxylase. As carbon dioxide is added to Glu to form Gla, the reduced form of vitamin K (vitamin K hydroquinone) is oxygenated to form vitamin K 2,3-epoxide. Another enzyme, vitamin K epoxide reductase (VKOR), is required to regenerate the vitamin K hydroquinone, completing the so-called vitamin K cycle (FIGURE 55.3).

VKCFD is an autosomal recessive disorder characterized by deficiency of all VKCFDs. It is a heterogeneous disorder with two subtypes. VKCFD1 is defined by defective γ-carboxylase (GGCX) activity, first demonstrated in Devon Rex cats.[115] Nineteen different mutations in the *GGCX* gene have been reported in human families, the majority of which are missense mutations (FIGURE 55.4). Two different homozygous point mutations (L394R and W501S) were found in three consanguineous families.[86,88,91] Another patient of nonconsanguineous parents[97] is a compound heterozygote with a splice site mutation that

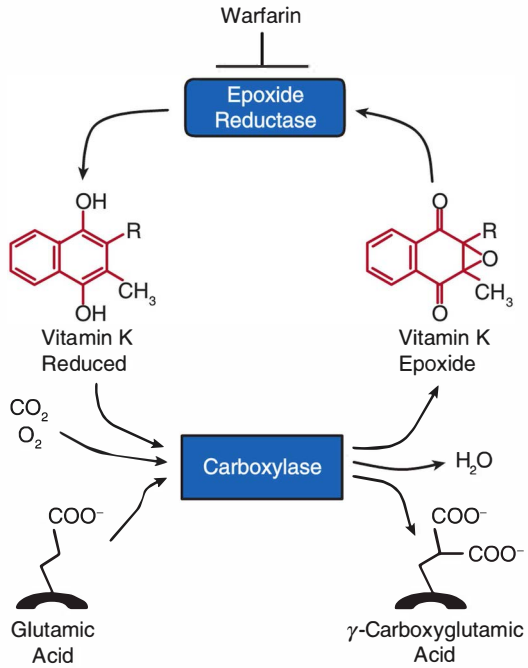

FIGURE 55.3 Vitamin K cycle. Vitamin K is an essential cofactor for γ-carboxylase (GGCX). GGCX adds a carbon dioxide to glutamic acid to form γ-carboxyglutamic acid at the same time that the reduced form of vitamin K (vitamin K hydroquinone) is oxygenated to form vitamin K 2,3-epoxide. VKOR is required to regenerate the vitamin K hydroquinone, completing the vitamin K cycle. Warfarin inhibits VKOR to limit the carboxylation of vitamin K–dependent coagulation factors. (Modified by permission from Sadler JE. Medicine: K is for koagulation. *Nature* 2004;427:493–494, Ref. [157], copyright (2003) Macmillan Publishers Ltd.)

results in early truncation of GGCX, and a missense mutation that changes a conserved arginine (R485P). GGCX carrying the L394R mutation demonstrated a threefold reduced activity compared with the wild-type protein.[86] L394 is in a 25–amino acid stretch of the enzyme that is highly conserved among all species with a known *GGCX* gene.[116] The primary defect associated with L394R appears to be reduced glutamate-substrate binding.[117,118] The W501S mutation appears to decrease the affinity of the carboxylase for the propeptide of its substrates.[88] The R485P mutation may affect vitamin K binding,[97] whereas the W157R and T591K mutations were shown to decrease carboxylase activity.[100] Other GGCX missense mutations have not yet been characterized in detail. In addition, splice site and intronic mutations have been reported.[92,97,101]

VKCFD2 results from functional deficiency of vitamin K 2,3-epoxide reductase (VKOR),[89,94] which is also the target of the oral anticoagulant warfarin.[119] VKOR activity was identified over 30 years ago.[120] However, the gene encoding the corresponding enzyme, vitamin K epoxide reductase complex subunit 1 (VKORC1), eluded identification until 2004.[121,122] Expression of *VKORC1* alone in insect cells confers warfarin-sensitive VKOR activity and the purified protein alone is sufficient for VKOR activity.[121,123] VKORC1 is a small protein of 163 amino acids (~18 kD) with four potential transmembrane domains.[124,125] Like γ-carboxylase, it is localized to the ER via

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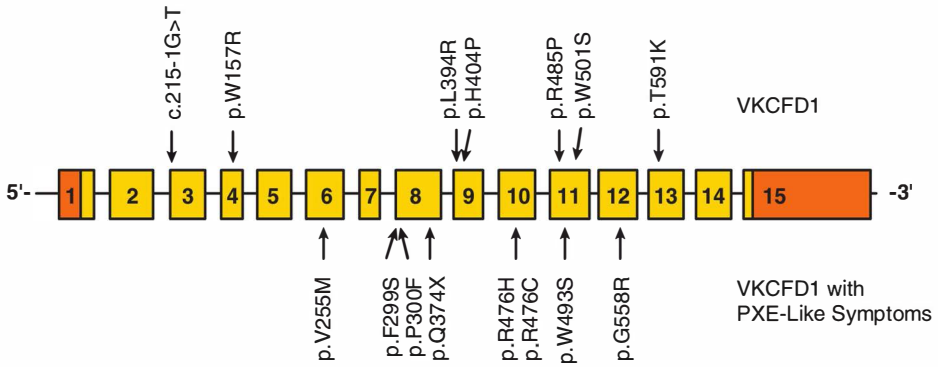

FIGURE 55.4 Structure of *GGCX* gene and mutations identified in VKCFD1. Mutations associated with VKCFD1 without the PXE-like phenotype are denoted above the gene. Mutations associated with the PXE-like phenotype are denoted below the gene. Exons are numbered and drawn to scale. *Shaded rectangles* represent the coding regions of the genes, with the open portions of exons 1 and 15 representing the 5′ and 3′ untranslated regions.

the *C*-terminal dilysine ER retention motif.[122] Current research is focused on identifying the redox partners of VKOR and the mechanism for electron transfer between VKOR and its partners.[126,127,128,129]

To date, only a recurrent homozygous *VKORC1* missense mutation (R98W) that decreases VKOR activity has been reported in families of distinct origins.[122,130] Multiple missense mutations were identified in warfarin-resistant rats and humans and shown to result in VKOR resistance to warfarin. Administration of warfarin during pregnancy results in warfarin embryopathy, characterized by a spectrum of birth defects including abnormal midfacial development, stippling of the epiphyses, and mental retardation.[89,131–133] Patients with *VKORC1* mutations do not resemble those of warfarin embryopathy, except for bone deformation in some patients.[89]

A subgroup of VKCFD with variable bleeding tendency is prominently associated with a pseudoxanthoma elasticum-like (PXE-like) phenotype, which is characterized by dystrophic mineralization of soft connective tissues, in particular the elastic fiber, skin, eye, and arterial blood vessels. Small yellowish papules coalesce on the skin, which also exhibits excessive folding with loss of recoil. Several mutations in *GGCX* (FIGURE 55.4) have recently been identified in VKCFD patients with PXE-like symptoms.[102,103,104] Decreased γ-carboxylase activity and accumulation of uncarboxylated MGP and osteocalcin, both of which are inhibitors of soft tissue calcification, have been documented in plasma and dermis.[102,103,134] It is not clear why only a subgroup of mutations in GGCX lead to a PXE-like phenotype.

The rarity of VKCFD and the fact that mostly missense mutations in GGCX and VKORC1 have been identified suggest that complete deficiency in either of these enzymes has more severe consequences. Indeed, deletion of the *GGCX* gene in mice results in partial embryonic lethality. Null mice surviving to term died uniformly of massive intra-abdominal hemorrhage at birth.[135] Similarly, *VKORC1* knockout mice died within 2 to 20 days after birth from extensive bleeding,[136] a lethality that can be rescued by the administration of vitamin K. These results exclude the existence of a redundant carboxylase pathway and indicate a role for γ-carboxylated proteins in early mammalian development.

Clinical Manifestations

Clinical symptoms of VKCFD vary widely, though they are roughly correlated with procoagulant protein levels.[94] Large amounts of vitamin K can partially correct the bleeding symptoms in most, but not all,[93,99] patients. Reported bleeding in VKCFD has included episodes of intracranial hemorrhage in the first weeks of life, sometimes leading to a fatal outcome. Hemarthroses and mucocutaneous bleeding may follow antibiotic therapy, due to decreased vitamin K production by gut bacteria. Skeletal defects have been reported in some probands, presumably resulting from undercarboxylation of bone Gla proteins.[84,89] Central nervous system abnormalities and mental retardation were also reported in some cases.[96,137]

Diagnosis and Differential Diagnosis

The hallmark of VKCFD2 is the simultaneous decrease of FII, FVII, FIX, and FX, though the levels range widely. Diagnosis of familial VKCFD2 requires differentiation from acquired forms of the disorder that can be caused by intestinal malabsorption of vitamin K or liver and renal dysfunction, or treatment with warfarin.

Treatment

Large doses of vitamin K (2 to 10 mg daily) are the standard treatment. Suspected patients should be started on vitamin K therapy as soon as possible. Response to vitamin K varies from complete correction to no effect. The underlying mechanism for this variability is unknown. Treatment with fresh-frozen plasma is the only alternative, if the response to vitamin K is inadequate. Large dose of vitamin K should be given to pregnant women suspected of carrying a fetus affected by VKCFD.

OTHER INHERITED DISEASES WITH MULTIPLE COAGULATION FACTOR DEFICIENCIES

Deficiencies of other combinations of coagulation factors have been reported,[1,59,138–152] most probably due to rare chance inheritance of two different disease genes[2,15,153] or occasionally due to chromosomal abnormalities as is the case of some combined

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FVII and FX deficiency.[153] However, several other inherited diseases may exhibit a secondary coagulation abnormality. Most notable are the congenital disorders of glycosylation (CDG), a rare but rapidly growing family of inherited diseases affecting many of the steps involved in glycosylation of proteins in the ER and Golgi.[154,155] CDG types 1a and 1b are associated with abnormal coagulation factor levels, including decreased activity of FXI and of the coagulation inhibitors antithrombin III and protein C,[156] probably due to hypoglycosylation of these factors.

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CHAPTER

56 Clinical Disorders of Fibrinolysis

DAPHNE B. STEWART, CHARLES W. FRANCIS, AND VICTOR J. MARDER

Excessive promotion or inhibition of fibrinolysis interferes with the hemostatic balance and can lead to clinical bleeding or thrombosis. Hyperfibrinolytic mechanisms leading to premature lysis of hemostatic clots may result in significant bleeding disorders. Conversely, if impaired fibrinolysis slows clot dissolution, local clot formation occurs unimpeded, and a procoagulant state ensues. The importance of the fibrinolytic enzyme system is underscored by the observations that constitutional abnormalities in plasminogen,[1–3] plasminogen activators (PAs),[4] or their release,[5] are associated with an increased risk for thrombosis, whereas defects in PA inhibitors[6] and α_2-antiplasmin (AP)[7] are associated with an increased bleeding risk. Disorders impairing and promoting fibrinolysis occur as inherited disorders, which are rare and typically caused by a single molecular defect. Acquired disorders are more common, and heightened or impaired fibrinolysis may be the primary or contributing mechanism to bleeding or thrombotic events that occur in the course of an underlying systemic disorder or as the result of pharmacologic therapy. Clinical management requires an appreciation of the potential for pathologic bleeding or clotting; the use of appropriate, sensitive diagnostic tests; and rational use of medical therapies.

HYPERFIBRINOLYSIS WITH BLEEDING

Inherited Disorders of Fibrinolysis with Bleeding

α_2-Antiplasmin Deficiency

Inherited deficiency of α_2-AP is a rare autosomal recessive condition characterized by clinical bleeding due to premature dissolution of hemostatic plugs, typically seen as rebleeding following trauma or invasive procedures (Table 56.1).[8,9–11] AP inhibits fibrinolysis by forming a stable inactive complex with plasmin, by binding to the lysine-binding site of plasminogen and competitively inhibiting plasminogen binding to fibrin, and by covalent binding to fibrin via FXIIIa to prevent fibrinolysis by plasmin.[12] In patients with clinical evidence of the disorder, typical screening coagulation tests are normal, but the euglobulin clot lysis time (ECLT) is short due to uninhibited plasmin activity. A mouse knockout model of AP deficiency demonstrates enhanced endogenous fibrinolytic activity, but the mice, unlike humans, do not have an overt bleeding tendency, reflecting the presence of compensatory mechanisms.[13] The gene for AP has been mapped to chromosome 17,[14] and several mutations causing congenital deficiency have been elucidated.[15,16,17,18,19] Type I deficiency represents a quantitative disorder, with similar decreases in functional and immunologic assays, while type II deficiency results from a structural change causing lower activity than antigen concentration.[20]

Homozygous type I AP deficiency was first reported in 1978[8] in a patient with severe lifelong bleeding complications that included hemothorax, hemarthrosis, gingival bleeding, easy bruising, and prolonged bleeding after minor injury. Only 40 cases have been reported; the phenotype is variable, with the most severely affected patients symptomatic in childhood, even presenting with umbilical hemorrhage.[21] Intramedullary hematoma in the diaphyses of long bones represents a unique site of bleeding.[22] Individuals with heterozygous AP deficiency may be asymptomatic or exhibit a mild bleeding tendency that worsens with advancing age,[23–25] mostly following trauma, dental extraction, or surgery. Homozygous AP deficiency patients generally have <10% normal AP and heterozygotes have levels of 20% to 50%.[23,26]

Treatment with the antifibrinolytic agents tranexamic acid (TXA) or epsilon aminocaproic acid (EACA) can prevent bleeding during and after invasive procedures and is effective for treating acute bleeding episodes.[21,27,28] While no standard protocol exists, Morimoto et al. suggests using TXA 7.5 mg/kg orally 3 hours prior to dental extraction and then every 6 hours for 7 days. For dental extractions requiring gingival incisions or removal of bone, six patients were treated with infusion of TXA, 1.5 mg/kg/h followed by oral dosing once hemostasis was obtained.[27] Infusion of fresh-frozen plasma (FFP) provides adequate and lasting treatment levels, as the *in vivo* half-life is >20 hours,[28] although solvent or detergent treatment of plasma inactivates AP.[29]

Plasminogen Activator Inhibitor-1 Deficiency

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), physiologically limiting fibrinolysis to a thrombus by generating irreversible, inactive complexes. Congenital PAI-1 deficiency may be quantitative or qualitative and is characterized by recurrent bleeding, primarily after surgery or trauma.[30,31,32,33–35] As with AP deficiency, screening coagulation tests are normal; the ECLT is short (<2 hours); and fibrinogen, plasminogen, and AP levels are reduced to variable degrees. Diagnosis may be aided by demonstrating PAI-1 activity of <1 U/mL combined with elevated plasmin–AP complexes.[36]

Quantitative deficiency in a family of 26 affected individuals was linked to a frameshift mutation,[33] and those individuals who were homozygous for the mutation had mild to moderate bleeding symptoms (epistaxis, menorrhagia, bleeding following trauma), while heterozygotes tolerated surgery without bleeding.[33] The incidence is perhaps underestimated, as low PAI-1 levels (<1 U/mL) have been found in 23% of patients investigated for a bleeding tendency.[37] Bleeding can be treated effectively with antifibrinolytic medications.

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Table 56.1

Hyperfibrinolysis with bleeding

Inherited Disorders	Clinical Characteristics
AP deficiency	Premature lysis of hemostatic plugs Autosomal recessive, chromosome 17 Homozygous: severe bleeding Short lysis time, decreased plasmin–AP complexes, and fibrinogen Prophylaxis and treatment: FFP, antifibrinolytics
PAI-1 deficiency	Homozygous deficiency with moderate to severe bleeding Absent PAI-1, short lysis time, low fibrinogen, and plasminogen Increased plasmin–AP complexes Treat with antifibrinolytics
Elevated t-PA	Continuous plasmin activation Short lysis time, decreased fibrinogen Elevated circulating t-PA Treat with antifibrinolytics
Quebec platelet disorder	Excess u-PA in platelet granules released into hemostatic plugs causing premature lysis Increased copy number of u-PA gene Moderate to severe delayed bleeding Treat with antifibrinolytics
Factor XIII deficiency	Non–cross-linked fibrin undergoes premature fibrinolysis Increased clot solubility, low FXIII Severe delayed bleeding Prophylaxis and treatment: FFP, FXIII concentrate, antifibrinolytics
Acquired Disorders	
Cirrhosis	Hyperfibrinolysis exacerbates bleeding tendency, increases mortality Impaired clearance of t-PA Impaired synthesis of AP, PAI-1, TAFI Pronounced fibrinolysis during anhepatic phase of orthotopic liver transplant
Primary hyperfibrinolysis	Circulating t-PA due to endothelial release or infusion Rapidly cleared If DIC absent, may treat with antifibrinolytics
APL	Blasts release t-PA, u-PA, elastase Dense surface expression of annexin II on blasts enhances t-PA activation of plasmin ATRA decreases annexin II expression
Menorrhagia (normal uterine anatomy)	Increased t-PA in endometrium with high local fibrinolysis Antifibrinolytic treatment decreases bleeding Progesterone intrauterine device (IUD) decreases bleeding
FXIII deficiency	Consumption or immune clearance May be medication related: isoniazid (INH) Treat with: FFP, FXIII concentrate, antifibrinolytics, immune suppression
Trauma	Increased fibrinolysis with severe hypoperfusion Degree of fibrinolysis correlates with mortality Early volume resuscitation with FFP may benefit

FFP, fresh-frozen plasma; u-PA, urokinase plasminogen activator; AF, antifibrinolytics; TAFI, thrombin-activatable fibrinolysis inhibitor; DIC, disseminated intravascular coagulation; ATRA, all-trans retinoic acid.

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Increased t-PA

A congenital hemorrhagic disorder due to increased plasma levels of PA has been described in a few patients, including one with fatal intracranial hemorrhage.[6,38] Laboratory findings reflect continuous plasmin activation, as evidenced by elevated plasmin–AP complexes, shortened ECLT, low fibrinogen, and increased plasma t-PA that can be inhibited by specific antibodies.[38] Bleeding occurs after surgery, trauma, or dental extraction, although some family members have no clinical bleeding despite laboratory evidence of increased clot lysis. In one report, treatment with EACA or TXA resulted in improvement in laboratory markers of hyperfibrinolysis with normalization of clot lysis times and an increase in fibrinogen and α_2-AP.[38]

Quebec Platelet Disorder

The Quebec platelet disorder is an autosomal dominant condition characterized by moderate to severe delayed bleeding, typically starting at 12 to 24 hours after hemostatic challenge.[39] Affected individuals experience large bruises, joint bleeds, and local bleeding following dental extractions or trauma. The disorder was initially designated as *Factor V Quebec* because of deficiency in platelet factor V content.[40] Additional platelet defects include reduced count, defective platelet aggregation to epinephrine, and absence of α-granule contents and intrinsic membrane proteins.[39,41–43] Platelets of affected individuals show excessive α-granule protein degradation due to excess platelet u-PA, but external membrane, dense granule, and cytoplasmic platelet proteins are not affected.[39,41,44–46] The bleeding state is caused by the excess u-PA that is released into hemostatic plugs, and bleeding episodes may be treated with fibrinolytic inhibitors.[44,47]

Genetic marker analysis links the abnormality to the u-PA gene (PLAU) on chromosome 10; no point mutations have been found,[48,49] but an increased gene copy number likely accounts for the excess u-PA expression.[50] It is likely that testing for PLAU amplification will facilitate diagnosis in the future.

Factor XIII Deficiency

Activated factor XIII (FXIII) cross-links fibrin strands and AP to fibrin, thus contributing to fibrin clot stabilization and protection against premature fibrinolysis.[51] Congenital FXIII deficiency is associated with severe, lifelong bleeding due to rapid, plasmin-mediated lysis of hemostatic clots. The first described case was a young man with severe bleeding and normal coagulation assays, who had clots that lysed readily; addition of normal plasma corrected the laboratory abnormalities.[52] Approximately 160 cases have been reported.[53] FXIII is a tetramer, formed from dimerization of two A subunits associated with two B subunits (see Chapter 17). Deficient cases typically have absent A subunit in plasma. Patients typically present with delayed bleeding, even from the umbilical stump, and suffer a lifelong predisposition to subcutaneous, intramuscular, and intracranial bleeding; impaired wound healing; and spontaneous abortion.[54] Bleeding generally is delayed after trauma, compatible with lysis of clots after initial hemostatic plug formation. Intracranial hemorrhage is the most frequent cause of bleeding-associated mortality.[53]

Routine clotting time assays are normal. Diagnosis is based on solubility of clots in 1% monochloroacetic acid and 5 M urea, and by quantitation of FXIII activity in platelets and plasma. Treatment of acute bleeding episodes may be accomplished by infusion of small volumes of FFP, as control of hemorrhage requires raising plasma concentration by only a few percent.[53] The plasma half-life of FXIII is 11 to 14 days, so prophylactic doses may be infused every 6 weeks. Patients are generally treated with lifelong FFP prophylaxis, with fibrinolytic inhibitors, or with FXIII concentrates. A recombinant product, FXIII-A2 homodimer, has been developed though not yet approved by the FDA.[55] Inhibitors to FXIII resulting from replacement treatment have been reported.[56]

Acquired Disorders of Fibrinolysis with Bleeding

Cirrhosis

Severe liver disease is frequently complicated by clinically significant bleeding, due to a combination of decreased synthesis and/or clearance of coagulation factors and inhibitors, and thrombocytopenia caused by splenic sequestration and marrow insufficiency (Table 56.1). Accelerated fibrinolysis is common; in some cases this may represent the predominant hemostatic abnormality and is attributable to impaired clearance of t-PA and decreased synthesis of fibrinolytic inhibitors.[11] Although elevated plasma levels of D-dimer suggest disseminated intravascular coagulation (DIC), macrovascular and microvascular thrombosis is less common in advanced cirrhosis compared with other conditions associated with DIC.[57] Rather, an accelerated fibrinolytic state typically exists, identified by a rapid ECLT, increased plasma t-PA, and decreased AP and PAI-1.[58,59,60,61,62] Elevated D-dimer levels are likely the result of degradation of plasma-soluble cross-linked fibrin.[63] Chronic systemic hyperfibrinolysis is present in 30% to 46% of patients with cirrhosis and is more prevalent with greater severity of liver failure.[64] In a study of 112 cirrhotic patients with esophageal varices but without clinical upper gastrointestinal bleeding who were followed for 3 years, multivariate analysis indicated that elevated t-PA and D-dimer were the only markers predictive of bleeding.[65] In another study of 86 patients with cirrhosis, 31% showed a rapid ECLT, which was proportional to the severity of hepatic dysfunction.[66] Treatment with fibrinolytic inhibitors such as EACA and TXA may be useful in patients with cirrhosis, particularly those with mucosal or gastrointestinal bleeding, but there are no prospective controlled studies to validate clinical observations.

Pathologic hyperfibrinolysis may contribute to severe hemorrhage in some cases of orthotopic liver transplantation (OLT), especially during the anhepatic phase of surgery.[67,68] Accelerated fibrinolysis during OLT has been associated with increased plasma t-PA, depletion of fibrinogen and AP, and elevated fibrin degradation products.[69] Both systemic release and reduced hepatic clearance of t-PA contribute to accelerated fibrinolysis, which improves after revascularization of the transplanted liver. Treatment with fibrinolytic inhibitors can be useful. In a double-blind, randomized, placebo-controlled study of 45 patients, those who received TXA had less intraoperative blood loss and reduced intraoperative need for plasma, platelets, and cryoprecipitate.[70] Porte et al.[71] showed in a double-blind study of OLT recipients that treatment with aprotinin reduced blood loss with no increase in thrombosis or mortality.

Primary (Systemic) Hyperfibrinolysis

Primary systemic hyperfibrinolysis follows the widespread endothelial release or the exogenous infusion of t-PA, which leads to hemostatic plug dissolution and bleeding at sites of

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vascular injury. Disparate clinical conditions may cause systemic hyperfibrinolysis, for example, the bleeding symptoms of dengue infection in which plasminogen is activated by the virus[72,73] and heat stroke, characterized by an elevated body temperature, an exaggerated acute-phase response and multiorgan failure,[74] which may present as a hemorrhagic diathesis.[75] A similar transient clinical bleeding disorder may occur acutely after coronary artery bypass grafting surgery,[76,77] or following the infusion of t-PA to achieve therapeutic thrombolysis. Laboratory findings of hyperfibrinolysis, including increased t-PA and D-dimer, decreased plasminogen, and increased plasmin–AP complexes, likely reflect an exaggerated release of endothelial cell t-PA causing this primary hemorrhagic disorder, without the thrombotic events that characterize DIC syndromes (see Chapter 98). The hemorrhage may be transient and self-limited, reflecting clearance of t-PA, and in the absence of concomitant DIC, therapy may be limited to an antifibrinolytic agent and replacement of consumed factors, especially fibrinogen. In most cases, t-PA release is of limited duration, and therapeutic intervention is directed to the underlying condition.

Secondary Fibrinolysis in DIC: Microcirculatory activation of fibrinolysis may be a secondary response to the marked hemostatic activation and microvascular thrombotic occlusion of DIC and serves an important compensatory function in maintaining vascular patency (see Chapter 98). The bleeding that complicates DIC is primarily due to consumption of procoagulant factors and platelets, and this local fibrinolytic response usually does not reach the systemic level of primary hyperfibrinolysis. Pharmacologic inhibition of the compensatory fibrinolysis of DIC with antifibrinolytic therapy must be considered carefully, since local activation of fibrinolysis serves the crucial role of maintaining microvascular patency in patients with DIC. Fibrinolytic inhibition may exacerbate thrombosis and worsen or precipitate ischemic symptoms.[78,79]

Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL) represents a malignant clonal expansion of immature myeloid cells characterized by a balanced translocation, t(15;17), and the expression of a chimeric protein derived from the fusion of genes for the nuclear retinoic acid receptor on chromosome 17 and a transcription factor (PML) on chromosome 15.[80] Before the introduction of therapy with all trans-retinoic acid (ATRA), hemorrhage was a major cause of mortality, occurring in 14% of 268 patients during induction therapy.[81] Thrombocytopenia, DIC, and fibrinolysis may all contribute to the bleeding diathesis.[82,83] Leukemic cells contain t-PA, u-PA, and elastase, and elevated plasma levels of these proteins have been found in patients with accelerated fibrinolysis and APL.[84–87] Leukemic blasts also produce inflammatory cytokines (TNF-α, IL-1β)[88,89] that downregulate endothelial anticoagulant properties and upregulate procoagulant tendencies and express annexin II, a cofactor for t-PA-mediated activation of plasminogen to plasmin.[90,91] Surface annexin II, plasminogen, and t-PA may cause plasminemia and hyperfibrinolytic hemorrhage,[90] especially as plasmin present on cell surfaces is protected from inhibition by AP.[92]

Chemotherapy typically exacerbates bleeding,[79,93] but ATRA normalizes hemostatic abnormalities in the 1st week of therapy,[81] at least partly by downregulating surface expression of annexin II.[94] Although studies demonstrating the benefits of fibrinolytic inhibitors were published before the introduction of ATRA for induction therapy, contemporary treatment should focus on the optimal use of ATRA, and fibrinolytic inhibitors should be considered only in patients who are refractory to treatment and who have significant bleeding manifestations. A retrospective analysis of 30 patients with newly diagnosed APL observed that AP was a useful marker of fibrinolysis and hemorrhage risk and permitted treatment of higher risk patients with a coagulopathy protocol of low-dose heparin, EACA, and blood products.[95]

Menorrhagia

Up to one-third of women undergoing hysterectomy for menorrhagia have an anatomically normal uterus.[96,97] However, increased t-PA is present in the endometrium and menstrual fluid in women with menorrhagia, compared to women with normal menstrual blood loss,[98] likely explaining the excessive bleeding in some patients. A meta-analysis of seven trials of antifibrinolytic agents (vs. placebo) for menorrhagia found that treatment was associated with a considerable reduction in blood loss.[96] Antifibrinolytic agents compared to other nonsurgical therapies, including mefenamic acid, norethisterone, and ethamsylate, show a strong, though nonsignificant, trend in favor of antifibrinolytic agents in the participants' perception of improvement of menstrual blood loss. Increased fibrinolytic activity has been identified in the endometrium of women with an intrauterine device (IUD) associated with menorrhagia, and treatment with antifibrinolytic agents has been effective in reducing menorrhagia.[99] In contrast to standard IUD, those that release progesterone more effectively mitigate menorrhagia, as shown in a study of 41 women with menorrhagia treated with a levonorgestrel-releasing intrauterine system of whom 100% had resolution of their menorrhagia by 6 months. Endometrial sampling confirmed significant local increases in PAI-1 and endothelial expression of the u-PA receptor, without effect on systemic hemostasis.[100]

Amyloidosis

Patients with systemic, monoclonal light-chain amyloidosis may present with bleeding due to acquired factor X deficiency[101] or hyperfibrinolysis secondary to increased t-PA.[102,103] Hemorrhage in such patients has also been attributed to amyloid angiopathy, bleeding from the weakened walls of blood vessels infiltrated with amyloid fibrils.[104] The clonal plasma cells responsible for amyloidosis contain u-PA, in contrast to clonal plasma cells from multiple myeloma patients.[105,101] One report showed resolution of hemorrhage following treatment with nafamostat mesilate, a u-PA inhibitor,[105] and others relate hyperfibrinolysis primarily to deficiency of AP.[102,106,107] Patients with amyloidosis and hyperfibrinolysis often respond well to antifibrinolytic therapy.[101–103,106,107]

Acquired Factor XIII Deficiency

Acquired inhibitory IgG antibodies to factor XIII are associated with high morbidity and a case fatality rate of 50%.[108–111] Among the reported cases, six were temporally related to isoniazid, five occurred in the setting of active autoimmune disease, and three in patients with monoclonal gammopathy of unknown significance (MGUS),[108,109] with the cause of the remaining cases obscure. The cause of bleeding is the same as in congenitally deficient states: impaired cross-linking with premature fibrin lysis.[51] Diagnosis may be made by mixing patient plasma with

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normal plasma, with quantification of inhibitor by Bethesda assay, using an ECLT endpoint. Treatment of acute hemorrhage includes FFP or purified FXIII concentrate and antifibrinolytic therapy, plus targeted elimination of the inhibitor through steroids, immunoglobulin therapy, and immunosuppressive agents.

FXIIII can also be consumed, for example during extensive surgery, and subnormal levels may increase the rate of hemorrhage. An assessment of postoperative FXIII levels in normal individuals undergoing neurosurgery showed that 33% of patients with symptomatic intracranial hematoma had an FXIII level <60% compared with only 7% of patients who did not suffer hematoma formation.[112] Bleeding is treated with FFP.

Trauma

Trauma patients presenting with shock show a coagulopathy that is proportional to the degree of organ hypoperfusion and have a fivefold increase in mortality.[113] The coagulopathy of hypoperfusion suggests both DIC and secondary fibrinolysis and plasmin generation.[114] In a retrospective study of 314 severe trauma patients, of 55 nonsurvivors, 44 had laboratory parameters consistent with hyperfibrinolysis, the severity of which was predictive of massive bleeding and death within 4 hours.[115] A greater degree of hyperfibrinolysis confirmed by rotational thromboelastography also confers a greater risk of death.[116] A cohort of 208 patients presenting with systemic organ hypoperfusion following traumatic injury showed a more profound than anticipated activation of fibrinolysis, independent of acidosis or hypothermia.[117] Although not yet clinically proven, the study by Brohi et al. concluded that aggressive correction of hypoperfusion would be the most effective way to minimize the bleeding due to hyperfibrinolysis. The choice of resuscitation medium may also be relevant. Whole-blood dilution studies confirmed preservation of fibrinolysis following massive dilution with FFP versus saline[118] and suggest that early volume resuscitation with plasma after trauma may prevent excessive bleeding from hyperfibrinolysis.

HYPOFIBRINOLYSIS WITH THROMBOSIS

Inherited Hypofibrinolysis

The contribution of defective fibrinolysis to thrombotic disease has been difficult to evaluate because screening tests such as the ECLT are less sensitive to decreased activity than increased activity (Table 56.2). However, there is suggestive evidence that impaired fibrinolysis contributes to thrombosis in both inherited and acquired hypofibrinolytic states. The MEGA study (Multiple Environmental and Genetic Assessment of risk factors for venous thrombosis) analyzed ECLT in 2,090 patients with a history of deep vein thrombosis (DVT) and in 2,564 control patients and found that the lowest quartile of ECLT (e.g., most prolonged) was associated with a twofold increased risk of VTE.[119] In a separate cohort study of 770 patients after first venous thrombosis and 743 healthy controls, elevated levels of PAI-1 and thrombin-activatable fibrinolysis inhibitor (TAFI) were associated with thrombosis, each conferring a 1.6-fold increased risk,[120] suggesting that a hypofibrinolytic state is a predisposing factor for thrombotic disease. In a study of 897 patients with a first episode of venous thromboembolism, elevated PAI-1 was an independent risk factor for recurrence during 10 years of follow up.[121]

Plasminogen Deficiency

Plasminogen is the zymogen of the active fibrinolytic enzyme plasmin, and deficiency could be expected to result in impaired fibrinolysis and pathologic thrombosis. Functional plasminogen deficiency (50% of normal) was described by Aoki et al.[122] in a patient with recurrent venous thrombotic disease, the defect being an amino acid substitution occurring near to the active site histidine.[123,124] Several other plasminogen variants have been characterized by normal or slightly reduced antigen concentration with decreased functional activity.[3,125–127] A study in Scotland found familial plasminogen deficiency at a frequency of 2.9 per 1,000.[128] A common missense mutation (K19E) was found in most of the propositi, but none of the individuals developed venous thrombosis. In a large referral center, plasminogen deficiency was identified in 23 of 1,192 subjects, with a DVT prevalence of 2.2% and arterial events in 1.4%.[129] Of the 23 plasminogen-deficient individuals, eight had additional thrombophilic defects and four had relevant circumstantial risk factors. In a large database of 2,132 consecutive patients with venous thromboembolism, 16 (0.75%) had plasminogen deficiency.[130] The volume of evidence suggests that plasminogen deficiency is not a strong thrombotic risk factor when present as a single defect.

Although the association with thrombotic disease is tenuous, severe type I plasminogen deficiency is clearly associated with ligneous conjunctivitis, manifest by pseudomembranes developing on ocular and extraocular mucosa,[131–133] impaired wound healing, hyperviscous nasopharygeal and bronchial secretions, and hydrocephalus. The conjunctival lesions contain fibrin without plasminogen, suggesting that deficient fibrinolysis in tears is the likely cause of the fibrinous pseudomembranes, and a plasminogen knockout mouse model confirms this association.[134] Schott et al.[135] demonstrated that the ligneous lesions could be eliminated by plasminogen replacement, with normal anatomy restored after 2 weeks. Recurrence was prevented by daily injections of plasminogen sufficient to achieve plasma concentrations to approximately 40%. Treatment with topical plasminogen has also been successful,[136] and two women treated with oral contraceptives showed marked increase in plasminogen and some resolution of pseudomembranes.[137]

Dysfibrinogenemia

Dysfibrinogenemia is usually asymptomatic but may be associated with bleeding, pregnancy loss, or thrombotic disease (see Chapter 54). Of 250 cases of dysfibrinogenemia reported by Haverkate and Samama[138] in 1995, 20% had a thrombotic phenotype. Specific structural defects in fibrinogen may predispose to thrombosis, for example, those that impair interaction with fibrinolytic proteins or that polymerize to form a less porous clot, both effects serving to limit optimal fibrinolytic action. Fibrinogen Chapel Hill III[139] and Fibrinogen Dusart[140] have been associated with familial DVT, likely related to impaired fibrin lysis, secondary to decreased plasminogen binding and reduced t-PA–induced plasminogen activation.[141–143] Both conditions were characterized as a single-base change, AαArg554Cys.[144] Fibrinogen New York I has a deletion of residues 9 to 69 of the β chain (exon 2 for the gene)[145] and is associated with abnormal fibrinolysis and

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Table 56.2

Hypofibrinolysis with thrombosis

Inherited Disorders	Clinical Characteristics
Plasminogen deficiency	Venous thrombosis uncommon Ligneous conjunctivitis resolves with plasminogen replacement
Dysfibrinogenemia	Abnormal fibrinogen variants form lysis-resistant clots Treat thrombotic events with standard anticoagulation
Defective vascular response	Impaired endothelial release of t-PA in response to venous occlusion Associated with thrombotic disease No prospective evaluation for screening
Antifibrinolytic protein polymorphisms	Lead to higher levels of gene expression Associated with thrombotic events
Elevated TAFI	Increased risk of ischemic stroke Increased risk for recurrence after first thrombosis
Elevated PAI-1	Increased risk for pregnancy loss Impaired response to therapeutic thrombolysis Increased risk for recurrent venous thrombosis
Acquired Disorders	
Use of antifibrinolytic agents	Risk of thrombosis in patient with thrombogenic stimulus Avoid in DIC Higher mortality in patients treated with aprotinin
Atherothrombotic disease	Increased risk of reinfarction after MI Risk for progression of CAD after MI Lower rate of recanalization after therapeutic t-PA Elevated PAI-1 common
Postoperative thrombosis	Elevated PAI-1 Hypofibrinolysis contributes to thrombotic risk PCD stimulates local fibrinolysis
Pregnancy APS	Elevated PAI-1, TAFI impair fibrinolysis Risk for venous and arterial thromboses Elevated PAI-1 Production of antibodies against t-PA, plasmin Antibodies against annexin II Antibodies to β_2-glycoprotein plasmin activation

DIC, disseminated intravascular coagulation; CAD, coronary artery disease; MI, myocardial infarction; t-PA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor 1; PCD, pneumatic compression device.

defective potentiation of plasminogen activation by t-PA.[146] An example of abnormal polymerization leading to tight fibrin gels with fibrinolytic resistance is Fibrinogen Tampere.[147] Fibrinogen Caracas V occurred in a Venezuelan family with thrombophilia and exhibited prolonged thrombin and reptilase times[148] and a decreased capacity to accelerate t-PA–induced plasminogen activation, caused by an AαSer532Cys mutation.[149]

Those patients with identified dysfibrinogenemia without clinical bleeding should be treated with routine thromboprophylaxis during surgery or periods of immobilization.[150] Following a documented thrombotic event, patients should receive standard anticoagulation therapy, although the benefit of prolonged anticoagulation is not well defined.[150]

Defective Vascular Response

Defective endothelial release or low endothelial stores of t-PA would theoretically produce a local deficiency and impaired fibrinolytic response to incipient clot formation that could increase the risk of pathologic thrombosis. Impaired local fibrinolysis after a venous occlusion has been observed in patients with idiopathic DVT,[151] and Johansson et al.[152] noted reduced plasma fibrinolytic activity after venous occlusion or DDAVP infusion in 12 out of 13 family members with DVT.[152] A similar defect with a high incidence of venous thrombotic disease was described by Jorgensen et al.[153] in three generations of a family; six members studied showed a subnormal increase in fibrinolytic activity after venous occlusion or exercise and suffered recurrent venous thrombosis. A report by Stead et al.[5]

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described a family with five members in two generations in whom venous thrombosis developed at an early age, and whose plasma fibrinolytic activity after venous occlusion was markedly deficient. Because it was associated with a subnormal increase in von Willebrand factor (vWF), a combined endothelial cell defect in processing or storage of vWF and t-PA was suggested. A case study comparing patients with idiopathic DVT and unaffected relatives analyzed for t-PA release after venous occlusion and PAI-1 activity at rest demonstrated impaired release in 9% of patients with DVT (8 of 91). Six of eight of these patients underwent family analysis, and 50% of family members tested also demonstrated impaired t-PA release, suggestive of familial clustering.[154] Although this putative causative factor is intriguing, it has not been critically evaluated prospectively.

Antifibrinolytic Protein Polymorphisms

TAFI is activated by thrombin and protects nascent clot against fibrinolysis by impairing plasminogen and t-PA binding. High plasma levels of TAFI have been seen in patients with acute ischemic stroke compared with healthy controls.[155,156] Although no definitive association has been demonstrated between a high baseline TAFI concentration and venous thromboembolism,[157,158,159] >75th percentile TAFI was associated with a twofold higher risk for VTE recurrence.[160] *PAI-1* polymorphisms that lead to higher levels of gene expression may be associated with thrombotic disease. In a small study of white women suffering two or more spontaneous pregnancy losses, homozygosity for polymorphisms of PAI-1 (4G) or FXIII (34Leu) was associated with a twofold increase in relative risk for pregnancy loss,[161] and a lower rate of cerebral recanalization following thrombolytic therapy after acute ischemic stroke has been noted in patients with high plasma PAI-1 levels.[162] However, a large, prospective cohort study of women found the PAI-1 4G/4G was associated with a significantly lower risk of cerebrovascular mortality,[163] demonstrating that the role of polymorphisms in thrombotic disease remains controversial.

Acquired Hypofibrinolysis with Thrombosis

Administration of Fibrinolytic Inhibitors

Thrombosis due to excessive inhibition of fibrinolysis may result from administration of antifibrinolytic agents such as EACA to predisposed patients, including those with DIC or with the thrombophilia of malignancy (Table 56.2). Intravascular coagulation normally leads to activation of compensatory fibrinolysis, presumably due in part to stimulation of PA release from endothelial cells. This response contributes to the maintenance of vascular patency, and inhibition of fibrinolysis in patients with DIC or those developing thrombosis may disrupt the balance between thrombosis and fibrinolysis and exacerbate the thrombotic process with serious morbidity.[164–166] Because EACA is excreted in the urine and can inhibit normal urinary fibrinolysis, thrombosis may develop in the urinary collecting system in patients who have urinary tract bleeding and may result in ureteral obstruction; therefore, antifibrinolytic therapy must be used with great caution in patients with hematuria.[164] This is also true for patients with severe hemophilia.[164]

Aprotinin administration in the context of coronary artery bypass surgery has been associated with excess mortality, not noted for the other antifibrinolytic agents, although an excess of thrombotic disease has not been definitively proven. The BART trial (Blood Conservation using Antifibrinolytics in a Randomized Trial) compared aprotinin to the lysine analogs and concluded that aprotinin afforded patients a lower risk for massive hemorrhage, but greater risk for death,[167] suggesting the more powerful hemostatic effect could cause death through distant thromboses. Secondary thrombotic outcomes following treatment with antifibrinolytic agents during cardiac surgery have not been definitive. Although a large observational study showed a significantly increased incidence of myocardial infarction (MI) (55% increase) and stroke after use of aprotinin,[168] a meta-analysis reported no difference between aprotinin, TXA or EACA, or placebo in the risk of MI or stroke.[169]

Atherothrombotic Disease

In atherosclerotic disease, fibrin deposition occurs in the arterial wall, and thrombotic events resulting from plaque rupture lead to vessel obstruction. The fibrinolytic system is an important regulator of fibrin deposition, and hypofibrinolysis might be expected to increase fibrin deposition and promote thrombus formation. Patients with coronary artery disease have elevated PAI-1,[170] which is a risk factor for reinfarction in young survivors of MI.[171,172] Juhan-Vague et al.[173] found correlations of elevated PAI-1 with subsequent MI or sudden coronary death in 3,043 patients with angina pectoris. Similarly, there was a strong correlation between low plasma fibrinolytic activity and coronary artery disease in the Northwick Park Heart Study.[174] A Swedish case–control study found that high plasma PAI-1 concentration predicted the occurrence of the first MI,[175] and high plasma PAI-1 concentrations were also associated with progressive coronary artery disease in young men with a history of MI.[176,177] Elevated levels of t-PA have also been associated with the development and progression of coronary artery disease.[178] However, most t-PA in the circulation exists in a complex with PAI-1, and high t-PA levels likely reflect elevated PAI-1 levels.[175,179–183] The principal features of the "metabolic syndrome" include insulin resistance, hyperinsulinemia, impaired glucose metabolism, hypertriglyceridemia, low high-density lipoprotein-cholesterol concentrations, obesity, and hypertension,[184] and since the original description in 1983, high plasma PAI-1 concentrations have also found a place in this cluster. Glucose and insulin increase PAI-1 synthesis by endothelial cells,[185,186] unsaturated fatty acids and very low density lipoprotein (VLDL) increase endothelial cell secretion of PAI-1,[187,188] and VLDL increases the production of PAI-1 by hepatocytes.[189] These relations are the result of regulatory elements in the PAI-1 gene that are responsive to insulin, lipids, and estrogens.[190] Individuals homozygous for the 4G allele of PAI-1 have higher plasma concentrations and are more sensitive to upregulation by hypertriglyceridemia,[190,191,192] perhaps explaining the higher prevalence of the 4G allele with cardiovascular disease in some studies.[193–195] Patients undergoing thrombolytic therapy who fail to recanalize the obstructed vessel may have an excess of PAI-1. For example, 13 of 43 patients with acute stroke treated with thrombolytic therapy who failed to recanalize had higher PAI-1 levels than did patients who did recanalize,[162] and conversely, PAI-1 deficiency was predictive of intracranial hemorrhage following thrombolytic therapy.[196]

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Postoperative Thrombosis

Surgical trauma results in rapid activation of hemostasis followed by a postoperative reduction of fibrinolytic activity related in part to an increase in PAI-1.[197–199] This pattern has clear benefit in preventing excessive bleeding at the surgical site, but may also contribute to the increased risk of postoperative venous thromboembolism. The development of postoperative venous thrombosis has been related both to preoperative deficiencies of fibrinolysis and to excessive inhibition of fibrinolysis postoperatively, although the association is not strong enough to be of clinical predictive value.[198–200] Stimulation of fibrinolytic activity may be one mechanism underlying the benefit of pneumatic leg compression for reducing the risk of postoperative venous thromboembolism.[201–207]

Pregnancy

Pregnancy induces significant changes in the hemostatic system with increases in procoagulants, decreases in free protein S, and alterations in fibrinolysis, all of which prepare for the hemostatic challenges of delivery but increase the risk of venous thromboembolic disease.[208–214] Although increases in PA levels occur during pregnancy, these are overwhelmed by greater increases in PAI-1 and PAI-2, the latter of which is produced by the placenta,[215–217] and by TAFI levels that progressively increase to a peak at 20 weeks of gestation.[218,219] Preeclampsia is associated with elevated PAI-1, in correlation with the severity of placental damage.[220–222]

Antiphospholipid Syndrome

The antiphospholipid syndrome (APS) is a hypercoagulable disorder characterized by arterial and venous thromboses and autoantibody production. Impaired fibrinolysis may be a central component of the clinical thrombotic disease, potential mechanisms being elevated PAI-1, anti-t-PA antibodies, antibodies against annexin II, and anti-β_2-glycoprotein-I that block activation of plasminogen by t-PA. Patients with asymptomatic APS have high levels of PAI-1 and diminished t-PA locally following venous occlusion, compared with healthy controls.[223] A common finding in APS is a high circulating level of lipoprotein (a), which competes with plasminogen for binding to fibrin, impairing plasmin-mediated fibrin degradation.[224] An observational study of 25 APS patients found 7 (28%) with IgG antiplasmin antibodies.[225] Another found that two patients with APS had anti-t-PA antibodies that blocked t-PA–mediated plasminogen activation,[226] and a third report found APS patients with antibodies to annexin A2 that correlated with the incidence of thrombosis.[227] Functional β_2-GP-I is a phospholipid-binding protein with a lysine-rich domain that is cleaved by plasmin to facilitate phospholipid binding.[228] This "nicked" β_2-GP-I binds plasminogen and inhibits activation to plasmin by t-PA.[229] While intact β_2-GP-I enhances t-PA–mediated plasminogen activation 20-fold,[230] depletion of intact β_2-GP-I, as with neutralization by autoantibodies in APS,[231] could diminish clot lysis by inhibiting t-PA function.

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CHAPTER

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Acquired Disorders of Coagulation: The Immune Coagulopathies

CHARLES R.M. HAY

The formation of a blood clot is the final result of a cascade of interactions among multiple plasma proteins that ultimately result in the conversion of fibrinogen to fibrin and the cross-linking of fibrin by activated factor XIII to form a stable clot. Quantitative or qualitative deficiencies of many of the coagulation factors involved in these reactions may be associated with a clinically significant bleeding disorder. Circulating immunoglobulin anticoagulants may arise in the plasma of individuals whose coagulation mechanism was previously normal. These inhibitor antibodies, usually but not exclusively immunoglobulin G (IgG), may function as a circulating anticoagulant when directed against a specific clotting factor protein. These are autoantibody inhibitors, in contrast to the alloantibody inhibitors, which arise in individuals with congenital factor deficiencies as a complication of replacement therapy.[1,2]

Although neutralizing autoantibody inhibitors have been described to neutralize each of the coagulation factor proteins, antibodies to factor VIII, von Willebrand factor (vWF), and factor X are rare, and autoantibodies to other clotting proteins are extremely rare. Nevertheless, when they do occur, they may produce life- and/or limb-threatening bleeding. Acquired hemophilia A (factor VIII:C deficiency) is the most common of the acquired autoantibodies against specific clotting factors but is much less prevalent than the lupus anticoagulant (LAC). In fact, the acquired autoantibody, which characterizes the LAC, is an *in vitro* anticoagulant only, and is actually associated with clinical hypercoagulability *in vivo*.

Autoantibodies targeting specific coagulation factor proteins are often associated with more severe bleeding manifestations and often demonstrate more complex pharmacokinetics and binding affinities than alloantibodies directed against the same protein. Although frequently found in subjects without any associated pathology, autoantibody inhibitors may arise in association with a number of benign diseases, such as autoimmune disorder, chronic inflammatory and infectious states, and in pregnancy. The development of autoantibody inhibitors may also complicate lymphoproliferative and, to a lesser degree, solid tumor malignancies. Incidental associations have also occurred in the context of certain antibiotics and bone marrow transplantation. Some associations are quite specific, for example, the association between myeloproliferative disease and hyperthyroidism and mild acquired von Willebrand disease (vWD) and the association between amyloid and factor X deficiency, but many patients have no obvious underlying pathology.

From a laboratory perspective, the diagnosis of autoantibody inhibitors is based on the inability of normal plasma to correct prolonged clotting assays produced by patient plasma in mixing studies. The potency of the inhibitor and the target of the inhibitor determine the mode of treatment of acute bleeding episodes.

ACQUIRED INHIBITORS TO FVIII

Acquired Hemophilia

Autoantibody FVIII:C neutralizing inhibitors appear spontaneously in subjects with previously normal hemostatic function causing *acquired hemophilia*.[1,2,3] This rare immune coagulopathy occurs with an incidence rate of 1.38 and 1.48 per 1 million per year in the only two studies of acquired hemophilia in which the patients are linked to a defined population.[4,5] The latter figure is as close to a true incidence as one can currently get, since it is based on a 2-year, cross-sectional, UK study in which considerable effort was expended to record all cases diagnosed in every hospital in the United Kingdom during that time. Even so, many low-titer (<5 BU) inhibitors may remain undetected unless patients experience severe bruising or bleeding after surgery or trauma. Some cases of mild disease may be missed because of the vagaries of diagnostic accuracy and the tendency for these patients to present to generalists, surgeons, and physicians. Acquired hemophilia is being diagnosed more frequently now that there is increased awareness that this syndrome complicates a wide variety of autoimmune and malignant diseases. Also, the availability of improved and specific treatment options for acquired hemophilia has increased the urgency of accurately diagnosing this potentially severe and morbid condition.

Epidemiology and Clinical Presentation

Acquired hemophilia has an equal sex distribution overall, though younger patients are more frequently female.[5] The condition may arise at any time in life but usually presents in the extremely elderly,[4,5,6,7,8,9,10,11] the median age at presentation having been reported by Collins to be 78 years (range 2 to 98 years).[4] Earlier series tend to report relatively younger patient groups, either because of recall, referral, and/or reporting bias[6,7] or because of the focus of the report is on the treatment of bleeding.[8,9] Presentation during childhood is exceptionally rare.[5,9,10]

In approximately 50% of individuals with auto antibodies to FVIII:C, there is no obvious underlying disease state ("idiopathic" inhibitors).[4,5,6,11] Older patients with acquired hemophilia are much less likely to have an associated disease state than younger patients.[5] Diseases associated with acquired hemophilia include autoimmune diseases, hematologic and nonhematologic malignancies, drug reactions, and pregnancy.[4,5,6] These are described in greater detail below:

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There is a strong inverse correlation between the age at presentation and the likelihood that the acquired hemophilia has arisen in relation to some underlying disease.[5] Collins found an underlying diagnosis in all five patients under the age of 40 years, 55% of those between 40 and 59 years, 42% of those between 60 and 79 years but only 23% of those aged 80 or over ($P < 0.01$).

Immune Disorders

Acquired hemophilia has not only been described particularly with chronic, long-standing, severe, rheumatoid arthritis, but also with other autoimmune disorders/collagen vascular diseases, including systemic lupus erythematosus (SLE), Sjögren syndrome, and less frequently with temporal arteritis, multiple sclerosis, myasthenia gravis, autoimmune hypothyroidism and hyperthyroidism, ulcerative colitis, graft versus host disease, and following vaccinations.[5,6,10–15] Autoimmune dermatologic diseases, including pemphigus and psoriasis have also been occasionally associated with acquired hemophilia. Individuals with autoimmune FVIII:C antibody inhibitors secondary to autoimmune disease usually have high-titer inhibitors (>5 BU) that do not usually resolve spontaneously and are commonly relatively resistant to treatment with corticosteroids.

Malignancy

Hematologic malignancies, usually but not exclusively lymphoproliferative malignancies, have also occasionally been associated with acquired hemophilia. These include chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma, multiple myeloma, Waldenstrom macroglobulinemia, myelofibrosis, and myelodysplastic syndromes.[5,6,17]

Solid tumor malignancies, including carcinoma of the prostate, breast, lung, stomach, pancreas, colon, head and neck, and renal cell[5,6,18] have all been associated with acquired hemophilia. Inhibitor antibodies secondary to solid tumors[17,18] tend to be of low-titer. There is no correlation between the titer of the autoantibody to FVIII:C and tumor size, aggressiveness, or likelihood of tumor response to chemotherapy or radiation. Acquired hemophilia complicating malignancy has a poor overall prognosis. Successful suppression of the autoantibody inhibitor occurs in only about 20% of these cases and may require tumor eradication.[17,18] If the autoantibody FVIII:C inhibitor does remit, it may recur and relapse of the inhibitor is not a reliable marker of tumor recurrence. Acquired hemophilia occurs much more frequently in association with lymphoproliferative malignancies than with solid tumors. CLL, non-Hodgkin lymphoma, multiple myeloma, and Waldenstrom macroglobulinemia are the most frequent hematologic malignancies associated with the development of autoimmune FVIII:C inhibitors. Although autoantibody and alloantibody inhibitors to FVIII:C are usually IgG, IgA and IgM monoclonal antibodies have been described in cases of multiple myeloma and CLL. Isolated cases of acquired hemophilia complicating myelodysplastic syndromes, myelofibrosis, and erythroleukemia, have also been reported.

Although acquired hemophilia has often been reported to follow drug administration, many of these drug associations may be coincidental and spurious.[5] Nevertheless, the literature lists associations with allergic diseases and drug reactions including asthma; allergies to penicillin, sulfonamides, phenytoin, chloramphenicol, and methyldopa; treatment with fludarabine, interferon-α; and antipsychotic medications.[19,20] Allergic reactions to medications, particularly sulfonamides, rarely penicillin and most recently clopidogrel,[29] have been implicated in the development of an autoimmune response to FVIII:C. The inhibitory antibody usually disappears shortly after withdrawal of the offending drug. Of interest is the fact that acquired inhibitors to FVIII:C have also been observed after treatment with medications known to interfere with the immune system, such as interferon-α and fludarabine.[19]

Pregnancy

The association between acquired hemophilia and pregnancy has been overstated in earlier publications in which pregnancy-associated acquired hemophilia accounted for up to 10% to 15% of total cases. This is probably accounted for by recall and reporting bias. The recent 2-year survey of Collins et al.[5] reported only three cases of pregnancy-associated acquired hemophilia (2% of the entire cohort and 4.3% of women reported). This represents 1 case per 350,000 births during that period. A similar incidence was reported from the Italian Registry.[23]

Acquired hemophilia arises in mostly primiparous women (80%), usually within 3 months postpartum, though cases have been described as late as 11 months postpartum, and presentation around the time of delivery or even antenatally is not uncommon.[5,8,9,22,24,26] Of the 42 cases of pregnancy-associated acquired hemophilia reported to the European acquired haemophilia (EACH2) registry, only 1 presented antenatally.[25] Late presentation may represent delayed diagnosis rather than late onset. Recurrence of the inhibitor in subsequent pregnancies has been reported but is not common, though mothers should be warned of this possibility. Although one study reported recurrence in four-sixth of subsequent pregnancies,[21] no relapses were reported in nine subsequent pregnancies in a further report[26] and no relapses were reported in the four patients reported in the Italian registry.[23] When acquired hemophilia occurs antenatally, transplacental transfer of IgG autoantibody may cause life-threatening bleeding in the baby.[23,27,28] Although spontaneous remission has been described in postpartum-acquired hemophilia patients, they usually require immunosuppressive therapy to achieve remission (see below). Severe uterine bleeding during labor or delivery may occur in those presenting antenatally and this sometimes requires hysterectomy. Nevertheless, the overall mortality of acquired hemophilia associated with pregnancy is relatively low (<5%).[22,26] These cases are usually sensitive to corticosteroids alone, though spontaneous remission may occur in up to 76%, if steroids are withheld. This contrasts with the relatively low spontaneous remission rate of <30% observed generally for autoimmune hemophilia.[22,23,24]

Spontaneous remissions of the autoimmune antibody inhibitor targeting FVIII:C is not uncommon, and may occur in approximately one-third of cases, usually after months to years of involvement.[6] More recent reports do not report spontaneous remission, because virtually all patients are offered immunosuppressive therapy to minimize the period during which they may be at hemorrhagic risk. Spontaneous remission in pregnancy-associated acquired hemophilia syndrome is said to be particularly common but, again, these women are usually offered immunosuppressive therapy to minimize the hemorrhagic risk.

Clinical Manifestations

The occurrence of an acquired inhibitor to FVIII:C typically is heralded by spontaneous, bruising and soft tissue bleeding in individuals who lack a previous bleeding history. Bleeding

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may sometimes be life- and limb-threatening. Hemarthrosis are unusual in acquired hemophilia, in contrast to congenital hemophilia. The commonest bleeding manifestation of acquired hemophilia is bruising, with often massive ecchymosis. Muscle bleeds occur in 40%, followed in frequency by gastrointestinal, urinogenital, and retroperitoneal bleeding. Spontaneous bleeding into the central nervous system is fortunately relatively infrequent.[5,6,7,8,9] Since the 1st obvious bleeding manifestation may be hematuria or melena, these patients may present to generalists and diagnosis may consequently be delayed, sometimes until after some surgical intervention.

Between 7.9% and 22% of cases have been reported to die from hemorrhage.[6,7,8,25] More recent studies reveal a trend toward decreasing hemorrhagic mortality, which may be related to the availability of improved management options.[8,9] However, the recent study of Collins et al. reported a 9.1% mortality rate from hemorrhage. Most hemorrhagic deaths are from gastrointestinal or retroperitoneal bleeding and occur within the 1st few weeks after presentation though patients remain at risk of life-threatening bleeding until the inhibitor has been eradicated.[5] Delayed diagnosis and inadvertent treatment, such as surgery, contribute significantly to the early high mortality rate, occurring mainly because these patients frequently present to surgeons and general physicians.[5,8]

Infectious complications, secondary to immunosuppressive therapy, account for most of the late deaths in patients with acquired hemophilia. Indeed, infection appears to be a commoner cause of death than hemorrhage in acquired hemophilia. Collins reported an 11% mortality rate from sepsis.[5] Indeed, 33% of patients from this cohort suffered from sepsis at some time in their course.

Although bleeding may be severe and life-threatening, the recent cross-sectional study of Collins showed that 34% of patients have only mild bleeding manifestations that did not require hemostatic replacement therapy.[4,5] This 2-year study attempted to capture all cases of acquired hemophilia presenting in any hospital in the United Kingdom during a 2-year period. This observation also accounts for the paradoxical observation that the prognosis for acquired hemophilia is better for patients managed outside a major teaching center because those with severe bleeding are more likely to be referred in to a teaching hospital.[5] Earlier reports tend to overstate bleeding severity either because of recall bias[6] or because they only report patients treated for bleeding.[8,9] Interestingly, at the time of presentation, neither the factor VIII level nor the inhibitor titer predicts the severity of subsequent bleeding events.[5,8] Patients who died from hemorrhage and those who required no hemostatic therapy at all had a similar presenting median (range) factor VIII level and inhibitor titer.[5]

Laboratory Diagnosis of Autoimmune FVIII:C Antibody Inhibitors

The clinical suspicion of acquired hemophilia, based on the recent onset of significant bruising or bleeding in a previously noncoagulopathic individual, is strengthened by the detection of a normal prothrombin time (PT) but prolonged activated-partial-thromboplastin time (aPTT). The aPTT initially corrects using 50:50 normal plasma, which suggests a factor deficiency, but the aPTT subsequently prolongs again on incubation at 37°C. An intrinsic factor screen will reveal a low factor VIII level, typically lower than 0.05 IU/dL, but may appear to show less dramatic reductions in factors XI, XI, and XII. This is a laboratory artifact caused by the inhibitor-depleting factor VIII from the XI-, XI-, and XII-deficient plasma used in these one-stage assays. Dilution experiments show an apparent increase in the concentration of factors IX, XI, and XII, whilst the factor VIII level remains low.[3,8,31,32] This may cause confusion with the LAC, which interferes nonspecifically with phospholipid-dependant one-stage assays but whose clinical presentation is dramatically different.[31] The diagnosis is confirmed using a formal Bethesda assay, possibly modified following the Nijmegen recommendations for low-titer inhibitors. Autoantibody factor VIII inhibitors in acquired hemophilia are usually polyclonal and have complex reaction kinetics. Consequently, patients with acquired hemophilia often have residual circulating factor VIII and the Bethesda assay is semiquantitative at best. This is linked with the clinical observation that bleeding severity is unrelated to the factor VIII level and inhibitor titer measured at the time of presentation. It is common practice to report the inhibitor titer based on the dilution closest to 50% inhibition. There have also been reports of anticoagulant treatment masking acquired hemophilia, possibly delaying diagnosis or being causally associated with the occurrence of acquired hemophilia. Patients on anticoagulants who present with bruising should therefore have both an aPTT and an International Normalized Ratio measured, for that reason.[33–35] An enzyme-linked immunosorbent assay (ELISA) assay may be useful in complicated cases.[3,30]

Immunochemistry of Autoantibody Inhibitors to FVIII:C

In contrast with the oligoclonal alloantibodies found in congenital hemophilia, and which are usually both IgG1 and IgG4, autoantibody inhibitors to FVIII:C is usually polyclonal IgG4. A further distinctive feature is that the autoimmune autoantibody inhibitors may rarely be monoclonal IgA or IgM antibodies and shifting immunoglobulin profiles have been observed, for example, shifting from IgM to IgG.[36]

Systematic epitope mapping has indicated that autoantibodies are usually directed against either the A2 or C2 domain of the FVIII:C molecule (62%) and much less frequently to both epitopes as observed with alloantibodies, which overwhelmingly interact with both A2 and C2 domains.[36,37] There appears to be preferential targeting of FVIII:C autoantibodies to the C2 domain (67%). The A3 epitope is targeted much less commonly. Both alloantibodies and autoantibodies bind to the same epitopes on the A2 and C2 domains. Autoantibodies may exert their inhibitory activity by interfering with thrombin cleavage sites on A2 or by impeding the interactions between FVIII:C and phospholipid or vWF protein on C2. This may result in increased FVIII:C catabolism and increased susceptibility of FVIII:C to degradation by activated protein C and factors IX and X.

Clinical Management

The management of acquired hemophilia involves treatment of any bleeding complications, simultaneous immunosuppression to abolish the inhibitor and investigation for and treatment of any underlying causative disorder. Patients with acquired hemophilia should be managed in or at least in collaboration with a hemophilia center experienced in managing the disorder, even if the initial presentation appears relatively benign. Invasive procedures and surgery should be avoided, if possible. Intramuscular injections should be avoided and venepuncture minimized since these can cause significant and troublesome

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bleeding. If procedures or surgery are unavoidable, they should be conducted in a hospital with an experienced hemophilia center and with adequate hemostatic cover. Patient education is also important, especially for patients managed as an outpatient. The patient and their relatives have to be made aware of the seriousness of the condition so that they present to hospital in a timely manner.[3,38,39]

Management of Bleeding Complications

FIGURE 57.1 shows an algorithm for treatment of bleeding complications.

Bleeding may be life- and limb-threatening. Morbidity and mortality are reduced by prompt and adequate hemostatic treatment supervised at an experienced hemophilia center. None of the hemostatic options available have predictable efficacy in acquired hemophilia. Neither the presenting inhibitor titer nor the residual factor VIII level, nor the factor VIII level achieved following the infusion of human or porcine factor VIII are predictive of clinical response.[3,8,9] Regular clinical review and appropriate imaging and laboratory monitoring are therefore vital when monitoring the efficacy of hemostatic therapy for this condition. An early assessment of hemostatic efficacy is very important so that if the treatment is not working a different agent may be tried with the minimum of delay. Dosing needs to be adequate and should continue for some time after bleeding is controlled, at a reducing dose, to optimize clinical response and minimize the risk of recurrent bleeding. This is particularly important for intracranial, intramuscular, and retroperitoneal bleeding. In contrast, many patients need no hemostatic treatment at all and subcutaneous bleeding and bruising can be managed conservatively, even if extensive. Mucosal bleeds may also benefit from the use of antifibrinolytic agents such as tranexamic acid or aminocaproic acid.

Bleeding is treated either with bypassing agents (currently, Factor Eight Inhibitor Bypassing Activity [FEIBA] (Baxter, Vienna) or NOVOSEVEN (rVIIa, Novo, Denmark) or with factor VIII-containing agents to raise the factor VIII level.[3] A recent survey from the EACH2 registry suggests that bypassing agents may be clinically more effective than factor VIII.[40,41] Porcine factor VIII has been unavailable for some years, however, and this comparison may have to be reevaluated when the current recombinant B-domain-deleted porcine factor VIII completes ongoing clinical trials in acquired hemophilia and gains a product license for this indication.

Factor VIII:C Concentrates

Previous reports of the treatment of bleeding in acquired hemophilia have shown that human factor VIII concentrate is the 1st agent used to treat bleeding in about one-third of cases.[6,8,9] The EACH2 registry data suggest that the response to factor VIII may be significantly worse than for bypass therapy in this group, probably because the half-life of factor VIII is severely curtailed in acquired hemophilia.[40,41] Human factor VIII may be used in large doses, when this is sufficient to overcome a relatively low-affinity inhibitor and an effective postinfusion factor VIII increment can be achieved. The use of factor VIII is, therefore largely limited to patients with low-affinity inhibitors. Although dose-calculating formula have been proposed, empirical dosing combined with close laboratory monitoring of the response and prompt dose adjustment is probably the best approach since the

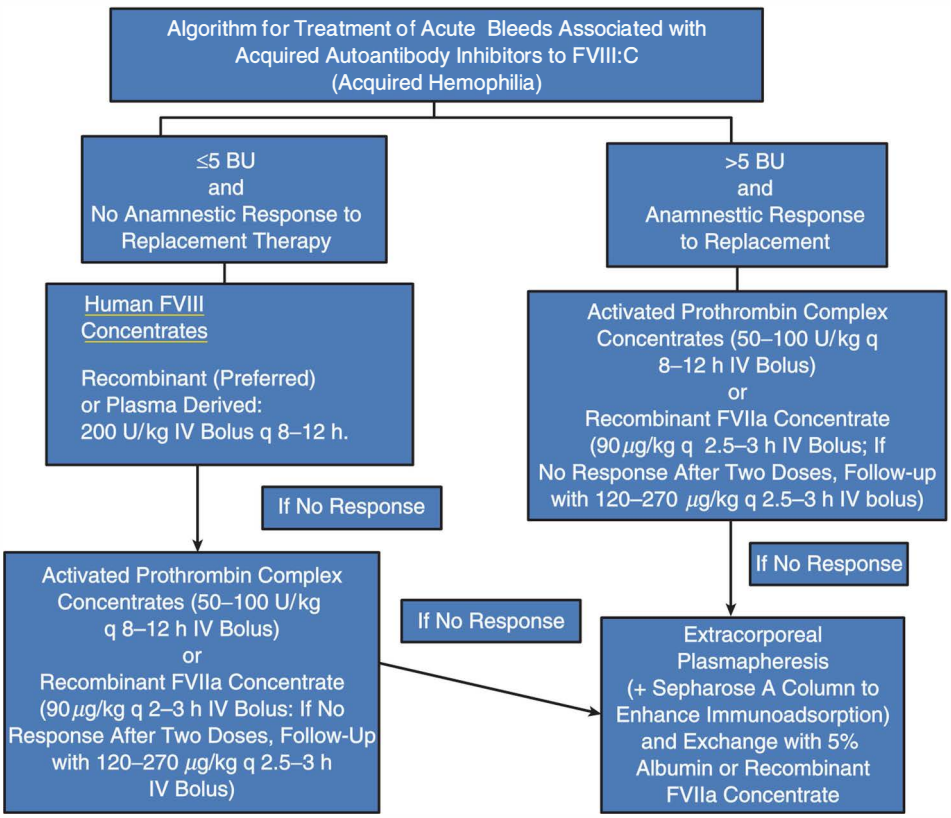

FIGURE 57.1 Algorithm for treatment of acute bleeds associated with acquired autoantibody inhibitors to FVIII:C. FIX, factor IX; BU, Bethesda units; rFVIIa, recombinant factor VIIa; PCC, prothrombin complex concentrate; DDAVP, 1-deamino-8-D-arginine vasopressin.

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Bethesda titer is not quantitative in this condition and therefore gives a poor guide to the likely response to factor VIII. Factor VIII replacement combined with immunoadsorption of the inhibitor antibody may also be effective, if this is available.[42,43] Human plasma-derived FVIII:C concentrates, which are characterized as being of intermediate purity by virtue of their substantial content of vWF protein, theoretically may be less susceptible to inactivation by the autoimmune FVIII:C inhibitor directed against the C2 domain[37]; however, this premise has not been confirmed in clinical studies. In contrast to congenital hemophilia, the use of factor VIII concentrate will not cause an anamnestic increase in the inhibitor titer.

Porcine Factor VIII

Another source of FVIII:C concentrate, which was very much more effective than human factor VIII, was porcine factor VIII concentrate. Cross-reactivity of antibodies in acquired hemophilia to porcine factor VIII was characteristically low, averaging 5% to 10%,[8,44,45] permitting porcine factor VIII to achieve hemostatic factor VIII levels in patients completely resistant to human factor VIII. Cross-reactivity varies considerably between individuals however, and so it is recommended to give an initial bolus of 75 to 100 IU porcine FVIII:C with subsequent dosing and dose intervals determined by the level of FVIII:C activity achieved and the durability of the response. FVIII:C levels of at least 30% to 50% are desirable, though clinical response has not been shown to correlate with the factor VIII level achieved.[8] Porcine factor VIII was reported to give a good or excellent response in 78% of 74 bleeds and failure in only 9%.[8] This response rate is comparable with that achieved using bypassing agents. More consistent high levels and improved pharmacokinetics may be achievable by administration using continuous infusion.[46] In contrast to congenital hemophilia, an anamnestic rise in the factor VIII inhibitor is not seen following exposure to factor VIII in acquired hemophilia. Specific antiporcine factor VIII antibodies may arise in 30% and these patients, however, rendering them resistant to porcine factor VIII.[45] Porcine factor VIII was manufactured from porcine plasma, which could not be rendered "virus free." Consequently, the product was withdrawn some years ago. An alternative, recombinant B-domain-deleted porcine factor VIII (Inspiration, California) has recently completed trials in congenital hemophilia[47] and multinational trials have begun in acquired hemophilia. This product looks very promising for the management of bleeding in acquired hemophilia but will probably not be available for general clinical use until 2013 to 2014.

Desmopressin (1-deamino-8-D-Arginine Vasopressin, DDAVP)

Administration of 1-deamino-8-D-arginine vasopressin (DDAVP) (Desmopressin) either intravenously or subcutaneously at the dose of 0.3 μg/kg induces a rapid rise of vWF and FVIII:C activities in normal individuals and those with mild uncomplicated hemophilia A and vWD.[47] DDAVP may also induce a rapid rise of FVIII:C in individuals with low-titer inhibitors, rendering the drug suitable for the treatment of minor, non–life-threatening bleeds or for prevention of potential bleeding secondary to mild surgical procedures in the context of acquired hemophilia.[48] The response is very variable, however. Of the 22 treatments reported, 11 patients with FVIII <5 IU/dL DDAVP resulted in an increase to between 15 and 140 IU/dL. Five patients with a Bethesda titer <2 BU/mL all responded with peak FVIII levels >80 IU/dL and a half-life of 4 to 6 hours. In contrast, no significant clinical response was reported for seven patients with a FVIII level <1 dL/mL.[48] The use of DDAVP in this condition is therefore very limited, and it should probably therefore be reserved for the treatment of minor bleeding in patients with a discernible circulating factor VIII level and low-level inhibitors.[3]

Bypassing Agents

At present the choice of bypassing agents is limited to FEIBA (Baxter Vienna) and Novoseven (recombinant activated factor VII, rVIIa, NovoNordisk, Denmark), although several manufacturers are currently developing analogs of rVIIa. Although non-activated prothrombin complex concentrates were used from the 1970s, these products have now largely fallen out of use for bleeding in acquired hemophilia. Both FEIBA and Novoseven have been shown to be effective in treating bleeding in acquired hemophilia.[9,40,41,49–51,52]

Although there are no direct comparative trials of FEIBA and Novoseven in acquired hemophilia, the data from the EACH2 registry and comparison of the published literature suggests that the two agents have similar efficacy.[9,40,41,49–51,52] National statistics suggest that FEIBA is used more frequently than Novoseven for acquired hemophilia, at least in the United Kingdom.[53] The choice of bypassing agent should depend, where possible, on the patient's previous response, the dosing schedule, and possibly cost. When first-line therapy fails, the alternative bypassing agent may be used. In cases of life- and limb-threatening bleeding, both bypassing agents may be used sequentially or together since they synergize and have greater hemostatic efficacy when used in combination.[53–54] The use of combination bypass therapy may be associated with an increased risk of both venous and arterial thrombosis, particularly in the elderly, and should therefore only be used for very serious bleeding, unresponsive to full doses of one or both bypassing agents.[3]

Both agents have been associated with a relatively small thrombotic risk.[39,52,56–60] Disseminated intravascular coagulation (DIC), myocardial infraction, and deep vein thrombosis have been associated with the use of FEIBA.[56] It is impossible to compare the thrombogenicity of the two products directly because most of the thrombotic events reported in relation to Novoseven are thought to relate to off-label use in nonhemophiliacs and FEIBA is currently only used in acquired and congenital hemophilia. However, the overall risk of thrombosis is low (~1 per 10^{4-5} infusions). Twelve (8.6%) of 139 patients with acquired hemophilia treated with rVIIa were reported to have had a thrombotic event, which was arterial in 10 cases.[52] Whether these events were directly related to rVIIa treatment is unclear and the authors point out that the methodology used would tend to overestimate the incidence of events.[52] EACH2 reported 8 (2.3%) arterial events amongst 337 patients.[40] One might expect that the thrombotic risk would be greater in patients with acquired rather than congenital hemophilia since the former are far more elderly and may have multiple risk-factors for arterial disease.

A limitation of bypassing agents is that they have no validated and readily available laboratory monitoring technique and dosing and dosing intervals must be adjusted based on clinical evaluation alone. Thrombin generation assays[60,61] and thromboelastography (TEG)[62,63] have both been used in a research

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setting to assess response and adjust dosage of bypassing agents. Neither is the test fully validated nor widely available and there are no published data in relation to the use of these tests to manage acquired hemophilia. It may not be possible to extrapolate evidence on the use of these tests from congenital to acquired hemophilia because of the differing immunochemistry and kinetics of the inhibitors involved. Further studies are required. Furthermore, preliminary evidence suggests that TEG does not predict clinical response consistently.[64]

FEIBA

The postulated mechanism of action of FEIBA is that of "bypassing" the obstruction to coagulation caused by the neutralizing inhibitor of FVIII:C. This is accomplished through their content of trace amounts of serine proteases (activated factors IX, X, VII, and thrombin). Retrospective analyses of patient cohorts with alloantibodies and autoantibodies have revealed efficacy rates of approximately 80% for both FEIBA (average dose of 70 IU/kg).[49] Furthermore, bolus doses higher than 200 IU/kg or frequent repeat dosing of activated prothrombin complex concentrates (aPCC) significantly increases the risks of thrombotic adverse events.[56] A more recent retrospective report of 34 severe and moderate bleeds treated mainly with 75 U/kg between 8 and 12 hourly reported an efficacy of almost 100% after a median of 6 infusions for moderate bleeding and 10 infusions for severe bleeding.[50] The EACH2 registry reported 94% efficacy for 64 bleeds treated with FEIBA.[40]

Recombinant FVIIa Concentrate

Recombinant FVIIa (rFVIIa, Novoseven) is generally administered as an intravenous bolus, resulting in supraphysiologic levels of FVIIa activity in blood. In individuals with normal coagulation, it is hypothesized that thrombin is generated after FVIIa interacts with tissue factor (TF) on mononuclear cell surfaces (monocytes and macrophages) and subsequently activates FX to participate in the tenase and prothrombinase complexes. This small quantity of thrombin is adequate only to activate platelets and to cleave vWF protein from FVIII:C, but this results in a self-amplification process in which rFVIIa binds to the surface of the activated platelet, activates FX in the presence of FVIII and FIX, and produces an extraordinary "thrombin burst" to affect fibrin formation.[65] Pertinent to the clinical situation with autoantibody FVIII:C or FIX inhibitors, large amounts of administered rFVIIa may bind nonspecifically to the platelet surface and activate FX in a TF-independent manner and in absence of FVIII:C and/or FIX.

Novoseven is normally given in a dose of 90 μg/kg, administered every 2 to 3 hours because of its short circulating half-life but a larger dose of 270 μg/kg has been shown to be safe and as effective as three 2-hourly doses of 90 μ/kg.[66,67] Once bleeding has stopped, the dose interval is lengthened but replacement therapy with rFVIIa should continue for several days to minimize the risk of re-bleeding. In the largest clinical trial in acquired hemophilia, rFVIIa, employed as first-line replacement therapy, reversed symptomatic bleeding 100% of the time, whereas rFVIIa, used as salvage or rescue therapy, yielded a 75% response rate.[25] A recent systematic review[52] reported a 95% efficacy for rVIIa, when used as first-line therapy for 103 bleeds. The EACH2 registry reported similar 91% efficacy for 170 bleeds treated with Novoseven as first-line therapy.[40] When used as second line therapy, the reported efficacy of Novoseven is lower at 86%.[52] The median duration of treatment has been reported to be 6 days (1 to 33).[52] If bleeding is not successfully stopped within the initial 24 hours of treatment with rFVIIa, the patient is likely to remain unresponsive to further rFVIIa and an alternative regimen should be considered.

Overall, very few side effects, including episodes of thrombosis, have been reported for rFVIIa concentrate use in autoimmune hemophilia. A finite risk does exist, however, and cases of myocardial infarction, DIC, and venous thromboembolism (VTE) have been reported.[40] No laboratory methods are available to monitor the hemostatic efficacy or safety of rFVIIa.

Immunosuppression Regimens

As soon as the diagnosis of acquired hemophilia has been established, the patient should be given some form of immunosuppressive treatment to eradicate the inhibitor.[3,30,38] Although up to 25% to 30% of patients may remit spontaneously,[22,23,24,68] they may suffer significant morbidity whilst awaiting spontaneous remission and are more likely not to remit. For that reason, the current consensus of opinion is that all patients with acquired hemophilia should be offered immunosuppressive therapy in the hope that this will not only optimize the remission rate but reduce the time taken to achieve remission, thus minimizing bleeding morbidity and mortality. However, when choosing an immunosuppressive regime, it should be remembered that infection is actually the commonest cause of death in acquired hemophilia[5] and aggressive immunosuppressive regimens may contribute to the morbidity and mortality associated with the condition. For this and other reasons, aggressive immunosuppressive regimens have fallen a little out of favor in recent years as a broad generalization. The options available for immunosuppression include administration of: corticosteroids alone; corticosteroids plus cyclophosphamide; rituximab (anti-CD20 monoclonal antibody); cyclosporin A; immunosuppression/immune tolerance induction regimens; high-dose intravenous immunoglobulin (IVIG); plasmapheresis/immunoadsorption; immune-tolerance regimens, and finally, corticosteroids combined with one or more cytotoxic agents cytotoxic agents (e.g., Cyclophosphamide, Azathioprine, Vincristine, 6-MP).

The various available regimens are difficult to compare directly because patient selection and end-points vary from report to report and most reports are of uncontrolled cohort studies. Almost all are case-reports, single center cohorts, or retrospective surveys. Furthermore, since most reports come from teaching centers, there is reporting bias toward more seriously affected cases. There may also be positive publication bias since failures are more difficult to publish.

Although there are no reports of relapse in older publications, patients with acquired hemophilia have presumably always relapsed and recent studies report a relapse rate of 10% to 20%.[5,69] Relapse occurred after a median of 7.5 months (0.25 to 14 months) in 20% of 102 patients.[5] This finding was reproduced in the EACH2 registry, which reported 18% relapse amongst those treated with steroids alone and 12% relapse in those treated with steroids and cyclophosphamide after a median of 4 months. All patients with acquired hemophilia therefore require long-term follow-up, even if they go into complete remission and should be warned of the possibility of recurrent bleeding symptoms. Furthermore, many patients require long-term maintenance immunosuppressive therapy to maintain remission or even partial remission and to prevent relapse.

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Corticosteroids with/without Cytotoxic Agents

For many years it was thought that prednisolone combined with cyclophosphamide offered a slightly higher remission rate and more rapid response, though this has recently been challenged.[5] Recent data and reexamination of the literature led one to the conclusion that the combination had no advantages over prednisolone alone, however.[3,5,41] The only randomized comparison recruited only 31 patients and was therefore underpowered. The patients were treated with prednisolone 1 mg/kg/d for 3 weeks. The 21 nonresponders were then randomized either to continue prednisolone or to continue prednisolone combined with cyclophosphamide.[70] There was no difference between treatment arms, though the number of subjects was very small.

The UK prospective cohort study[5] permitted a prospective but nonrandomized comparison of patients treated with prednisolone or with prednisolone plus cytotoxic agents. The study design (an all-inclusive cohort study of all patients presenting in the United Kingdom over 24 months) should prevent selection bias. This showed that the 34 patients treated with prednisolone alone had a 76% complete remission rate after a median of 49 (31 to 62) days treatment, compared to a 78% complete remission rate after 39 (34 to 57) days for 45 patients treated with steroids and cytotoxics. Neither the remission rate nor the time to remission were significantly different, though the comparison is probably, again, statistically underpowered.

Aggregation of data from uncontrolled cohorts suggested similar results.[41] However, a systematic review[31] of 20 publications showed that there was a higher remission rate in patients treated with prednisolone combined with cyclophosphamide. This was not associated with a lower mortality rate, however, perhaps because of the greater toxicity of the combination than prednisolone alone.[31] An analysis of the 331 patients reported to the EACH2 registry has been conducted, in which the groups were matched for potential confounding variables such as age, gender, inhibitor titer, factor VIII level, and underlying etiology.[71] This group reported an odds ratio (95% CI) of 3.25 (1.51 to 6.96) $P < 0.001$ in favor of combination therapy. There were peculiarities in this data in that the remission rate for steroids alone was much lower, at 58%, than reported in the aggregate literature, whereas the 80% remission rate reported for combination therapy is comparable with what has been published before if not higher than average. This difference was not reflected in outcome. Mortality and sustained remission rate was similar in both groups.[5,31,71] This suggests that although combination treatments offer a better short-term remission rate they do not improve long-term outcome.[71] The median time to remission is reproducibly 5 weeks, so treatment must be given for more than 3 weeks before an assessment of success or failure may be made.

Rituximab

Rituximab (anti-CD20 antibody) has been a popular treatment for acquired hemophilia, particularly since the publication of a report of its use in three patients in combination with other drugs[72] and the first-line use of rituximab in 10 patients, of whom eight achieved rapid complete remission.[73] The remaining two patients responded to cyclophosphamide. A further study reported a complete remission rate of 100% in six patients treated with rituximab combined with prednisolone with or without cytotoxics after response times of 1, 2, 4, 8, 36, and 52 weeks, which are similar to those reported for other regimens.[74] Rituximab has proved to be a disappointing drug used as a second-line agent and there is no convincing evidence that is superior to other regimens.[31,75] Franchini[38] reviewed 71 patients with acquired hemophilia treated with rituximab, mostly in combination with a variety of other immunosuppressive agents. Although these authors reported a response rate in excess of 90% the data was difficult to interpret with confidence and they still suggested that rituximab should be used as a second-line agent in combination with steroids. The EACH2 data would support such a cautious approach since only 30 of the 51 patients (59%) treated with rituximab in combination achieved a stable remission, no better than steroids alone.[70] Only 42% of the patients treated with rituximab alone achieved remission.[70] Since rituximab is usually given in combination with one or more other immunosuppressive drugs, the data is difficult to interpret. However, there is no convincing evidence to suggest that it improves the remission rate or reduces the relapse rate and is therefore currently not recommended as first-line therapy.

Cyclosporine

This drug is used as a second-line agent, usually in combination with steroids, and there are a number of anecdotal reports of its successful use in inducing remission. [41,76,77]

Intravenous High-Dose Immunoglobulin

High-dose immunoglobulin enjoyed a vogue during the late 1980s and early 1990s, when reports of its successful use 1st appeared.[78] The early promise was never realized, however. A prospective study of 16 patients treated with IVIG reported that only three subjects (one also treated with steroids) achieved a remission and all three had inhibitor titers ≤1 BU/mL.[79] This 12.5% response rate is actually lower than the reported spontaneous remission rate.[68] Neither the UK Cohort study[5] nor the EACH2 registry nor the systematic review of Delgado et al.[31] show any additional benefit from including IVIG in the treatment regimen for acquired hemophilia and so high-dose immunoglobulin is currently not recommended for this condition.[3]

Extracorporeal Immunoadsorption

Forty-five patients were reported to have been treated with a combination of cyclophosphamide, prednisolone, IVIG, and daily immunoadsorption using a sepharose column combined with 200 IU/kg of factor VIII daily.[80] Bleeding was controlled rapidly and the inhibitor titer also declined rapidly achieving undetectable levels after a median of only 3 (95% CI 2 to 4) days. Remission was reported in 88% of patients after a median of 14 days. This is clearly a very effective approach to the management of bleeding, which may be useful in the treatment of resistant bleeding in centers equipped for immunoadsorption. The response remission rate is not convincingly superior to conventional therapy, however, and the observations are uncontrolled. The rationale for the use of the factor VIII in this regimen, other than to control bleeding is unclear. Since patients with acquired hemophilia do not usually elicit an anamnestic increase in their inhibitor following factor VIII infusion, it is difficult to understand in what way factor VII will act as an immunomodulatory agent in such a regimen.

Immune Tolerance Induction

The rationale for immune tolerance induction is that factor VIII may stimulate antibody-producing cells to divide, thus making them susceptible to cytotoxic immunosuppressants.[81] This

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hypothesis seems implausible given that the patient is making factor VIII constantly anyway and given that an anamnestic increase in inhibitor titer does not usually follow factor VIII infusion in acquired hemophilia. None of the studies of this approach were controlled and factor VIII was invariably administered in combination with an aggressive combination of cytotoxic immunosuppressive drugs and so the results are very difficult to interpret. Lian et al. reported a 92% remission rate in 12 patients treated with steroids, vincristine, and cyclophosphamide given thrice weekly with factor VIII after 1 to 3 cycles of this treatment.[79] This group subsequently reported very similar results (83% remission) in six patients treated with the same immunosuppressive regimen but without factor VIII.[82]

Nemes and Pitlik[83] reported on the use of a more aggressive regimen where factor VIII was administered daily (30 IU/kg) in a reducing regimen over 3 weeks combined with cyclophosphamide and methyl prednisolone. Although they reported a 93% remission rate after a median of 4.6 weeks compared to a 67% remission at a median of 28.3 weeks in six historical controls, the control group is inadequate since the number of subjects and controls is too small to provide an adequate comparison. The results of these studies do not provide sufficient evidence of additional benefit from the inclusion of factor VIII in the immunosuppressive regimen to recommend this approach.

In conclusion, although there is general agreement that all patients with acquired hemophilia should be offered immunosuppressive therapy to attempt to eradicate the inhibitor, in the absence of clinical trials, there is no consensus as to the best regimen to use. Furthermore, the choice of regimen does not appear to influence the prognosis. Prednisolone combined with cyclophosphamide is probably superior to prednisolone alone. Rituximab and cyclosporine are probably second-line approaches and combined cytotoxic regimens are probably third-line. A balance must be struck, in this predominantly elderly population, between adequate immunosuppression on the one hand and excessive treatment-related morbidity on the other.

INHIBITORS OF VON WILLEBRAND FACTOR PROTEIN

Acquired von Willebrand Disease

Autoantibodies to vWF protein give rise to a clinical disorder resembling congenital vWD, acquired von Willebrand disease or syndrome (AvWS). In the absence of any large prospective cross-sectional studies, the incidence of this condition is unknown and it is almost certainly also still under-diagnosed.[84,85] The condition may arise at any stage of life but occurs most frequently in the elderly at a median age of 62 years (range 2 to 96 years.[86] AvWS is usually, but not invariably, an epiphenomenon of an underlying disease, most commonly lymphoproliferative or myeloproliferative disorders, which account for between 48% and 63% of cases.[85,86,87,88,89–108] The condition is seen in a wide variety of lymphoproliferative disorders including lymphoma, monoclonal gammopathy of unknown significance (MGUS), myeloma, and Waldenstrom macroglobulinemia.[86,87,88,89–103] Mohri et al.[88] reported acquired hemophilia in 10% of 260 prospectively studied patients with hematologic disorders. AvWS has also been reported in association with autoimmune diseases such as SLE[85,86,109–112] and hypothyroidism,[86,113–117] cardiovascular disorders[86,118,119,–127] and solid tumors.[85,86,128–130] The association with cardiovascular disorders, in particular, has been increasingly recognized, accounting for 46% of cases in one recent retrospective study.[118] Since 1st being recognized in association with congenital heart disease,[119,120] AvWS has also been described in relation to a variety of acquired cardiac defects including aortic stenosis with gastric angiodysplasia (Heyde syndrome) and ventricular assist devices.[121–127]

Less frequent etiologic associations with AvWS have included chronic graft versus host disease post bone marrow transplantation[112]; post vaccination prodromes[16]; solid tumor nonhematologic malignancies such as Wilms tumors[128–131] and peripheral neuroectodermal tumors.[132] Finally, AvWS has iatrogenic associations with the use of valproic acid,[133,134] ciprofloxacin,[135] griseofulvin,[136] and hydroxyethyl starch.[137,138]

It is important to identify the underlying condition causing AvWS, not just because the condition may itself require treatment, but also because successful treatment of the underlying condition often leads to resolution of the acquired hemophilia. It seems likely that milder cases of AvWS may escape detection because they resolve when the underlying condition is successfully treated and may only come to light if the patient undergoes a surgical procedure prior to treatment of the underlying condition. It has therefore been argued that a bleeding history should be taken and appropriate tests conducted to exclude AvWS prior to surgical intervention in patients with myeloprololiferative and lymphoproliferative disorders and untreated hypothyroidism.

Clinical Presentation

The clinical presentation is very variable, reflecting the considerable heterogeneity of AvWS. Most individuals present with a mild bleeding pattern of easy bruising and epistaxis. In some cases abnormal bleeding may only be observed following surgery or injury, however. More severely affected individuals bleed spontaneously, with bruising and bleeding predominantly involving the gastrointestinal tract and other mucous membranes, such as epistaxis.[84] Bleeding may, occasionally, be life threatening.

Diagnosis

The diagnosis is based partly on the history of recent onset bleeding in a patient with no previous or family history of bleeding. The discovery of an "underlying disorder" may be coincidental however and, in order to distinguish AvWS from hereditary vWD it will sometimes be necessary to test not only the patients but their first-degree relatives.

The laboratory diagnosis of AvWS is based on measurement of factor VIII, von Willebrand antigen and activity (Ristocetin cofactor activity or collagen binding assay). Multimeric analysis in plasma and platelets and von Willebrand propeptide has also been used to diagnose AvWS. It is important always to test for anti-vWF antibodies, because this is of clinical importance.[86,88,118] This can be carried out by conducting mixing experiments, measuring von Willebrand activity before and after incubation at 37°C for 1 to 4 hours. Some sandwich ELISA assays have also been described.[86,140] This assay is sensitive but its specificity requires further evaluation. Tiede et al.[118] evaluated this diagnostic approach retrospectively in 35 patients, reporting a high (86%) sensitivity of vW mutimeric analysis for AvWS similar to the combination of all the other assays combined. Another way of looking at this would be to say that the other

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assays are diagnostically sufficient, particularly since most laboratories do not have ready access to multimer testing.

There is also an argument for taking a careful bleeding history and possibly testing asymptomatic individuals with high-risk disorders prior to surgical intervention.[141]

Pathophysiology

There are several, very different proposed mechanisms for the development of AvWS. The mechanism and even vW subtype cannot be reliably predicted by identifying the underlying disorder, though some disorders are more strongly associated with specific mechanisms and vW types than others. Indeed, more than one mechanism may be operating in an individual at any one time. In general, vWF is normally secreted in AvWS, the exception being hypothyroidism, which is associated with decreased synthesis or secretion. Various mechanisms result in increased clearance, either of the entire native vWF giving rise to type 1 vWD or to selective clearance of high molecular weight (HMW) multimers, giving rise to type 2A vWD, though type 3 vWD is also occasionally reported.[85,86,139] Type 3 AvWS is rare but has been observed in association with Wilms tumors and essential thrombocytosis. The etiologies for type III AvWS respond to chemotherapy and/or tumor resection.[93,120,122,142] The mechanisms underlying AvWS are as follows:

Immune-mediated

Autoantibodies to vWF form immune complexes and increase clearance by Fc-bearing cells. This may reduce the concentration of vWF overall, giving rise to subtype 1 or selectively deplete HMW multimers, giving rise to subtype 2A. Binding to functional epitopes reducing von Willebrand activity has also been described.[85,109–112,139] This mechanism is associated with lymphoproliferative disorders, autoimmune disorders such as SLE and some patients with myeloproliferative disorders.[85,109–112,139] This mechanism sometimes causes severe depletion of vW factor (type 3).

Direct Adsorption of HMW Multimers

Adsorption of vWF protein, particularly the HMW multimeric forms, onto surfaces of tumor cells, platelets, or endothelial cells of abnormal blood vessels is a common mechanism giving rise to acquired type 2 VWD. This is the predominant mechanism in myeloproliferative disorders and solid tumors but has also been described in cardiovascular disease with angiodysplasia and lymphoproliferative disorders. This mechanism gives rise to type 2A AvWS. It has been thought that there may be adsorption of vWF to platelets when the platelet count is particularly high in myeloproliferative disorders, improving when the platelet count falls below 1,000.[107,108]

Increased Viscosity of Blood, Proteolytic Degradation of HMW Multimers

This mechanism, associated with circulating proteases, is also found in patients with cardiovascular disease, myeloproliferative and lymphoproliferative disorders, and solid tumors. It gives rise to type 2A AvWS.[85,86]

Sheer Stress-Induced Proteolytic Digestion

This results in selective depletion of HMW multimers, giving rise to 2A vWD in cardiovascular disorders.[86,109–112] Specific proteases such as the neuramidase produced by Wilms tumors may also result in type 2A AvWD[128–130] resolving when the tumor is successfully treated.

Decreased Synthesis/Secretion

This is the cause of AvWS in hypothyroidism, giving rise to type 1 AvWS[109,110] which resolves when the condition is treated with thyroxin.[116]

Treatment of Acquired von Willebrand Syndrome

There are two strands to the treatment of AvWS, identification and treatment of the underlying condition and management of bleeding. Management of bleeding depends to some extent on the subtype of AvWS and on the mechanism(s) causing the AvWS. Treatment of the underlying condition will usually lead to resolution of the AvWS. Unfortunately, an underlying diagnosis is not always identified and when it is it may sometimes be untreatable or, in the case of MGUS, for example, may not normally be treated. In such cases, bleeding must be managed according to one of the strategies described below.

Bleeding has been managed using a variety of strategies alone or in combination including Desmopressin (DDAVP), vWF/FVIII concentrate, high-dose IVIG, steroids, rituximab, and other immunosuppressive agents and Novoseven.[141]

Desmopressin (DDAVP) and vWF/FVIII Concentrates

Consistent with the mechanisms responsible for the decreased vWF activities in type II AvWS, treatment with Desmopressin (DDAVP), cryoprecipitate, or vWF/FVIII:C concentrates may yield poor and unsustained vWF incremental increases and relatively poor clinical responses. Success rates for DDAVP varied according to the nature of the underlying disorder. Federici et al.[86] reported 10% response in cardiovascular disorders, 21% in myeloproliferative disorders, 33% in autoimmune disorders, 44% in lymphoproliferative disorders, and 75% in patients with solid tumors. In 10 patients with MGUS, vWF activity improved 30 minutes after DDAVP administration in all patients, returning to baseline at 4 hours postinfusion.[142] In contrast, type 1 AvWS in untreated hypothyroidism, in which the synthesis of vWF is decreased but in which FVIII:C and vWF has a normal circulating half-life responds well to administration of Desmopressin (DDAVP)[113] and their vWF activities can be normalized by treatment with thyroxin.[113–117]

High doses of vWF concentrate and/or continuous infusion may be required to compensate for increased clearance of HMW vW multimers. The half-life may be very short, particularly in patients with MGUS or inhibitor antibodies.[141–143] Very close monitoring of the response is required for the dose-rate to be optimized.

Novoseven (recombinant factor VIIa, rVIIa, Novo Nordisk, Denmark)

Novoseven has been used successfully in standard dosage (90 μg/kg) in patients with AvWS, with a reported response rate of 96%.[141,144] Few adverse events have been reported though some caution should be exercised since elderly patients may be at greater risk of thrombosis. One case of myocardial infarction has been reported in a patient with type 2A vWD following treatment with Novoseven.[145]

Antifibrinolytic Drugs

The antifibrinolytic agents, ε-aminocaproic acid and tranexamic acid may be useful, particularly in combination with either DDAVP or Novoseven, for mucous membrane, gastrointestinal, and urinogenital bleeding. For minor bleeding, antifibrinolytic

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agents may be tried on their own. The use of antifibrinolytic agents may also reverse vWF proteolysis associated with activation of fibrinolysis induced by mechanical or damaged heart valves.

High-Dose Immunoglobulin

Standard doses of IVIG (1 g/kg/d for 2 days or 0.4 g/kg/d for 5 days) can be more effective than either DDAVP or concentrate in producing a sustained increase in vWF activity, normalization of the multimeric structure, and shortening of the bleeding time in patients with IgG MGUS.[141,143,146] This effect usually becomes apparent within 12 to 24 hours and so is not immediate.[141,143,146] Other hemostatic strategies may have to be used in the actively bleeding patient while waiting to see if immunoglobulin is going to be effective. This effect, though transient, reaches a maximum after 4 days, returning to baseline over 2 to 3 weeks.[143] It is unclear whether this improvement is related to binding of the offending IgG by anti-idiotype antibodies in the intravenous gammaglobulin, or whether improvement is due to blockade of the Fc receptors in the reticuloendothelial system with decelerated clearance of the vWF from the circulation. Low-dosage IVIG regimens have also proven to be effective for planned surgery.[98,147] As would be anticipated, high-dose intravenous gammaglobulin has been shown to be effective in other lymphoproliferative and autoimmune disorders but is ineffective in patients with IgM-MGUS states.[98,141]

Steroids and Other Immunosuppressive Strategies

Although immunosuppressive strategies might be expected to be effective in AvWS of autoimmune etiology, the response to steroids alone and in combination and also to rituximab have all been somewhat disappointing and do not have a big role to play.[141]

Plasmapheresis

Plasmapheresis has been reported to be useful in patients with IgM-MGUS.[141] Plasmapheresis should be conducted with fresh frozen plasma (FFP) as replacement material to prevent depletion of fibrinogen. Plasmapheresis may also be usefully combined with other treatment modalities such as DDAVP or vWF/FVIII replacement therapy.

ACQUIRED FACTOR X DEFICIENCY

Acquired factor X deficiency is a rare condition associated usually with significant bleeding. Almost all cases are caused by rapid clearance of factor X by adsorption onto amyloid fibrils in the vasculature and organs of the reticuloendothelial system in amyloid light-chain (AL) amyloidosis.[147–152] Only a few, isolated, cases of true, circulating, autoantibodies to factor X have been reported.

Factor X deficiency is the most common coagulopathy acquired in association with systemic light chain (AL) amyloidosis.[147–152,154] The factor X level in these cases is usually between 0.02 and 0.5 IU/L and bleeding and bruising tend not to occur until the factor X level falls to below 0.25 IU/L.[213] The factor X deficiency is typically an isolated abnormality, but occasional patients have combined deficiencies with other vitamin K-dependent clotting factors, including factor IX[153,155,157] or factor VII.[156]

It is thought that the bleeding tendency in patients with systemic amyloidosis cannot be attributed solely to acquired factor X deficiency or to combined clotting factor deficiencies but also to a synergistic contribution to impaired hemostasis by the physical changes in the integrity of the microvasculature caused by the amyloid deposition.[158,159]

There are approximately 10 published reports of transient factor X deficiency unassociated with amyloid and probably caused by a circulating inhibitor antibody.[160–162] Evidence of specific factor X inhibition was demonstrated in the plasma of only one of these transient cases, a woman with pneumonia following the use of erythromycin.[162] Otherwise, specific autoimmune antifactor X inhibitory antibodies are extremely rare[160–163] and have been detected in three elderly, previously healthy individuals who experienced sudden onset of bleeding[163,164,166] without any obvious underlying disease; three cases of inflammatory bowel disease or gastrointestinal malignancy; a child with extensive burns;[165] and a woman whose pneumonia was being treated with erythromycin.[162] In three cases, the antifactor X inhibitor was characterized as an IgG antibody.[163,164,166] The best characterized IgG autoimmune factor X inhibitor antibody inhibited activation of factor X by VIIa/TF, by IXa/VIIIa/phospholipid complex, and by Russell viper venom.[164] The antibody failed to inhibit the proteolytic activity of factor Xa toward a chromogenic substrate. However, under reaction conditions of limited factor Xa availability, the IgG could be shown to impair hemostatic functions of factor Xa that require the participation of its light chain: activation of prothrombin by prothrombinase; activation of factor VII bound to TF; and inhibition of VIIa/TF activity by factor Xa/TF pathway inhibitor complexes.[164]

Nonspecific nonneutralizing inhibitors of factor X activity, which were associated with transient factor X deficiency and major hemorrhagic complications, have been detected in two individuals in conjunction with a lupus-like anticoagulant.[224] Because no specific neutralizing antibodies were isolated from their plasmas, it was surmised that the nonspecific, nonneutralizing antibodies formed factor X–IgG immune complexes and mediated the accelerated clearance of factor X from the circulation.[167]

Diagnosis

The diagnosis rests on the emergence of abnormal bleeding in an individual with no previous personal or family history of bleeding. Screening tests reveal a prolonged PT and aPTT that corrects with normal plasma and is associated with a reduced factor X level.[168] The thrombin time (TT) may also be prolonged as may the reptilase.[168,169] Where present, prolongation of the TT and reptilase usually reflect hepatic amyloid infiltration and/or the nephrotic syndrome.[168,169] The prolonged PT is most predictive of clinical bleeding but was independent of whole body amyloid load. The prolonged PT and aPTT are explained by reduced factor X activity without evidence of an inhibitor; however, the corresponding factor X antigen levels are discordantly and consistently higher than the corresponding decreased factor X activity levels (median FX:Ag/FX:C = 2.5). The etiology of this phenomenon has not been explained.[168] Because multiple clotting factor deficiencies may occur in conjunction with decreased factor X activity in the context of systemic amyloidosis, for example, factors VII, IX, and V,[225] other specific clotting factor assays may be called for. In those whose decreased factor X levels do not correct with mixing studies, serial dilutions for specific clotting factor assays will indicate if a specific

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neutralizing autoimmune antifactor X antibody inhibitor or a nonspecific lupus-like antibody exists.

Treatment of Acquired Factor X Deficiency

Treatment strategies for patients with acquired factor X deficiency must be individualized and depends on the etiology of the coagulopathy. Treatment of bleeding due to acquired factor X deficiency secondary to systemic amyloidosis is often challenging because infused factor X is extremely rapid cleared. The administration of prothrombin complex concentrates (PCCs) (inactivated or activated) may be useful even though they may only be transiently effective. Splenectomy has been reported to result in a significant rise in the plasma factor X level; but surgery may be hazardous since adequate hemostasis cannot be guaranteed. There are anecdotal reports of the successful use of Novoseven to prevent bleeding and to facilitate splenectomy.[170,171] FFP may be utilized in plasmapheresis and exchange protocols intended to expedite factor X replacement, or when the physical removal of autoimmune antifactor X antibody inhibitors is deemed necessary to control bleeding[172] but should not be used as a replacement therapy for acquired factor X deficiency since they will be ineffective. Thompson et al.[173] reported 60 patients with acquired factor X deficiency of all severities who underwent 112 surgical procedures using a variety of hemostatic strategies. Only six of these individuals were severely affected (X < 0.1 IU/L). This report is remarkable for not using PCC in any of the 13 major invasive procedures reported, though only 4 of those individuals had a factor X level of <0.1 IU/L. Two patients bleed excessively following splenectomy, one with a baseline X of 0.03 IU/L and the other with a baseline of 0.06 IU/L, both despite the use of Novoseven. Milder cases (X > 0.1 IU/L) were generally given no hemostatic replacement therapy and did not bleed abnormally.[173]

The usual course of the coagulopathy is linked to the ability to eradicate the underlying plasma cell dyscrasia in systemic amyloidosis, although spontaneous resolution of amyloid-associated factor X deficiency does, very rarely, occur.[174] Therefore, aggressive treatment with high-dose melphalan chemotherapy, with or without corticosteroids and stem cell rescue, has emerged as the treatment of choice for systemic amyloidosis. This approach usually reverses or at least ameliorates the factor X deficiency.[158,175,176]

Acquired circulating autoantibody inhibitors of factor X are usually transient and so long-term eradication strategies are not usually necessary in those cases.[163,164] Active bleeding is treated optimally with PCCs or rVIIa,[162] and/or daily plasmapheresis combined with IVIG and steroids.[166] Intravenous γ globulin may sometimes facilitate the disappearance of factor X antibody inhibitors when used alone.[165]

ACQUIRED AUTOIMMUNE INHIBITORS TO FIBRIN FORMATION AND POLYMERIZATION AND TO FACTOR XIII

The final stages of blood coagulation consist of fibrin formation, mediated by thrombin cleavage of fibrinopeptides A and B from fibrinogen, followed by polymerization of fibrin monomers, concluding with the covalent cross-linking of the α and γ chains of adjacent fibrin molecules by factor XIIIa, which stabilizes the fibrin clot.[177] Spontaneously acquired, neutralizing inhibitory antibodies have been identified that specifically target each of these reactions associated with the development of severe clinical bleeding.

Although alloantibody inhibitors that target fibrinogen have, rarely, been observed following replacement therapy with FFP or cryoprecipitate for hereditary afibrinogenemia,[178] acquired autoimmune antibodies are considerably commoner. These may complex with fibrinogen and fibrin monomers inhibiting fibrin polymerization and thrombus stabilization. Acquired polyclonal IgG autoantibodies have also been shown to inhibit both thrombin-mediated release of fibrinopeptide A from fibrinogen[179] and fibrin monomer formation[180] without interfering with fibrin polymerization or cross-linking. The affected individuals presented with a severe hemorrhagic syndrome reminiscent of congenital afibrinogenemia. Antibodies targeting fibrinogen should be suspected when the aPTT, PT, TT, and reptilase time are all prolonged and chronometric assays for fibrinogen are reduced. Mixing studies with the TT show significant prolongation.

Impaired fibrin polymerization has also been reported in patients with monoclonal and polyclonal IgG autoantibody inhibitors. These antibodies specifically targeted fibrinopeptide epitopes on fibrinogen[181–184] and/or fibrin monomers.[182–184] Clinical bleeding was consistent with dysfibrinogenemia, but their purified fibrinogens were functionally and structurally normal and, when isolated, their inhibitors did not interfere with fibrinopeptide cleavage or fibrin cross-linking.[182,183] These acquired autoimmune neutralizing autoantibody inhibitors were detected in individuals with *de novo* coagulation defects arising in association with chronic aggressive hepatitis, postnecrotic cirrhosis, and ulcerative colitis,[182] SLE,[183] and benign and malignant monoclonal gammopathies.[184–186] In one study, autoantibodies directed specifically against fibrinogen and fibrinogen fragments X, Y, and D were detected in pregnant and postpartum women, particularly in those who are Rh immunized, and their newborns.[187] Laboratory abnormalities were observed in these cases, but the significance of these "physiologic" antibodies remains unclear because no hemorrhagic diathesis was apparent.

Acquired antibodies that inhibit cross-linking of fibrin polymers might be expected to target epitopes on the factor XIII molecule or the cross-linking sites on fibrin. Only isolated case-reports of alloantibody inhibitors complicating replacement therapy of congenital factor XIII deficiency have appeared in the literature. Acquired factor XIII deficiency, caused by autoantibody inhibitors has been described complicating a wide variety of mainly autoimmune conditions[188–193] and drug therapies.[194–197] These have included SLE, Henoch-Schonlein purpura, inflammatory bowel diseases, psoriatic arthritis, IgG monoclonal gammopathies, and acute leukemias (promyelocytic, lymphoblastic, and myeloid).[198,199] Long-term isoniazid treatment for TB and the use of procainamide, penicillins, diphenylhydantoin, and practolol have also been implicated in the development of clinically relevant autoimmune antifactor XIII antibody inhibitors.

Autoimmune factor XIII deficiency can be produced by predominantly IgG autoantibody inhibitors directed against factor XIII. These neutralize factor XIII clotting activity by: inhibition of factor XIII activation by thrombin (type I inhibitor)[190,200]; or interference of factor XIIIa-mediated transamidase activity and fibrin polymer cross-linking (type II); or blockade of factor XIIIa binding sites on fibrin (type III).[201–204]

Acquired factor XIII deficiency can also develop by non-immunologic mechanisms. The venom of caterpillars in the *Lonomia* species produces a severe hemorrhagic syndrome due

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to direct proteolysis of the factor XIII molecule.[205,206] The crude salivary gland extract of the giant Amazon leech, *Haementeria ghilianii*, contains a unique peptide, named tridegin, which specifically inhibits the transglutaminase function of plasma factor XIIIa.[207] This is the most potent, naturally occurring inhibitor of factor XIIIa yet described. Synthetic, low molecular weight inhibitors (nonspecific thiol reagents and specific azol derivatives and azolium salts) exert their factor XIII inhibitory effects at nanomolar concentrations.[189]

The presence of inhibitor antibodies and nonimmunologic inhibitors of factor XIIIa can usually be detected *in vitro* with mixing experiments, using patient plasma or purified IgG fraction to inhibit the cross-linking of α chains of fibrin in normal plasma.[190,208] There is also reduced fibrin clot stability in dissociating agents, such as 5 M urea (urea clot solubility test).[190,208] Global coagulation assays, such as aPTT, PT, TT, and fibrinogen levels, are usually normal although the thromboelastogram may show a pattern consistent with factor XIII deficiency. Decreased factor XIII activity can be quantitated specifically by the dansylcadaverine incorporation assay.

Clinical Presentation and Management

The severe, recurrent, and spontaneous hemorrhagic complications[191–202,209] caused by acquired inhibitors to factor XIII or fibrin stabilization are associated with a high mortality rate. Gastrointestinal, genitourinary, pulmonary, and intraperitoneal bleeding often occurs in combination with dramatic soft tissue hematomas and ecchymoses; intracranial hemorrhage has also has been described[194] and emphasizes the need for prompt diagnosis and treatment. The day to day bleeding pattern of acquired factor XIII deficiency is considerably more severe than congenital factor XIII deficiency and includes hemarthroses, poor wound healing, recurrent miscarriage, though fatal intracranial bleeds may occur in both.

When treating acute hemorrhage, one must attempt to overcome the inhibitor and replace factor XIII activity or fibrinogen substrate in plasma. Cryoprecipitate or fibrinogen concentrate or factor XIII concentrate is likely to be much more effective than FFP but neither may be adequate to overcome the inhibitor. Administration of large doses of plasma-derived, viral-attenuated factor XIII concentrate (Fibrogammin P, factor XIII concentrate, ZLB Behring,) appears to be the safest and most effective and efficient alternative method of controlling or preventing acute episodes of bleeding in patients with acquired FXIII deficiency.[210,211] Plasmapheresis or protein A column immunoadsorption, to physically remove the inhibitor, followed by exchange transfusion with factor XIII concentrate, cryoprecipitate, or FFP, are sometimes necessary to reverse refractory bleeding. Longer-term care is directed at eradication of antibody production, which can be approached with immunosuppressive drug regimens employing corticosteroids and alkylating agents.[193,203] Spontaneous remission may be observed, particularly if the problem is drug-induced and the offending drug is discontinued.

Inhibitors of Prothrombin and Thrombin

Acquired inhibitors of thrombin or prothrombin may cause bleeding diathesis but more rarely, thrombosis, whereas antibodies to prothrombin are frequently associated with thrombosis. Nonneutralizing, antiprothrombin antibodies lead to a prothrombotic state associated with the LAC.[212] Antiphospholipid antibodies are a heterogeneous group of antibodies that can be detected as anti–β_2-glycoprotein I antibodies, antiprothrombin antibodies, and the LAC.[213] These antibodies are relatively common; Nojima et al.[214] reported such antibodies in 51% of 96 patients with SLE. These authors showed that the coexistence of IgG antiprothrombin antibodies and LAC activity is the most significant risk factor for VTE in patients with SLE.[214] Rarely, however, severe bleeding may occur in patients with SLE because of antibodies causing acquired hypoprothrombinemia-lupus anticoagulant syndrome.[215] Immunosuppressive therapy may be helpful in such cases.

Generally, inhibitor antibodies to coagulation factors specifically bind to coagulation factors and either neutralize their procoagulant activity,[216] accelerate their clearance,[217] or proteolytically degrade them.[218] A novel mechanism has been described of an autoantibody directed toward prothrombin and induced protease-like activity to facilitate a stable stoichometric complex formation with its physiologic inhibitor antithrombin without thrombin conversion.[219] This resulted in potent inhibition of coagulation associated with recurrent bleeding, retroperitoneal, and pulmonary hematoma, in the absence of an underlying disease.

In contrast to antiprothrombin antibodies, neutralizing autoantibodies against thrombin are very rare. Most appear in patients exposed to topical preparations of bovine thrombin or fibrin "glue" during major surgery and these antibodies consequently react mainly with bovine coagulation proteins.[220–223] Acquired inhibitors directed primarily against human thrombin are usually associated with predisposing, usually autoimmune, conditions such as rheumatoid arthritis or SLE[222,224–227] and are extremely rare in patients without an underlying disease. Some patients with either monoclonal or polyclonal gammopathies occasionally have inhibitors of thrombin.[228–231] Isolated cases of antithrombin autoantibodies have been reported in patients with liver cirrhosis, or even without any apparent disease.[227,232] Sometimes these inhibitors cause a serious bleeding disorder[217,233–235] but in cases where the antibody binds to a site involved in the molecule's anticoagulant function, thrombotic disease results.[236]

Apart from these sporadic cases, most examples of acquired thrombin inhibitors occur in patients exposed to bovine topical thrombin or fibrin-glue preparations during major surgery.[222] Prospective studies found evidence of immunization to bovine thrombin and/or factor V in a 3rd to one-half of such patients.[223,237] Most of these patients are asymptomatic, but some bovine thrombin inhibitors cross-react strongly with human thrombin causing severe and even life-threatening bleeding.[237]

Antithrombin antibodies should be suspected if coagulation tests show a prolonged PT, a prolonged aPTT, and a markedly prolongedTT. Antithrombin inhibitors more typically cause bleeding than antiprothrombin antibodies and the TT is also more prolonged than the PT. Factor-activity levels are markedly decreased, and mixing studies show a poor correction with normal plasma, suggesting the presence of an inhibitor of thrombin. Given the possibility of an unfavorable outcome, in the presence of prolonged PT, aPTT, and TT, with normal platelet count and fibrinogen, a search for an inhibitor of thrombin should be pursued in patients with either bleeding or thrombosis. Timely diagnosis and the initiation of prompt aggressive treatment may reduce morbidity and mortality.

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Acute bleeding episodes associated with autoantibodies to prothrombin or thrombin may be difficult to reverse and there is no consensus on the best approach to replacement therapy. The administration of "bypassing" agents, such as aPCC and rFVIIa concentrate, used alone or in combination with each other or with plasmapheresis and exchange transfusions with FFP, probably constitutes the best strategy.[238,239] The concurrent use of antifibrinolytic agents, such as ε aminocaproic acid or tranexamic acid and human-derived fibrin glues may increase efficacy. Corticosteroid use has also been reported to be helpful for the long-term attempt to reverse the coagulopathy and to suppress autoantibody formation.[238]

ACQUIRED INHIBITORS TO FACTOR V

Etiology and Natural History

Alloantibody inhibitors of factor V are rare, even after repeated replacement therapy in individuals with congenital factor V deficiency.[240] Neutralizing autoantibodies targeted specifically at factor V are extremely rare. Acquired factor V inhibitors occur primarily in elderly individuals[241] who have received treatment with β lactam or aminoglycoside antibiotics (streptomycin and Gentamicin) or who have malignant diseases.[242,243,244] More recently, approximately two-thirds of factor V inhibitors were reported to have developed following the application of topical bovine thrombin alone or fibrin glue prepared with bovine thrombin for surgery.[242,243] Both of these bovine products are contaminated with small amounts of bovine factor V, stimulating the production of antibovine thrombin or factor V antibodies, which cross-react with human thrombin and factor V.[245] Hemorrhagic complications, due to acquired neutralizing factor V antibodies, usually occur after repeated exposures to bovine thrombin products but have also been reported after single exposure.[246] The prognosis for factor V inhibitors is generally good. Patients exposed to bovine thrombin have the best prognosis and "idiopathic" inhibitors the worst prognosis.

Most factor V inhibitors are polyclonal IgG, although mixed IgG associated with IgM[247] or IgA inhibitors[248] have also been reported. Autoimmune factor V antibody inhibitors usually disappear spontaneously within 8 to 10 weeks after their 1st appearance. Their mechanism(s) of inhibition are variable and resemble antibodies to FVIII:C. This is not surprising given the considerable base-sequence homology between the two molecules. The inhibitor may be directed to epitopes on the C2 domain of factor V, thereby interfering with the interaction of factor Va with phospholipids and disrupting formation of the prothrombinase complex.[249] Alternatively, antifactor V IgG autoantibodies have been detected in both patient plasma and platelets and in neutralized factor V procoagulant activity by reacting with an epitope contained on the factor V light chain.[250]

Curiously, acquired antifactor V autoantibodies may rarely cause a paradoxical hypercoagulable state. Thrombotic rather than hemorrhagic manifestations were described in a woman in whom severe activated protein C resistance arose because of the blockade of the factor Va proteolytic cleavage sites by an acquired antifactor V antibody. The patient's factor V coagulation protein activity was unaffected; however, her purified antifactor V antibody produced a "factor V_{Leiden}-like" defect when incubated *in vitro* with normal plasma.[251]

Acquired factor V deficiency, unassociated with the presence of an autoantibody inhibitor, has been described as a complication of primary systemic amyloidosis.[252]

Clinical Presentation

In general, the bleeding manifestations of autoantibody inhibitors to factor V are similar to those observed in congenital factor V deficiency. The clinical presentation of factor V deficiency due to autoantibody inhibitors ranges from mild epistaxis, or gingival bleeding, to life-threatening retroperitoneal hemorrhage.[253] Other reported presentations include hematospermia, hematuria, and severe gastrointestinal bleeding.[250] The severity of clinical bleeding varies considerably and typically correlates with the inhibitor titer and degree of factor V deficiency. Some patients have little if any bleeding even with surgery, despite overtly abnormal laboratory findings.[253] The propensity to clinical hemorrhage induced by antifactor V antibodies may be related to the content of factor V in the α-granule of the platelet and the susceptibility of that factor V to neutralization by the circulating antibody.[254,255] Platelet factor V may be relatively protected from neutralizing antifactor V antibodies even though plasma factor V is completely neutralized.[254] Platelet transfusion may be particularly useful in treating bleeding in acquired factor V deficiency for this reason.[255] In contrast, severe bleeding complications have been observed when antifactor V IgG was present in both the patient's plasma and platelets.[250]

Diagnosis

An inhibitor to factor V should be suspected when mixing studies with patient and pooled normal plasma do not correct the abnormally prolonged aPTT and PT in patient plasma. Factor V activity should be absent in the patient plasma and the mixture with normal plasma but progressive dilutions of patient plasma with factor V-deficient plasma will eventually allow for the expression of factor V activity as the inhibitor is sufficiently diluted out. A further confirmatory laboratory assay for autoimmune factor V antibody inhibitors includes a prolonged dilute Russell viper venom time, which does not shorten after incubation with an excess of phospholipid, in contrast to LACs.

Treatment of Factor V Inhibitors

Patients with acquired factor V inhibitors can usually be managed adequately with transfusions of FFP or platelets.[255,256] Platelet transfusions appear to be an especially effective means of "bypassing" the inhibitor because platelet factor V does not become accessible to the inhibitor until platelets are activated.[255] Salvage therapy for unresponsive patients includes the administration of FEIBA or rFVIIa to bypass the inhibitor to factor V. Alternatively, both high-dose IVIG[257] and plasmapheresis with extracorporeal immunoadsorption methods have been used.[258]

Because most autoimmune factor V antibody inhibitors are transient, disappearing spontaneously over weeks to months, it is uncertain whether immunosuppressive regimens actually hasten the disappearance of the inhibitor. Combination treatment with plasmapheresis and corticosteroids;[238] corticosteroids, Azathioprine, and plasmapheresis;[251] or prednisone and cyclophosphamide have all been reported to reverse the coagulation

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defect over time, but the effect of these agents on the natural history of this disorder is not yet known.

AUTOIMMUNE ANTIBODY INHIBITORS TO FACTOR XII

Most cases of autoantibodies directed against factor XII have been described in the context of primary or secondary antiphospholipid syndrome (APS) with a LAC.[259,260,261] Twenty-one of forty-two patients with APS in one study were reported to have either IgG or IgM autoantibodies targeting factor XII and four of these had decreased factor XII levels.[261]

The clinical significance, if any, of these autoantibodies is obscure, since factor XII deficiency does not cause bleeding and probably does not cause thrombosis.[262–265] Unsurprisingly, autoantibody inhibitors to factor XII do not cause bleeding but may be either a marker or epiphenomenon of hypercoagulability and fetal loss in patients with APS,[266,267] independent of a concurrent LAC or β_2-glycoprotein antibodies. Acquired inhibitors directed against factor XII have also been described in association with various liver diseases: metastatic liver disease from gastric carcinoma, autoimmune hepatitis, and severe chronic hepatitis B.[184] The coagulation abnormalities corrected after partial hepatectomy, corticosteroid treatment, and hydroxychloroquine therapy, respectively.[268] Replacement therapy is therefore not indicated, but heparin and aspirin may improve the outcome of pregnancy if the patient gives a history of recurrent fetal loss.

ACQUIRED AUTOIMMUNE ANTIBODY INHIBITORS OF FACTOR XI

Autoimmune antibody inhibitors typically arise in association with an underlying collagen vascular disease, such as SLE [274,275,269–273] and less frequently in association with hematologic malignancy,[276,277] autoimmune gastrointestinal diseases[278], or treatment with phenothiazines.[272,279]

The autoimmune antibody inhibitor against factor XI usually results in apparent reductions in all intrinsic coagulation factors, though excessive bleeding is not usually observed. Factor XI inhibitors are typically detected only when coagulation is tested for some other reason and shows a prolonged aPTT. Autoantibody-induced factor XI deficiency has been shown to improve following corticosteroid therapy in patients with SLE[273] and chemotherapy of CLL.[276] A patient with chronic myelomonocytic leukemia and a factor XI inhibitor has also been reported to respond to Novoseven (rVIIa) given for an invasive procedure.[277]

ACQUIRED DEFICIENCIES AND INHIBITORS OF FACTOR IX

Autoimmune Acquired Factor IX Inhibitors

Spontaneously acquired factor IX autoantibody inhibitors are extremely rare. [274,275,280–285] The associated disease states are quite similar to those underlying acquired factor VIII inhibitors. Most autoimmune factor IX inhibitors resolve spontaneously within 1 to 7 months, but it is not clear whether the natural history is altered by immunosuppressive therapy. Corticosteroids with or without high-dose IVIG (400 mg/kg/d for 5 consecutive days by intravenous infusion), has been reported anecdotally to be successful.[265] Furthermore, removal of an associated malignant tumor may allow for spontaneous resolution of factor IX antibodies.[286] Recurrence of the autoantibody is unusual following successful treatment. As a general rule the strategies for treatment of acquired autoimmune hemophilia A can be applied for acquired autoimmune factor IX antibody inhibitors.

Acquired Nonimmune Factor IX Deficiency

Acquired isolated factor IX deficiency due to nonimmune mechanisms leading to loss or increased clearance of factor IX are more common than autoantibody inhibitors causing factor IX deficiency. This coagulopathy has been associated with heavy proteinuria in nephrotic syndrome,[287,288] primary amyloidosis,[289] Gaucher disease,[290] and pituitary insufficiency.[291] Laboratory assessment confirms the absence of an inhibitor and bleeding manifestations are treated with factor IX replacement in the form of PCCs (activated or unactivated), ultra-high purity plasma-derived or recombinant factor IX concentrates, or rFVIIa for salvage therapy. The replaced factor IX protein usually has accelerated plasma clearance in these situations, except for pituitary insufficiency, which may be treated successfully with administration of thyroxin.

Laboratory Evaluation of Factor IX Inhibitors

Allo- and auto-antibodies to factor IX present with prolongation of the aPTT and a low factor IX activity assay, which does not correct in mixing studies with normal plasma. Both allo- and auto-antibodies to factor IX are immediate-acting with no progressive loss of activity following prolonged incubation at 37°C.[282,292,293] Antifactor IX alloantibodies may also form circulating immune complexes capable of precipitating anaphylactic reactions when the patient is reexposed to factor IX containing replacement products. This has not been described with autoantibodies. Specificity of the factor IX alloantibody or autoantibody inhibitor can be confirmed in factor IX coagulation assays, and the degree of inhibition can be quantified using a modified Bethesda assay.[294,295]

Patients with factor IX alloantibodies present most commonly with hemarthroses. In contrast, hemorrhagic complications with autoimmune antifactor IX inhibitors are similar to those reported for acquired hemophilia A, with few hemarthrosis, severe and extensive ecchymoses, and intramuscular bleeds, as well as gastrointestinal and genitourinary bleeding.[296]

The mainstay of therapy for patients with hemophilia B and with high-titer factor IX inhibitory alloantibodies, is the administration of recombinant or plasma-derived factor IX for low-level inhibitors and activated PCCs (FEIBA) or rVIIa for higher level inhibitors[297–301] Novoseven (rFVIIa) is the treatment of choice in patients who have had anaphylaxis since these patients cannot be given factor IX concentrate. These treatment products are used in regimens similar to those for FVIII:C alloantibody inhibitors and are equally effective[296–301] with an 80% complete and partial response rate.[302] Treatment failures require alternative strategies, such as combining the PCCs with rFVIIa.[303] Patients who are high responders with low-titer alloantibodies may experience an anamnestic response to any source of infused factor IX protein within 2 to 5 days. Occasionally, in high-titer inhibitor situations, plasmapheresis or extracorporeal

immunoadsorption is necessary to transiently decrease inhibitor titers and facilitate control of acute bleeding.[296]

ACQUIRED FACTOR VII DEFICIENCY

Acquired factor VII deficiency is common, though seldom arising in isolation, associated with Coumadin therapy, broad-spectrum antibiotic therapy, liver disease, and vitamin K deficiency caused by diet or malabsorption. In contrast, isolated, acquired factor VII deficiency caused by specific autoantibodies to factor VII is exceedingly rare, an anecdotal phenomenon and clinical curiosity.

The pattern of bleeding in individuals with acquired autoimmune factor VII deficiency tends to be more severe and is more likely to be life-threatening than in congenital factor VII deficiency.[303–307] This may reflect publishing bias. Hemarthroses are uncommon in both congenital and autoimmune factor VII deficiencies. Acquired autoimmune factor VII deficiency has developed in association with various clinical situations, including aplastic anemia with the use of antithymocyte globulin [308]; administration of drugs such as penicillins, cephalosporin's, and interleukin 2 (IL-2) therapy[307,309]; septicemia[310]; pancreatitis[311]; and malignancy, such as multiple myeloma.[312] A specific factor VII autoimmune antibody has also been isolated from the plasma of an AIDS patient with a concurrent lupus-like anticoagulant.[313]

These acquired factor VII autoantibody inhibitors are predominantly neutralizing IgG antibodies.[306,314] Rarely, factor VII deficiency has occurred because of IgG–factor-VII complex formation with accelerated clearance of the immune complex.[308] Alloantibody formation has also been reported in individuals with severe congenital factor VII deficiency after treatment with rFVIIa concentrate.[315]

Acquired deficiency of factor VII, unrelated to autoimmune antibody inhibitor formation, is most commonly due to vitamin K deficiency states associated with hepatic diseases (severe cirrhosis, ethanol or acetaminophen toxicity, Dubin-Johnson syndrome, Rotor syndrome, Gilbert disease, etc.)[316,317] or with medications classified as vitamin K antagonists (Warfarin and Coumadin derivatives). Factor VII deficiency has also accompanied amyloidosis,[318,319] presumably caused by adsorption of the clotting factor to amyloid fibrils.

Laboratory analysis of plasma samples from individuals with specific autoimmune factor VII antibody inhibitors usually reveals isolated prolongations of the PT assays and factor VII activity assays, which do not correct with mixing studies performed with normal plasma. The aPTTs are typically normal. In contrast, for factor VII deficiency due to hepatic disease or Warfarin effects, mixing studies with patient and normal plasmas should show immediate normalization of prolonged PTs and aPTTs (reflecting deficiencies in all vitamin K-dependent clotting factor proteins) and depressed factor VII activity assays.

Because of the rarity of autoimmune acquired factor VII deficiency due to circulating inhibitors, there is no consensus on the best treatment strategy for significant bleeding complications. Anecdotal reports indicate that eradication of the associated underlying disease state is clearly crucial over the long term, for example, normalization of autoimmune factor VII deficiency that occurred after an allogenic bone marrow transplant-cured aplastic anemia[308] and after tumor regression by chemotherapy. Factor VII activities decreased again when the tumor relapsed.[312]

For significant active bleeding, administration of plasma-derived or genetically engineered factor VII concentrates is probably the most efficient and consistently effective first-line treatment strategy to overcome the inhibitor. FFP has limited usefulness because of the large volumes required, the prolonged administration time, the possible transmission of blood-borne pathogens, and the low likelihood that the factor VII inhibitor can be overwhelmed. PCCs may be of value but also may induce hypercoagulability with repeated doses. When these strategies are unsuccessful in reversing bleeding or if achieved factor VII increments after replacement therapy are short lived because of the inhibitory antibody, extracorporeal plasmapheresis with or without concomitant intravenous gammaglobulin may be lifesaving.[316,320] Antifibrinolytic agents and fibrin glues may be useful adjunctive therapies.

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CHAPTER

58

Laboratory Markers of Activated Coagulation and Its Regulation

KENNETH A. BAUER AND HELEN A. IRELAND

The development of blood tests to predict thrombotic events, or to confirm or exclude the presence of thrombosis, has been a goal of scientists working in the field for many years. The hemostatic mechanism comprises platelets, vessel wall components, and procoagulant, inhibitory, and fibrinolytic proteins. Advances in our knowledge of the biochemistry of these processes have facilitated the development of sensitive and specific assays that are able to detect platelet activation, generation of coagulation enzymes, products of intravascular fibrin formation or dissolution, and activated inhibitory proteins in addition to the inactive coagulation factors (zymogens) and their inhibitors. This chapter focuses on measures of activated markers of hemostasis and their relationship to thrombotic disease. While many of these assays have not found a place in clinical practice, their use as research tools has furthered our understanding of the hemostatic mechanism and its perturbation in many disease states.

Due to the short half-life of most of the hemostatic enzymes, it has not been possible to measure directly the levels of most of the active forms *in vivo*. Many of the enzymes are neutralized rapidly by naturally occurring protease inhibitors or bound to cellular receptors in the locale in which they are generated. Faced with these obstacles, immunochemical assays have been developed for peptides that are liberated with the activation of coagulation zymogens or for enzyme–inhibitor complexes. The assays that have been developed, along with the reported *in vivo* half-lives for some of the species, are listed in Table 58.1 and described in detail in a later section of this chapter. Most of these assays were initially developed in research laboratories, but some are now available commercially in kit form using either polyclonal or monoclonal antibodies.

The assay formats for these markers are generally of two types. The first approach that has been used is a competitive radioimmunoassay procedure. This requires an antibody population that recognizes antigenic determinants on the activation fragment or enzyme–inhibitor complex that are hidden in the parent zymogen or inhibitor. The need for highly specific antibodies to the marker of interest could sometimes be obviated by devising sample processing procedures that efficiently remove the cross-reacting species in plasma before assay. The use of the radioimmunoassay has, however, waned due to the requirement of radioactive tracers. The second approach, used more commonly today, is the enzyme-linked immunosorbent assay. In most instances, immunoglobulin G (IgG) or F(ab′) two fragments directed toward epitopes on one region of the marker are bound to wells of plastic microtiter plates to "capture" the antigen from the plasma specimen. After washing away unbound species, a second IgG or F(ab′) two population recognizing epitopes in a different domain of the marker molecule is added to the wells. These antibodies are coupled to an enzyme capable of cleaving a suitable colorimetric substrate, allowing the development of a titration curve.

Successful execution of clinical investigations seeking to establish a relation between markers of coagulation, fibrinolysis, and thromboembolic disease requires, first, that the selected assays are specific for the products of interest and possess sufficient sensitivity. Second, the tests must be standardized so that the assays perform in a reproducible manner. Third, care must be taken to ensure that technical factors do not introduce *in vitro* artifacts that can significantly alter the immunoassay results (e.g., drawing of blood samples through indwelling intravenous catheters). These include venipuncture quality, choice of anticoagulant, sample processing procedures (if required), and plasma storage conditions.

ASSAYS FOR COAGULATION ACTIVATION

Many of the protein components of the hemostatic mechanism are inactive zymogens that are converted to their active forms, serine proteases. For coagulation system enzymes to be generated at any significant rate, a zymogen, a cofactor, and a converting enzyme must form a multimolecular complex on a natural surface. These transformations are suppressed if the converting enzyme is inhibited, the protein cofactor is destroyed, or the surface receptors that are essential for the assembly of the macromolecular complex are sequestered. For an overview of the blood coagulation mechanism, see Chapter 8.

Assays for Contact System Activation

Contact system activation can be monitored by immunoassays for factor XIIa, factor XIIa–C1 inhibitor complex, kallikrein–C1 inhibitor, and kallikrein–α_2-macroglobulin. Factor XI activation can be measured as factor XIa and factor XIa–factor XIa inhibitor (α_1-antitrypsin, antithrombin, C1 inhibitor, and α_2-antiplasmin) complexes.[1–3,6,7]

Assays for Intrinsic and Extrinsic Pathway Activation

The activation of factor IX can be monitored by measuring the levels of the factor IX activation peptide[10,47] or factor IXa–antithrombin complexes.[11] These assays reflect the action of factor XIa, the factor VII–tissue factor complex, or both, on factor IX (FIGURE 58.1). Factor X activation mediated by the extrinsic or intrinsic pathways can be monitored by measuring the factor X activation peptide.[12]

Table 58.1

Immunochemical and enzymatic markers of coagulation and fibrinolytic system activation

Biochemical Step	Marker (Reference)	T1/2 (Reference)
Coagulation		
Factor XII–factor XIIa	Factor XIIa[1]	
	Factor XIIa–C1 inhibitor complex[2,3]	
Prekallikrein–kallikrein	Kallikrein–C1 inhibitor complex[3,4]	
	Kallikrein–α_2-macroglobulin complex[5]	
Factor XI–factor XIa	Factor XIa–XIa inhibitor complexes[6,7]	
Factor VII–factor VIIa	Activated factor VII[8,9]	
Factor IX–factor IXa	Factor IX activation peptide[10]	15 min
	Factor IX–antithrombin complex[11]	30 min
Factor X–factor Xa	Factor X activation peptide[12]	30 min
	Factor X–antithrombin complex[13]	
Prothrombin–thrombin	Prothrombin activation fragment F1+2[15–18]	90 min[14]
	TAT complex[19,21,22]	15 min[20,23]
Protein C–activated protein C	Protein C activation peptide[24]	5 min
	Activated protein C–protein C inhibitor complex[26,27]	40 min[25]
	Activated protein C–α_1-proteinase inhibitor (antitrypsin)[27]	140 min[25]
Fibrinogen–fibrin	Fibrinopeptide A[28–30]	3–5 min[29]
	Fibrinopeptide B[31,32]	
Fibrinolysis		
Plasminogen–plasmin	Plasmin–α_2-antiplasmin complex[22,33–38]	
	Plasmin–α_2-macroglobulin complexes[34]	
Plasmin action on fibrin I	Bβ1–42 fragment[39,40]	
Plasmin action on fibrin II	Bβ15–42 fragment[41]	
Plasmin action on fibrinogen/fibrin	Fibrinogen (fibrin) degradation products[42,43]	
Plasmin action on cross-linked fibrin	D-dimer[44,45]	8 h[46]

An assay for factor VIIa uses a clotting assay utilizing recombinant tissue factor that has undergone a *C*-terminal truncation.[8,9] This tissue factor mutant maintains cofactor activity toward factor VIIa but does not support factor VII activation.

Assays for Measuring *In Vivo* Thrombin Generation

Prothrombin activation fragment F1+2 (F1+2): Thrombin generation takes place at an appreciable rate under physiologic conditions only in the presence of factor Xa, factor Va, calcium ions, and activated platelets. During this process, the amino-terminus of the prothrombin molecule is released as F1+2 (see FIGURES 58.1 and 58.2).

Thrombin–antithrombin complex measurement: Thrombin and other serine proteases within the coagulation pathway can be rapidly inhibited by circulating antithrombin, in a mechanism potentiated by endogenous heparan sulfate. Thrombin–antithrombin (TAT) complex can be measured in plasma and used as a marker of thrombin generation[19,21,48] (FIGURE 58.2).

Assays for Thrombin Activity

Once evolved, the serine protease, thrombin, converts fibrinogen into fibrin, releasing fibrinopeptides A and B from the amino-terminal regions of the Aα and Bβ chains of fibrinogen, respectively[49] (see FIGURE 58.3A). The rate of fibrinopeptide A release from fibrinogen is considerably faster than that of fibrinopeptide B.[31,49,50] Cleavage of fibrinopeptide A from fibrinogen generates an intermediate known as *fibrin I* that can polymerize but cross-links poorly.[51,52] Further proteolysis of fibrin I can occur through the action of either thrombin or plasmin. If thrombin action predominates, fibrinopeptide B is released, and fibrin II is formed. Immunoassays for fibrinopeptide A [28,30,53,54,55] and fibrinopeptide B[31,32] provide an index of thrombin action on fibrinogen.

ASSAYS FOR INHIBITOR COMPLEXES AND ACTIVATED INHIBITORS OF COAGULATION

Protein C Pathway

Thrombin activity and generation can also be inhibited rapidly by activation of the protein C pathway. Thrombin binds to

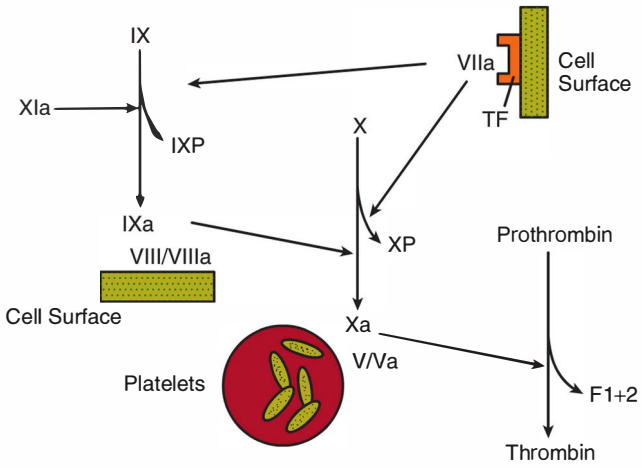

FIGURE 58.1 Pathways of coagulation activation. The activation of factor IX by factor XIa or the factor VII–tissue factor (TF) mechanism liberates the factor IX activation peptide (IXP). The conversion of factor X to factor Xa by the factor IXa–factor VIII/VIIIa–cell surface complex releases the factor X activation peptide (XP). The generation of thrombin from prothrombin is mediated by factor Xa in the presence of factor Va and activated platelets. During this process, the F1+2 fragment is released.

thrombomodulin on vascular endothelial cells and once bound can no longer participate in procoagulant mechanisms, either to activate platelets or to cleave fibrinogen. Endothelial protein C receptor, which lies adjacent to thrombomodulin within the caveolae region of the endothelial membrane, augments this mechanism by capture of protein C from the circulation, particularly in large vessels, and presents protein C to the thrombin–thrombomodulin complex. Thrombin–thrombomodulin activates protein C, which then inactivates factor Va and factor VIIIa by cleavage, thereby also regulating thrombin generation. Assays have been developed to monitor this pathway (FIGURE 58.2). Immunoassays to measure protein C activation include measurements of the protein C activation peptide,[24] an immunoactivity assay for activated protein C,[56,57,58] and activated protein C–inhibitor complexes.[26,27,59]

ASSAYS FOR ACTIVATED FIBRINOLYSIS AND ITS INHIBITION

Tissue plasminogen activator (tPA), produced by endothelial cells, converts circulating plasminogen to plasmin, the enzyme responsible for degradation of the fibrin clot. This process can be suppressed by complex formation between tPA and circulating plasminogen activator inhibitor 1 (PAI-1).

Fibrinopeptides Released by Plasmin

Plasmin action on fibrin I releases Bβ1–42 from the amino-terminus of the Bβ chain and cleaves the carboxyl terminus of the Aα chains, thereby generating fragment X[60] (FIGURE 58.3B). In contrast, plasmin proteolysis of fibrin II results in the release of Bβ15–42 rather than Bβ1–42 because fibrinopeptide B has already been removed by the action of thrombin. Plasma levels of Bβ1–42[39,40] and Bβ15–42,[41] peptides released by the proteolytic action of plasmin on fibrinogen and fibrin, respectively, have been used as indices of *in vivo* plasmin activity. The antisera used in these assays show significant, but not absolute, specificity for the various fibrinopeptides relative to fibrinogen. Consequently, cross-reacting fibrinogen must be removed from plasma samples before analysis, using techniques that do not alter the levels of the peptides.[29,61]

The initial assay for Bβ1–42 was indirect and involved measurement of fibrinopeptide B immunoreactivity before and after thrombin addition to fibrinogen-depleted plasma. The resultant increase in immunoreactivity, termed thrombin-increasable fibrinopeptide B, reflects the presence of Bβ1–42, a fibrinopeptide B-containing fragment.[62] Assays were subsequently developed that directly measured the levels of Bβ1–42 with either a polyclonal[40] or a monoclonal antibody.[39] These antisera are specific for Bβ1–42 and do not cross-react with fibrinopeptide B or Bβ15–42. An alternative approach is to use an antiserum raised against Bβ15–42 that cross-reacts completely with Bβ1–42 because it recognizes the carboxyl-terminal region of these peptides.[63] However, the Bβ1–42/Bβ15–42 assay may underestimate the circulating levels of Bβ1–42 because release of the carboxyl-terminal arginine residue by circulating carboxypeptidases decreases the immunoreactivity of the peptide when measured with a carboxyl-terminal–directed antisera.[40]

Using a monoclonal antibody, a specific assay for Bβ15–42 has been developed[41] that provides a marker of plasmin action on fibrin II. However, two factors limit the utility of this test. First, because the antibody exhibits some cross-reactivity with Bβ1–42, the assay may not be a specific marker of fibrin proteolysis in patients with marked fibrinogenolysis.[64] Second, only low levels of Bβ15–42 are generated during thrombolysis,[65,66] probably because the Bβ42–43 bond of fibrin II is not cleaved as readily by plasmin as is the same bond of fibrinogen or fibrin I. Exploiting this phenomenon, the fibrin specificity of thrombolytic agents has been increased by linking them to monoclonal antibodies against the Bβ15–42 domain of fibrin, thereby targeting them to fibrin.[67,68]

Assays for Large Fragments of Fibrinogen and Fibrin

The levels of fibrinogen or fibrin degradation products (FDPs) can be measured using a variety of immunologic techniques[42,43] or by use of the staphylococcal clumping reaction.[69] Most of the antisera used in these assays cross-react with fibrinogen, and the tests therefore must be performed on serum samples. These assays, like those for fragment E,[70,71] a degradation product found in most high molecular weight derivatives produced by plasmin proteolysis of fibrinogen or fibrin, do not differentiate between fibrin and fibrinogen degradation products. Furthermore, serum FDP measurements can be problematic[72] because incomplete removal of fibrinogen in the formation of serum falsely elevates FDP levels, whereas adsorption of degradation products to the clot results in a spurious reduction in FDP values.[73] These limitations have prompted the development of antibodies against neoantigenic determinants on plasmin-derived fibrinogen or fibrin fragments that do not cross-react with fibrinogen. Both polyclonal and monoclonal antibodies against neoantigens on fragments D and E[74–77] have been developed for this purpose. Although some clinical testing has been done with these assays,[78,79] they have largely been replaced by assays for D-dimer.

Plasmin-induced lysis of cross-linked fibrin results in the formation of a variety of degradation products, D-dimer being

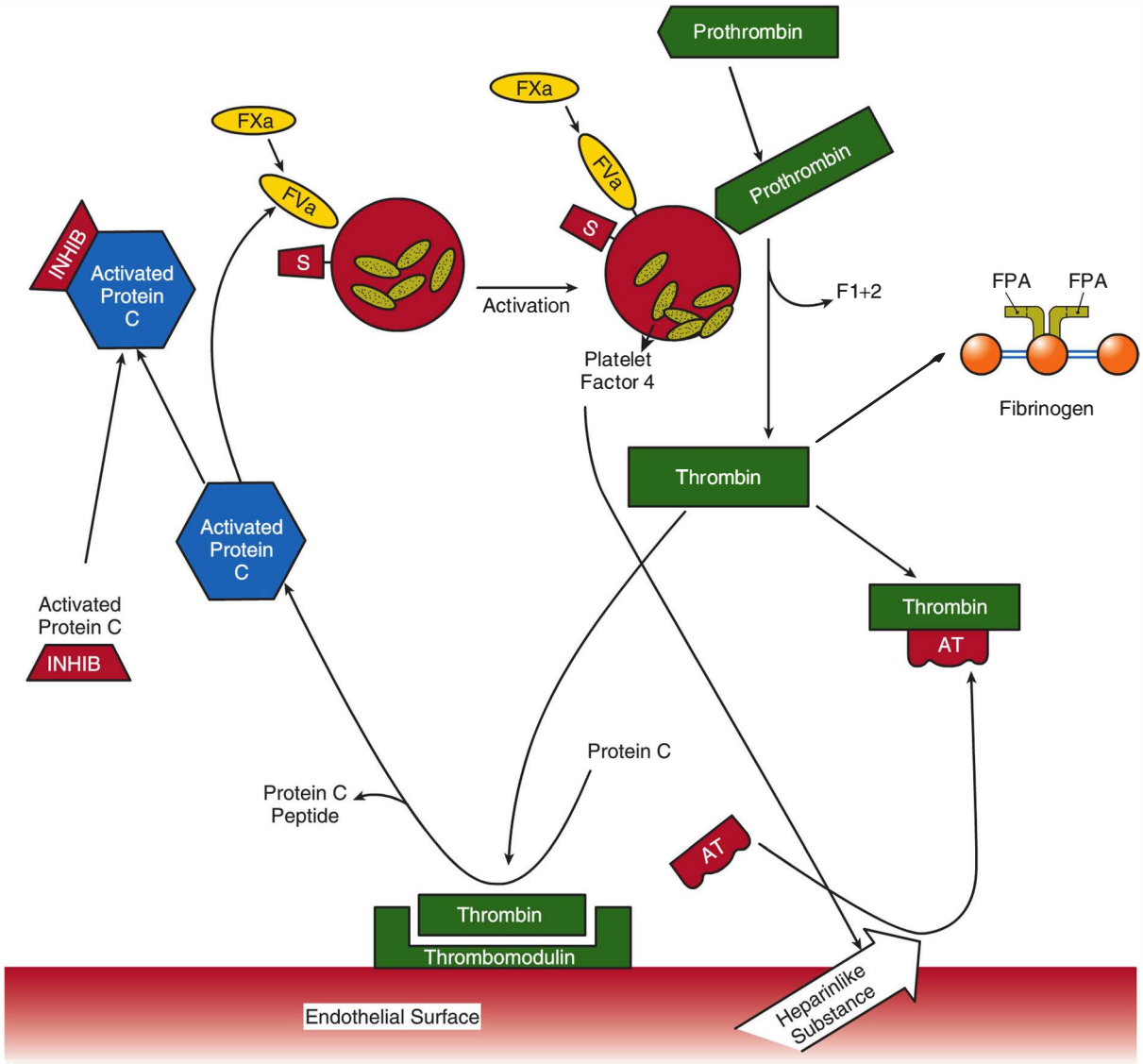

FIGURE 58.2 Regulation of thrombin generation by the natural anticoagulant mechanisms of the endothelium. Factor Xa, factor Va, protein S, prothrombin activation fragment F1+2, fibrinopeptide A, antithrombin, and activated protein C inhibitor(s) are designated as FXa, FVa, S, F1+2, FPA, AT, and activated protein C inhibitor, respectively. Thrombin may be inactivated by forming 1:1 stoichiometric complexes with its major physiologic inhibitor, antithrombin, thereby resulting in the formation of TAT complexes. Activated protein C can be neutralized by inhibitors of activated protein C (e.g., protein C inhibitor, α_1-proteinase inhibitor, and α_2-macroglobulin), which results in the generation of activated protein C inhibitor complexes.

the smallest. This fibrin derivative is comprised of two fragment D moieties covalently linked by their γ chains.[80,81] D-dimer levels in whole blood can be measured by red cell agglutination, whereas levels in plasma can be quantified using an enzyme immunoassay (EIA) or latex bead agglutination. All assays use monoclonal antibodies that recognize neoepitopes on D-dimer that are not expressed on the D-domains of non–cross-linked fibrinogen or fibrin.[44,45] Although agglutination assays can be done more rapidly than the EIA, latex agglutination assays are less sensitive and provide only semiquantitative results.

The performance of each D-dimer assay differs, depending on the specificity of the monoclonal antibodies used in the test, variability in the cutoff value selected to identify positive results, and characteristics of the patient populations in which the test was evaluated.[82–84] Most commercial D-dimer assays use authentic D-dimer as the reference standard, others use fibrinogen. When a fibrinogen standard is used, results are reported as fibrinogen equivalent units, with two of these units approximately equivalent to a single D-dimer. The use of different standards makes it difficult to compare study results because the units for D-dimer measurement are rarely specified.

The concept that D-dimer is derived only from the breakdown of cross-linked fibrin in thrombi has been challenged, because it was observed that the levels of D-dimer in patients undergoing coronary thrombolysis are higher than those that would be predicted given the volume of the coronary

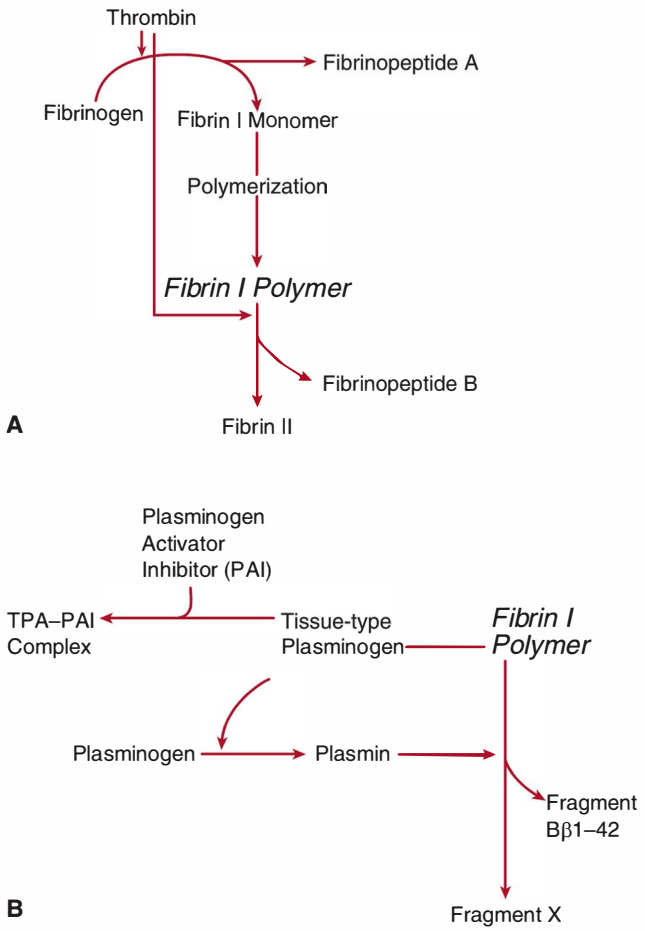

FIGURE 58.3 The actions of thrombin **(A)** and plasmin **(B)** on fibrin I polymer. **A:** The conversion of fibrinogen to fibrin II polymer by thrombin. **B:** The action of plasmin on fibrin I polymer. The fibrinolytic capacity of human plasma is determined primarily by the activity of tissue-type plasminogen activator (tPA) and its inhibitor (plasminogen activator inhibitor 1 [PAI-1]), which are released from vascular endothelial cells. tPA is able to bind to fibrin I polymer, which allows plasminogen to be activated to plasmin at an increased rate.

thrombus.[85] In addition, D-dimer levels can be elevated in the absence of concomitant evidence of angiographic reperfusion after thrombolysis.[85] There are two potential explanations for these findings. The first is that a flaw in the design of D-dimer assays can limit its specificity and lead to higher than expected plasma levels.[64,86] Although a monoclonal antibody is used to "capture" the D-dimer in plasma, a panspecific antibody is sometimes used to identify the bound antigen. Cross-reactivity of this "tag" antibody with FDPs would lead to an overestimate of D-dimer levels in the setting of thrombolytic therapy. Rather than reflecting clot lysis, a second possibility is that the elevated D-dimer levels after thrombolytic therapy may reflect large amounts of circulating soluble cross-linked fibrin,[64] thereby explaining the increased D-dimer levels that occur in the absence of angiographic evidence of reperfusion.[64]

As an alternative to D-dimer, plasma levels of soluble fibrin monomer, a complex of fibrin monomer with fibrinogen, can be measured as an index of thrombin action on fibrinogen. Early assays used protamine sulfate to precipitate soluble fibrin monomers,[87] but newer tests use monoclonal antibodies against fibrin-specific epitopes on the α or γ chains, or examine the cofactor activity of fibrin monomer in a tPA-induced plasminogen activation assay.[88] The two types of assays yield different results, highlighting the need for standardization. Theoretically, assays for soluble fibrin monomer may be superior to D-dimer assays if patients with thrombosis have reduced fibrinolytic activity, as has been suggested by some investigators.[89]

Assays for Plasmin–α_2-Antiplasmin

α_2-antiplasmin, the major plasma inhibitor of plasmin, forms a 1:1 stoichiometric complex with the enzyme.[90–94] The reaction between plasmin and α_2-antiplasmin is extremely rapid and the enzyme–inhibitor complex is stable and devoid of enzyme activity.[33,95,96] Assays that measure the plasma levels of plasmin–α_2-antiplasmin complexes have been developed and can be used as an index of *in vivo* plasmin generation.[22,33,34,35–38] Increased levels of complex have been detected in patients with intravascular coagulation,[97] consistent with the activation of fibrinolysis that occurs in this disorder.

APPLICATIONS OF ASSAYS

Influence of Physiologic Factors and Organ Dysfunction

A number of variables can result in overlap of assay results between individuals with a pathologic condition or altered physiologic status and health. Although values further from the mean of the normal distribution are more likely to be abnormal, appropriate interpretation requires an appreciation of the factors that can influence measurements.

The normal aging process alters coagulation activation in a predictable fashion.[18,98,99] With advancing age from 45 to 70 years, an increasing number of patients who are otherwise healthy exhibit elevated levels of F1+2. This reflects increased thrombin generation because clearance of radiolabeled F1+2 is unchanged.[98] The levels of the factor IX and X activation peptides also increase with advancing age.[10] Significant, but somewhat less striking, positive correlations have been observed between increasing age and the levels of fibrinopeptide A and protein C activation peptide.[98] In a study of 25 healthy Italian centenarians, coagulation and fibrinolysis measurements were compared with two control groups of healthy adults ranging from 18 to 50 years and 51 to 69 years.[99] Older controls generally had slightly higher values of several measurements than younger controls. Centenarians had striking signs of heightened coagulation enzyme activity as assessed by plasma measurements of factor VIIa ($P < 0.01$ compared with either control group) or the activation peptides of factor IX, factor X, and prothrombin and TAT complexes ($P < 0.001$ for all). Heightened coagulation enzyme activity was accompanied by enhanced formation of fibrin (high fibrinopeptide A and D-dimer, $P < 0.001$) and secondary fibrinolysis (plasmin–antiplasmin complex, $P < 0.001$).

It has been reported that strenuous exercise in the form of long-distance running leads to increased TAT complex levels without elevations in the levels of fibrinopeptide A.[100] Cigarette smoking has been associated with elevations in activation markers of blood coagulation.[101]

Certain medications that are not known to have a direct effect on blood coagulation can alter the activity of the coagulation mechanism. Caine et al.[102] showed that administration of conjugated equine estrogen daily to menopausal women for 3 months increased the levels of F1+2 in a dose-dependent manner. The activity of fibrinopeptide A also increased, and levels of protein S and antithrombin were decreased as compared to placebo treatment. In women who had undergone oophorectomy, Kroon et al.[103] confirmed that 0.625 mg of conjugated equine estrogen, as well as 0.05 transdermal 17β-estradiol daily for 6 weeks increased levels of F1+2. Scarabin et al.[104] found that 2 mg of estrogen valerate daily with cyclic progesterone increased F1+2 levels and decreased antithrombin activity, whereas transdermal estrogen (2.5 mg 17β-estradiol daily with cyclic progesterone) did not. The treatment of patients with coronary heart disease with gemfibrozil, a drug used to lower serum cholesterol and triglyceride concentrations, reduces F1+2 levels by approximately 25%.[105]

In healthy volunteers, postprandial elevations in the levels of factors VIIa and IX activation peptide have been demonstrated, but no changes were observed in the levels of factor XIIa or indices of thrombin generation.[106–108] The roles of factors XII, XI, and IX in factor VII activation were evaluated by investigating patients with isolated deficiencies of these factors after a fatty meal.[106,108] Factor VIIa levels increased postprandially in patients with factor XII deficiency but did not change in those with factor IX deficiency. In patients with factor XI deficiency, Miller et al.[106] found that factor VIIa levels rose postprandially, whereas Silveira et al.[108] did not observe significant alterations. From these studies, it can be concluded that factor IX plays a critical role in the events linking postprandial lipemia to factor VII activation, but factor XII does not. This implies that factor XII is not involved in the activation of factor VII by lipolysis of triglyceride-rich lipoproteins.

Dysfunction in normal physiologic clearance mechanisms can also result in substantial elevations in the levels of activation peptides. For F1+2, this has been demonstrated in patients with chronic renal failure on dialysis.[109,110] Caution is required, therefore, in interpreting an elevated level of a marker as evidence of heightened coagulation or fibrinolytic activity in disorders associated with renal (e.g., thrombotic thrombocytopenic purpura, systemic lupus erythematosus, nephrotic syndrome, renal transplant rejection) or hepatic dysfunction. In a prospective epidemiologic study of healthy middle-aged males, the Second Northwick Park Heart Study (NPHSII) measured the levels of F1+2, fibrinopeptide A, factor IX activation peptide, factor X activation peptide, factor VIIa, and factor XIIa. The factor IX activation peptide was the only coagulation activation marker found to correlate with creatinine.[111]

To determine the influence of inflammation on levels of activated markers of coagulation, correlation analysis was done between C-reactive protein (CRP) and factor XIIa, factor VIIa, factor IX activation peptide, factor X activation peptide, F1+2, and FPA in healthy individuals within NPHSII.[111] Only weak significant correlations (Pearson correlation 0.15, $n >$ 1,135) were observed for each analyte with CRP, suggesting that inflammation does not contribute a great deal to levels of coagulation activation markers. The Pearson correlation between CRP and fibrinogen in the same individuals was 0.45, suggesting a large influence of inflammation upon fibrinogen. Stronger correlations were, however, observed between the coagulation activation markers downstream from FVIIa-tissue factor. Factor IX activation peptide and factor X activation peptide were highly correlated (0.47, n = 1,335), while the correlation coefficients between factor X activation peptide and prothrombin F1+2 or factor VIIa were 0.23 and 0.21, respectively.

Disseminated Intravascular Coagulation

A number of factors can trigger disseminated intravascular coagulation (DIC), and increased coagulation system activation and secondary fibrinolysis are cardinal features of the disorder. The aforementioned assays should therefore be sensitive markers of acute or chronic DIC syndromes, and elevations in levels of factor X activation peptide,[12] protein C activation markers,[27,59,112] F1+2,[16,24] TAT complex,[16,113–115] fibrinopeptide A,[24,29,30,53,55,116] fibrinopeptide B,[31,32,62] and Bβ1–42[62] have been observed in such patients. However, the sensitivity and specificity of these assays for the diagnosis of DIC have yet to be determined, and the diagnosis of DIC can usually be made with simpler laboratory tests.

Nossel et al.[62] used radioimmunoassays for fibrinopeptide A, B, and Bβ1–42 (measured as thrombin-increasable fibrinopeptide B) to investigate the pathophysiology of DIC in patients receiving hypertonic saline to terminate pregnancy. Immediately after intrauterine infusion, there was a marked increase in fibrinopeptide A levels, an index of thrombin action on fibrinogen. This was followed by a rise in the concentration of Bβ1–42, a measure of plasmin action on non–cross-linked fibrin I polymer. On the basis of these observations, it was hypothesized that the relative rates of proteolysis of the Bβ chain of fibrinogen by thrombin and plasmin were determinants for venous thrombosis.[60] Supporting this concept, a study of patients undergoing neurosurgery and craniotomy found that individuals who developed venous thrombosis exhibited fibrinopeptide A levels that were considerably greater than those of Bβ1–42 during the 4 days preceding the onset of this disorder. These observations suggest that thrombosis occurs at a time when thrombin action predominates over that of plasmin. Studies have also been carried out to determine whether elevated levels of plasmin–α_2-antiplasmin complexes before surgery are predictive of postoperative venous thrombosis.[117,118] These data suggest that patients subjected to major abdominal surgery, who have enhanced fibrinolytic activity preoperatively, have a lesser tendency to develop postoperative deep venous thrombosis.

Venous Thromboembolism

Levels of F1+2,[14,119,120] TAT complex,[113,115,119,120] fibrinopeptide A[29,53,121,122] and B,[31,32] and protein C activation peptide[24] are frequently elevated in symptomatic patients with venous thrombosis, pulmonary embolism, or both. Although these parameters are sensitive indices of hemostatic enzyme generation, the tests are not specific for intravascular thrombosis. Elevated values have been reported in nonpathologic, as well as in pathologic conditions, including localized and generalized infections, cancer, and connective tissue disease (Table 58.2).

D-dimer testing has emerged as a cost-effective strategy to help exclude the diagnosis of acute venous thromboembolism, thereby obviating the need for serial imaging studies (see Chapter 83). The D-dimer tests with the highest sensitivity are those that use the EIA format. Although initial EIA technology

Table 58.2

Conditions associated with elevated levels of markers of coagulation activation (excluding thrombosis or DIC)

Condition	Marker (Reference)
Physiologic	
Normal aging	F1+2,[18,98,99] FPA,[98] PCP,[98] factor IX activation peptide,[10] D-dimer[99]
Strenuous exercise	TAT[100]
Alimentary lipemia	Factor VIIa,[106–108] factor IX activation peptide[108]
Normal pregnancy	F1+2,[123] D-dimer[124]
Pathologic	
Cigarette smoking	Activation peptides of factor IX and X, F1+2[101]
Postmenopausal estrogen use	F1+2, FPA[102]
Systemic lupus erythematosus	FPA[30,124]
Malignancy	F1+2, FPA[12,125,126]
Acute myelogenous leukemia	F1+2, FPA[24,127,128]
Sickle cell disease	F1+2, FPA, D-dimer[129–132]
Prothrombin complex concentrate administration in hemophilia B	Factor X activation peptide, F1+2, TAT, FPA[133,134,135]
Hemolytic uremic syndrome in children	F1+2, TAT[136]

FPA, fibrinopeptide A; PCP, protein C activation peptide; TAT, thrombin–antithrombin complex.

was cumbersome, time-consuming, and only amenable for batch assays, current assays can be done rapidly and samples can be assayed individually.[137–139] The accuracy of rapid EIA methods is similar to that of the original, more cumbersome assays, and these tests are considered to be the gold standard for measuring D-dimer levels.

The results of quantitative D-dimer assays, measured at the end of warfarin therapy and then 1 month after its discontinuation may also help stratify patients with an initial unprovoked episode of venous thromboembolism with respect to recurrence risk.[140–146] A meta-analysis of seven studies including a total of 1,888 patients who completed at least 3 months of anticoagulation found that a D-dimer level of less than approximately 500 ng/mL ("a negative test") was associated with a 3.5% (95% CI 2.7 to 4.3) annual risk of recurrence, while a D-dimer over this level ("a positive test") was associated with an 8.9% (95% CI 5.8 to 11.9) risk in each of the first 2 years.[147] Potential difficulties in applying this approach in the clinic, however, are lack of agreement and different cutpoints for different commercially available D-dimer assays (there is no D-dimer "standard"). Clinicians must therefore rely on an assay manufacturer's cutpoint for a positive or negative D-dimer test based upon published studies assessing recurrence risk.[148]

Although assays for fibrin monomer have accuracies similar to those for D-dimer,[149–151] they have yet to be rigorously tested in clinical management studies. It is important to point out that each assay is different, and results obtained with one assay cannot be extrapolated to another.[82,152] Consequently, each individual assay must be evaluated in prospective management studies before it can be adopted into clinical practice.

Sepsis

Septicemia frequently results in disturbances of the hemostatic mechanism. In septic shock, DIC can lead to the deposition of fibrin in the microvasculature and the failure of multiple organs. Endotoxin, a lipopolysaccharide present in the outer membrane of gram-negative bacteria, plays an important role in the development of the clinical and laboratory manifestations of septicemia. The intravenous administration of *Escherichia coli* endotoxin to healthy volunteers under controlled conditions increases thrombin generation, as monitored by F1+2 and TAT complex assays, that is maximal 3 to 4 hours after infusion without evidence of contact system activation.[153] Evidence exists that this activation is driven by the tissue factor pathway (see subsequent text). Factor XI, interposed between the contact system and factor IX, is activated after endotoxin infusion.[6] The fibrinolytic system also is triggered by endotoxin, as evidenced by increased levels of tPA and plasmin–α_2-antiplasmin complex at 2 to 3 hours.[153,154] This rise in fibrinolytic activity subsequently is offset by the release of PAI-1.

Many of endotoxin's biologic effects appear to be mediated by cytokines synthesized and released by macrophages and monocytes. High levels of cytokines, tumor necrosis factor (TNF), and interleukin-6 (IL-6) have been observed within the first 2 hours of administering endotoxin to healthy subjects.[153–156] Bauer et al.[157] showed that TNF has a potent procoagulant effect *in vivo* by studying the changes in the levels of F1+2, protein C activation peptide, and fibrinopeptide A after the administration of the recombinant cytokine to patients with cancer. In healthy volunteer studies, the early dynamics and route of

coagulation activation in response to TNF administration were investigated.[158] This resulted in the early activation of factor X, probably by the factor VII–tissue factor pathway. Infusion of recombinant IL-6 into patients with renal cell carcinoma results in elevated plasma concentrations of F1+2 and TAT complexes; fibrinolysis is not affected.[156]

Because the immunoreactivities of coagulation system zymogens and their respective activation peptides are virtually identical in humans and chimpanzees, it has been possible to reproduce a coagulopathic response to intravenous endotoxin in this animal that is similar to that in humans.[159] To investigate the molecular pathways elicited by endotoxin, pentoxifylline, a xanthine oxidase inhibitor that interrupts "immediate early" gene activation by monocytes, or a potent monoclonal antibody that rapidly neutralizes tissue factor–mediated initiation of coagulation, was administered to chimpanzees just before infusing endotoxin. Pentoxifylline markedly inhibited the endotoxin-elicited increases in serum levels of TNF and IL-6, as well as the effects on coagulation (as measured by assays for F1+2, TAT complex, and fibrinopeptide A) and fibrinolysis (as monitored by assays for plasminogen activator activity and plasmin–α_2-antiplasmin complexes). In contrast, the monoclonal antibody to tissue factor completely abrogated the augmentation in thrombin generation, but had no effect on cytokine levels or the fibrinolytic response. These data indicate that the fibrinolytic response triggered by endotoxin is not dependent on the generation of thrombin and that cytokines may be important in mediating the activation of both the coagulation and the fibrinolytic mechanisms *in vivo*. In addition, treatment with an antibody to IL-6 prevents coagulation activation after administration of a low dose of endotoxin to chimpanzees and does not affect activation of the fibrinolytic system.[156] IL-6 probably augments coagulation by an indirect mechanism, because this cytokine has not been reported to influence the hemostatic properties of vascular endothelium or monocytes *in vitro*. Studies of administration of live *E. coli* to primates (baboons) indicate that, although an antibody to factor XII can inhibit activation of the contact system, it has no effect on the manifestations of DIC (see Chapter 15B).

Hereditary Deficiencies of Coagulation Factors

The investigation of patients with hereditary coagulation factor deficiencies using the activation peptide assays has generated information about the pathways responsible for coagulation activation *in vivo* under basal conditions (i.e., the absence of thrombosis or provocative stimuli). Patients with factor VII deficiency, but not factor XI deficiency, have reduced levels of factor IX activation,[10,11] whereas patients with deficiencies of factor VIII or factor IX have normal levels of factor X and prothrombin activation.[12] The administration of relatively small doses of recombinant factor VIIa (10 to 20 μg/kg of body weight) to patients with factor VII deficiency results in substantial elevations in the plasma concentrations of the factor IX activation peptide, factor X activation peptide, and F1+2.[160] Therefore, these data demonstrate that the factor VII–tissue factor pathway is largely responsible for the activation of factor IX, as well as factor X in the basal state.[10,12,160] Using a clotting assay for factor VIIa, patients with severe factor IX deficiency were found to have circulating levels of factor VIIa <10% of those in healthy individuals.[8,161] This suggests that factor IXa is the principal activator of factor VII *in vivo* under basal conditions. These observations were corroborated in NPHSII, in which a haplotype in the F7 gene was associated with particularly reduced factor VII coagulant activity levels, and significantly reduced factor VIIa and factor IX activation peptide levels; this suggests that under basal conditions factor VIIa is also a principal activator of factor IX.[162]

To provide direct proof that basal level activation of the hemostatic mechanism occurs via factor VIIa–dependent activation of factor X, a monoclonal antibody directed against human tissue factor was administered to normal chimpanzees before infusing relatively high concentrations of recombinant factor VIIa (~50 μg/kg body weight).[163] The monoclonal antibody used in this study is a potent inhibitor of factor VIIa–tissue factor complex function *in vitro*.[164] The administration of antibody before factor VIIa abolished the significant increases in the plasma levels of factor IX activation peptide, factor X activation peptide, and F1+2 mediated by the infused recombinant protein alone.

Infusions of highly purified factor IX concentrate to individuals with hemophilia B increase plasma factor IX activation peptide levels that are initially greatly decreased, but do not change factor X activation peptide or F1+2 measurements.[160] Administration of highly purified factor VIII concentrates to patients with hemophilia A results in no significant change in concentrations of factor X activation peptide and F1+2.[160] These observations therefore indicate that the factor IXa–factor VIIIa–cell surface complex is unable to activate factor X under basal conditions. In response to vascular injury or thrombotic stimuli, it is surmised that increased formation of free thrombin or factor Xa via the action of the factor VII–tissue factor pathway generates factor VIIIa on a natural surface (e.g., activated platelets) on which assembly of the factor IXa–factor VIIIa complex takes place. This hypothesis is consistent with the severe bleeding tendency of most patients with factor VIII or factor IX deficiency and with the insensitivity of the factor X activation peptide and F1+2 assays to significant deficiencies of these two proteins.

The earlier mechanistic findings derived from studies of patients with coagulation factor deficiencies have significant potential implications regarding the utility of basal coagulation system markers in diagnosing patients with prethrombotic disorders.[160] It follows that the conversion of a prethrombotic state to a thrombotic event occurs due to small increases in the generation rates of hemostatic enzymes that exceed the inhibitory threshold of an individual's endogenous anticoagulant mechanisms. It remains to be determined whether persons with elevated basal levels of coagulation system markers are more likely to respond in a hypersensitive fashion to environmental stimuli. Because the activity of the blood coagulation mechanism in such individuals is closer to the threshold of normal inhibitory processes, they may generate slightly more thrombin by the extrinsic pathway. This thrombin could then be used to ignite the dormant intrinsic cascade, which could ultimately result in the generation of large amounts of free thrombin and the development of arterial or venous thrombosis.

Hereditary Thrombotic Disorders

Hereditary deficiencies of proteins C, S, and antithrombin, as well as activated protein C resistance caused by the factor V–Arg506Gln variant have all been associated with hypercoagulable states, and coagulation activation has been investigated in patients with each of these disorders. In asymptomatic individuals with heterozygous deficiencies of protein C or protein S, the mean F1+2 level is significantly increased as compared

to age-matched controls.[165,166,167] Approximately one third of patients have levels greater than the upper limit of normal controls (defined as the mean +2 standard deviations).[165,166] The elevated F1+2 concentrations are not caused by diminished clearance of the polypeptide.[165] Fibrinopepetide A levels are elevated in approximately 20% of subjects.[165,166]

Protein C activation as measured by its peptide or an immunoenzymatic assay for activated protein C is reduced to approximately 50% of normal in asymptomatic persons with heterozygous protein C deficiency.[24,56] In two adult patients with homozygous protein C deficiency, it was shown that the levels of protein C activation peptide as well as F1+2 can be normalized by administration of a monoclonal antibody–purified protein C concentrate.[168] Therefore, augmented activity of the protein C anticoagulant pathway can inhibit prothrombin activation *in vivo*, and protein C activation by the thrombin–thrombomodulin complex is a tonically active mechanism in the regulation of coagulation system activation.

Markers of thrombin generation have been measured in asymptomatic nonanticoagulated patients from thrombophilic families with the factor V–Arg506Gln variant. Martinelli et al.[169] reported that the median levels of F1+2 and TAT complex were significantly elevated in 69 heterozygotes from 23 families as compared to age- and sex-matched controls (for F1+2 and TAT complexes, 1.24 nM and 3.3 ng/L vs. 1.08 nM and 2.4 ng/mL, $P < 0.005$, respectively). Similar results were obtained by Zöller et al.[167] for F1+2 in 38 nonanticoagulated patients (1.7 vs. 1.3 nM, $P < 0.001$). In the NPHSII, a prospective cardiovascular survey of healthy men, aged 50 to 61, with no antecedent history of thrombosis, the mean levels of F1+2 were found to be significantly increased among heterozygous carriers of either the factor V–Arg506Gln variant or the prothrombin G20210A variant.[170]

In asymptomatic patients with hereditary antithrombin deficiency (levels ~50% of normal), it was initially reported that F1+2 levels are frequently increased as compared to age-matched unaffected siblings,[14] while fibrinopeptide A measurements were similar in both groups. Subsequently, it was shown that the high concentrations of the fragment resulted from an *in vitro* effect due to the presence of low amounts of heparin (final concentration of 4 U/mL) in the anticoagulant cocktail used for sample collection in the presence of reduced blood levels of antithrombin.[171] Other studies of antithrombin-deficient subjects did not demonstrate significant elevations in plasma F1+2 or fibrinopeptide A levels.[166,172] An investigation of a single large family with functional antithrombin deficiency (antithrombin Hamilton, a type II mutation having diminished serine protease reactivity) found significantly higher levels of F1+2 in affected family members than in those without the mutation.[173] However, the mean F1+2 level was within the normal range, and the majority had normal levels. The mean levels of TAT complex and fibrinopeptide A were not significantly different between the antithrombin-deficient individuals and their unaffected family members.[173] The reason for this apparent paradox in patients with heterozygous antithrombin deficiency, which is generally considered as the most prothrombotic disorder among the hereditary thrombophilias, is unknown.

Lupus Anticoagulants

Ginsberg et al.[174] found that the presence of anticardiolipin antibodies in patients with systemic lupus erythematosus is associated with a prothrombotic state as measured by the F1+2 and fibrinopeptide A assays. This relation was maintained even after excluding from analysis those individuals with prior thrombotic histories. Another study by Tripodi et al.[175] of patients with systemic lupus erythematosus and lupus anticoagulants found significant elevations in fibrinopeptide A, but not F1+2.

Acute Coronary Syndromes

This section reviews data about coagulation activation markers in patients with acute coronary syndromes (ACS) and describes how this information has helped to clarify the pathophysiology of coronary artery thrombosis. Most patients with acute myocardial infarction and unstable angina have high plasma levels of fibrinopeptide A.[176,177] The levels of fibrinopeptide A in spot urine samples and 24-hour urine collections from these patients also have been found to be elevated.[178,179] These findings are consistent with the results of angiographic, angioscopic, and pathologic studies that have clearly shown that intracoronary thrombosis plays a pivotal role in the pathogenesis of these coronary syndromes.[180]

Abnormally high plasma F1+2 or fibrinopeptide A levels are found in approximately 50% of patients during the acute phase of the disease.[181] No difference in the magnitude of the elevation in the levels of these peptides has been observed between patients with unstable angina than in those with acute myocardial infarction.[181] This may indicate that plasma F1+2 and fibrinopeptide A levels are not dependent on the characteristics of the thrombus (which is nonocclusive and platelet-rich in unstable angina, but occlusive and fibrin-rich in myocardial infarction),[182] but rather reflect a systemic condition of hypercoagulability.

The degree of coagulation activation seems to be related to prognosis. Ardissino et al.[183] measured both plasma and urinary fibrinopeptide A levels in patients with unstable angina on hospital admission and found that elevated plasma levels were associated with a higher risk of developing primary (death or myocardial infarction) or secondary (refractory angina requiring emergency coronary revascularization) clinical end points during hospitalization. Granger et al.[184] found that higher baseline levels of F1+2 were associated with an increased likelihood of death or myocardial reinfarction during a 30-day follow-up period in patients with myocardial infarction receiving thrombolytic therapy.

The serial measurement of coagulation activation peptides over time has provided interesting information concerning the pathophysiology of ACS. Merlini et al.[181] measured F1+2 and fibrinopeptide A plasma levels in patients with unstable angina or acute myocardial infarction during the acute phase and 6 months after their initial acute presentation. Both analytes were elevated during the acute ischemic episode, thereby reflecting the presence of an intracoronary thrombosis. However, in patients with an uneventful clinical course at 6 months, fibrinopeptide A levels had returned to the normal range, whereas F1+2 levels remained high. This observation indicates that increased thrombin generation persists after ACS episodes but is not sufficient to initiate fibrin formation and deposition. It is not known whether the presence of such biochemical hypercoagulability state is a risk factor for the occurrence (or recurrence) of coronary events, but studies to address this question in both healthy populations without prior cardiac events, as well as patients with recent prior coronary events are ongoing.

Coronary angioplasty mechanically causes plaque disruption that exposes the procoagulant matrix of the plaque itself to blood flow, giving rise to thrombosis. Abrupt coronary artery occlusion still represents one of the major complications of the procedure. Marmur et al.[185] measured arterial blood F1+2 and fibrinopeptide A during uncomplicated coronary angioplasty. Although no changes occurred in most patients, seven of 32 showed an increase in F1+2, which was associated with an increase in fibrinopeptide A in four. These data show that most patients do not experience heightened coagulation system activation during uncomplicated coronary angioplasty under antithrombotic treatment with heparin; however, a few do, and this could be due to the biochemical characteristics of the plaque itself[186] or to deeper trauma of the vessel wall induced by the angioplasty procedure. In another series of patients undergoing mechanical revascularization, Oltrona et al.[187] showed that a failure to normalize fibrinopeptide A levels during the procedure (heparin resistance) was associated with a higher risk of complications. In a study comparing fibrinopeptide A, F1+2 and TAT complex following plain old balloon angioplasty, coronary stenting, and cutting balloon angioplasty, coagulation was found to be less activated following cutting balloon angioplasty.[188]

Atrial Fibrillation

Chronic atrial fibrillation predisposes to thromboembolism, and cardioversion itself also is associated with cerebral, systemic, and pulmonary embolic events. The mechanism underlying the occurrence of embolic episodes after the restoration of sinus rhythm in these patients is poorly understood. The reduction in the incidence of cardioversion-related embolic events induced by the administration of warfarin has led to the widespread recommendation that anticoagulation should be prescribed for several weeks before and after cardioversion in patients with chronic atrial fibrillation.[189] The use of anticoagulants, however, is not recommended in patients with acute atrial fibrillation lasting <48 hours who undergo cardioversion, although definitive data about the risk of postcardioversion embolism in these patients are scanty. The finding of atrial thrombosis in 14% of patients with recent-onset atrial fibrillation suggests that these individuals also are at risk for developing embolic events.[190] Oltrona et al.[191] measured the plasma concentrations of TAT complex and fibrinopeptide A in a series of patients with acute nonvalvular atrial fibrillation and found a significant increase in the plasma concentrations of both markers early after pharmacologic cardioversion as compared to hospital admission; the levels decreased toward baseline values 1 month after restoration of sinus rhythm. This finding of a hypercoagulable state early after cardioversion in patients with recent-onset atrial fibrillation thereby raises the important question as to whether prophylactic antithrombotic treatment also should be adopted for these cases.

Predicting Risk of a Heart Disease Event in Health

Geometric mean levels for coagulation activation markers were assessed at baseline in the prospective study, NPHSII, and analyzed for those who went on to have a myocardial infarction (clinical and silent), and those who went on to have coronary intervention procedures. There was no indication that increased thrombin generation occurred in the baseline samples of those who developed a coronary ischemic event, neither was there any indication that increased factor XIIa contributed to future development of coronary ischemic events. However, geometric mean levels of FVIIa were significantly lower in each coronary heart disease (CHD) group compared with those who had no event, while factor IX activation peptide levels were significantly higher in those who had a future myocardial infarction (MI). Further analysis confirmed that low factor VIIa and high factor IX activation peptide levels were independently associated with future development of MI or total coronary ischemic events when included in the same model. While these results seem rather counterintuitive, further analysis of NPHSII using haplotype data for the factor VII gene has also revealed a haplotype associated with low levels of FVII (factor VII antigen and particularly low factor VIIc), but also resulting in low factor VIIa levels, and the haplotype was associated with increased risk of a future coronary ischemic event. However, this factor VII haplotype, as mentioned previously, was associated with reduced factor IX activation peptide levels.[156] One of the variants in this factor VII haplotype codes for the amino acid substitution R353Q. This variant has also been identified in patients with factor VII deficiency, particularly associated with a functional deficiency,[192] and interestingly, thrombotic events were also recorded in some of these patients.[193,194] Whether these findings suggest the possibility that a dampening of the tissue factor stimulated blood coagulation can lead to a coronary ischemic event, or whether these findings are specific to factor R353Q polymorphism, requires further study.

Antithrombotic Therapy

Intravenous heparin, a mainstay in the treatment of patients with ACS, acts by inhibiting thrombin and activated factors X and IX, and less potently activated factors XI and XII. The anticoagulant action of heparin is due primarily to its ability to bind antithrombin, thereby accelerating the rate of inhibition of the major coagulation enzymes, particularly thrombin. Biochemical studies of patients with ACS have demonstrated clearly that intravenous heparin rapidly inhibits the action of thrombin on fibrinogen and lowers plasma fibrinopeptide A levels to within the normal range.[195–197] Merlini et al.[198] showed that intravenous heparin at doses giving an activated partial thromboplastin time of more than double its baseline value reduced plasma fibrinopeptide A levels, but did not lower F1+2 levels in most patients. Studies have shown that hirudin, a high-affinity direct thrombin inhibitor, does not decrease thrombin generation in patients with stable angina or ACS.[199] Because thrombin plays a critical role in the amplification of the coagulation cascade by activating factor V and factor VIII, persistent thrombin generation may contribute partly to the persistent thrombotic risk that exists despite anticoagulation. Plasma levels of both fibrinopeptide A and F1+2 have been found to be higher in patients developing infarction, reinfarction, or refractory ischemia, suggesting that the presence of a persistent hypercoagulable state in patients with ACS, notwithstanding heparin therapy at the usual dose, still may be associated with an unfavorable outcome.[198,200]

The measurement of coagulation peptides after the discontinuation of heparin or hirudin has revealed the presence of a so-called rebound phenomenon. Gallino et al.[196] have shown that, although a decrease in plasma fibrinopeptide A levels occurs during heparin infusions, a sharp increase occurs once the infusion is over, which suggests a reactivation of coagulation

system activity. This finding has been confirmed by Granger et al.,[200] who found the same phenomenon for F1+2 and activated protein C levels (which also reflect, in part, increased thrombin generation). A similar effect has been observed after the discontinuation of argatroban, another direct thrombin inhibitor.[201] It is tempting to speculate that the increase in clinical events observed after the cessation of heparin or argatroban therapy is linked to this rebound in coagulation,[201,202] most likely due to the persistence of the original procoagulant activity that necessitated anticoagulation in the first place.

Therapy with oral anticoagulants such as warfarin suppresses prothrombin activation *in vivo* as measured by the F1+2.[203,204,205] In patients with prior thrombotic histories and high F1+2 levels, Conway et al.[203] demonstrated that stable anticoagulation at moderate intensity as reflected by international normalized ratios (INRs) of 2.5 to 3.5, produces 5- to 10-fold reductions in the extent of prothrombin activation. It also has been shown that the mean values of F1+2 decrease in parallel with the intensity of warfarin therapy. A study has demonstrated that a target INR range as low as 1.3 to 1.6 results, on average, in a 50% reduction in prothrombin activation from baseline levels.[205] It is important to note that the findings of these studies cannot be translated into clinical practice because the F1+2 level that confers antithrombotic protection has yet to be determined.

Although F1+2 levels often are suppressed below normal in stably anticoagulated patients on oral agents, this has not been observed for TAT complexes and fibrinopeptide A. However, it has been observed that plasma fibrinopeptide A concentrations increase above the normal range in patients with a history of myocardial infarction several weeks after the cessation of the drug.[206]

On the basis of these data, clinical studies have been carried out in situations in which the monitoring of coagulation system function by standard methods has been unsatisfactory (e.g., the prophylaxis of subacute thrombosis after stent implantation). The main problem of intracoronary stenting is subacute thrombosis, which often occurs unpredictably even in conditions of optimal anticoagulation according to classic standard coagulation tests. It has been shown that an increase in F1+2 levels, despite optimal anticoagulation, is a sensitive indicator of subacute thrombosis.[207] In a case–control study,[208] a study population on oral anticoagulants was investigated using F1+2 measurements, as well as classic coagulation tests and compared to a control population in which only the classical tests were used to monitor the level of anticoagulation. It was found that the incidence of subtotal occlusion was 3.5% in the study group, but 17% in the control group. For elective and nonelective stenting, the monitoring of F1+2 levels therefore may be associated with a reduced rate of subacute thrombosis.[208]

Extracorporeal Circulation

Using activation markers of coagulation, Boisclair et al.[52,109,172] have examined whether extracorporeal circulation in patients undergoing hemodialysis for chronic renal failure or cardiopulmonary bypass for coronary artery bypass grafting activates the contact system. They did not find that exposure of blood to foreign surfaces triggers contact activation and rather postulated it to be a consequence of defective neutralization of downstream coagulation proteases (e.g., factor Xa, thrombin) caused by the absence of normal vascular endothelium. In patients undergoing cardiopulmonary bypass, it was suggested that the surgery itself was primarily responsible for increased factor VII–tissue factor mechanism function and the subsequent generation of factor Xa and thrombin.[47] These findings, however, must be reconciled with the observations of increased kallikrein—and factor XIIa–C1 inhibitor complexes during bypass (see Chapter 15B). The fibrinopeptide assay was found to be a useful marker of thrombin activity in the extracorporeal circuit and has been used for objectively monitoring the required dose of heparin, low molecular weight heparins, and heparinoids,[209–215] and assessing activated coagulation over the longer term in hemodialysis.[216]

Thrombolytic Therapy

The availability of biochemical markers that reflect the activation of the coagulation and fibrinolytic systems has led to a number of important observations in patients receiving thrombolytic therapy. The measurement of coagulation activation markers during thrombolytic therapy in patients with acute myocardial infarction has demonstrated the prothrombotic effect of thrombolytic therapy. In patients receiving streptokinase or recombinant tPA, there is an increase in the plasma levels of fibrinopeptide A, TAT complex, and F1+2.[217–220] Although the concomitant administration of heparin prevents fibrinopeptide A levels from increasing,[221] F1+2 levels increase in most patients despite the associated anticoagulant therapy.[222] These findings raise the question of the role of hypercoagulability in the pathogenesis of the failure of reperfusion or postthrombolytic reocclusion. The possibility exists that the ability of plasmin to activate platelets contributes to this. In patients receiving thrombolytic therapy, the behavior of coagulation activation markers after treatment seems to be predictive of outcome. Gulba et al.[223] have shown that patients in whom TAT complex levels increase 90 minutes after the start of thrombolytic therapy are those with no reperfusion or in whom reocclusion occurs. Granger et al.[184] have assessed the relation of fibrinopeptide A and F1+2 levels with subsequent reinfarction and death in patients who received either recombinant tPA or streptokinase; lower levels of fibrinopeptide A or F1+2 after 12 hours were associated with a trend toward a lower likelihood of subsequent reinfarction.

Merlini et al.[224] found that there was an increase in F1+2 levels in patients with unstable angina who received thrombolytic therapy in addition to heparin. In the patients who developed refractory angina or infarction, higher fibrinopeptide A levels also were measured, thereby suggesting that the hypercoagulable state induced by thrombolytic therapy may be detrimental in the setting of the nonocclusive thrombosis typical of unstable angina. This perhaps explains the unfavorable results of this therapy in such patients.

The validity of using plasma fibrinopeptide A and B levels as specific markers of thrombin activity in the setting of thrombolytic therapy has been questioned on the basis of observations that tPA and urokinase have direct catalytic activity against fibrinogen. Under plasminogen-free conditions, tPA releases fibrinopeptides A and B from fibrinogen, but unlike thrombin action on fibrinogen, the rate of tPA-induced fibrinopeptide B release is faster than that of fibrinopeptide A, and release of both fibrinopeptides is unaffected by heparin.[225] Urokinase also has catalytic activity against fibrinogen but only releases fibrinopeptide B.[226] Although pharmacologic concentrations of tPA may directly cleave fibrinopeptide A from fibrinogen, the observation

that therapy with either streptokinase or tPA leads to increased levels of fibrinopeptide A[218–220] that are rapidly lowered with high doses of heparin[220] suggests that thrombin is the major mediator of the elevated fibrinopeptide A levels in this setting.

Using concomitant measurements of Bβ1–42 and Bβ15–42 in patients receiving tPA therapy, Eisenberg et al.[65] demonstrated that the fibrinolytic effect of tPA therapy persists for some time after fibrinogenolysis ceases. This finding is consistent with the results of studies in experimental animals showing that tPA-induced clot lysis continues after the drug is cleared from the circulation,[227] likely reflecting enzymatically active tPA bound to the thrombus. In accord with these findings, it is of interest to note that the rapid administration of tPA in an animal model produces an enhanced thrombolytic effect with reduced bleeding, as compared to more prolonged infusions.[227] Despite the affinity of tPA for fibrin, its administration to patients with acute myocardial infarction or venous thromboembolic disease causes significant fibrinogenolysis. Using plasma levels of Bβ1–42 as an index of fibrinogenolysis, Owen et al.[228] demonstrated that streptokinase, a plasminogen activator without fibrin specificity, as well as tPA produced significant fibrinogen proteolysis. Although it was predicted that high doses of tPA would produce systemic fibrinogen breakdown, studies have provided a mechanism by which even physiologic concentrations of tPA can induce fibrinogenolysis. It has been shown that soluble FDPs, particularly (DD)E, potentiate tPA-induced plasminogen activation by binding both plasminogen and tPA.[229,230] These findings are not surprising given the potentiating activity of cyanogen bromide fragments of fibrin or fibrinogen[231–235] that are frequently used as stimulators in commercial tPA activity assays. Studies indicate that tPA binds to (DD)E with an affinity similar to fibrin. Likewise, plasminogen also binds to (DD)E with high affinity, suggesting that, like fibrin, (DD)E serves as a template to which both tPA and plasminogen bind.[236]

The interaction of tPA with (DD)E is mediated by its second kringle domain.[240] In contrast, tPA binds to fibrin mainly by its finger domain.[236,237] These findings rationalize the greater fibrin selectivity of vampire bat plasminogen activator (b-PA) relative to tPA. Although structurally similar to tPA, b-PA lacks a second kringle domain.[238] Consequently, unlike tPA, b-PA does not bind to (DD)E.[236]

Other Conditions Associated with Elevated Levels of Markers of Coagulation Activation

Elevated levels of markers of coagulation activation have been reported in settings in which thrombosis or DIC is not evident clinically. A list of some of these disorders is shown in Table 58.2.

CONCLUSION

This chapter has summarized the results of clinical studies that indicate that a biochemical imbalance between procoagulant and anticoagulant mechanisms can be detected in the blood of humans before the appearance of thrombotic phenomena. The continuing application of biochemical and clinical approaches to investigations of the hemostatic system should generate a greater appreciation of the mechanisms that lead to venous and arterial thrombotic disorders in humans.

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CHAPTER 59

Overview of Acquired and Inherited Clinical Platelet Disorders

JOEL S. BENNETT

An insufficient number of platelets (thrombocytopenia) or impaired platelet function (thrombocytopathia) can produce bleeding. Both situations are encountered frequently in clinical practice, engendering substantial and appropriate concern about the risk and prevention of bleeding. A summary of key points to be taken from this overview are listed in Table 59.1.

QUANTITATIVE PLATELET DISORDERS

Definition of Thrombocytopenia and Risk of Bleeding

The normal range for platelet counts in healthy individuals is 150,000 to 350,000/μL. However, as discussed in Chapter 60, mean platelet counts are higher in women than men, independent of the presence of iron deficiency, and decline with age, especially after age 60.[1] Although the significance of severe thrombocytopenia is usually readily apparent, the significance of borderline thrombocytopenia, defined as a platelet count between 100,000 to 150,000/μL, is often unclear. Some platelet counts in this range are outliers of the normal platelet count distribution, but they can also represent an early manifestation of an unrecognized disease. Many patients with borderline thrombocytopenia carry a diagnosis of autoimmune thrombocytopenia (ATP), but in a prospective study of 217 healthy individuals with incidental borderline thrombocytopenia, 88% still had a platelet count of >100,000/μL after 5 years without a specific diagnosis being established.[2] Based primarily on this study, the International Working Group of the Vicenza Consensus Conference has set the threshold for the diagnosis of idiopathic thrombocytopenia (ITP) as a platelet count of <100,000/μL.[3]

The number of circulating platelets is the composite of the rate of platelet production, the rate of platelet destruction, and platelet sequestration in the spleen. In humans, the platelet lifespan is 7 to 10 days and reflects both nonrandom platelet senescence and random platelet consumption.[4] Platelet senescence is likely explained by an intrinsic apoptotic pathway that acts as an "internal timer," determining when an aged platelet should be removed from the circulation.[5,6] Based on measurements of platelet kinetics in patients with marrow hypoplasia, it has been predicted that there is a fixed platelet requirement of approximately 7,100 platelets/μL/d and that these platelets are used to support the integrity of the vasculature.[7] There is also a pool of platelets in the spleen that accounts for approximately 30% of the platelet volume in normal individuals and that is in dynamic equilibrium with platelets in the circulation.[8,9] However, >30% of the platelet volume may be sequestered in an enlarged spleen due to conditions such as portal hypertension or infiltrative diseases such as in myeloproliferative neoplasms.[10]

The normal platelet count is far in excess of the number of platelets required for hemostasis. Although the cutaneous bleeding time, a measure of platelet function *in vivo*, can be increased at platelet counts <100,000/μL,[11] significant spontaneous bleeding does not usually occur in an otherwise hemostatically normal individual until the platelet count declines to <5,000/μL.[12] Clinical trials of patients receiving chemotherapy or being treated for aplastic anemia indicate that a threshold of 10,000 platelets/μL is sufficient to minimize the risk of spontaneous bleeding,[13] and hemorrhage following trauma or surgery usually does not occur if the platelet count is >50,000/μL.[14,15] Nonetheless, there is no absolute threshold for spontaneous bleeding due to thrombocytopenia, and it can occur at higher platelet counts when fever, sepsis, severe anemia, or other hemostatic defects are present or when platelet function is impaired, for example, by medications.

Differential Diagnosis of Thrombocytopenia

From a practical clinical standpoint, thrombocytopenic disorders can be divided into those having an immunologic basis and those that do not.

Antibody-Mediated Thrombocytopenia

Immune Thrombocytopenia

Immune thrombocytopenia, also known as ITP or ATP, is an acquired disorder caused by antiplatelet antibodies.[16,17,18] As discussed in Chapter 61, ITP is characterized by the presence of an isolated platelet count of <100,000 in the context of normal red and white cell counts, a peripheral blood smear only remarkable for a decreased number of platelets, and the absence of splenomegaly.[17] In addition, a diagnosis of ITP requires that there be no family history of thrombocytopenia and no use of medications that can cause thrombocytopenia.[17,19] Because there is no standard diagnostic laboratory test for ITP, it is a diagnosis of exclusion.[20] Thus, when a patient's clinical presentation deviates from the paradigm given above, the diagnosis of

Table 59.1

Key points regarding thrombocytopenia and thrombocytopathia

- The platelet count of most asymptomatic patients with borderline thrombocytopenia (platelet counts of 100,000–150,000/μL) remains stable without a specific diagnosis.
- Significant spontaneous bleeding usually does not occur in an otherwise hemostatically normal individual until the platelet count declines to <5,000/μL.
- ATP is characterized by an isolated platelet count <100,000 in the context of normal red and white cell counts, a peripheral blood smear only remarkable for a decreased number of platelets, and the absence of splenomegaly. When a patient's clinical presentation deviates from this paradigm, the diagnosis of ATP must be reconsidered.
- The differential diagnosis of ATP includes pseudothrombocytopenia, drug-induced thrombocytopenia, underlying autoimmune disease, lymphoproliferative disorders, immunodeficiency, myelodysplasia, and infections with hepatitis C virus, human immunodeficiency virus, or *H. pylori*.
- Although many medications impair platelet function *in vitro*, only a few, including aspirin, the thienopyridines and the $\alpha_{IIb}\beta_3$ antagonists, regularly cause clinically significant bleeding.
- Nonimmune mechanisms can cause thrombocytopenia by decreasing platelet production by the bone marrow, platelet sequestration in an enlarged spleen, or by decreasing the lifespan of circulating platelets.
- Inherited thrombocytopenia is rare but must be considered in the differential diagnosis of a thrombocytopenic patient.
- Hereditary disorders of platelet adhesion, aggregation, and platelet secretion must be considered in the evaluation of easy bruising, menorrhagia, and excessive postoperative and postpartum blood loss.
- Abnormal hemostasis specifically due to perturbed platelet function is a feature of the chronic myeloproliferative disorders (PV, ET) and of dysproteinemias (multiple myeloma).

ITP must be reconsidered. ITP can occur at any age, in either sex, and can present with either spontaneous mucocutaneous bleeding or unexplained asymptomatic thrombocytopenia. Characteristically, the bone marrow of patients with ITP is normal, except for a compensatory increase in megakaryocytes, so that bone marrow examination is rarely necessary or helpful. Although assays are available to detect antiplatelet antibodies, they lack sensitivity and specificity and are rarely useful diagnostically.

In approximately half of the patients who are considered to have ITP, platelet antibodies are secondary to another disorder, for example, autoimmune diseases (systemic lupus erythematosus), lymphoproliferative disorders (chronic lymphocytic leukemia), immunodeficiency disorders (common variable immunodeficiency), myelodysplasia, and infections with hepatitis C virus, human immunodeficiency virus, or *Helicobacter pylori*.[20]

In adults, ITP is generally a chronic disease; only 15% of patients remit within a year of onset. By contrast, childhood ITP is often preceded by a febrile illness and more than 70% remit within 6 months, regardless of treatment; only 5% to 10% of children go on to develop chronic disease.[20] The goal of treatment of ITP is to maintain a safe, but not necessarily normal, platelet count with minimal toxicity from therapy.[17] As bleeding is usually minimal or absent until the platelet count is <30,000/μL, asymptomatic patients with a platelet count >30,000/μL can be followed with no treatment. Nonetheless, maintenance of a hemostatically effective platelet count may require the chronic administration of a low dose of corticosteroids (i.e., 10 mg of prednisone daily), splenectomy, or immunosuppression with drugs such rituximab, azathioprine, or cyclophosphamide. For patients refractory to therapy, treatment with the thrombopoietin receptor agonists romiplostim or eltrombopag can be considered.[21] When patients with ITP present emergently with severe thrombocytopenia (<5,000/μL) and/or major or life-threatening hemorrhage, treatment usually includes high-dose corticosteroids and/or intravenous immunoglobulin (IVIgG).[22] Anti-D immune globulin may be substituted for IVIgG in Rh-positive patients who have not undergone splenectomy. Platelet transfusions are generally unable to increase platelet counts in patients with severe ITP because of the rapid clearance of antibody-coated transfused platelets, but the concurrent transfusion of IVIgG and platelets has been reported to increase platelet counts in most patients.[23,24]

Alloimmune Thrombocytopenia

Alloimmune thrombocytopenia results from sensitization to platelet alloantigens such as PI^{A1} following platelet or red cell transfusion to a patient lacking this antigen (post-transfusion purpura, PTP) or maternal sensitization to a foreign platelet antigen during pregnancy, similar to Rh incompatibility (neonatal alloimmune thrombocytopenia, NATP).[25,26] PTP causes profound thrombocytopenia 7 to 10 days after transfusion and can be treated with IVIgG or plasma exchange. NATP causes severe thrombocytopenia and bleeding in neonates and is treated with platelet transfusion, corticosteroids, and IVIgG.

Drug-Induced Thrombocytopenia

Antiplatelet antibodies induced by sensitization to drugs can cause acute platelet destruction and must be considered in the differential diagnosis of thrombocytopenia[27] (see Chapter 62). Although there is a long list of drugs reported to cause antibody-mediated platelet destruction, the evidence for the 24 drugs listed in Table 59.2 is particularly strong.[19] In general, drug-dependent antibody binding to platelets requires initial reversible binding

Table 59.2

Drugs associated with antibody-mediated thrombocytopenia

Abciximab
Acetaminophen
Amiodarone
Ampicillin
Carbamazepine
Eptifibatide
Haloperidol
Ibuprofen
Irinotecan
Naproxen
Oxaliplatin
Phenytoin
Piperacillin
Quinidine
Quinine
Ranitidine
Rifampin
Simvastatin
Sulfisoxazole
Tirofiban
Trimethoprim/Sulfamethoxazole
Valproic acid
Vancomycin

Adapted from Reese JA, Li X, Hauben M, et al. Identifying drugs that cause acute thrombocytopenia: an analysis using 3 distinct methods. *Blood* 2010;116:2127–2133, with permission.

of a drug to platelet membrane glycoproteins.[28,29] Uncommonly, drugs such as penicillin act as haptens by covalently binding to platelet membrane glycoproteins or drugs such as gold salts, levodopa, and procaine amide induce platelet-specific neoantigens and a clinical scenario mimicking ITP.[30] In another variation of drug-induced thrombocytopenia, platelet counts may decrease immediately after administration of the chimeric human/murine anti-$\alpha_{IIb}\beta_3$ monoclonal antibody fragment abciximab due to preexisting antibodies against the murine portion of the drug, or thrombocytopenia can be delayed for 5 to 10 days during which time new antibodies are generated.[31] Similarly, acute thrombocytopenia can follow the administration of the small molecule $\alpha_{IIb}\beta_3$-antagonists eptifibatide and tirofiban.[31] Here, the thrombocytopenia is thought to be due to naturally occurring antibodies recognizing platelet neoepitopes induced by antagonist binding. Emergent treatment for severe drug-induced thrombocytopenia with bleeding includes stopping the likely offending medication, platelet transfusion, corticosteroids, and IVIgG, similar to the emergent treatment for severe ITP.

Heparin-induced thrombocytopenia (HIT) is a special case of drug-induced thrombocytopenia since it is associated with arterial and venous thrombosis rather than bleeding (see Chapter 108). Depending on the clinical population at risk, HIT occurs in approximately 1% to 5% of patients given unfractionated heparin for 5 to 10 days and is due to the formation of antibodies against a complex of heparin and the platelet α granule protein platelet factor 4.[32] HIT is a clinical diagnosis with criteria that include an acute decrease in platelet count of 50% or to >150,00/μL; the diagnosis is confirmed by appropriate testing for heparin-related antibodies.[32,33] If HIT is a possibility, all heparin administration should be stopped and alternative anticoagulation instituted with a direct thrombin inhibitor such as argatroban, at least until the platelet count returns to normal.[33] Warfarin should not be used for acute HIT because it can be associated with venous limb gangrene, a syndrome similar to warfarin-induced skin necrosis that may lead to amputation.[34]

Nonimmune Thrombocytopenia

Nonimmune mechanisms can cause thrombocytopenia by decreasing platelet production by the bone marrow, by decreasing the lifespan of circulating platelets, or by increasing platelet sequestration in an enlarged spleen.

Inherited Thrombocytopenia

Inherited thrombocytopenia can present in childhood in association with developmental disorders (Chapter 63). However, the diagnosis is easily overlooked in adults when thrombocytopenia is the major finding. Inherited thrombocytopenia often presents as macrothrombocytopenia, as in the Bernard-Soulier syndrome (BSS)[35] and MYH9-related disorders[36] (Chapters 63 and 65). Exceptions are the Wiskott-Aldrich syndrome and its variant X-linked thrombocytopenia that are characterized by small platelets[37] and rare reports of isolated familial thrombocytopenia with normal-sized platelets linked to mutations on chromosome 10.[38]

Nonimmune Causes of a Shortened Platelet Lifespan

These disorders include sepsis, disseminated intravascular coagulation (DIC), thrombotic thrombocytopenic purpura/hemolytic uremic syndrome (TTP/HUS), preeclampsia/eclampsia, cardiopulmonary bypass, and giant cavernous hemangiomas (Kasabach-Merritt syndrome) (see Chapter 64). The thrombocytopenia in these disorders resolves following treatment of the underlying disorder and platelet transfusion is rarely necessary. As noted for HIT, TTP/HUS is primarily a thrombotic, not a bleeding, disorder. DIC evolves first as a thrombotic disorder, with bleeding manifestations following consumption of platelets and coagulation factors.

Thrombocytopenia Due to Decreased Platelet Production

Thrombocytopenia due to decreased platelet production is caused by bone marrow disorders such as acute leukemia, aplastic anemia, myelodysplastic syndromes, and myelophthisic processes that results from bone marrow replacement by tumor (carcinoma, lymphoma, myeloma) or fibrous tissue, as well as by bone marrow suppression by chemotherapy, radiation, ethanol toxicity, and infection (HIV, measles, CMV, varicella). These disorders should be readily apparent by history and by careful examination of a peripheral blood smear. When the cause of decreased platelet production cannot be readily reversed,

long-term prophylactic platelet transfusion is frequently considered, but such treatment may lead to alloantibody development and refractoriness to platelet transfusion. To minimize the risk for refractoriness to transfusion, a threshold of 10,000 platelets/μL has been found to be sufficient for prophylactic transfusion to prevent spontaneous hemorrhage in patients being treated with cytotoxic chemotherapy, undergoing bone marrow transplantation or being followed for aplastic anemia.[13]

Thrombocytopenia Due to Platelet Sequestration in an Enlarged Spleen

Approximately 30% of the circulating platelet mass is normally present in the spleen,[8,9] but a greater percentage can be sequestered when the spleen is enlarged.[10] Platelet counts in this situation are generally in the range of 40,000 to 50,000/μL, but bleeding due to thrombocytopenia alone is unusual.

Pseudothrombocytopenia

Platelet clumping in a blood sample anticoagulated with ethylenediaminetetraacetic acid (EDTA) can cause spurious thrombocytopenia when platelet counts are measured in automated counters.[39,40] This *in vitro* artifact is caused by platelet-agglutinating antibodies that recognize epitopes predominantly on $\alpha_{IIb}\beta_3$ that are exposed at low calcium concentrations.[41] It can be distinguished from true thrombocytopenia by examining a blood film prepared from a finger stick or by performing a platelet count in blood anticoagulated with citrate or oxalate (FIGURE 59.1). The prevalence of pseudothrombocytopenia is 0.11% to12.5% of patients with a platelet count of <100,000/μL.[42]

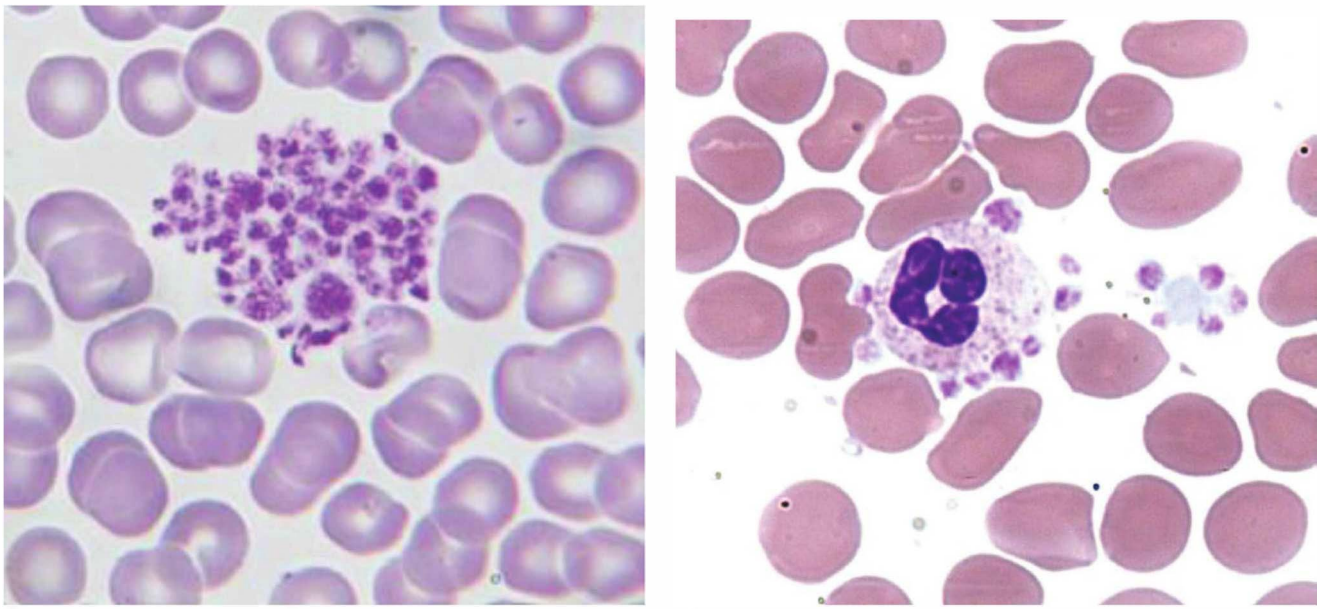
QUALITATIVE PLATELET DISORDERS

A prolonged bleeding time in a patient with a normal platelet count suggests a diagnosis of von Willebrand disease (Chapter 52) or an inherited or acquired qualitative platelet disorder (Chapters 65 and 66, respectively).

Inherited Qualitative Platelet Disorders

Inherited Disorders of Platelet Membrane Glycoproteins

Inherited abnormalities in the amount or function of the platelet membrane glycoprotein receptors for collagen (GPVI and the integrin $\alpha_2\beta_1$)[43–48] and for von Willebrand factor (vWF) (GPIb-IX-V)[35] impair platelet adhesion to the subendothelial connective tissue matrix under low shear and high shear conditions, respectively. The most common and best studied of these adhesion abnormalities is the autosomal recessive BSS caused by mutations in genes for GPIbα, GPIbβ, or GPIX.[35] The resulting deficiency or perturbed function of GPIb-IX-V produces macrothrombocytopenia, a markedly prolonged cutaneous bleeding time, absent ristocetin- and botrocetin-induced vWF binding to platelets, and mucocutaneous bleeding of variable severity that begins in infancy or childhood.[49] Glanzmann thrombasthenia (GT) is a rare autosomal recessive bleeding disorder in which platelets do not aggregate because of deficiency or abnormal function of the platelet fibrinogen receptor $\alpha_{IIb}\beta_3$ (also known as GPIIb/IIIa).[50] As with BSS, GT presents with mucocutaneous bleeding in infancy, but unlike BSS, the platelet count and



A B

FIGURE 59.1 Pseudothrombocytopenia. **A**: Peripheral blood smear from a patient with no history of bleeding, a normal hemoglobin, and a normal white cell count but a platelet count of 10,000/μL measured using an automated counter and blood anticoagulated with EDTA. The blood smear shows extensive platelet clumping. When the platelet count was repeated using blood anticoagulated with sodium citrate, it was normal. (Adapted from Chia J, Hsia CC. Pseudothrombocytopenia. *Blood* 2011;117:4168, with permission, Ref. [90]). **B**: An example of platelet satellitism in which platelets rosette around neutrophils due to antibody binding to EDTA-exposed epitopes on both platelet $\alpha_{IIb}\beta_3$ and neutrophil FcγIII receptors.

platelet morphology are normal and platelets in plasma agglutinate in the presence of ristocetin and botrocetin, but do not aggregate in response to ADP, collagen, or thrombin.[51]

Platelet Granule Disorders

Platelets contain three types of granules that are extruded from activated platelets in the platelet release reaction.[52] The granules include dense or δ granules containing ADP, ATP, calcium, serotonin, and pyrophosphate; α granules containing proteins either obtained from the plasma or synthesized and packaged by megakaryocytes; and lysosomes containing acid hydrolases and microperoxisomes. In these disorders, mucocutaneous bleeding can result from a decrease in the number of granules, a decrease in granular contents, or perturbations of granule secretion.

α granule deficiency is a rare cause of mild to moderate mucocutaneous bleeding and bone marrow fibrosis in which platelets have a gray appearance on peripheral blood smears due to the absence of α granules (gray platelet syndrome, GPS).[53] GPS is associated with mutations in the gene *NBEAL2* that encodes a protein involved in vesicular trafficking.[54–56] Dense granule deficiency (δ storage pool disease, δSPD) is the most common platelet secretion disorder and can be subclassified into δSPD associated with albinism (the Hermansky-Pudlack and Chediak-Higashi syndromes) and δSPD in otherwise normal individuals.[57] Patients with δSPD have a normal platelet count, a prolonged bleeding time, and abnormal platelet aggregation studies.[58] An increased ratio of platelet ATP to ADP because of absence of the dense granule pool of ADP is diagnostic.[58] Although bleeding in patients with secretion disorders can be controlled by platelet transfusion, this is seldom necessary because DDAVP can shorten the bleeding times and improve hemostasis.[59]

Disorders of Platelet Secretion

These are a heterogeneous collection of disorders of stimulus-response coupling in which the platelet function of affected individuals resembles that of individuals who have been given platelet inhibitory drugs such as aspirin. Thus, there may be marked impairment of aggregation and secretion in response to weak platelet agonists (ADP, epinephrine, low concentration of collagen), while responses to stronger agonists may be normal; the bleeding time is usually prolonged. Although inherited abnormalities in platelet signal transduction are rare, they have been particularly informative in understanding normal platelet physiology.

Disorders of Platelet Coagulant Activity

The plasma membrane of activated platelets provides a procoagulant surface for the assembly of the "tenase" and "prothrombinase" complexes that activate coagulation factor X and prothrombin, respectively (see Chapter 35). At a minimum, this activity results from the calcium-induced exposure of acidic phospholipids on the platelet surface, thought to be brought about by a membrane-associated scramblase.[60–62] Isolated deficiency of platelet procoagulant activity, designated Scott syndrome, is exceedingly rare[63] and results from the inability of platelets to translocate acidic phospholipids from the inner to the outer leaflet of the platelet plasma membrane. Mutations in the protein TMEM16F linked to scramblase activity may be causative of Scott syndrome.[62,64]

Acquired Disorders of Platelet Function

Acquired platelet function disorders are relatively common, but are usually asymptomatic or mild. Nonetheless, they can be of clinical importance when they occur concurrently with another hemostatic abnormality.

Drugs

Although a wide variety of drugs can impair platelet function *in vitro*, only a small number regularly produce clinically significant bleeding (see Chapter 66). These include aspirin,[65] other NSAIDs,[66–68] the thienopyridines and other $P2Y_{12}$ inhibitors,[69] and the $\alpha_{IIb}\beta_3$ antagonists abciximab, tirofiban, and eptifibatide.[70] Aspirin prevents platelet synthesis of thromboxane A2.[71] This has a minimal effect on hemostasis in normal individuals[72] but prolongs the bleeding time and can precipitate hemorrhage in individuals with other hemostatic defects such as von Willebrand disease.

Nonhematologic Disorders

Platelet dysfunction can be a prominent feature of liver disease, uremia, and cardiopulmonary bypass (Chapters 126, 127, and 129). Uremia regularly causes platelet dysfunction, but this has been mitigated to a great extent by effective dialysis. However, when uremia-induced thrombocytopathia does occur, it is treated with the vasopressin analog desmopressin (DDAVP)[73]; conjugated estrogens[74] and cryoprecipitate[75] have been used as well. Correction of anemia with transfusion or erythropoietin can also improve hemostasis in patients with renal failure.[76,77] Hemostatic abnormalities in liver disease are complex, and the contribution of platelet function abnormalities is uncertain.[78] Platelet dysfunction following cardiopulmonary bypass results from the interaction of platelets with the bypass circuit and from hypothermia induced during bypass[79] and generally resolves spontaneously in 2 to 24 hours.

Abnormal hemostasis related to abnormal platelet function occurs in patients with chronic myeloproliferative disorders such as polycythemia vera (PV) and essential thrombocythemia (ET), with dysproteinemias such as multiple myeloma and macroglobulinema, with myelodysplasia and leukemia, with acquired forms of von Willebrand disease, and in the presence of antiplatelet antibodies. Both thrombosis and hemorrhage are complications of the myeloproliferative disorders and are associated with increases in whole blood viscosity in PV, elevated platelet counts, intrinsic platelet function abnormalities, and leukocyte and/or endothelial cell dysfunction.[80,81] Some studies indicate that gain-of-function JAK2 mutations in ET confer an increased risk of thrombosis[81] (see Chapter 102). Marked increases in platelet count increase the risk for bleeding, likely due to the acquired von Willebrand disease that results from adsorption of vWF to the platelet surface.[82] Reducing the elevated red cell mass in PV into the normal range significantly reduces the hemostatic complications of this disorder.[83] Features predicting high-risk disease in patients with ET include age >60, a history of thrombosis, and platelet counts $>1.5 \times 10^6/\mu L$.[84] Although both hydroxyurea and anagrelide can reduce the platelet count in patients with myeloproliferative disorders, a randomized trial of patients with ET indicated that treatment with hydroxyurea was superior to anagrelide with a decreased rate of arterial thrombosis, major bleeding, and transformation to myelofibrosis.[85] Aspirin has been found to be beneficial in patients with PV, but it can also exacerbate a bleeding tendency in myeloproliferative patients with acquired von Willebrand disease.[86]

The platelet dysfunction associated with dysproteinemias improves when the concentration of paraprotein is decreased by plasmapheresis or chemotherapy. Although thrombocytopenia is the major cause of bleeding in acute leukemias and myelodysplastic syndromes, bleeding may occur even in patients with normal platelet counts because of platelet dysfunction. Causes of acquired von Willebrand disease include the depletion of hemostatically effective high molecular weight vWF multimers,[87–89] antibodies directed against functional domains in vWF, antibody-accelerated vWF clearance, shear stress–induced vWF proteolysis, cellular adsorption and removal of vWF by malignant or other cells, and decreased vWF synthesis. Administration of DDAVP, the transfusion of vWF concentrates, and IVIgG can improve hemostasis in these patients.[89]

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Full Reference Lists can be found online

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CHAPTER

60 Differential Diagnosis of Thrombocytopenia

JOEL S. BENNETT AND CHARLES S. ABRAMS

Thrombocytopenia is encountered frequently in clinical practice, engendering substantial and appropriate concern about its etiology and the consequent risk and prevention of bleeding. For example, in patients admitted to intensive care units (ICUs), approximately 30% to 50% manifest thrombocytopenia at some point during their ICU stay.[1,2,3] In turn, the thrombocytopenia has been associated with increased bleeding, increased transfusion of blood products, more prolonged ICU stays, increased ICU and hospital mortality, and often extensive, usually fruitless, efforts to identify its etiology. In this chapter, we discuss the definition of thrombocytopenia, the lifespan of circulating platelets and mechanisms of platelet senescence, the risk of bleeding in thrombocytopenic patients, and mechanisms for the development of thrombocytopenia in clinical disorders. The salient points of this discussion are provided in Table 60.1.

DISTRIBUTION OF PLATELET COUNTS IN NORMAL INDIVIDUALS

The normal range for platelet counts in healthy individuals has been reported as 150,000 to 350,000/μL[4] and is essentially the same regardless of the type of automated hematology analyzer used to measure the platelet count.[5] As determined from an analysis of the cross-sectional Third National Health and Nutrition Examination Survey of the United States population from 1988 to 1994 (NHANES III), mean platelet counts of 26,327 individuals were higher in women than men, independent of the presence of iron deficiency, declined with age, especially after age 60, and were greater in non-Hispanic blacks as compared to non-Hispanic whites and Mexican Americans.[6] Similar differences had been identified in a large cohort of men and women in Israel,[7] in a population study of whites, Africans, and Afro-Caribbeans in London,[8] and in 3,311 HIV-negative Ugandans.[9] In a study of platelet counts in 12,517 individuals in 10 relatively isolated villages in the Ogliastra area of Sardinia, the prevalence of thrombocytopenia (defined as a platelet count <150,000/μL) in different villages ranged from 1.5% to 6.8%, and there was a progressive decrease in platelet number with aging.[10] It is noteworthy that the prevalence of thrombocytosis also differed between villages and was inversely related to the prevalence of thrombocytopenia. Thus, the normal range for platelet counts varied from village to village, implying that the normal range for platelet counts can be influenced by genetic factors and is consistent with the finding of significant heterogeneity in the distribution of single-nucleotide polymorphisms among the villages. Genetic polymorphisms that affect the platelet count in a negative and positive fashion have been identified in the genes for interleukin-6 and the thrombopoietin receptor c-mpl.[11–13] Although these differences in mean platelet count affect populations rather than individuals, and are relatively mild so that they are unrelated to the efficiency of hemostasis, it is important to be cognizant of the fact that there are differences in the normal range of platelet counts when analyzing the effect of medications, therapy, and devices that can affect the platelet count such as immunization.[14]

Although the clinical significance of severe thrombocytopenia is usually readily apparent, the nature and significance of borderline thrombocytopenia, that is, platelet counts between 100,000 and 150,000/μL, can be a vexing problem. With the advent of automated cell counting, asymptomatic individuals with incidentally discovered borderline thrombocytopenia are being identified with reasonable frequency. While some of these individual, by necessity, represent outliers of the normal distribution of platelet counts, the thrombocytopenia of others could represent an early manifestation of an unrecognized disease. In the absence of positive findings, many of these individuals are given a diagnosis of idiopathic autoimmune thrombocytopenia (ITP). To address the paucity of information about the fate of individuals with borderline thrombocytopenia, Stasi et al.[15] prospectively studied 217 apparently healthy individuals incidentally discovered to have borderline thrombocytopenia as defined above. When followed for 6 months, two of the subjects were found to have myelodysplasia and a third systemic lupus erythematosus (SLE). The platelet counts of 23 others increased into the normal range for at least 3 months, whereas the platelet counts of 11 declined to <100,000/μL, and they were classified as having ITP. One hundred ninety-one subjects who continued to manifest borderline thrombocytopenia at 6 months were then followed for a median duration of 64 months. Thirteen subjects achieved stable platelet counts >150,000/μL and 109 continued to have stable borderline thrombocytopenia. Platelet counts of 12 subjects declined to <100,000/μL, and these subjects met the study's criteria for ITP. Of the 11 subjects initially thought to have ITP, five subsequently developed autoimmune disorders. Overall, the probability of developing ITP in these subjects at 10 years was calculated to be 6.9%, and the probability of developing another autoimmune disorder at 10 years was 12.0%. However, the majority of subjects continued to manifest borderline thrombocytopenia without developing another disorder. Thus, it is likely that they simply represent the lower end of the normal platelet count distribution. Moreover, based primarily on the results of this study, as well as on the observations discussed above that platelet counts <150,000/μL are frequently found in apparently healthy individuals in some non-Western populations, the International Working Group of the Vicenza Consensus Conference set the threshold for the diagnosis of ITP at a platelet count of <100,000/μL.[16]

Table 60.1

Clinical implications of thrombocytopenia

Although the range of normal platelet counts in healthy individuals is 150,000–350,000/μL, the platelet counts of many individuals with borderline thrombocytopenia, that is, platelet counts of 100,000–150,000/μL, remain stable over time and most likely represent outliers of the normal platelet count distribution
The normal lifespan of circulating platelets is 7–10 d, but measured platelet lifespan decreases as the platelet count decreases
Most platelets are removed from the circulation because of senescence, but approximately 18% of platelet removal is random. It has been postulated that the function of randomly removed platelets is to support endothelial integrity
Approximately 30% of the normal platelet volume is present in the spleen. The amount of platelets sequestered in the spleen can increase with splenic enlargement
Platelets contain an endogenous apoptotic pathway that may be largely responsible for their senescence
Clinical experience suggests that a platelet count of 10,000/μL is a sufficient threshold for prophylactic platelet transfusions to minimize the risk of spontaneous bleeding in the absence of fever, infection, renal failure, and medications that impair hemostasis
Platelet counts of at least 50,000/μL are sufficient for most surgical procedures, but procedures involving the brain or eyes may require platelet counts >100,000/μL. Nonetheless, bleeding complications often do not correlate well with preprocedure platelet counts
Clinically relevant thrombocytopenia can result from decreased platelet production by bone marrow megakaryocytes, from accelerated platelet removal, or from platelet sequestration in an enlarged spleen

Nonetheless, we have encountered a number of patients with borderline thrombocytopenia who have either intermittently or progressively developed symptomatic thrombocytopenia consistent with a diagnosis of ITP. While it may be inappropriate to label all patients with borderline thrombocytopenia with a diagnosis of ITP when their thrombocytopenia is first discovered, these patients should be monitored to determine if ITP does eventually develop.

PLATELET KINETICS

Measurements of platelet survival in humans using autologous platelets labeled *in vitro* with either chromium-51[17] or indium-111[18,19] reveal that the lifespan of platelets in the circulation ranges from 7 to 10 days with a platelet turnover of 40,000 to 50,000/μL/day.[20] ^{111}In complexed with 8-hydroxyquinolone (oxine) is currently the preferred radionuclide for these studies because of its shorter half-life, higher γ photon yield, and the greater affinity of the oxine complex for platelets.[19,21] These features of ^{111}In-oxine have enabled the measurement of autologous platelet survival in patients with ITP who have platelet counts of <20,000/μL.[22] Estimating platelet lifespan from the decrease in circulating platelet-associated radioactivity is accomplished by fitting the survival curve into one of several platelet survival models.[23] The most commonly applied mathematical formulation is the multiple hit (or γ function) model developed by Murphy and Francis,[24] based on the hypothesis that a platelet is repeatedly subjected to multiple external environmental insults or "hits" before it is removed from the circulation. However, the validity of this hypothesis has been questioned because of the observation that platelets contain an internal apoptotic pathway that may be largely responsible for their senescence[25] (see below). Nonetheless, when the survival of autologous ^{51}Cr-labeled platelets was compared in normal subjects and patients with stable thrombocytopenia due to bone marrow failure, platelet lifespan correlated with the platelet count, decreasing as the platelet count decreased (FIGURE 60.1).[26] Based on a mathematical model in which a fixed platelet lifespan was reduced by a degree of random platelet destruction, the authors calculated that there was random destruction of approximately 7,100 platelets/μL/day.

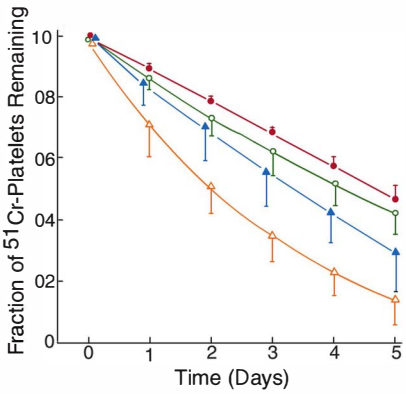

FIGURE 60.1 Effect of platelet count on the lifespan of platelets labeled with ^{51}Cr. Platelet lifespan was measured in normal individuals (*closed circles*, platelet lifespan 9.6 ± 0.6 days) and patients with bone marrow hypoplasia and stable platelet counts ranging from 50,000 to 100,000/μL (*open circles*, platelet lifespan 7.0 ± 1.5 days), platelet counts <50,000 (*closed triangles*, platelet lifespan 6.0 ± 1.7 days), and patients with more severe thrombocytopenia (platelet counts of 19,000 ± 6,000/μL) using labeled donor platelets (*open triangles*, platelet lifespan 3.4 ± 1.1 days). (Adapted from Hanson SR, Slichter SJ. Platelet kinetics in patients with bone marrow hypoplasia: evidence for a fixed platelet requirement. *Blood* 1985;66:1105–1109, with permission.)

This accounted for approximately 18% of the platelet turnover in normal individuals, while the remainder of platelet turnover occurred by senescent mechanisms. They also postulated that the shortened platelet lifespan they observed as platelet counts decreased resulted from an increase in the proportion of platelets removed by random processes and that the function of the randomly removed platelets is to support endothelial integrity.

Sixty to seventy percent of infused labeled platelets are recovered in the circulation of normal individuals, but this increases to nearly 100% when they are infused into individuals who have undergone splenectomy.[27–29,30] Thus, there is a splenic platelet pool that amounts to approximately 30% of the platelet volume in normal individuals. The splenic pool is in dynamic equilibrium with the circulating platelet pool. For example, the splenic pool is depleted in parallel with the circulating platelet pool during platelet apheresis.[31,32] Epinephrine infusions decrease splenic blood flow and the exchangeable splenic platelet pool and prolong the intrasplenic platelet transit time from its normal value of 10 to 12 minutes. This indicates that splenic blood flow is a major determinant of the size of the exchangeable splenic platelet pool.[33] Moreover, because the intrasplenic transit time of platelets is 5 to 6 times greater than that of erythrocytes, it is likely that by virtue of their smaller size, platelets are required to traverse a complex pathway through the splenic cords.[30]

PLATELET SENESCENCE

Analyses of measurements of platelet survival suggest that the platelet lifespan is determined by a combination of random platelet removal and a nonrandom aging mechanism that appears to result in the removal of senescent platelets by the reticuloendothelial (RE) system. Although the factors responsible for platelet aging are not known with certainty, there is accumulating evidence that the intrinsic apoptotic pathway may act as an "internal timer" that regulates platelet senescence.[25,34] Platelets contain several members of the antiapoptotic Bcl-2 family, as well as the proapoptotic proteins Bax and Bak.[34,35] Mason et al.[34] found that hypomorphic mutations of the antiapoptotic protein Bcl-x_L caused thrombocytopenia in mice due to increased platelet clearance, whereas inactivating other antiapoptotic proteins, Bcl-2, Bcl-w, and Mcl-1, in platelets did not. Furthermore, treating mice with the BH3 mimetic ABT-737, which antagonizes Bcl-x_L, caused acute thrombocytopenia. By contrast, absence of the proapoptotic proteins Bak and Bax was associated with a marked increase in platelet count and prolongation of the platelet lifespan.[36] Since the half-life of Bcl-x_L in platelets is shorter than Bak,[34,37] it has been proposed that as the inhibitory effect of Bcl-x_L declines, a threshold is reached, at which point uninhibited Bak is able to cause platelet apoptosis.[25] Nonetheless, how platelet apoptosis could cause platelet removal by the RE system is still unclear. Apoptosis causes phosphatidylserine (PS) exposure on cell surfaces, which can be a signal for phagocytosis of the apoptotic cells. Although apoptosis causes PS exposure on the platelet surface, agonist stimulation also exposes PS as a component of platelet procoagulant activity by a pathway that is independent of the apoptotic mechanism.[38] It remains to be established whether PS exposure plays a role in the clearance of senescent platelets, in addition to its role in supporting the formation of procoagulant complexes.

Supporting senescence as the major factor that determines platelet lifespan are purported decreases in platelet size, density, metabolic activity, and function as they age in the circulation.[39–42] Platelets contain a pool of mRNA that can be stained with thiazole orange ("reticulated platelets").[43] Because thiazole orange appears to preferentially label the youngest circulating platelets, and because the lifespan of "reticulated platelets" in mice is approximately one-third of the overall platelet survival, these findings support the notion that platelets age in the circulation.[43–45] However, others have studied the relationship between platelet size and platelet age, and while they confirmed heterogeneity in the size of circulating platelets and found a small correlation between platelet volume and lifespan, they could not confirm that platelet size decreases over time as they circulate. They concluded that the heterogeneity in platelet size results from factors related to platelet production, rather than aging.[46] This point is reinforced by the observations that patients with the Wiskott-Aldrich syndrome, and its variant X-linked thrombocytopenia, have small platelets with a shortened lifespan, whereas platelet survival in a kindred with inherited macrothrombocytopenia and megakaryocytic hyperplasia was normal.[47]

As noted above, the signals telling the RE system to remove senescent platelets are not known. However, studies of the mechanism responsible for the accelerated clearance of transfused platelets stored at cold temperatures revealed that desialylation of platelet surface glycoproteins occurred when the platelets were rewarmed prior to transfusion.[48,49] Loss of sialic acid results in the exposure of penultimate galactose residues, predominantly on platelet GPIbα and causes rapid platelet clearance, largely via hepatic asialoglycoprotein (Ashwell-Morell) receptors.[50] Whether these observations are relevant to the normal clearance of senescent platelets is not clear, in particular because clearance has been attributed to RE cells rather than to hepatocytes. However, it is noteworthy that the depletion of hepatic and splenic macrophages had little effect on the lifespan of freshly transfused platelets, suggesting that desialylation as platelets age could play a role in the recognition of senescent platelets.[51]

RISK OF BLEEDING DUE TO THROMBOCYTOPENIA

The number of platelets circulating in normal individuals is far in excess of the number required for normal hemostasis. Moreover, although meticulous cutaneous bleeding time measurements indicated that primary hemostasis is progressively impaired as platelet counts decline below 100,000/μL,[52] measurements of spontaneous fecal blood loss in patients with aplastic anemia revealed that blood loss did not substantially increase until platelet counts were <5,000/μL (FIGURE 60.2),[53] a number similar to the number of platelets thought to be required to maintain the integrity of the endothelium.[26]

Consistent with the latter observation, randomized and observational studies of patients treated with cytotoxic chemotherapy, undergoing bone marrow transplantation, or followed for aplastic anemia indicate that a threshold of 10,000 platelets/μL is sufficient for prophylactic platelet transfusion to minimize the risk of spontaneous bleeding in the absence of fever, infection, renal failure, and medications that impair hemostasis.[54–56,57] However, it may be best to avoid prophylactic platelet transfusions in patients with chronic stable thrombocytopenia, who have platelet counts as low as 5,000 to 10,000/μL, and who are free of serious bleeding to avoid alloimmunization and platelet refractoriness.[58] Issues that remain to be addressed are whether

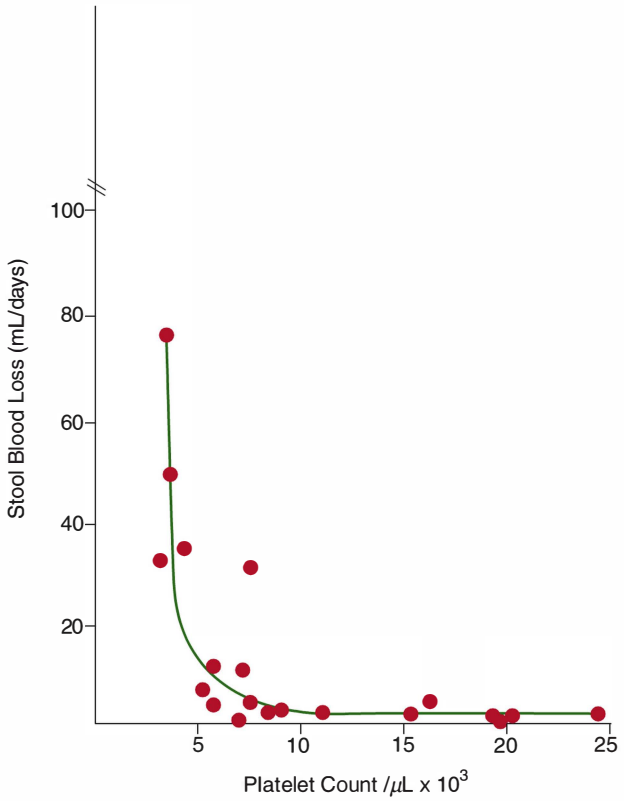

FIGURE 60.2 Relationship between stool blood loss and platelet count. Blood loss was measured using red cells labeled with ^{51}Cr in 28 patients with aplastic anemia. A threshold of increased blood loss was detected at a platelet count of approximately 5,000/μL. (Adapted from Beutler, E. 1993. Platelet transfusions: The 20,000/μL trigger. *Blood* 81:1411–1413, with permission.)

the platelet count trigger point for prophylactic transfusion can be safely lowered to 5,000/μL, and whether transfusion for clinical bleeding may be as effective as prophylactic transfusion.[59]

There is scant evidence upon which to base therapeutic decisions regarding platelet transfusion in other clinical situations. Guidelines have been proposed based on clinical experience and expert opinion.[55,58,60,61] Platelet counts of at least 50,000/μL are sufficient for most surgical procedures. However, procedures involving the brain or eyes may require platelet counts >100,000/μL. A bone marrow biopsy can be performed without platelet support. It has been reported that a lumbar puncture can be safely performed with meticulous technique at platelet counts >20,000/μL,[55,62] a liver biopsy at platelet counts >50,000/μL,[63] and gastrointestinal endoscopies and bronchoscopies at platelet counts >20,000/μL.[64,65] Platelets counts >50,000/μL have been recommended for dental extractions and central venous catheter insertions.[55] However, it should be noted that bleeding complications often do not correlate well with preprocedure platelet counts.[66,67]

MECHANISMS OF THROMBOCYTOPENIA IN CLINICAL DISORDERS

Thrombocytopenia can result from decreased platelet production by bone marrow megakaryocytes, from accelerated platelet removal, or from platelet sequestration in an enlarged spleen. A scheme for the systematic evaluation of patients who present with thrombocytopenia is shown in FIGURE 60.3. Since clinical disorders resulting in thrombocytopenia are described in detail in the subsequent chapters, they will only be briefly considered here.

Thrombocytopenia Due to Decreased Platelet Production

As discussed in Chapter 64, decreased platelet production occurs in diseases of the bone marrow, such as acute leukemia, aplastic anemia, myelodysplasia, and paroxysmal nocturnal hemoglobinuria; when bone marrow is replaced by metastatic carcinoma, fibrosis, or multiple myeloma; from the effects of chemotherapy, radiation therapy, or drugs that affect megakaryocyte number or function; from ethanol toxicity; and from infections with viruses such as HIV, measles, CMV, and varicella. Thrombocytopenia due to decreased platelet production is often accompanied by the perturbation of other blood cell counts, and thus, may be readily apparent after reviewing a peripheral blood smear. When thrombocytopenia in this setting is accompanied by bleeding, the bleeding usually requires treatment by platelet transfusion. Furthermore, when decreased platelet production cannot be readily reversed, prophylactic platelet transfusion to prevent bleeding is often considered. However, prophylactic platelet transfusion is complicated by the short platelet lifespan, the 5-day shelf-life of untransfused platelets, and platelet immunogenicity. As discussed above, the recommended threshold for prophylactic platelet transfusion is currently 10,000/μL, although it remains to be determined whether a threshold of 5,000/μL would be sufficient, and whether transfusion for bleeding rather than prophylaxis would be just as efficacious. In many patients with chronic stable thrombocytopenia, bleeding symptoms are minimal, even at platelet counts of 5,000 to 10,000/μL, so that prophylactic platelet transfusions may not be necessary. When they are transfused, using single-donor apheresis platelets and/or platelet donors who are human leukocyte antigen-identical to the recipient should be considered to minimize the risk of alloimmunization.

Thrombocytopenia Due to Increased Platelet Destruction

In the absence of splenomegaly, the presence of megakaryocytes in an otherwise normal bone marrow implies that thrombocytopenia is due to increased platelet destruction. Both nonimmune and immune processes can lead to a shortened platelet lifespan. Nonimmune causes, discussed in Chapter 64, include sepsis, disseminated intravascular coagulation, cardiopulmonary bypass, and giant cavernous hemangiomas (Kasabach-Merritt syndrome). The thrombocytopenia in these disorders resolves when the disorders resolve, and platelet transfusion is rarely necessary. As discussed in Chapter 99, the thrombocytopenia in patients with thrombotic thrombocytopenic purpura/hemolytic uremic syndrome is associated with thrombosis rather than bleeding, and platelet transfusion is only indicated in rare instances when significant bleeding occurs. Although there are reports of clinical deterioration, even death, following platelet transfusion, this remains a controversial issue.

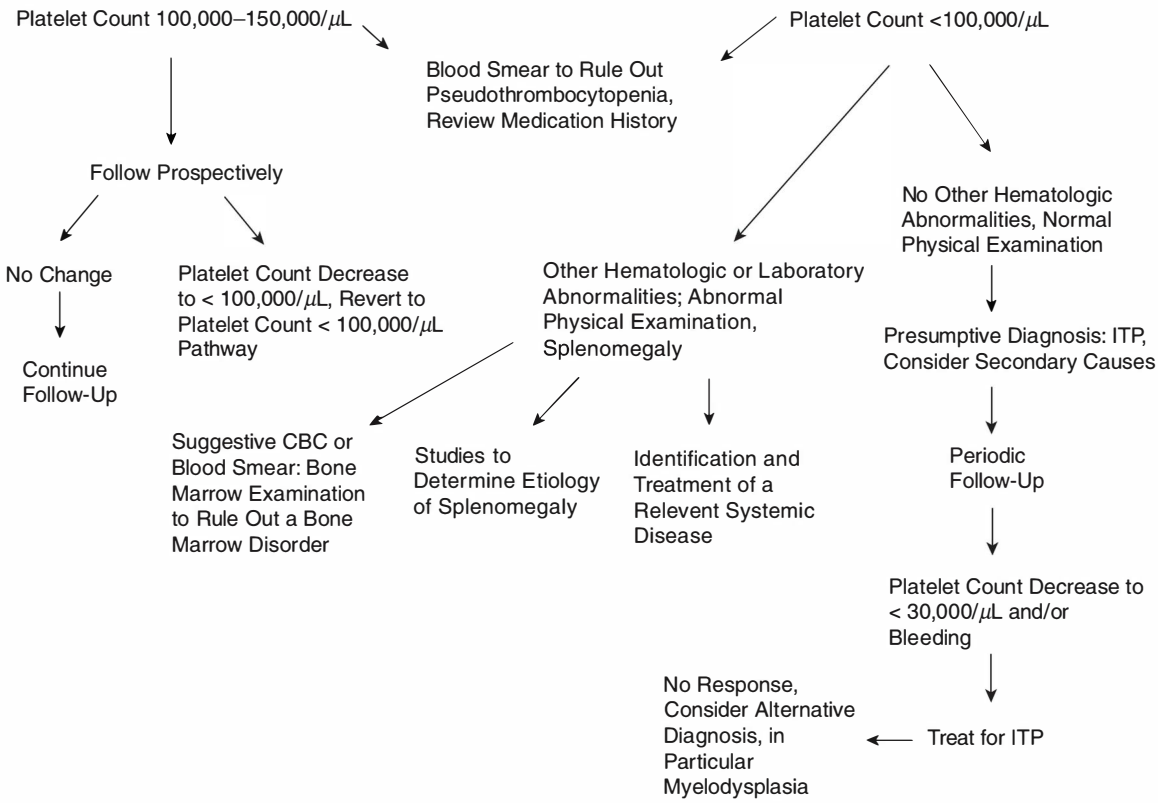

FIGURE 60.3 A scheme for the systematic evaluation of a patient with thrombocytopenia.

Immune-mediated platelet destruction can be due to medication, alloimmune sensitization following transfusion or pregnancy, and autoimmunity (see Chapters 61 and 62). As noted in Chapter 62, the list of medications reported to cause immune-mediated platelet destruction is long, but the most frequently implicated drugs include quinine and quinidine, sulfonamides, and gold salts. Nonetheless, medications should always be considered as a possible etiology for thrombocytopenia. Besides stopping the offending medication, emergent treatment for severe thrombocytopenia with bleeding includes platelet transfusion, as well as corticosteroids and intravenous immunoglobulin (IV IgG), which is similar to emergent treatment for autoimmune thrombocytopenia (see below).

Heparin-induced thrombocytopenia (HIT), discussed in Chapter 108, is a special case of drug-induced thrombocytopenia. HIT occurs in 1% to 4% of patients given unfractionated heparin for 5 to 10 days and is due to antibodies against a complex of heparin and the platelet α-granule protein platelet factor 4 (PF4). Patients with HIT rarely bleed, but rather the complex of heparin, PF4, and antibody activates platelets, causing thrombosis in both the arterial and venous circulations. HIT must always be considered when thrombocytopenia is detected in a hospitalized patient.

Alloimmune thrombocytopenia due to sensitization to alloantigens, such as Pl^{A1}, results from transfusion (posttransfusion purpura [PTP]) or from maternal sensitization during pregnancy (neonatal alloimmune thrombocytopenia [NATP]). PTP causes profound thrombocytopenia 7 to 10 days after transfusion and can be treated with IV IgG or plasma exchange. NATP can cause severe thrombocytopenia and bleeding in neonates, and it is treated with platelet transfusion, corticosteroids, and IV IgG.

Autoimmune thrombocytopenia, also known as idiopathic thrombocytopenic purpura (ITP), is due to circulating antiplatelet autoantibodies. An ITP-like picture can also occur in autoimmune diseases such as SLE, in patients with low-grade lymphoproliferative disorders such as chronic lymphocytic leukemia and well-differentiated lymphocytic lymphoma, and in patients with HIV infections. ITP can occur at any age, in either sex, and it presents with either spontaneous mucocutaneous bleeding or unexplained asymptomatic thrombocytopenia. Red cell and white cell counts are normal, splenomegaly is absent, and peripheral blood smears are only remarkable for a decreased number of platelets, some of which may be larger than normal. Although the presence of megakaryocytes in the bone marrow of patients with ITP confirms that their thrombocytopenia is due to accelerated platelet clearance, a bone marrow examination is rarely necessary in the absence of findings suggesting a bone marrow disorder such as myelodysplasia. Assays are available to detect antiplatelet antibodies, but they lack sensitivity and specificity, and they are rarely helpful diagnostically.

The diagnosis and treatment of ITP in children and adults is discussed in detail in Chapter 61. Suffice it to say, treatment is guided by symptoms and platelet count. Bleeding is usually minimal to absent until platelet counts decline to <30,000/μL. Thus, asymptomatic patients with platelet counts >30,000 can be followed without treatment. When bleeding is present and/or the platelet count is <30,000/μL, initial treatment is directed to inhibiting antibody-mediated platelet clearance

using corticosteroids and/or high doses of IV IgG or anti-D immunoglobulin. Most patients respond to this treatment, and platelet counts increase toward normal over days to weeks. Platelet transfusions alone are generally ineffective at increasing platelet counts in patients with severe ITP, because of antibody-mediated clearance of the transfused platelets, but the concurrent transfusion of high-dose immunoglobulin and platelets has been reported to rapidly increase platelet counts in most patients, and to control bleeding as well.[68,69] In adults with chronic ITP, splenectomy can produce long-term complete remissions in approximately 66% of patients, and some benefit in another 10% to 15%.[70]

Thrombocytopenia Due to Increased Platelet Sequestration

As noted above, approximately 30% of the circulating platelet mass is normally sequestered in the spleen.[30] Additional platelets may be sequestered when the spleen is enlarged due to portal hypertension or infiltrative diseases.[30] In general, patients with thrombocytopenia due to an enlarged spleen (hypersplenism) have palpable spleens, and there is a correlation between the degree of splenomegaly and the extent of thrombocytopenia. Platelet lifespan in patients with hypersplenism is normal or nearly normal.[30] Platelet counts in patients with hypersplenism are generally in the range of 40,000 to 50,000/μL, and bleeding due to thrombocytopenia alone due to platelet sequestration is unusual.

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CHAPTER

61 Immune Thrombocytopenia and Posttransfusion Purpura

HOWARD A. LIEBMAN, DOUGLAS B. CINES, AND JAMES B. BUSSEL

IMMUNE THROMBOCYTOPENIA

Immune thrombocytopenia (ITP) is a heterogeneous autoimmune disorder characterized by thrombocytopenia with or without mucocutaneous bleeding manifestations. Because of its complex pathophysiology and association with a variety of other disorders, ITP is best characterized as a syndrome sharing the common clinical phenotype of immune-mediated thrombocytopenia.[1] While the clinical manifestations of ITP have been known for over a century, only in the last 20 years has our increased understanding of normal immune regulation led to better understanding of the pathophysiology of ITP involving aberrant interactions of antigen presenting cells, B lymphocytes, and regulatory thymic lymphocytes leading to increased platelet destruction and ineffective platelet production.[2,3] This new understanding has resulted in new therapeutic interventions with improved treatment outcomes, particularly for patients refractory to traditional therapies.

Definition and Epidemiology

ITP is an acquired disorder characterized by isolated thrombocytopenia with a peripheral blood platelet count $<100 \times 10^9$/L in the absence of any obvious other initiating or underlying cause, although ITP may be associated with other predisposing conditions (see "Secondary ITP").[4] This definition was the result of a recent consensus opinion by an International Working Group of ITP experts.[4] Therefore, the diagnosis of ITP is one of exclusion and is based principally on patient history, physical examination, complete blood count, and review of the peripheral blood smear.[5] Historically, the disorder was defined as either acute, with resolution of thrombocytopenia occurring within 6 months, or chronic persisting >6 months.[5] Acute ITP was a disorder observed predominately in children and chronic ITP (CITP) in adults. However, the recent International Working Group in ITP consensus report has divided the disorder into three phases: newly diagnosed ITP with a duration up to 3 months, persistent ITP 3 months to 1 year, and CITP >1 year.[4] During the time period encompassing newly diagnosed and persistent ITP, 85% of children and approximately 15% of adults will have a spontaneous remission. Spontaneous remissions are rare beyond a year.

ITP can be further characterized as either primary (idiopathic) or secondary to a number of associated disorders such as immunodeficiency diseases, autoimmune-rheumatologic disorders, lymphoproliferative diseases, and certain chronic infections.[4] It is estimated that approximately 20% of ITP cases in the United States are secondary to other disorders.[1] However, the incidence of secondary ITP worldwide may vary greatly, particularly in populations with high incidence of human immunodeficiency virus (HIV), hepatitis C virus (HCV), or *Helicobacter pylori* (*H. pylori*) infection. There are few data on the incidence or prevalence of ITP in populations other than in North America and Europe. The prevalence of ITP in adults is significantly greater than that of children due to the chronic nature of the disease in adults. The incidence of newly diagnosed ITP in adults ranges from 1.6 to 3.9 per 100,000 persons per year.[6–8] While older retrospective studies suggested a younger peak incidence for CITP in adult patients, more recent studies find an increased incidence and prevalence with advancing age.[6,7,9] In nearly all studies, the female-to-male ratio ranges from 1.2 to 1.9, with the female predominance more evident among young to middle age adults.[6–9]

Pathophysiology of ITP

Antiplatelet Autoantibodies

Harrington et al. were the first to provide experimental evidence that a plasma factor, later shown to be an antibody, was responsible for premature platelet destruction in ITP.[2,10,11] Early assays designed to detect platelet-bound antibodies were very sensitive, but had inadequate specificity. Using newer assays specific for platelet autoantibodies directed against specific platelet glycoproteins, such antibodies can be detected in 40% to 80% of patients with clinical ITP.[2,12,13] However, absence of detectable antibodies does not rule out a diagnosis of ITP, since many patients without detectable antibodies respond to traditional ITP therapies and antibodies may occur in patients with liver disease and other conditions without clinical ITP.

The antigenic target for these antiplatelet autoantibodies are prevalent platelet glycoproteins such as platelet GPIIb/IIIa in nearly two-thirds of patients, platelet GPIb/IX in approximately 20% of patients and in the remaining patients, antibodies are directed against both of these platelet glycoprotein complexes or other platelet antigens.[13] The B- and T-cell response against these common platelet glycoproteins can be directed against various antigenic sequences on same protein resulting in a polyclonal and polyspecific response. Also, antibodies against specific antigenic sequences on certain platelet glycoproteins may have different pathogenic properties. For example, a unique autoantibody observed in patients with HIV-associated ITP directed against platelet GPIIIa amino acid residues 49 to 66 appears capable of inducing platelet fragmentation by generating platelet superoxides, at least *in vitro*.[14] As stated above, some patients with assumed ITP may not have detectable antibodies due to a misdiagnosis such as early myelodysplasia in an older patient; antibodies directed against a less prevalent platelet antigen; low titer of antibody; clearance of the autoantibodies

with treatment; or the thrombocytopenia resulting from T lymphocyte–mediated platelet cytotoxicity.[15] In view of the variability of antibody detection in patients with suspected ITP, current guidelines do not recommend screening patients for platelet antibodies to make an initial diagnosis.

The spleen, in addition to being the major site of antibody-mediated platelet destruction, is a major site of autoantibody production as shown by *in vitro* studies where ITP splenic cells produced IgG that bound to platelets[16–18] and megakaryocytes.[19] Since in some ITP patients, autoantibody persists after splenectomy, there must be additional production sites, for example, bone marrow–derived B lymphocytes.[20]

Role of the Thymic Lymphocyte in ITP

Production of autoreactive IgG against a protein antigen, like all immunoglobulin isotype–switched IgG antibody responses, is initiated by activated T helper cells that recognize their antigens on antigen presenting MHC class II positive dendritic cells (DCs) or macrophages.[3,21–23] DCs from patients with ITP have upregulated costimulatory molecules enhancing autoreactive T- and B-cell responses against platelets.[3] The ongoing process of macrophage-mediated platelet destruction and further presentation of new platelet proteins to T and B lymphocytes contributes to the epitope spreading observed over time in patients with ITP and may contribute to a more refractory disease in patients with ITP of longer duration.[1]

Adults and children with CITP have a serum cytokine profile (IL2/IL10/interferon gamma) suggesting a Th0/Th1 activation pattern.[24–29] Oligoclonal accumulation of CD4+ T helper cells that frequently use Vβ3, 6, 10 and 13.1 to 14 genes for their T-cell receptors have been found in the peripheral blood of patients with CITP by some investigators.[30] These studies suggest that CITP is accompanied by a loss of T lymphocyte tolerance against platelet antigens with the accumulation of abnormal T lymphocyte clones that can direct autoreactive B cells to differentiate and secrete IgG autoantibodies.[30] Recent studies have clearly demonstrated that ITP patients have significant defects in immune self-tolerance.[1,3] Autoreactive cytotoxic CD8-expressing T lymphocyte clones capable of directly damaging platelets and possibly megakaryocytes have also been demonstrated[15] and are likely to proliferate under the influence of similar T helper lymphocytes.

A distinct population of T lymphocytes, essential to maintaining peripheral self-tolerance, preventing autoimmune disease, and limiting chronic inflammatory diseases,[31] termed Tregs, are usually reduced in number and/or function in patients with ITP.[32,33] Quantitative and qualitative defects in the Treg population appear to correlate with persistence of CITP. For example, an association between an improvement in the peripheral blood Treg number and function and remission induction with ITP treatment has been reported.[34]

One of the many factors responsible for maintaining proliferation of B lymphocytes is B cell–activating factor (BAFF).[35,36] BAFF is a member of the tumor necrosis factor family and binds to several receptors, one of which, BR3 is important for B cell–dependent Th1 responses. BAFF mRNA is increased in patients with active ITP compared to healthy controls, and values return to normal during remission. Addition of BAFF to cocultures of ITP lymphocytes and platelets reduced apoptosis of both B and T lymphocytes in patients with ITP, but not in normal subjects.[37] Apoptosis was prevented by the addition of a soluble decoy of the BAFF receptor. Also, in cocultures of ITP platelets and ITP B cells, addition of BAFF increased platelet apoptosis, which was again ameliorated using the decoy receptor.[37]

Suppression of Platelet Production

The dogma of ITP as exclusively a disorder of accelerated platelet destruction was challenged in the early 1980s by a number of investigators.[38–40] Using indium-111–labeled autologous platelets, these investigators found evidence not only of increased platelet clearance, but also impaired platelet production.[38–40] Recent studies have demonstrated inhibition of *in vitro* megakaryocyte growth and maturation by antibodies from some ITP patients.[41,42] Evidence that patients with ITP have autoreactive cytotoxic thymic (T) lymphocytes capable of directly lysing platelets suggests that these lymphocytes may also induce megakaryocyte injury and contribute to defective platelet production.[15] While megakaryocyte numbers and appearance in bone marrow specimens appear normal in most patients with ITP when viewed under light microscopy, electron microscope studies of bone marrows from ITP patients show extensive damage in the majority of megakaryocytes.[43,44]

Thrombopoietin (TPO) is the major physiologic regulator of megakaryocyte growth and differentiation.[45,46] TPO is synthesized and secreted by the liver and production is constant with no translational or posttranslational regulation.[47] When TPO is released into the circulation, most of the protein binds to TPO receptors on platelets and is cleared with the platelets with the remaining TPO binding to receptors on bone marrow precursors.[48] TPO levels are not increased above the normal range in the majority of patients with ITP and this observation provided the theoretical basis for treatment with TPO-receptor agonists.[45,48,49]

Clinical Presentation and Natural History of ITP in Adults

Adult patients with ITP are more likely to manifest disease with a chronic course. Overall, approximately 15% of patients remit within a year after disease onset, but rare late remissions occur, even among patients who have failed splenectomy.[7,50–52] While bleeding manifestations are the most common presenting symptoms in patients with ITP, the increased use of automated blood counts results in the frequent identification of asymptomatic patients or patients with minimal bleeding. Therefore, it is uncertain whether there is a period of mild-to-moderate thrombocytopenia in adults prior to the development of severe thrombocytopenia with bleeding. In a study of 217 healthy individuals with incidentally discovered thrombocytopenia and platelet counts between 100×10^9/L and 150×10^9/L, the 10-year probability of developing ITP as defined by platelet counts $<100 \times 10^9$/L was 6.9% (95% CI 4.0% to 12.0%). The 10-year probability of developing autoimmune disorders other than ITP was 12% (95% CI 6.9% to 20.8%).[53]

Bleeding manifestations vary widely among patients. Mucocutaneous bleeding, including petechiae, purpura, epistaxis, and gum bleeding, is the most common initial manifestation. The feared complications of internal bleeding or fatal intracranial hemorrhage are rare and more likely to occur in older individuals with additional comorbidities.[7,50–52] Symptomatic bleeding is to certain degree related to the platelet count, with nearly all major bleeding occurring with platelet counts under 30×10^9/L.

Only in patients with hepatitis C–related thrombocytopenia does major bleeding appear to occur with some frequency at higher platelet counts.[54]

The risk of bleeding and fatal hemorrhage in adults with severe CITP, defined as a platelet count of $<30 \times 10^9$/L at least 1 year following diagnosis, was analyzed in a pooled analysis of published clinical series comprising 1,800 patients.[55] The annual incidence of fatal hemorrhage was 1.6 to 3.9 cases per 100 patient years, with a lower risk in patients under 40 years of age (0.4% per year) compared to patients >60 years of age (13% per year).[55] The risk of nonfatal hemorrhage was estimated to be 3% per year in patients <40 years of age and 71% per year for patients >60 years of age. However, it should be noted that this case series dated from 1954 to 1991 and therefore, did not include patients treated with newer agents (rituximab, TPO receptor agonists) or managed with laparoscopic splenectomy that has a documented lower complication risk.

The prognosis of ITP in adults may be better than previously thought, with a majority of patients eventually obtaining a safe platelet count off treatment. In an Italian study of 310 children and adults (median age 40 years; range 8 to 87 years) with CITP, defined as a platelet count $<150 \times 10^9$/L 6 months after diagnosis, only 34 (11%) patients continued to have a platelet count under 30×10^9/L with a median follow-up of 121 months (range 7 to 434 months); 19 of the severely thrombocytopenic patients were receiving no treatment.[56] Major bleeding occurred in 9 (3%) patients with only 1 fatal intracranial hemorrhage.[56] While only 26 (8%) patients who received medical therapy alone achieved a sustained complete remission, 125 patients were able to maintain a platelet count over 50×10^9/L with low-dose prednisone or other treatments. Of the 109 patients who underwent splenectomy, 72 (66%) had a sustained response and 37 were either refractory or relapsed later.[56]

A single institution study of 152 adult ITP patients accrued over a 20-year period reported a complete response (defined as a platelet count over 100×10^9/L at 2 years off all treatment) in 90 (59%) patients and 114 (75%) had a platelet count $>30 \times 10^9$/L.[51] Among the patients who had a complete response, 46/90 (51%) underwent splenectomy to obtain remission. Only nine of these patients had a relapse during an extended follow-up. Four patients with severe thrombocytopenia died during the first 2 years of observation. Only one patient died of a bleeding complication (intracerebral bleed) and three from infectious complications (two with gram-negative sepsis and one with cytomegalovirus pneumonia); all four of these patients were older than 65 years. Compared to the age-matched general population, the mortality risk in adults with ITP was estimated at 1.5% (95% CI 1.1 to 2.2).[51] Patients with secondary ITP had a higher risk of dying of 6.0% (95% CI 2.5 to 14.5), most likely reflecting the morbidity and mortality associated with the underlying diseases. Patients with severe thrombocytopenia (platelets $<30 \times 10^9$/L) were more likely to require hospitalization, averaging 1.2 hospital admissions during the first 2 years following diagnosis.[51]

ITP diagnosed in elderly patients (age >65 years) is generally believed to be more refractory and associated with an increased risk of major bleeding, as well as higher mortality.[57] However, a recent retrospective single institution study of 178 consecutive elderly patients (age >65 years) presenting over a 17-year period appears to refute some of the earlier observations.[50] All patients had platelet counts $<150 \times 10^9$/L and clinical and bone marrow studies consistent with a diagnosis of ITP. One hundred twelve patients (63%) were not treated at presentation and 90 patients (50.6%), including 12 patients (6.8%) with platelet counts $<50 \times 10^9$/L, received no treatment for the duration of their follow-up.[50] Five untreated patients had a spontaneous complete remission. Eleven untreated patients developed other pathology during a median follow-up of 47 months (range 2 to 96 months). This included antiphospholipid antibody syndrome (APLS) (four patients); myelodysplastic syndrome (three patients); gastric lymphoma (one patient); rheumatoid arthritis (one patient); monoclonal gammopathy (one patient); and ovarian carcinoma (one patient). Therapy with low dose prednisone was initiated in 66 patients (37%) on presentation. The mean platelet count in these patients was 15×10^9/L (range $1–43 \times 10^9$/L). Within 1 month of treatment 49 patients (63.6%) increased their platelet count to over 50×10^9/L, with 16 patients attaining a complete remission. Eighteen of the 49 patients (36.7%) relapsed during the period of follow-up.[50] Only one nonresponding patient died of intracerebral bleed. As in other studies, the risk of bleeding appeared to be greatest among the patients with platelet counts persistently $<30 \times 10^9$/L.[34]

In a prospective analysis of 183 patients (25 children and 158 adults) treated with splenectomy during a 10-year period, 53 patients (29%) were refractory to this treatment.[58] The single significant clinical prognostic factor predictive of refractoriness to splenectomy was a platelet count below 50×10^9/L 10 days after surgery ($P < 0.001$). Of the 53 patients, 6 were lost to follow-up; there after, 47 refractory patients (26.6%) were followed prospectively for a median of 7.5 years (5 to 15 years). Thirty seven refractory patients (77%) achieved a durable platelet count $>100 \times 10^9$/L, 9 without treatment and 27 with low-dose corticosteroids or azathioprine. Six patients (13%) continued to have moderate thrombocytopenia with platelet counts of 35×10^9/L to 100×10^9/L and five patients failed to respond to any treatment and had platelet counts $<20 \times 10^9$/L. Three of the five severely refractory patients died of bleeding.[58]

McMillan and Durette[52] reported on the long-term outcome of 114 adult patients who failed to obtain a stable platelet count $>30 \times 10^9$/L after splenectomy; 105 patients were available for long-term follow-up. With a median follow-up of 110 months, a majority of patients (71.4%) achieved a complete or stable partial remission with a median of 46 months. Thirty patients (29.6%) remained unresponsive to treatment, failing to obtain a platelet count over 30×10^9/L. Eleven patients (10.2%) died of bleeding due to refractory thrombocytopenia. Six patients (5.6%) died of treatment-related complications, most often infection.[52]

A systemic review of the literature on the efficacy of splenectomy in ITP reported a 64% complete durable response with a mean follow-up of 7.25 years (range 5 to 12.75 years) with few late relapses.[59] Therefore, extrapolating from the studies of Bourgeois et al. and McMillan and Durette, an estimated 95% of adult patients with ITP can achieve a safe platelet count when splenectomy is incorporated into the ITP treatment algorithm. Except in the highly refractory patients (persistent platelet counts $<30 \times 10^9$/L), the risk of fatal hemorrhage is quite low and morbidity appears to reflect a combination of nonfatal hemorrhage and treatment-related toxicities.

Clinical Presentation and Natural History of ITP in Children

The peak age of presentation of ITP in children is between 5 and 6 years with 70% of cases presenting between ages of 1 and 10 years.[60] Approximately 50% to 60% of children will have a febrile illness that preceded the discovery of thrombocytopenia. Numerous viral infections, including rubella, varicella, mumps, rubeola, and Epstein-Barr virus, as well as immunizations with MMR vaccine have been associated with the subsequent development of ITP in children.[1,60–62] As with adults, review of the blood smear is essential. Signs of mucocutaneous bleeding most often occur without other systemic symptoms and the child frequently does not appear ill.[63,64] Bleeding manifestations vary from little or none, to the typical findings of purpura, petechiae, epistaxis, and menorrhagia in older females.[63–66] Most children present with platelet counts under 20×10^9/L.[66] In a study of 863 children with newly diagnosed ITP, no or mild bleeding manifestations were reported in 77% of patients, moderate bleeding occurred in 20%, and severe bleeding in 3%.[66] Life-threatening bleeding is rare and the estimated risk of intracranial hemorrhage is between 0.1% and 0.5% in newly diagnosed cases.[60,66,67] A retrospective systematic review of medical literature to ascertain the characteristics of children with intracranial hemorrhage in acute CITP found the median time to a hemorrhagic event from diagnosis was 32 days (range 0 to 8 years), with 72% occurring within the first 6 months and with 71.5% of the children having platelet counts $<10 \times 10^9$/L at the time of the bleed.[68]

Most children have a spontaneous resolution of thrombocytopenia within weeks irrespective of treatment.[69,70] However, therapy may shorten the duration of severe thrombocytopenia. Approximately 65% to 70% of children remit by 6 months and another 15% to 20% by 12 months.[60,71] Only 5% to 10% of children develop chronic disease. Children who develop CITP tend to be older, are more often female, and usually present with a higher platelet count.[69–73] Severe or life-threatening bleeding manifestations are rare in children with CITP.[69,73]

Diagnostic Approach to Patients with Suspected ITP

In almost all cases, a presumptive diagnosis of ITP can be made by a careful history, physical examination, complete blood count, and review of the blood smear.[5,74] Response to initial treatment with corticosteroids, intravenous immunoglobulin (IVIG), or anti-RhD supports the diagnosis and confirms the immune nature of the thrombocytopenia. However, additional investigation is necessary to exclude secondary ITP and provide additional information to assist with patient management.

Patient history should be directed toward excluding nonimmune disorders associated with thrombocytopenia, such as primary bone marrow disorders, active infection, cirrhotic liver disease with portal hypertension, drugs, and supplements. A careful drug history should be taken since a large number of medications have been associated with thrombocytopenia (see Chapter 62).[75] A family history of thrombocytopenia or a bleeding disorder may suggest inherited thrombocytopenia (see Chapter 63). A history of recurrent infections may suggest an underlying immune deficiency disorder that may be a contraindication to immunosuppressive therapy. A history of diabetes or psychiatric disorder may be a contraindication to high-dose corticosteroid treatment. Any constitutional symptoms such as fever, night sweats, or weight loss suggest an alternate diagnosis or a secondary cause of ITP.

Physical examination may reveal bleeding manifestations consistent with the severity of the thrombocytopenia, but should otherwise be normal. Finding splenomegaly, hepatomegaly, or adenopathy strongly suggests a diagnosis other than primary ITP. Some physical findings such as a malar rash or joint abnormalities may suggest that thrombocytopenia is secondary to an autoimmune-rheumatologic disorder.

A complete blood count should reveal isolated thrombocytopenia with normal white and red blood cell numbers. Anemia may exist if there has been significant bleeding and findings consistent with a microcytic anemia due to iron deficiency may exist in a female due to excessive menstrual blood loss. In patients with anemia, a reticulocyte count should be performed to determine whether anemia is due to recent blood loss or decreased red cell production. In some patients, concomitant autoimmune hemolytic anemia (Evan syndrome) may be diagnosed by finding an increased reticulocyte count, an elevated MCHC, and spherocytes on the peripheral blood smear.

A careful review of the peripheral blood smear is mandatory to exclude abnormalities in other cellular elements of the blood. Abnormalities in erythrocyte or leukocyte morphology may suggest an underlying bone marrow disorder, such as the myelodysplastic syndrome, or a congenital cause of thrombocytopenia. For example, the presence of a MYH9-related disorder may be suggested by finding leukocyte inclusion bodies. Many congenital thrombocytopenic disorders are associated with very large platelets (macrothrombocytopenia) that may also be reflected in an abnormally high mean platelet volume. Increased numbers of small lymphocytes may suggest a diagnosis of chronic lymphocytic leukemia (CLL) with secondary ITP. Increased numbers of lymphocytes with granular inclusions can suggest a diagnosis of large granular lymphocytic leukemia with secondary ITP. Pseudothrombocytopenia due to platelet clumping should be excluded: if platelet clumping is seen on the blood smear, a repeat platelet count using a blood sample collected in citrate or heparin rather than EDTA should be performed. Thrombocytopenia resulting from thrombotic thrombocytopenic purpura or hemolytic uremic syndrome must be considered if the blood smear shows significant numbers of schistocytes.

The International Working Group on ITP recommended a number of additional laboratory tests to further assist in the diagnosis and management of patients with primary ITP.[74] These recommendations are listed in Table 61.1. Bone marrow examination has been recommended in patients refractory to ITP-therapy or who relapse after an initial remission, patients with systemic symptoms or atypical physical findings, and prior to splenectomy.[74] A bone marrow aspirate and biopsy should be performed in patients older than 60 years and include cytogenetic studies and flow cytometry.[74]

Three chronic infections are commonly associated with the development of secondary ITP and their identification and treatment may affect the clinical outcome.[1,74,76,77,78] *Helicobacter pylori* infection, as detected by either the urea breath test or stool antigen, is recommended in all patients with a presumptive diagnosis of ITP. If these tests are unavailable or if patients are receiving proton pump inhibitors for ulcer treatment or prophylaxis, serologic detection can be considered if the patient has

Table 61.1

International ITP working group consensus recommendations for the diagnosis of ITP in children and adults

Basic Initial Evaluation	Addition Studies of Potential Value	Studies of Unproven Benefit
Patient history	APLAs (patients with history of thrombosis or fetal loss)	TPO assay
Family history	TSH or antithyroid antibodies	Reticulated platelets
Medication history	ANA in children	Platelet-associated immunoglobulins
Physical examination	Pregnancy test in women of child bearing years	Serum complement
Complete blood count	Viral PCR for parvovirus and CMV (selected refractory, febrile or leukopenic patients)	Bleeding time
Quantitative immunoglobulins	Platelet glycoprotein-specific antibodies	Platelet survival studies
Blood group (Rh)		
DAT		
Helicobacter pylori		
HIV		
HCV		
Bone marrow (in selected patients)		

Adapted from Glanz J, France E, Xu S, et al. A population-based, multisite cohort study of the predictors of chronic idiopathic thrombocytopenic purpura in children. *Pediatrics* 2008;121:e506–e512.

not received recent treatment with IVIG. Testing in children is not supported by the literature.[74]

Thrombocytopenia associated with infection with the HIV or the HCV can result in thrombocytopenia years before the development of viral-related symptoms. Thrombocytopenia associated with both viruses is indistinguishable from primary ITP and can response to standard ITP treatments. However, optimal treatment for HIV- and HCV-related thrombocytopenia should be directed toward treatment of the viral infection. Since both infections may be silent in early stages, screening for these infections is recommended regardless of patient background or reported lifestyle.[74] Patients should also be screened for hepatitis B infection since treatment with prednisone and/or rituximab may result in viral reactivation and fulminant hepatitis.

The international ITP working group recommends obtaining baseline immunoglobulin levels (IgG, IgM, and IgA). Low levels can be found in patients with common variable immunodeficiency, which is accompanied by a surprisingly high incidence of autoimmune complications including immune thyroid disease, autoimmune hemolytic anemia, and ITP.[79] Immune suppression in these patients should be avoided as should splenectomy, if possible. Selective IgA deficiency can also be associated with the development of ITP, with many patients lacking a history of recurrent or chronic infection. If required to treat ITP, these patients should receive IVIG preparations that are IgA depleted to avoid severe infusion reactions.

A direct antiglobulin test (DAT, direct Coomb test) should be considered if there is evidence of unexplained anemia associated with a high reticulocyte count. A positive DAT has been reported in up to 22% in patients with ITP, most often without overt evidence of hemolysis.[70] Therefore, it should also be performed along with red cell Rh typing in patients who are to be treated with anti-RhD, since the presence of a pretreatment antibody on the red cell could increase the risk of intravascular hemolysis.[74,80]

Several other tests have been suggested in the consensus reports that may be of clinical utility in some patients (Table 61.1).[74] Screening for antiphospholipid antibodies (APLAs) (lupus anticoagulant [LA], anticardiolipin [ACL] and anti-β_2-glycoprotein antibodies) should be considered in any patient with a prior history of thrombosis or fetal loss. While APLAs can be found in up to 40% of ITP patients, they have no apparent effect on the outcome of ITP treatment and the majority of patients with such antibodies do not develop clinical manifestations of the APLS.[76] Therefore, routine testing for these antibodies is not recommended unless there is a history suggestive of the APLS. A positive antinuclear antibody assay (ANA) may be present in 15% to 25% of patients with ITP, however independent of patients with clinical systemic lupus erythematosus, a positive test in primary ITP has not been shown to predict response to treatment or prognosis in adults.[81] Therefore, an ANA test is not routinely recommended in adults. However, a positive ANA test may be a predictor of chronicity in childhood ITP.[82]

Clinical hypo- and hyperthyroidism is reported in up to 20% of patients with ITP and antithyroid antibodies are present in nearly 40% of patients.[81,83] Mild to moderate thrombocytopenia has been reported in patients with both hypothyroidism and hyperthyroidism and platelet counts return to normal when patients become euthyroid.[83] Also, patients with ITP and clinical hypo- or hyperthyroidism maybe refractory to standard ITP treatment and only become responsive when their thyroid disease is treated.[83] Therefore, it can be useful to screen refractory patients with an assay for thyroid stimulating hormone (TSH)

to rule out associated thyroid disease.[74,83] In selected patient populations, testing for antibodies to thyroglobulin or other thyroid antigens may identify patients at risk of developing clinical thyroid disease.[74]

Secondary ITP

CITP has been classified either as a primary autoimmune disorder (idiopathic thrombocytopenic purpura) or as secondary to a number of underlying disorders.[4] Common conditions associated with secondary ITP include immunodeficiency disorders, lymphoproliferative disorders, and chronic infection with HIV, *H. pylori*, and HCV.[1,4,76,77,81] Other autoimmune rheumatologic disorders are associated with secondary ITP, of which systemic lupus erythematosus (SLE) and APLS are the most common.[1,4,76,77,81]

Systemic Lupus Erythematosus

Although laboratory evidence of autoimmunity, such as a positive ANA, is common in ITP, few patients are likely to develop SLE or related conditions.[1,84] The pathophysiology of ITP in SLE and APLS is similar to that of primary ITP and in most cases is mediated by antiplatelet glycoprotein autoantibodies. Decreased platelet production, as observed in primary ITP, may also be involved in the pathogenesis of ITP in patients with this secondary form of the disorder.[1,38]

SLE is a systemic inflammatory disease that can affect any organ system, including the hematologic system, which is 1 of 11 criteria for diagnosis.[84] Hematologic manifestations include leukopenia, hemolytic anemia, and thrombocytopenia, any or all of which can be present at the onset of the disease or can develop later. Twenty to twenty-five percent of patients with SLE have platelet counts $<100 \times 10^9$/dL, although <10% of patients have platelet counts $<30 \times 10^9$/L.[85,86] A recent study from China reported an incidence of significant thrombocytopenia in SLE of 24.2%.[87] In patients with SLE, thrombocytopenia with platelet counts $<100 \times 10^9$/L is more prevalent when patients have high titer ACL antibodies.[88] However, it is unclear whether these patients also fulfill criteria for APLS.

Potential mechanisms for the pathogenesis of thrombocytopenia in patients with SLE include antibody-mediated platelet destruction by anti-GPIIb/IIIa and anti-GPIb/IX antibodies or antiplatelet glycoprotein autoantibodies that induce megakaryocyte apoptosis.[2] Lupus vasculitis, thrombotic microangiopathy, and hemophagocytosis are immunopathologic disorders associated with SLE in which platelets are destroyed secondary to a microvascular inflammatory process. Thrombocytopenia in these disorders reverses with correction of the primary process. There are no studies of plasma TPO levels in patients with SLE and thrombocytopenia, but it can be assumed that they are similar to those reported in patients with ITP.[89]

A low platelet count in SLE can also result from mechanisms other than autoimmunity. These include drug-induced thrombocytopenia and infection. Antimalarial agents, commonly used to treat SLE, have been reported to induce thrombocytopenia, although recent data suggest improvement of platelet counts with hydroxychloroquine.[60,90] Infections with *H. pylori*, HCV, HIV, and other viral illnesses can further contribute to the risk of developing thrombocytopenia.[1] Two disorders associated with thrombocytopenia, Evans syndrome and Graves disease, are both reported to be more frequent in patients with SLE compared to the general population.[2,91] Thrombocytopenia in pregnant patients with SLE can result from the HELLP syndrome; in the postpartum period, SLE flares can add to the difficulty in diagnosis.[92]

When patients with SLE have associated ITP, corticosteroids remain the primary treatment modality, although every effort should be made to switch to a steroid-sparing therapy to minimize the long-term exposure. Immunosuppressive agents such as methotrexate, azathioprine, leflunomide, dapsone, cyclosporine, mycophenolate mofetil, and cyclophosphamide are often used as disease modifying and steroid-sparing agents in SLE.[93] Each of these agents has also been used to treat patients with CITP.[5,59] Several case series indicate that B lymphocyte depletion with rituximab, an agent with documented responses in refractory ITP, is also of clinical benefit in patients with refractory SLE.[94] Splenectomy, if possible, should be avoided, particularly in patients with active SLE who will require further immunosuppressive therapy and therefore be at risk of infection.

Antiphospholipid Antibody Syndrome

APLS is characterized by recurrent fetal loss and recurrent venous and/or arterial thromboembolic events.[95] In the most severe form, catastrophic antiphospholipid syndrome, multiple organ dysfunction occurs.[95] Although frequently associated with SLE, over half of the patients with APLS do not have clinical evidence of SLE.[96,97]

Thrombocytopenia in association with APLS is more common in patients with SLE than in primary APLS (PAPS), although it is well described in both.[98] The reported incidence of thrombocytopenia varies between 22% and 42%.[2,96–99] Thrombocytopenia in APLS tends to be less severe than that seen with ITP; platelet counts usually range from 70 to 120K and it does not usually require intervention.[99] Accordingly, it is less commonly associated with bleeding manifestations than ITP. APLS may present with isolated thrombocytopenia without other signs and symptoms. The presence of thrombocytopenia does not seem to affect the thromboembolic complications of the disorder, as rates of thrombosis are similar with and without thrombocytopenia.[96,100] While the findings of cardiac valvular vegetations and myocardial infarction are not associated with thrombocytopenia, there is a strong statistical association between valvular leaflet thickening and thrombocytopenia.[97] Chorea, arthritis, and livido reticularis, other clinical manifestations of APLS, are also associated with the presence of thrombocytopenia.[97]

The incidence of the LA, a defining characteristic of the disorder, is higher in APLS than in ITP. Antibodies to specific glycoprotein receptors such as GPIb/IX and GPIIb/IIIa are detectable in 40% of ITP patients with APLAs.[99–102] Godeau et al.[102] noted that in 11/15 patients with APLS and platelet counts <50K, antiplatelet antibodies to specific platelet glycoproteins could be identified. In patients with both PAPS and SLE-associated APLS, antiplatelet antibodies were found much more frequently in patients with thrombocytopenia compared to patients without thrombocytopenia. In addition, there was a strong association between PAPS with thrombocytopenia and ACL antibodies, the LA, and specific platelet glycoprotein antibodies.[103–105] It has been proposed that binding of antibodies to β_2 glycoprotein-1 induces dimerization of the protein, thereby stimulating phosphorylation of the abetalipoprotein E receptor. In turn, this results in activation of p38MAP kinase, generating

thromboxane A_2 as well as phosphatidylinositol-3-kinase and Akt and inducing platelet activation and sensitization to other agonists.[2,103] APLAs bind also to anionic phospholipids normally found on the inner platelet membrane. This implies that platelet activation or aggregation is needed to expose these phospholipids on the outer membrane surface, thereby enabling antibody binding.[104] The contribution of APLA *per se* to thrombocytopenia remains uncertain.

Thrombocytopenia in APLS rarely requires treatment unless the platelet count is below 30K. However, thrombocytopenia in patients with the APLS will respond to corticosteroid therapy and most other ITP treatments. In addition to prednisone, there are case reports indicating a response to rituximab with improvement in the platelet count as well as reduction or disappearance of ACL antibodies.[106–108]

Lymphoproliferative Disorders

Thrombocytopenia is a well-recognized complication of lymphoproliferative disorders. In addition to decreased platelet counts due to splenic sequestration in patients with splenomegaly and decreased production due to bone marrow replacement and/or systemic chemotherapy, immune platelet destruction can occur in 1% to 5% of patients.[109,110] While cases of ITP have been reported with nearly all lymphoproliferative disorders, the majority of cases are reported in patients with CLL, Hodgkin disease (HD), and large granular T-lymphocyte (T-LGL) clonal proliferations.

Retrospective studies have reported ITP in 1% to 2% of patients with CLL.[111–116] The incidence of ITP appears to be significantly less than that of CLL-associated autoimmune hemolytic anemia.[112,113,115] A multicenter study of 3,150 CLL patients found ITP in 35 patients (1%).[113] Older age and advanced CLL stage requiring therapy were found to be independent risk factors for autoimmune hemolytic anemia, but it is uncertain whether this is also true for ITP.[113] The presence of active CLL does not correlate with the onset or severity of ITP, which is distinctly different from autoimmune hemolytic anemia.[113,114] ITP may precede a diagnosis of CLL, and flow cytometry of bone marrow specimens from older patients with ITP may reveal small clones of monoclonal CLL phenotypic CD19/CD5 positive lymphocytes.[111,115]

A recent retrospective cohort study identified ITP in 69/1,270 patients (5%) with CLL.[114] ITP occurred at any time in the course of the disease with the median time to a diagnosis of ITP of 13 months (0 to 81 months). Laboratory characteristics of the CLL patients with ITP included a higher frequency of unmutated IgVh genes (54% vs. 18%), more frequent involvement of the Vh1 family (43% vs. 21%), and a lower frequency of the Vh4 family (4% vs. 22%).[114] The predominance of unmutated B lymphocytes and the unique Vh gene distribution may reflect a CLL subset with a greater propensity to autoimmune phenomena. As might be predicted by the greater frequency of patients with unmutated CLL, the patients with ITP had a lower 5-year survival (64% vs. 82%).[114] CLL patients with ITP were also less likely to respond to IVIG and prednisone treatment alone (19/35) when compared to the responses observed with the use of cytotoxic chemotherapy (27/41).

The reported prevalence of ITP in HD is estimated at 0.2% to 1%.[116–119] A review of 4,090 patients with HD in the British National Lymphoma Investigation (BNLI) registry identified only eight cases.[119] The majority of reported HD–ITP cases occur after the diagnosis of HD.[116–119] The median time to the development of ITP after the diagnosis of HD in the BNLI registry was 23 months (3 to 57), with six of the eight cases developing ITP while in remission.[119] However, a large population-based case-controlled epidemiologic study in Scandinavia found a statistically significant increased risk of Hodgkin lymphoma in patients with a personal history of ITP.[120]

T-LGL proliferation is a clonal proliferation of mature T lymphocytes that predominately express the CD8 phenotype.[121] T-LGL clonal proliferation is frequently found in patients with rheumatologic disorders such as rheumatoid arthritis, but can occur independent of other diseases.[121,122] Neutropenia and anemia are frequently reported in association with T-LGL.[121,122] Mild thrombocytopenia can occur in up to 20% of patients, but severe thrombocytopenia was observed in only 1% of patients.[122] The development of severe thrombocytopenia is most often due to suppression of megakaryopoiesis by cytotoxic T-LGL lymphocytes.[106–108] Amegakaryocytic thrombocytopenia frequently develops in conjunction with other cytopenias.[123–125] Treatment should be directed against the T-LGL clone employing cytotoxic therapy (cyclophosphamide) or cyclosporine.[123–125]

Human Immunodeficiency Virus Infection

Thrombocytopenia was first associated with the acquired immune deficiency syndrome (AIDS) before the discovery of the HIV virus.[126] Prior to the use of highly active antiretroviral therapy (HAART), thrombocytopenia (*HIV-ITP*, platelet count $<150 \times 10^9$/L) was identified in approximately 5% to 30% of HIV-1 infected patients.[77] Thrombocytopenia is more prevalent in patients with advanced HIV infection as defined by a CD4-lymphocyte count of $<200/\mu$L and clinical AIDS.[77] An increased incidence and severity of thrombocytopenia in HIV-infected intravenous drug users compared to HIV infected homosexuals has also been reported.[127,128] These differences may be explained, in part, by the higher incidence of coinfection with hepatitis C and underlying liver disease in HIV-infected intravenous drug users.[129]

Most data were obtained prior to the widespread use of HAART in patients with early HIV infection. There are few data on the current prevalence of thrombocytopenia in patients under active antiviral treatment. However, recent prospective data from the Women's Interagency HIV study have documented a reduction in the incidence of anemia and neutropenia in HIV-infected women on HAART therapy.[130,131] This is in accord with the impression that there has been a similar reduction in the incidence of thrombocytopenia, especially platelet counts $<50 \times 10^9$/L in compliant patients.

The pathogenesis of thrombocytopenia is partially dependent on the viral burden as defined by the severity of immunodeficiency. HIV-associated thrombocytopenia developing early after infection more often resembles classical ITP in which thrombocytopenia is mediated primarily by peripheral destruction, whereas thrombocytopenia in patients with immunologic AIDS (CD4 lymphocytes $<200/\mu$L) is predominately attributable to decreased platelet production and ineffective hematopoiesis.[132,133] While platelet counts may improve with antiretroviral therapy in both patient populations, patients with advanced disease are less likely to respond to classic primary ITP therapy such as corticosteroids, IVIG, or anti-RhD.

HIV-seropositive patients can develop thrombocytopenia several years before the development of overt AIDS, and the

early disease is clinically indistinguishable from classic ITP.[77] However, the clinical picture of HIV-ITP is often mild, with only a minority of patients having platelet counts $<50 \times 10^9$/L.[77] Major bleeding is rare and only a few cases of fatal hemorrhage have been reported. There has been greater variability in HIV-associated thrombocytopenia in patients with hemophilia A.[77] Severe thrombocytopenia in patients with advanced HIV infections is frequently associated with additional cytopenias.[77] HIV-infected drug users are also more likely to have antibodies to both hepatitis B and C and to have abnormal liver function studies.[129]

HIV-ITP is generally responsive to therapeutic interventions used in classical ITP. Therapy with prednisone produces a major hematologic response (platelet count $>100 \times 10^9$/L) in over half of patients, although only a minority will maintain platelets $>50 \times 10^9$/L after cessation of steroids.[134] No deleterious effect of short-term treatment with prednisone has become evident. However, long-term treatment with corticosteroids should still be avoided and other coinfections such as tuberculosis, cytomegalic virus, or hepatitis C should be excluded before initiating treatment with corticosteroids. IVIG and anti-RhD are equally effective in increasing platelet counts acutely in severely affected patients,[77] but a crossover study clearly demonstrated a longer duration of response with the latter agent.[135] Splenectomy has proven to be safe and effective in refractory patients with HIV-ITP.[77] After splenectomy, there is a transient increase in the peripheral blood CD4 lymphocyte count, which reflects redistribution from the splenic pool to the circulation, rather than an improvement in the patient's immunologic status.

HIV-related cytopenias have been found to correlate with plasma viral load.[136] Effective antiretroviral therapies (HAART) have resulted in improvement in several HIV-related cytopenias, including HIV-ITP.[136,137] Use of HAART in both *de novo* and zidovudine-refractory HIV-ITP has induced sustained platelet responses in association with effective viral suppression.[77,137]

Responses to zidovudine and HAART may be more limited in HIV-infected intravenous drug users, possibly reflecting the impact of associated liver disease and infection with HCV.[77] In a prospective placebo-controlled, double-blind, randomized trial, 12 of 14 zidovudine refractory HIV-infected intravenous drug users with elevated serum alanine aminotransferase suggestive of underlying liver disease and who were treated with interferon-α (IFN-α) had a statistically significant increase in their platelet counts by week 4 of therapy.[138] An unblinded open label trial of IFN-α in a cohort of predominately homosexual men documented responses in 9/16 patients, with responses occurring as early as 2 weeks after the initiation of treatment.[139] Such rapid responses preclude the possibility that improvement in the platelet count is due solely to suppression of concomitant HCV infection. There are no reported studies on the association of *H. pylori* infection and thrombocytopenia in HIV infected patients.

Helicobacter Pylori Infection

Helicobacter pylori is a gram-negative spiral-shaped bacterium that colonizes the mucous layer of the human stomach.[140] It has been causally linked with a diverse spectrum of gastrointestinal disorders, including gastritis, peptic ulcer disease, dyspepsia, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma. Recent studies have provided strong evidence for an association between *H. pylori* infection and ITP in certain populations.[1,76,78]

A number of hypotheses have been advanced about the mechanisms by which *H. pylori* may cause ITP. One proposes molecular mimicry in which *H. pylori* induces antibodies that cross-react with epitopes on platelets proteins. The possible role of genetically and antigenically diverse cytotoxicity antigen A (CagA) gene products as candidates for evoking ITP antibodies was reported in two recent studies.[141,142]

No differentiating features have been identified in prospective series between the clinical presentation in patients with infection from those with classical ITP, aside from an older age in *H. pylori*–positive cases and in some studies higher platelet counts.[76] A uniform association has not been observed between *H. pylori* infection and symptoms of dyspepsia.[76] A cross-sectional study by Fukui et al.[143] did not find a correlation between *H. pylori* infection and thrombocytopenia during pregnancy.

A systematic review of studies on the treatment of *H. pylori* infection in 1,555 patients with ITP found a complete response (platelets $>100 \times 10^9$/L) and an overall response (platelets $>30 \times 10^9$/L or a doubling of the baseline platelet count) of 42.7% (95% CI 31.8% to 53.9%) and 50.3% (95% CI 41.6% to 59.0%), respectively.[78] Responses ranged from 0% in American series[144] to 100% in one Italian series.[145] In an Italian-English study, eradication therapy was administered to *H. pylori*–positive patients who either had a platelet count of $<50 \times 10^9$/L or symptoms of dyspepsia.[146] Platelet responses were observed in 17/52 patients (33%) who received treatment as compared with 0/12 patients who did not. Responders were younger than nonresponders (55 ± 8 years vs. 60 ± 14 years), had a higher baseline platelet count ($48 \pm 21 \times 10^9$/L vs. $31 \pm 20 \times 10^9$/L), a shorter disease duration (14 ± 7 months vs. 34 ± 23 months), and had either not received prior therapy for ITP or had received only prednisone or IVIG.[146] Platelet responses to eradication therapy appear to be sustained in the majority of successfully treated patients.[78] Adverse events from eradication therapy have been described as mild, usually consisting of abdominal pain and diarrhea, leading to discontinuation of treatment in <5% of cases. Patients who fail to respond to antimicrobial therapy should be retested to determine if eradication treatment was effective.

Hepatitis C Infection

HCV is the most common blood borne chronic viral infection in the United States. The third National Health and Nutrition Examination Survey estimated that nearly 3.2 million persons in the US population are infected with HCV.[147] Worldwide, an estimated 145 million individuals or 2.2% of the world's population are infected.[148] A significant proportion of HCV infected individuals are unaware of their illness and many only present to their physicians when they develop overt symptoms. In addition to cirrhotic liver disease, HCV is associated with the development of a variety of immune diseases and lymphoma.[1,76,77] Chronic HCV infection has also been associated with the development of thrombocytopenia.[1,76,77] Thrombocytopenia may be present even in the absence of clinically evident liver disease or splenomegaly and may be mistakenly diagnosed as primary ITP.[77]

Six cross-sectional studies have reported serologic evidence of HCV infection in 159/799 patients (20%) with ITP.[76,77] In the largest series published to date, HCV antibodies were identified in 76 of 250 patients (30%) fulfilling the American Society of Hematology guideline criteria for ITP.[54] There were significant differences in demographic characteristics of HCV-positive

compared with HCV-negative ITP patients. HCV-positive patients were older (54.9 ± 8 years vs. 40.3 ± 8 years) and there was no female preponderance as there is in HCV-negative ITP.[54] Pockros et al.[149] retrospectively identified 7 ITP cases among 3,440 new HCV patients seen over a 56-month period. They estimated that the prevalence of CITP among their HCV patients was much greater than would be expected by chance. Even in the presence of active liver disease, patients with HCV infection presented with lower platelet counts compared to patients with hepatitis B or alcoholic liver disease.[54,150]

There have been conflicting data reported with respect to the presence of specific antibodies in platelet eluates from patients with HCV-related thrombocytopenia.[76] In one report, 66% of HCV infected patients had detectable antiplatelet glycoprotein antibodies. However, their presence did not correlate with the patient platelet counts.[151] A similar frequency of antiplatelet glycoprotein antibodies was found in patients with alcoholic cirrhosis, indicating these antibodies are not necessarily specific for HCV infection.[151] In contrast, a cross-reactive antibody to an HCV protein and platelet GPIIIa has been described by Zhang et al.[152] that is capable of inducing peroxide-mediated platelet fragmentation, similar to an antibody found in patients with HIV-associated thrombocytopenia.

In one study from Japan,[153] platelet counts in HCV-positive patients were lower than in HCV-negative patients (26 ± 9 vs. 49 ± 30 × 10^9/L, respectively). Conversely, in an American series,[54] HCV-positive patients had less severe thrombocytopenia, as defined by a platelet count ≤10 × 10^9/L (4% vs. 46% for ITP). However, 74% had a platelet count ≤50 × 10^9/L. Symptoms and signs of thrombocytopenia were less frequent in HCV-positive ITP, but major bleeding was more frequent (25% vs. 10%,). Serum cryoglobulins and ACL antibodies were more frequent in HCV-positive ITP (90% and 62%) than in HCV-negative ITP (7% and 15%).[54] In another study, the characteristics of ITP in HCV-positive patients did not differ from those without a positive serology. However, this study evaluated only patients with platelet counts ≤25 × 10^9/L.[154]

A combined analysis of available studies indicates that responses to corticosteroids are significantly lower in HCV-positive than in HCV-negative patients (OR 0.39, 95% CI 0.17 to 0.86).[61] However, this result is entirely attributable to a single study from Sakuraya et al.,[153] in which none of the 10 HCV-positive patients treated with prednisolone achieved a response. Other case series have reported a >50% platelet response to steroids.[76] The response to splenectomy was not found to differ significantly between the HCV-positive and HCV-negative CITP patient populations (OR 0.84, 95% CI 0.19 to 3.61).[76]

Rajan et al.[54] noted that only a minority of HCV-positive patients received some form of treatment (29 [38%] vs. 158 [91%] for HCV-negative ITP).[54] Four of seven patients treated with prednisone responded, but six developed elevated hepatic transaminases of greater than twice pretreatment levels while on therapy and all six had a documented increase in HCV viral load. Two patients developed elevated serum bilirubin levels, with one patient developing overt jaundice. Treatment with either IVIG or anti-RhD IgG has proven to be effective in increasing platelet counts in both the HCV seropositive and seronegative patients.[54,76]

Approximately half of adult ITP patients with HCV and treated with IFN-α responded with a rise in platelet count.[76] Responders to IFN-α could be distinguished from the nonresponders by a decrease in HCV quantitative RNA, hepatic transaminases, and cryoglobulins.[54] Iga et al.[155] reported significant increases in the platelet counts of 12 HCV infected patients who were complete responders to IFN-α treatment, but no improvement in the platelet counts of 11 patients who failed IFN-α therapy as assessed by viral load.

IFN-α–based therapy can suppress platelet production. In a phase II study, Eltrombopag, an oral TPO receptor agonist, increased platelet counts in the majority of patients with cirrhosis associated with HCV infection to >100 × 10^9/L, permitting continuation of IFN-based therapy.[156] Response was dose dependent, with 9/17 responding to 30 mg/day, 15/19 to 50 mg/day, and 20/21 to 75 mg/day. None of the 17 patients treated with placebo increased their platelets to >100 × 10^9/L.[156]

Immune Deficiency

Immunodeficiency disorders are well known to be associated with ITP. The commonest form and best understood is common variable immune deficiency (CVID), also known as hypogammaglobulinemia. At least 10% of patients with CVID develop ITP and another 10% develop autoimmune hemolytic anemia.[157] Among patients with CVID, autoimmunity to platelets and red blood cells occurs more frequently in those with mutations in TACI, a B-cell growth factor receptor.[158] Furthermore, ITP does not appear to develop in patients with X-linked agammaglobulinemia (Brutons), presumably because there are no abnormal circulating B and T cells to instigate the autoimmune response to platelets. ITP is also common in patients with autoimmune lymphoproliferative syndrome (ALPS or Canale-Smith syndrome), an apoptosis defect in autoreactive T and B cells, although the pathophysiology has not been established.[159] Recent studies have suggested that overt or a "forme fruste" of ALPS may underlie a substantial fraction of pediatric cases of Evan syndrome (concurrent autoimmune hemolytic anemia and ITP).[160]

Management of Primary ITP in Adults

A number of important clinical factors must be considered before treatment is initiated in adults with primary ITP. Consideration must be given to the bleeding risk, toxicities of specific therapies, patient age and comorbidities, and patient preference and life style.[74] Management of adult CITP is different from that of ITP in children. For children with ITP, when treatment is given, it is initially aimed at rapidly increasing the platelet count to a safe level, then maintaining an adequate and safe count with the assumption that the child will have a spontaneous remission. For adults, most of whom will have CITP, the therapeutic goals are generally different. Therapies to increase the platelet count to a safe level and/or prevent further bleeding should be well tolerated and given over a limited period of time. More aggressive early intervention may be justified if proven to have curative potential. Since attaining a spontaneous remission becomes much less likely a year after diagnosis, short-term therapies, such as IVIG or anti-RhD infusions may be used to avoid severe thrombocytopenia while awaiting the response to a "curative" treatment. Also small changes in the platelet count may be clinically significant. An increase in the platelet count from 5–10 × 10^9/L to 20–25 × 10^9/L can have an important impact on the risk and occurrence of bleeding. Clinical experience and recent clinical trial data indicate that the risk of major bleeding is quite low at platelet counts of >30 × 10^9/L.

In some patients with thrombocytopenia without significant bleeding manifestations, observation alone with occasional "rescue treatment" with IVIG, anti-RhD, or pulsed steroids may be an appropriate choice. The obvious advantages of this approach are that there is less toxicity from therapy, patients have less frequent physician visits, spend less time focused on their disease, and the cost of treatment is reduced. The success of this approach is dependent upon the patient and the severity of disease. If patients have bruising, oral bleeding, or heavy menses, it may prove to be impossible to "ignore" their ITP and the patient may have heightened anxiety, restrict their social interactions, and return urgently to the hematologist for management. More serious bleeding may occur in the context of other illness or trauma; a low platelet count may prevent use of medications to treat other conditions, such as aspirin for coronary artery disease or pegylated interferons for the treatment of hepatitis C infection and could lead to the avoidance of other medical or dental care. For these reasons, periodic visits are optimal to maintain a patient–physician dialogue and prevent disease denial that may have serious consequences.

Therefore "observation and no treatment" is done with the understanding that the patient will have further follow-up and the physician is available to manage bleeding events should they arise. It is clear that if the platelet count is sufficiently low ($<20 \times 10^9$/L) and if there is bleeding, then the risk of serious hemorrhage is appreciable. Certain issues need to be considered at this juncture before discussing available treatments. First, what other reasons exist to treat patients even if they do not appear to be at immediate risk of life-threatening hemorrhage. Second, how completely has the patient been evaluated for secondary forms of ITP and what investigations are required to distinguish a case of "refractory" ITP from that of another etiology such as myelodysplasia. Third, where should the balance be between raising the platelet count in the absence of bleeding, side effects of therapy, improvement of health-related quality of life, and a policy of reasonable, cost-effective patient follow-up.

If a patient with CITP is being seen for the first time, it is worthwhile to review the items in the Table 61.1 to ensure the diagnosis of CITP is correct. Among the primary considerations is the exclusion of secondary ITP in which resolution of the primary, underlying disease would impact on the management of the thrombocytopenia.

The rest of this chapter is devoted to treatment of patients with CITP with the understanding that management paradigms are changing as new clinical trials and therapeutic agents are introduced. The consensus of ITP experts is that treatment should be considered when the platelet threshold is 20–30 $\times 10^9$/L, but occasionally it is appropriate to select a higher target count.[74] For secondary ITP, treating the underlying disease, such as infection or an immune defect, is usually the preferred approach. This section will assume that appropriate evaluation has been undertaken and patients with secondary causes of ITP will have been managed accordingly.

Corticosteroid Therapy

Corticosteroids are the standard initial therapeutic agents.[74] The corticosteroids used most often include prednisone, parenteral methylprednisolone, and dexamethasone. These medications are frequently used in combination with IVIG and IV anti-RhD to rapidly increase platelet counts in patients with significant thrombocytopenia ($<5 \times 10^9$/L) and extensive bleeding manifestations.[74]

Prednisone is the most frequently used corticosteroid. The drug is given orally at a dose of 0.5 to 2 mg/kg daily. Platelet counts will increase to $>50 \times 10^9$/L in a few days to several weeks in responders, depending upon the baseline platelet count and whether IVIG or anti-RhD is used in conjunction with the drug. Bleeding may cease before a rise in the platelet count is seen. When used alone, 65% to 70% of patients respond.[161–163] Responses in adults are rarely durable with only 13% to 17% of patients having an unmaintained normal platelet count at 1 year after treatment with prednisone alone.[161–163]

High dose methylprednisolone has been used to increase platelet counts more rapidly in patients not responding to oral prednisone.[164,165] There is no evidence that this improves long-term outcomes and most responding patients transition successfully to prednisone. Recent trials have shown significant efficacy for the initial treatment of ITP with pulse dexamethasone.[166,167] Previous studies on the use of high dose dexamethasone for the treatment of refractory ITP were abandoned due to significant toxicity and marginal efficacy.[168] A study of a single course of high dose dexamethasone (40 mg/day × 4 days) in newly diagnosed patients reported a high initial response (85%), with half of the responding patients maintaining their response at 6 months.[166] A multicenter trial of dexamethasone (40 mg/day × 4 days) repeated every 14 days for four courses produced at 86% response rate (platelets >50 10^9/L) and a 65% complete response rate (platelets $>100 \times 10^9$/L).[167] At 15 months median follow-up, 60% of responding adults had not relapsed and for both adults and children who obtained a complete response, 87% remained in remission at 15 months median follow-up.[167] However, a recently reported randomized trial comparing one or two cycles of the high dose dexamethasone to 1 mg/kg prednisolone failed to demonstrate superiority of the dexamethasone regimen.[169] Additional studies will be needed to prove this approach provides superior long-term benefit.

Intravenous Immunoglobulin

A number of controlled treatment trials have clearly demonstrated 65% to 80% of patients treated with IVIG show an increased platelet counts within 24 to 48 hours.[5,74,170,171] Duration of response is rarely >10 to 14 days. Initial treatment regimens, in which 0.4 g/kg was infused daily for 5 days have been supplanted by a higher dose regimen of 1 g/kg daily for 1 or 2 days. This latter regimen is associated with longer daily infusion time and greater toxicity, including infusion reactions, headaches, occasional aseptic meningitis, hemolytic anemia due to alloantibodies, acute renal failure, and rare thrombotic events primarily in the elderly. The concomitant use of corticosteroids may enhance the platelet response, reduce infusion reactions, and result in fewer headaches.

Intravenous Anti-RhD

Patients who are Rh positive, not significantly anemic, and who retain their spleens are candidates for treatment with anti-RhD. Before initiating treatment with anti-RhD, patients should be Rh typed and have a DAT performed. Treatment with the FDA-approved dose of 50 μg/kg can result in an increase in the platelet count to over 50×10^9/L in nearly 80% of patients, but requires 48 to 72 hours for a clinically significant response.[135,172,173] Infusions of 75 μg/kg have been associated with a more rapid

increase in the platelet count, equivalent to that reported with IVIG treatment.[173] Duration of response to anti-RhD therapy is usually greater than observed with IVIG treatment, averaging 21 days.[135] Treatment with anti-Rh immunoglobulin typically results in a mild hemolytic anemia with an average decrease in hemoglobin of 1 to 1.5 g. Occasional episodes of intravascular hemolysis resulting in renal failure and rare cases of disseminated intravascular coagulation have been reported,[174,175]. Because of these reactions, anti-RhD is no longer available in Europe for the treatment of ITP.

Splenectomy

Splenectomy is the historical "gold standard" for the treatment of CITP. A systematic review of the medical literature has clearly shown that 65% of patients will have unmaintained remissions lasting 10 years or longer. In these patients, the procedure appears to be curative.[59] However, outcomes beyond this time frame have not been well studied. The response rate in the 1st week is nearly 85%, with 20% of patients relapsing within weeks to years.[59] However, even after relapse, patients appear to be less refractory to medical treatment.[59] Patients older than 45 years appear to have a lower response to splenectomy.[59] At present there are no readily available clinical or laboratory markers that are predictive of response to splenectomy, although one report suggests that hepatic sequestration of autologous indium-111–labeled platelets maybe predictive of a poor response.[176]

Laparoscopic splenectomy is associated with lower complication rates and faster recovery, no greater need for accessory splenectomy, and no loss in efficacy. The efficacy of splenectomy decreases with increasing age above age 45 years; this means that the higher response rates to splenectomy are in patients with the greatest potential for late complications due to their longer lifespan postprocedure. The risk of postsplenectomy sepsis has been greatly reduced by immunization for pneumococcus, haemophilus influenza, and meningococcus.[59] Patients should have yearly influenza vaccinations and if a fever occurs, the patient should immediately receive parenteral broad spectrum antibiotics that are effective against pneumococcus. This is true no matter how many years postsplenectomy and despite immunization. Other long-term outcomes after splenectomy are unknown; particularly in regard to uncommon events such as pulmonary hypertension or vascular dementia.[177–179]

Anti-CD20 Therapy (Rituximab)

B lymphocyte depletion using rituximab, a humanized anti-CD20 monoclonal antibody, has shown limited, but reproducible efficacy in patients with CITP.[180,181,182] Nearly 60% of patients will have an initial increase in their platelet count, with an estimated one-third of patients obtaining an initial (1 year) unmaintained complete response (platelets $>100 \times 10^9$/L) with the use of the standard lymphoma regimen of 375 mg/m^2 rituximab weekly for 4 weeks.[180,181,182] Nearly all partial responders will relapse within 1 year, while nearly all complete responders will maintain their response for at least 1 year. Approximately one half of complete responders will relapse within 4 more years. Although repeat treatment of the relapsed complete responder can result in a high rate of response,[183] the safety of this approach has not been fully evaluated. Concerns with the use of anti-CD20 therapy include development of progressive multifocal leukoencephalopathy[184] and reactivation of hepatitis B, but the risk may be lower than reported in patients with lymphoma and other systemic autoimmune disorders since patients with ITP are generally not intrinsically immunosuppressed by their disease or receiving additional immunosuppressive therapies. Common, but less serious, toxicities include first infusion reactions such as fever and chills and serum sickness, the latter especially common in children.[185] Although total immunoglobulin levels rarely decrease, patients cannot mount a normal humoral antibody response to some vaccines for months after treatment. The optimal dose and frequency of rituximab administration for the treatment of ITP remains unknown.[186]

Other Second-Line Therapies with Documented Efficacy

Azathioprine has documented efficacy for ITP patients' refractory to first-line treatment. In one retrospective series, the overall response rate was 64% with 45% of patient obtaining a normal platelet count. The median time to response was 4 months. Responses tend to be maintained, but reported follow-up was short.[187] Toxicities can include nausea, leucopenia, hepatitis, and occasionally pancreatitis.

Cyclosporin A has been effective when given in combination with prednisone.[188] Improvement in platelet count occurred in 80% of patients with 42% of patients obtaining a complete response. Some remissions maybe durable even after the drug is discontinued.[189] Side effects include hypertension, neuropathy and renal toxicity.

Cyclophosphamide, given orally (1 to 2 mg/kg daily for at least 16 weeks) or intravenously (0.3 to 1 g/M^2 every 2 to 3 weeks) has demonstrated efficacy in the treatment of adults with refractory ITP who have failed splenectomy, with reported response rates ranging from 24% to 85%.[190–192] Toxicities may be significant, including severe leucopenia, hemorrhagic cystitis, possible sterility in men and the rare late complication of acute leukemia.

Danazol is an attenuated androgen analogue that is often effective as a corticosteroid-sparing agent when administered orally at doses of 200 to 800 mg daily.[193] Approximately 50% to 65% of patients respond, but nearly all relapse within 1 year of stopping the drug. Toxicities include virilism in women, elevated liver enzymes, skin rashes, myalgia, and rare hepatic adenomas. There may be an enhanced risk of venous thrombosis when higher doses are used.

Mycophenolate mofetil is an immunosuppressive agent predominately used to reduce the risk of solid-organ transplantation rejection. It inhibits inosine monophosphate dehydrogenase activity resulting in inhibition of T and B lymphocyte proliferation. Several case series have documented responses in refractory patients.[194,195] In one report, 7/18 refractory patients sustained an increased platelet count of $>50 \times 10^9$/L.[195] Treatment dose ranged from 250 mg twice daily to 500 mg twice daily in adults and 250 to 750 mg/day in children.[195] Toxicities include headaches, nausea, vertigo, and low white blood cell count.

Thrombopoietin Receptor Agonists: Romiplostim and Eltrombopag

Romiplostim was the first TPO receptor agonist to enter clinical trials.[196] Subsequently two randomized, placebo controlled studies were published, one in splenectomized patients and the other study in nonsplenectomized patients.[197] In both studies, the same dosing regimen was used with progressive weekly increases in dose depending on the platelet count, beginning at 1 μg/kg and increasing to 18 μg/kg. Rescue medications were allowed for overt bleeding or very low counts associated with a high risk of

bleeding. Concomitant medications such as prednisone could be decreased or stopped as the platelet count increased.

In both trials, unequivocal efficacy was demonstrated. Durable response (platelets ≥50 × 10^9/L for 6 of the last 8 weeks and no rescue therapy was seen in 38% of splenectomized patients and 61% in nonsplenectomized patients. The overall response rate (platelet counts ≥50 × 10^9/L on 4 of 24 weeks) was 79% in splenectomized patients and 88% in nonsplenectomized patients. There were no responses in the splenectomized patients and only 14% in nonsplenectomized patients taking placebo. There were statistically significant reductions in the use of concomitant medications such as prednisone and reduced use of rescue medications (IVIG, anti-RhD, or prednisone). No tachyphylaxis was seen over the 6 months of study and no important toxicities emerged, such as an increased incidence of thromboembolic events or malignancies. Rare instances of increased bone marrow reticulin were observed in patients at the higher doses: for this reason the upper dose recommended for treatment is now 10 μg/kg. "Rebound" thrombocytopenia with platelet counts decreasing to less than pretreatment baseline levels occurred in up to 5% to 10% of patients who stopped treatment abruptly. Romiplostim was licensed in August 2008 based on these two studies.

Patients who entered one of the two previous studies were eligible to enter a long-term maintenance study that validated the efficacy and safety of romiplostim use for up to 3 years of follow-up.[198] Overall, this study showed that long-term use of romiplostim as a maintenance therapy was feasible. Platelet counts could be maintained for years without a need to significantly increase the dose. Many patients learned to give weekly injections at home. No important new toxicities were identified with extended use. Nine cases of bone marrow reticulin fibrosis were identified, but no cases of myelofibrosis/myeloid metaplasia. Whether this incidence is significantly higher than occurs in patients with ITP successfully treated with other agents requires additional study.

An open-label 52-week randomized trial of romiplostim versus standard of care reported a higher rate of platelet response, lower incidence of treatment failure and splenectomy, and less bleeding in the romiplostim-treated patients.[199] However, utility beyond 52 weeks compared to patients who were treated with splenectomy is unknown. Another study has reported safety and efficacy in 22 children.[200]

Eltrombopag is the first oral TPO receptor agonist to complete clinical trials and to be licensed for use in the United States. In the initial published study, eltrombopag was given for 6 weeks in daily doses of placebo, 30, 50, and 75 mg and results were compared to patients given placebo.[201] Responses were seen in 70% to 80% of patients at the two highest eltrombopag doses compared to 11% in those receiving placebo.[201] No important toxicity was seen and bleeding decreased as the platelet counts increased. A second study reported similar results for thrombocytopenic patients with hepatitis C infection, allowing continued antiviral chemotherapy without dose reduction or interruption.[156]

A third confirmatory study was a 6-week randomized placebo controlled trial comparing 50 mg of Eltrombopag to placebo.[202] It demonstrated a 59% response rate in the Eltrombopag arm, based on an increase in platelet count to >50 × 10^9/L, compared to a 16% increase in platelet count in those taking placebo. Bleeding manifestations were decreased in responders, but returned to baseline levels after the medication was discontinued and platelet counts declined. In this study, rebound thrombocytopenia upon drug withdrawal was rare and no statistically significant organ toxicity was seen. However, some patients developed significant liver enzyme increases requiring cessation of medication. Accordingly, the FDA has required a black box warming concerning the need to monitor liver function in patients taking Eltrombopag.[202]

Other Eltrombopag studies have been reported in only abstract form. These include a 6-month randomized study trial comparing Eltrombopag to placebo with results comparable to the 6-month pivotal trial of romiplostim, as well as a long-term extension study.[203] Also long-term follow-up of all patients enrolled in the eltrombopag trial found a lower frequency of thromboembolic events than suggested in earlier reports.[204]

Single agent treatment of refractory ITP with azathioprine, danazol, cyclophosphamide, cyclosporine A, and mycophenolate mofetil has limited efficacy; no single agent induces unmaintained remission in more than 30% of refractory cases and this is particularly true in patients refractory to splenectomy.[205] Even autologous stem cell transplants have only been found to have a 25% to 33% response rate and are without multiyear follow-up.[206] Therefore, the higher response rates reported in postsplenectomy refractory patients treated with TPO receptor agonists suggest that these would be the drugs of choice for this group of patients. These agents also lessen the need for immunosuppressive drugs, thereby reducing the risk of infection in this high-risk patient population.

Combination chemotherapy has been utilized in some situations to increase response rate and decrease the toxicity of single agents. The first report involved a patient with HD and refractory ITP whose thrombocytopenia responded to six cycles of CHOP chemotherapy. Treatment of additional patients was associated with significant toxicity, but good responses were seen in some patients.[207] Long-term follow-up showed that all, but one responder, maintained the response over many years.[208] Boruchov et al. studied a multiagent therapy regimen in highly refractory patients using initial IVIG and methylprednisolone or intravenous anti-RhD and vincristine, or all four agents together, followed by danazol plus azathioprine in combination. The latter combination was effective in 13 of 17 patients, but the duration of response was brief in many patients.[209]

POSTTRANSFUSION PURPURA

Posttransfusion purpura (PTP) is a syndrome characterized by the development of severe thrombocytopenia 5 to 10 days following the transfusion of blood products. The vast majority of cases occur in women (90%), nearly all of whom have a history of multiple pregnancies or transfusion of blood product.[210,211,212] The initiating transfusion most often consists of red blood cells, but episodes of PTP have occurred after platelet transfusion and even plasma infusion.[210,211,212,213] In 80% of cases, the recipient of the transfusion is homozygous for the Pl^{A2} [HPA-1b] platelet GPIIIa polymorphism. In this case, patient serum is found to contain an alloantibody against the Pl^{A1} [HPA-1a] platelet antigen.[210,212,214] Alloantibodies against other platelet antigen polymorphisms, and occasionally HLA antigens, have been reported in other cases of PTP, though the latter remains controversial.[210,212,215–217] Prior sensitization in women occurs most frequently during pregnancy. Alloimmunization to the Pl^{A1}

antigen has been shown to occur in 1 of every 1,000 pregnancies in the white population or in approximately 6% of homozygous Pl^{A2} female carriers.[218] In a study of transfusion-induced platelet reactive antibodies, 8.1% of previously untransfused patients developed a mix of pan-reactive antibodies against the glycoproteins IIb/IIIa, Ia/IIa, and Ib/IX.[219] A conservative estimate is that PTP occurs after 1:50,000 to 100,000 transfusions, suggesting that many cases go unreported or misdiagnosed.[220] The lower than expected frequency of PTP may also be explained by genetic restriction on antibody production. PTP and neonatal allo ITP have been linked to the HLA B8 and DRB30101.[218,221,222]

Despite the clear serologic finding of platelet alloantibodies in the majority of cases of PTP, the pathophysiologic mechanism by which ITP develops in patients with PTP remains unknown. Although a number of hypothetical mechanisms have been proposed, none have been definitively demonstrated in the majority of patients. A hypothesis that PTP is initiated by the binding of antibody–antigen immune complexes to autologous platelets has never been convincingly demonstrated[223] nor has the proposal that Pl^{A1} antigen is absorbed by autologous platelets and platelets are subsequently destroyed *via* an innocent bystander mechanism.[224,225] Moreover, neither of these mechanisms account for the observation that prior to the use of plasmapheresis and IVIG therapy, thrombocytopenia in some cases of PTP has persisted for >30 days.[226,227] A more tenable hypothesis is that the infusion of foreign platelet antigens results in the transient development of a cross-reactive antiplatelet autoantibody.[228,229] Infusions of Pl^{A1}-negative (Pl^{A2} restricted) platelets did not increase the platelet counts in some patients with PTP, suggesting an antigenic target different from this polymorphism.[230,231] Moreover, in one report, reinfusion of plasma from a patient with PTP after platelet recovery failed to reinduce thrombocytopenia.[224] Also, repeat transfusion of Pl^{A1}-positive blood into a patient with a prior history of PTP has failed to induce a new episode of thrombocytopenia.[232]

Once thrombocytopenia develops in PTP, platelet counts rapidly fall to levels that are usually below 10×10^9/L.[210,211,212] Bleeding manifestations are common, including epistaxis, petechiae, ecchymosis, gingival bleeding and genitourinary bleeding.[210,211,212,227] Intracranial bleeding has been reported in 5% to 8% of patients.[210,211,212,227] The severity of bleeding appears to be greater than that observed with primary ITP or drug-related thrombocytopenia. This may be due in part to the fact that PTP frequently develops in the postsurgical setting following blood transfusion and in patients with significant comorbidities. A number of cases have been misdiagnosed as heparin-induced thrombocytopenia or other drug-induced thrombocytopenia based upon the time of onset of thrombocytopenia and the fact that many patients are simultaneously receiving medications associated with the development of thrombocytopenia.[233]

While the diagnosis of PTP is primarily based on patient history and clinical finding, serologic studies are required to confirm the presence of platelet alloantibodies and the absence of the corresponding antibody on the patient platelets following recovery.[210,211,212,215,216,223] International reference sera are now available to detect antihuman platelet alloantigens and alloantibodies and many referral centers have donors that are known to be HPA-1b homozygotes or that lack or possess other phenotypes occasionally implicated in PTP.[234,235] Patient platelet genotype can be determined with serologic or molecular methods.[240] Patient platelet genotyping may be of further value in family counseling regarding screening, for example, female siblings of child-bearing age.

PTP is self-limited. However, prolonged severe thrombocytopenia places patients at risk of potentially fatal bleeding. Therefore, PTP, though rare, should always be considered in the relevant clinical context and treated as an emergency. The efficacy of corticosteroids cannot be adequately determined due to the variable natural history.[210,211,212,236,237] Exchange transfusion appears to be effective in shortening the duration of severe thrombocytopenia. However, recurrent thrombocytopenia can occur once exchanges have been stopped and the procedure is both labor intensive and cumbersome.[210,230,238] IVIG, as used in the treatment of ITP, is highly effective and safe for the treatment of PTP and is the treatment of choice.[210,211,212,239] When given with corticosteroids, infusion reactions are rare and most patients respond by increasing platelet counts to a safe level within 2 to 7 days.[239] An occasional patient will relapse, but will respond to a second course of high dose immunoglobulin.[239]

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Full Reference Lists can be found online

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CHAPTER

62 Drug-Induced Thrombocytopenia

RICHARD H. ASTER AND JAMES N. GEORGE

BACKGROUND

Drugs induce thrombocytopenia through various mechanisms. The inhibition of platelet production is an expected result of the dose-dependent toxicity of chemotherapeutic agents. Rarely, pancytopenia (aplastic anemia) results from idiosyncratic drug hypersensitivity. A few drugs suppress platelet production selectively. A much larger number cause thrombocytopenia by promoting platelet destruction that can be acute and severe and is usually caused by drug-dependent antibodies. Mechanisms by which drugs are known to cause thrombocytopenia are summarized in Table 62.1.

In this chapter, we consider drug-induced thrombocytopenia according to pathogenesis, clinical presentation, diagnosis, and treatment. The drug implicated most often as a cause of thrombocytopenia is heparin. Heparin-induced thrombocytopenia (HIT) has a unique pathogenesis, and thrombosis, rather than thrombocytopenia, is the major concern in patients with this condition. HIT is discussed in Chapter 108 and is mentioned here only briefly.

INCIDENCE

The annual incidence of drug-induced thrombocytopenia has been estimated in four studies to be approximately 1 case per 100,000 annually[1,2,3,4] (Table 62.2). However, these data are the result of reporting to national surveillance groups or reviewing hospital records. It can be reasonably assumed that many patients suspected of having drug-induced thrombocytopenia are not reported and that affected patients often do not require hospitalization. It is likely therefore that published estimates greatly underestimate the frequency of this condition. Another cause of underestimation may be the tendency to misdiagnose drug-induced thrombocytopenia as acute autoimmune thrombocytopenic purpura (AITP).[5]

SUPPRESSION OF PLATELET PRODUCTION BY DRUGS

Chemotherapeutic Agents

Thrombocytopenia is a common, dose-dependent side effect of chemotherapy used for treatment of cancer and for immunosuppression. All myeloid elements are likely to be affected, but thrombocytopenia is often dose limiting. Various cytokines and leukokines, including IL-6 and IL-11, have been used to reduce the severity of thrombocytopenia in patients receiving chemotherapy,[6] but only IL-6 has been approved for this purpose. Thrombopoietin receptor agonists are not yet approved but show promise and are in various stages of evaluation.[7] Profound thrombocytopenia sometimes occurs in patients treated with antiviral agents such as azidothymidine used to treat human immunodeficiency virus infection.

It should not be assumed a priori that thrombocytopenia associated with chemotherapy is caused by inhibition of megakaryocytopoiesis. Patients have been described who experienced an acute and very severe drop in platelet levels when treated with agents such as oxaliplatin, cyclosporine A, irinotecan, and suramin despite the demonstration of adequate numbers of megakaryocytes in aspirated bone marrow.[8] In such instances, appropriate serologic testing may demonstrate the presence of drug-dependent, platelet-reactive antibodies capable of causing platelet destruction when drug is administered. Systemic reactions such as chills, fever, and hypotension occurring at the time of drug administration can provide a clue to the presence of such an antibody.

Drug-Induced Aplastic Anemia

Numerous medications are thought to be capable of causing aplastic anemia on an idiosyncratic basis. Implicated drugs include anticonvulsants, nonsteroidal anti-inflammatory drugs, and sulfonamides. The pathogenesis of this complication is poorly understood,[9,10] and there is at present no means of identifying patients at risk to develop this complication.

Drugs that Selectively Inhibit Megakaryocytopoiesis

Alcohol

Thrombocytopenia, sometimes severe, is common in persons who ingest large quantities of alcohol for long periods of time.[11] Although vitamin deficiency and/or splenomegaly and cirrhosis contribute to the lowering of platelet levels in some cases, there is evidence that ethanol can directly affect megakaryocytopoiesis. In general, thrombocytopenia is mild in patients with chronic alcoholism, but very low platelet counts and bleeding have been described. After abstinence for a few weeks, platelet levels usually return to the normal range, but moderate thrombocytopenia persists for longer periods in some patients.

Estrogens

Large doses of estrogens adversely affect megakaryocytopoiesis, leading to profound thrombocytopenia in some species of animals, especially dogs.[12] Rare cases of thrombocytopenia associated with megakaryocyte dysplasia in humans treated

Table 62.1

Classification of drug-induced thrombocytopenia

Decreased Platelet Production
Generalized suppression of hematopoiesis (dose-dependent toxicity; e.g., chemotherapeutic agents)
Idiosyncratic aplastic anemia (e.g., anticonvulsants, sulfonamides, nonsteroidal anti-inflammatory drugs)
Selective suppression of platelet production (e.g., anagrelide, valproic acid)
Platelet destruction through nonimmune mechanisms (e.g., desmopressin, protamine sulfate)
Platelet Destruction Through Immune Mechanisms
Hapten-dependent antibodies (penicillin?)
"Quinine-type" immune thrombocytopenia (e.g., quinine, sulfamethoxazole, vancomycin, piperacillin
Drug-induced, platelet-specific autoantibodies (e.g., gold salts, procaine amide)
Thrombocytopenia in patients treated with ligand-mimetic platelet inhibitors (e.g., tirofiban, eptifibatide)
Abciximab-induced thrombocytopenia
Drug-induced immune complexes (heparin)

with estrogens have been described. It has been suggested that estrogens can exacerbate thrombocytopenia in women with autoimmune thrombocytopenia.

Histone Deacetylase Inhibitors

Histone deacetylase inhibitors (HDACi), a new class of chemotherapeutic drugs, regularly produce a degree of thrombocytopenia in treated patients, an effect that limits their use in some individuals.[13] Animal studies suggest that HDACi act directly on megakaryocytes to inhibit maturation and proplatelet formation[13] through a mechanism not fully understood. Preclinical studies provide evidence that HDACi-induced thrombocytopenia can be ameliorated by treatment with a thrombopoietin receptor agonist. Valproic acid, although mainly used as an anticonvulsant, is an HDACi and causes mild thrombocytopenia in up to one-third of treated individuals.[14] Rarely, thrombocytopenia is acute and severe.

Linezolid

Linezolid is an oxazolidinone antibiotic used for treatment of drug-resistant bacterial infections. Low-grade thrombocytopenia occurs in 30% to 40% of individuals treated with this drug for more than 10 days.[15] Patients with renal dysfunction and those given the drug intravenously[15] are more likely to experience a reduction in platelet levels. There is evidence that linezolid acts to suppress megakaryocytopoiesis through a dose-dependent mechanism.

Imatinib Mesylate

Thrombocytopenia, often accompanied by neutropenia, occurs in up to 70% of patients treated with imatinib mesylate for chronic myelogenous leukemia.[16] The mechanism of this effect is poorly understood. Responses to granulocyte colony stimulating factor and IL-11 have been described.

Anagrelide

The quinazoline compound anagrelide causes isolated thrombocytopenia with little effect on other aspects of hematopoiesis.[17] Animal studies suggest that anagrelide interferes with megakaryocyte maturation, possibly through the action of a metabolite, an effect that makes it useful for treating thrombocythemia in patients with myeloproliferative conditions. In rare cases, profound thrombocytopenia has occurred in patients given anagrelide for this purpose.

Table 62.2

Incidence of drug-induced thrombocytopenia

Country	Years	Method	Annual Incidence
Sweden[a]	1966–1970	Reports to Swedish Adverse Reaction Committee	1/100,000
Denmark[b]	1968–1991	Reports to Danish Committee on adverse drug reactions	1/100,000
USA[c]	1972–1981	Hospital records, Puget Sound Group Health Cooperative	0.6/100,000
USA[d]	1983–1991	Hospital records, case control study	1.8/100,000

[a]Bottiger LE, Bottiger B. Incidence and cause of aplastic anemia, hemolytic anemia, agranulocytosis and thrombocytopenia. *Acta Med Scand* 1981;210:475–479.
[b]Pedersen-Bjergaard U, Andersen M, Hansen PB. Drug-specific characteristics of thrombocytopenia caused by noncytotoxic drugs. *Eur J Clin Pharmacol* 1998;54:701–706.
[c]Danielson DA, Douglas SW III, Herzog P, et al. Drug-induced blood disorders. *JAMA* 1984;252:3257–3260.
[d]Kaufman DW, Kelly JP, Johannes CB, et al. Acute thrombocytopenic purpura in relation to the use of drugs. *Blood* 1993;82:2714–2718.

Interferons

Low-grade thrombocytopenia is a common side effect of interferon therapy. Profound thrombocytopenia and bleeding have been described in patients with chronic hepatitis C given interferon α and pegylated interferon-2b.[18] Interferons α and γ inhibit megakaryocyte proliferation in cell culture models and stem cell differentiation in animals, but whether this is relevant to thrombocytopenia developing in patients treated with interferons is uncertain.

DRUG-INDUCED PLATELET DESTRUCTION THROUGH NONIMMUNE MECHANISMS

Leukokines and Hematopoietic Growth Factors

An acute drop in platelet levels has been described in patients treated with granulocyte–macrophage colony-stimulating factor. Activation of macrophages and monocytes in the liver and spleen has been implicated as a possible cause. Macrophage colony-stimulating factor may exert a similar effect in rare individuals.

Tumor Necrosis Factor α and Interferon γ

Acute, severe thrombocytopenia has been described in a small number of patients following treatment of Ewing sarcoma with tumor necrosis factor α combined with interferon γ. A direct effect of the cytokines on endothelium, leading to increased platelet clearance has been postulated as a possible mechanism.

Porcine Factor VIII

Patients given porcine factor VIII for treatment of hemophilia often experience mild thrombocytopenia. Occasionally, a profound drop in platelet levels occurs. Hypersensitivity symptoms such as chills, fever, and urticaria sometimes accompany these reactions. Bovine von Willebrand factor (vWF) contaminating the Factor VIII preparations has been implicated. Whether xenoantibodies specific for porcine factor VIII contribute to this effect has not been established.

Desmopressin

Desmopressin (DDAVP) is used for treatment of some patients with von Willebrand disease (vWD) because of its ability to induce vWF release from endothelial cells.[19] Patients with type 2B vWD, who possess a vWF mutant that binds to the platelet vWF receptor with high affinity, sometimes experience severe thrombocytopenia after treatment with DDAVP. Patients with "platelet-type" vWD, who have a mutant vWF platelet receptor, can also develop thrombocytopenia following DDAVP administration.

Protamine Sulfate

Protamine sulfate is widely used to neutralize circulating heparin following open-heart surgery. Mild thrombocytopenia often develops after protamine administration, but it can be difficult to ascertain whether this is a consequence of protamine infusion or is representative of low-grade thrombocytopenia routinely seen after this procedure. However, sequestration of indium 111–labeled platelets in the liver was demonstrated in a cohort of patients given protamine. Protamine is known to bind to chondroitin sulfate normally found on the platelet surface[20] but whether this interaction is related to transient platelet sequestration following protamine infusion is uncertain.

Amrinone

The pyridine phosphodiesterase-3 inhibitor amrinone is widely used for treatment of heart failure. Up to one-third of individuals given amrinone experience low-grade thrombocytopenia soon after starting the drug. The mechanism of this effect is uncertain, but the *N*-acetyl metabolite of the drug was implicated in one study.

DRUG-INDUCED PLATELET DESTRUCTION THROUGH IMMUNE MECHANISMS

Pathogenesis

As summarized in Table 62.3, drug-dependent, antibody-mediated immune thrombocytopenia is caused by several distinctly different mechanisms.

Hapten-Dependent Antibodies

It is generally thought that low molecular weight compounds like drugs induce antibodies only when they are covalently linked to a larger carrier molecule, usually a protein. Accordingly, it was assumed for many years that immune thrombocytopenia in patients sensitive to drugs is caused by antibodies raised against drug covalently linked to a platelet membrane glycoprotein. However, antibodies identified in such patients did not exhibit the behavior expected of a hapten-dependent immunoglobulin since they could not be inhibited by excess concentrations of drug and they failed to react with platelets pretreated with drug and then washed. It is now thought that classical "hapten-dependent antibodies" rarely, if ever, cause immune thrombocytopenia. However, penicillin and penicillin derivatives, which spontaneously form covalent linkages with cell membrane proteins, do on occasion induce true hapten-dependent antibodies capable of causing immune hemolytic anemia.[10] By analogy, it is possible that thrombocytopenia occurring rarely in patients treated with penicillin and structurally similar drugs is caused by this mechanism. However, this has not been confirmed experimentally.

"Quinine-Type" Immune Thrombocytopenia

Sudden and sometimes life-threatening thrombocytopenia following treatment with medication was described as a clinical entity more than 120 years ago. The responsible antibodies are unusual in that they are completely harmless in the absence of drug, but in its presence, bind tightly to specific platelet membrane glycoproteins and cause platelet destruction.[8,21] Several hundred different drugs have been implicated, but quinine, quinidine, various antibiotics, nonsteroidal anti-inflammatory drugs, and the chemotherapeutic drug oxaliplatin are among those that trigger this condition most often.[8,21,22] Since quinine is the prototypic drug associated with this condition, we will refer to it as "quinine-type" thrombocytopenia. Antibodies found in patients with "quinine-type" thrombocytopenia are unique among pathogenic immunoglobulins in several respects: (a) They bind tightly to epitopes on platelet membrane glycoproteins at the highest

Table 62.3

Mechanisms of drug-induced immune thrombocytopenia and commonly implicated medications[a]

Designation	Mechanism	Examples
Hapten-dependent antibody	Drug (hapten) links covalently to membrane protein and induces a drug-specific immune response.	Penicillin, cephalosporin antibiotics?
"Quinine-type" thrombocytopenia	Drug induces antibody that binds to membrane protein only in the presence of soluble drug.	Quinine, many antibiotics, nonsteroidal anti-inflammatory drugs, anticonvulsants
Autoantibody induction	Drug induces antibody that reacts with platelets in the absence of drug.	Gold salts, L-Dopa, procainamide
Fiban-induced thrombocytopenia	Drug (ligand) reacts with membrane glycoprotein IIb/IIIa and induces a conformational change recognized by naturally occurring antibody?	Eptifibatide, tirofiban
Drug-specific antibody	Naturally occurring or induced antibody is specific for the murine component of abciximab, a chimeric Fab fragment specific for GP IIIa.	Abciximab
Immune complex	Drug binds to PF4 to produce a complex for which antibody is specific. The resulting immune complex activates platelets via Fc receptors.	Heparin

[a]Adapted from Aster RH, Curtis BR, McFarland JG, et al. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis, and management. *J Thromb Haemost* 2009;7:911–918.

achievable drug concentration, rather than being inhibited as might be expected of a "hapten-dependent" antibody. (b) They fail to recognize platelets pretreated with the sensitizing drug. (c) They recognize only glycoproteins found on human or primate platelets. (d) They dissociate from the target glycoprotein upon being suspended in buffer in the absence of drug.[8,23]

Various mechanisms have been postulated to explain how a soluble drug might promote high-affinity but reversible binding of this type of antibody to a target glycoprotein. This issue is not fully resolved, but it has been shown experimentally that the reaction is mediated by the Fab, rather the Fc, immunoglobulin domain, ruling out the possibility that the antibodies form some type of drug-dependent "immune complex" that reacts with platelets via Fc receptors as was formerly thought. An alternative concept advanced to explain this interaction proposes that the drug binds to a specific site on a membrane glycoprotein and somehow reconfigures it so that an epitope recognized by antibody is created. If this were the case, it would be expected that binding of drug to the target would be sufficiently stable to allow cells pretreated with drug to be recognized by antibody, but this does not hold for the great majority of drugs known to cause "quinine-type" thrombocytopenia. A recently advanced proposal supported by experimental data calls for drug to act by enhancing the "fit" between low avidity, naturally occurring immunoglobulins, and localized domains on platelet glycoproteins, especially GP IIb/IIIa and GP Ib/IX[8,21,24] (FIGURE 62.1). This concept proposes that one structural element of drug reacts with the antigen-combining site of antibody and another binds to the target glycoprotein, improving the fit between the two macromolecules sufficiently for binding to occur at concentrations of the three reactants achievable in the circulation when drug is administered. By analogy, it is possible that a drug-dependent fit between a B-cell receptor and a platelet glycoprotein is involved in the induction of "quinine-type" antibodies, but there is no direct evidence to support this.

Drug-Induced, Platelet-Specific Autoantibodies

About 1% of patients injected with gold salts, formerly used to treat rheumatoid arthritis and other inflammatory conditions, developed immune thrombocytopenia that behaved clinically like autoimmune thrombocytopenia unrelated to drug exposure. A condition resembling AITP clinically occurs occasionally in patients treated with levodopa, procaine amide, and other drugs.[25] Patients have been described who developed both drug-dependent antibodies and drug-independent autoantibodies simultaneously.[25] The mechanism by which drugs sometimes induce platelet-specific autoantibodies is not understood.

Thrombocytopenia in Patients Treated with Ligand-Mimetic Platelet Inhibitors

RGD (arginine-glycine-aspartic acid)-mimetic fibrinogen receptor antagonists (fibans) inhibit platelet function by binding to the RGD recognition site on the platelet GP IIb/IIIa glycoprotein complex and blocking its interaction with fibrinogen. The inhibitors tirofiban and eptifibatide are widely used to reduce posttreatment complications in patients undergoing percutaneous transluminal coronary angiography, and other drugs of this class are in development. Acute, severe thrombocytopenia

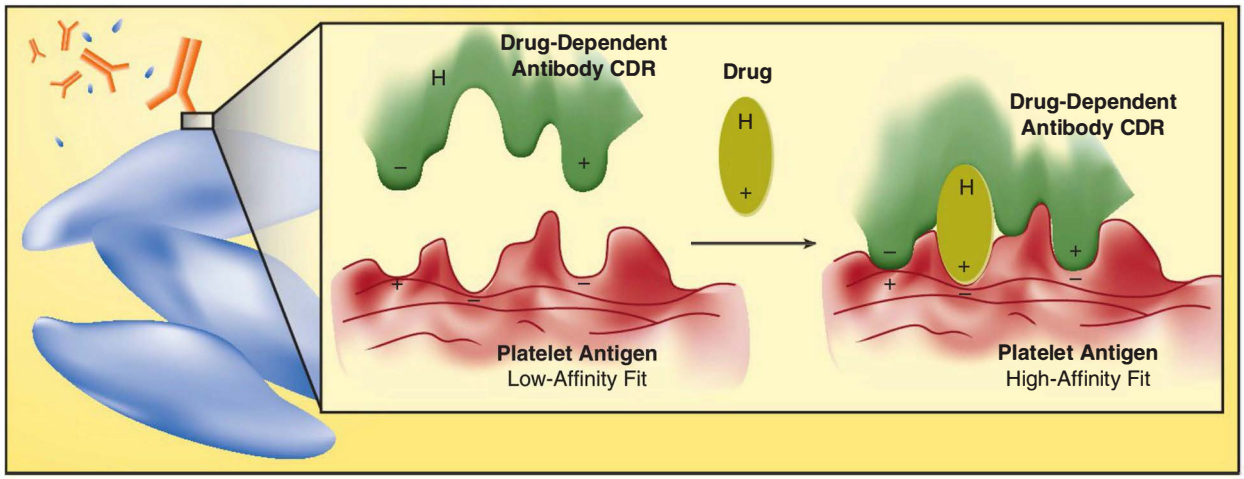

FIGURE 62.1 Model for the binding of a drug-dependent antibody to an epitope on a platelet glycoprotein. Antibodies capable of causing drug-dependent thrombocytopenia react weakly with an epitope on a target glycoprotein. The binding affinity (Ka) for this interaction is too low to allow a sufficient number of antibody molecules to bind in the absence of the drug ("low-affinity fit"). The drug contains structural elements that are complementary to a negatively charged site on the glycoprotein and a hydrophobic site (H) on the complementarity-determining region (CDR) of the antibody. The drug interacts with these sites to improve the fit between the two proteins, increasing the Ka to a value that permits binding to occur at levels of antibody, antigen, and drug achieved in the circulation after ingestion of the drug ("high-affinity fit") (From Aster RH, Bougie DW. Drug-induced immune thrombocytopenia. *N Engl J Med* 2007;357:580–587.)

occurs in 0.1% to 1% of patients given fiban-type platelet inhibitors, often within a few hours of the first exposure.[26,27] Although most affected patients recover uneventfully, fatal bleeding has been described. Paradoxical thromboembolic complications have occurred in some patients with fiban-induced thrombocytopenia and may be related to the tendency of some antibodies to activate platelets through Fc receptors, thereby inducing procoagulant activity.[28] When RGD and RGD-mimetic compounds bind to the RGD recognition site on GP IIb/IIIa, the integrin undergoes structural changes that are recognized by certain monoclonal antibodies specific for "ligand-induced binding sites." By analogy, it is possible that antibodies causing thrombocytopenia in patients treated with fiban drugs are specific for neoepitopes induced in the integrin by binding of the RGD mimetic. However, this has not been confirmed by direct experiment. Remarkably, antibodies capable of causing severe thrombocytopenia in patients treated with fiban drugs can be present in patients never before exposed to one of these agents.

Abciximab-Induced Thrombocytopenia

Abciximab is a "humanized" Fab fragment that inhibits platelet function by binding to a site on GP IIIa close to the fibrinogen recognition site.[29] About 2% of patients given abciximab for the first time and a higher percentage of those given the drug a second time experience acute thrombocytopenia within 6 to 12 hours of exposure. Most patients recover uneventfully but intracranial hemorrhage and deaths have been described.

Abciximab-induced thrombocytopenia following first exposure to the drug is caused by naturally occurring antibodies that bind to abciximab-coated platelets.[30] Evidence suggests that these antibodies recognize murine peptide sequences incorporated into the abciximab Fab fragment to confer specificity for GP IIb/IIIa. Thus, abciximab-induced thrombocytopenia appears to be the only known example of a drug-induced immune thrombocytopenia in which the responsible antibodies react directly with drug bound to a platelet membrane glycoprotein.

In some patients, thrombocytopenia develops 5 to 10 days after abciximab administration. It is known that abciximab remains detectable on circulating platelets for as long as 2 weeks after a single dose, indicating that the drug can transfer from one platelet to another. Delayed thrombocytopenia following abciximab treatment is caused by abciximab-specific antibodies newly induced by drug exposure that bind to abciximab-coated platelets persisting in the circulation.[31] Such antibodies are often very potent and can cause life-threatening bleeding, often after discharge from the hospital and while other platelet inhibitors such as clopidogrel are being taken. It is prudent to advise patients given abciximab about this possible complication.

Patients given abciximab sometimes manifest "pseudothrombocytopenia," the result of platelet clumping in a blood sample anticoagulated with ethylenediamine tetraacetic acid. The mechanism responsible for this phenomenon is not well understood. Pseudothrombocytopenia is an *in vitro* artifact that should be distinguished from true thrombocytopenia by performing a platelet count in blood anticoagulated with citrate or by examining a blood film prepared from a finger stick.

Drug-Induced Immune Complexes

In theory, immune complexes present in the circulation could bind to platelet FcγRII receptors, causing platelet activation and thrombocytopenia. However, the extent to which this actually occurs is uncertain because of competition for the receptor between immune complexes and the very high concentration of monomeric IgG normally present in the circulation. A clear

Table 62.4

Clinical criteria to document a drug-induced etiology for thrombocytopenia

Criterion	Description
1. Therapy with the candidate drug preceded thrombocytopenia and recovery from thrombocytopenia was complete and sustained after therapy with the drug was discontinued.	
2. The candidate drug was the only drug used before the onset of thrombocytopenia, *or* other drugs were continued or reintroduced after discontinuation of therapy with the candidate drug with a sustained normal platelet count.	
3. Other causes for thrombocytopenia were excluded.	
4. Reexposure to the candidate drug resulted in recurrent thrombocytopenia.	
Level of evidence	
Definite:	Criteria 1, 2, 3, and 4 met.
Probable:	Criteria 1, 2, and 3 met.
Possible:	Criterion 1 met.
Unlikely:	Criterion 1 not met.

exception is HIT in which antibodies react with the chemokine platelet factor 4 (PF4) when it forms a complex with heparin or with chondroitin sulfate, the major glycosaminoglycan on the platelet surface.[32] The resulting immune complexes activate platelets despite competition from normal plasma IgG because they are formed in close proximity to the Fc receptor. HIT is the most common form of drug-induced immune thrombocytopenia, but the reduction of platelet levels is rarely sufficient to cause bleeding. HIT is discussed in detail in Chapter 108.

Drugs Implicated in Immune Thrombocytopenia

As noted, a large number of drugs has been implicated in immune thrombocytopenia. When the connection is supported only by one or two case reports, the relationship could well be coincidental. George et al. regularly monitor the English language literature for reports of drug-associated thrombocytopenia using a defined set of criteria to evaluate the likelihood of a cause-and-effect relationship between drug exposure and a drop in platelet count on a scale of 1 to 4 (Table 62.4). These criteria are structured so that a score of 3 makes drug-induced thrombocytopenia "probable" and a score of 4 makes it "definite." This database is periodically updated and is available on the internet at http://www.ouhsc.edu/platelets. George et al. recently undertook a comprehensive analysis of drugs implicated in the pathogenesis of thrombocytopenia utilizing three independent databases: (a) published case reports of drug-associated thrombocytopenia analyzed by the criteria shown in Table 62.4; (b) detection of drug-dependent, platelet-reactive antibodies in a laboratory having extensive experience with this type of testing; (c) analysis of the adverse event reporting system of the Food and Drug Administration using "data mining" algorithms.[22] Twenty-three drugs were identified as having a possible or probable cause-and-effect relationship with thrombocytopenia by all three methods (Table 62.5). An extensive summary of these analyses involving a total of 1,444 drugs can be accessed on line as an appendix to Reese et al.[22] or at http://www.ouhsc.edu/platelets.

Detection of an antibody that binds to platelets only in the presence of a drug implicated as a cause of thrombocytopenia provides strong evidence for a cause-and-effect relationship. In the decade 1998 to 2008, the Platelet Neutrophil Immunology Laboratory of Blood Center of Wisconsin identified drug-dependent, platelet-reactive antibodies specific for 64 different medications in patients suspected of having drug-induced immune thrombocytopenia (Table 62.6). Antibodies detected

Table 62.5

Drugs found to be significantly associated with thrombocytopenia by three independent methods: analysis of published case reports, detection of drug-dependent, platelet-reactive antibodies, and data mining of the adverse events reporting system of the U.S. Food and Drug Administration[a]

Abciximab	Piperacillin
Acetaminophen	Quinidine
Ampicillin	Quinine
Carbamazepine	Ranitidine
Eptifibatide	Rifampin
Ethambutol	Simvastatin
Haloperidol	Sulfisoxazole
Ibuprofen	Tirofiban
Irinotecan	Trimethoprim–Sulfamethoxazole
Naproxen	Valproic Acid
Oxaliplatin	Vancomycin
Phenytoin	

[a]Adapted from Reese JA, Li X, Hauben M, et al. Identifying drugs that cause acute thrombocytopenia: an analysis using 3 distinct methods. *Blood* 2010;116:2127–2133.

Table 62.6

Drug-dependent, platelet-reactive antibodies detected 1998–2008[a]

Drug Category	#	Individual Drugs
ACE inhibitor	1–3	Lisinopril
Analgesic	1–3	*Acetaminophen*, propoxyphene
Antibiotic	>15	Sulfamethoxazole, vancomycin
	4–15	Ceftriaxone, levofloxacin, nafcillin, piperacillin, rifampin, trimethoprim
	1–3	Ampicillin, amoxicillin, cefazolin, cefadroxil, cefepime, cefpodoxime, ceftazidime, ceftizoxime, cefpodoxime, ciprofloxacin, ethambutol, lisinopril, loracarbef, metronidazole, nitrofurantoin, sulfisoxazole
Anticonvulsant	>15	Carbamazepine
	4–15	Phenytoin
	1–3	Lamotrigine, lorazepam, valproic acid
Antidepressant, antipsychotic	1–3	Amitriptyline, bupropion, Haldol, olanzapine, paroxetine, sertraline
Antithyroid	1–3	Propylthiouracil
Beta-blocker	1–3	Atenolol, propranolol
Cardiac	4–15	Amiodarone
	1–3	Dobutamine
Chemotherapeutic agent	4–15	Oxaliplatin
	1–3	Geldanamycin, irinotecan, suramin
Cinchona alkaloid	>15	Quinine, quinidine
Diuretic	4–15	Furosemide
GP IIb/IIIa inhibitor	>15	Abciximab, eptifibatide, tirofiban
	4–15	Orbofiban, xemilofiban
Histamine receptor antagonist	1–3	Fexofenadine, ranitidine
Narcotic	1–3	Fentanyl
Nonsteroidal anti-inflammatory	4–15	*Naproxen*
	1–3	Celecoxib, *ibuprofen*, oxaprozin
Proton pump inhibitor	1–3	Esomeprazole, lansoprazole, pantoprazole
Thrombin inhibitor	1–3	Argatroban
Vasodilator	1–3	Papaverine

#Indicates number of DDAbs identified. Italics = metabolite-specific antibodies
[a]Adapted from Aster RH, Curtis BR, McFarland JG, et al. Drug-induced immune thrombocytopenia: pathogenesis, diagnosis, and management. *J Thromb Haemost* 2009;7:911–918.

most often were specific for the cinchona alkaloids quinine and quinidine, 23 different antibiotics, particularly vancomycin, sulfamethoxazole, rifampin, piperacillin, and ceftriaxone, the anticonvulsants carbamazepine and phenytoin and the platelet inhibitors abciximab, eptifibatide, and tirofiban.[8]

Drug Metabolites as Triggers for Thrombocytopenia

Although detection of a drug-dependent, platelet-reactive antibody by a laboratory skilled in the required techniques provides strong evidence that the antibody caused thrombocytopenia, it is not unusual for negative test results to be obtained despite a history strongly suggestive of drug-induced immune thrombocytopenia. An important reason for this is that a drug metabolite formed *in vivo* can induce antibodies that are detectable only when the appropriate metabolite is used for testing. Since drugs often have numerous metabolites, many of which are not readily available, establishing that a drug metabolite caused immune thrombocytopenia is not a simple matter. However, it has been convincingly shown that two metabolites of acetaminophen (the glucuronide and sulfate conjugates), a metabolite of para-amino salicylic acid, the glucuronide conjugate of naproxen, and unidentified metabolites of ibuprofen and diclofenac can, when added to a reaction mixture containing serum from a patient with drug-induced thrombocytopenia, cause antibody binding to target platelets.[8,33,34] Further studies to define the range of drug metabolites causing sensitization leading to drug-induced thrombocytopenia

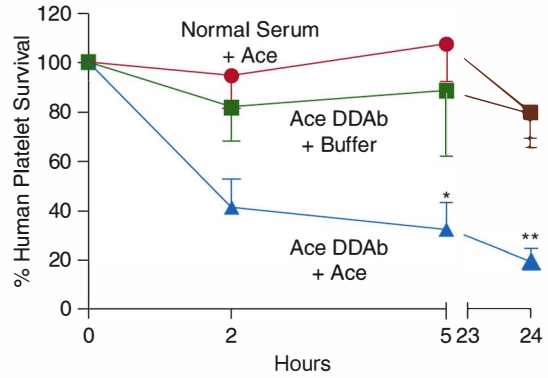

FIGURE 62.2 A drug metabolite produced *in vivo* promotes clearance of platelets by a metabolite-specific platelet antibody. Human platelets suspended in serum containing an antibody specific for acetaminophen glucuronide were transfused to NOD/scid mice. Acetaminophen was injected intraperitoneally at 0 and 5 hours. Platelet survival was significantly shortened in mice given both antibody and acetaminophen (*triangles*) but not in mice given antibody (*squares*) or drug (*circles*) with normal serum. Average of triplicate experiments 1 SEM. *$P < 0.05$, **$P < 0.01$, Ace, acetaminophen, DDAb, drug-dependent antibody. (From Bougie DW, Nayak D, Boylan B, et al. Drug-dependent clearance of human platelets in the NOD/scid mouse by antibodies from patients with drug-induced immune thrombocytopenia. *Blood* 2010;116:3033–3038.)

and other cytopenias could greatly improve laboratory diagnosis. Bougie et al.[35] recently adapted the NOD/scid mouse, in which human platelets circulate normally, to address this question. In this model, it was shown that following injection of the drugs acetaminophen and naproxen, metabolites were produced by the mouse that diffused into the circulation in quantities sufficient to cause destruction of platelets by a metabolite-dependent human antibody (FIGURE 62.2). Further use of this model may facilitate identification of other drug metabolites that cause sensitization leading to thrombocytopenia.

Clinical Presentation

Drug-induced immune thrombocytopenia is uncommon but should be considered in any patient who presents with unexplained acute thrombocytopenia. Patients should be asked specifically about exposure to quinine and sulfamethoxazole, which are among the most common triggers for immune thrombocytopenia. Nondrugs such as foods and herbal remedies have been shown to cause severe thrombocytopenia in rare instances. In seriously ill hospitalized patients who may have other causes for thrombocytopenia such as bacterial sepsis, it is easy to overlook a drug-induced etiology. In this situation, the antibiotics vancomycin,[36] piperacillin,[37] ceftriaxone, and rifampin should be given particular attention.

Patients with drug-induced thrombocytopenia are often considered initially to have autoimmune (idiopathic) thrombocytopenia (ITP). It is not uncommon for such individuals to be treated with corticosteroids, IV γ globulin, and platelet transfusions and for recovery to be attributed to these therapies rather than to withdrawal of drug. Extreme examples of patients, who presented as many as five times for acute "idiopathic" thrombocytopenia before a drug-induced etiology was documented, have been described.[5]

Patients presenting with acute, drug-induced thrombocytopenia have nearly always been exposed to the sensitizing drug during the preceding 24 hours. It has been noted that the platelet inhibitors abciximab, tirofiban, and eptifibatide are an exception to this rule, since "naturally occurring" antibodies capable of causing thrombocytopenia when these drugs are administered are present in a subset of normal persons.[26] Acute, immune thrombocytopenia after first exposure to piperacillin[37] and vancomycin[36] has also been described, but is rare. It is common for systemic symptoms such as flushing, chills, fever, and dizziness to occur soon after drug exposure. Severely affected patients often present with widespread cutaneous petechiae and ecchymoses as well as bleeding from the gums or the urinary or gastrointestinal tracts. Hemorrhagic bullae in the buccal mucosa indicate a very low platelet count. Some patients report to a dentist because of bleeding gums after brushing the teeth. When the responsible drug is discontinued, bleeding symptoms usually improve within 48 hours and the platelet count is restored to normal within 1 week. Occasionally thrombocytopenia persists for 1 or 2 weeks. In patients with vancomycin-induced thrombocytopenia, this may be a consequence of slow clearance of drug from the circulation, especially if there is renal dysfunction. Occasionally, patients present with coexisting immune hemolytic anemia and/or granulocytopenia; in such cases several different antibodies, each specific for one of the affected cell types, can sometimes be demonstrated.

Patients with thrombocytopenia caused by sensitivity to quinine and, rarely, other drugs sometimes present with profound renal failure and microangiopathic hemolytic anemia, a picture typical of the hemolytic uremic syndrome (HUS).[38] Why HUS sometimes accompanies drug-induced immune thrombocytopenia is unknown. Patients with this complication often require hemodialysis for several weeks before renal function is restored. HUS in patients presenting with drug-induced immune thrombocytopenia should not be confused with a similar condition seen after treatment with certain chemotherapeutic and immunosuppressive drugs in which spontaneous recovery is relatively uncommon. Patients treated with the platelet function inhibitors ticlopidine and clopidogrel for several weeks or longer occasionally experience severe thrombocytopenia together with other hematologic abnormalities and symptoms typical of thrombotic thrombocytopenic purpura (TTP) that often do not resolve spontaneously when drug is discontinued. The mechanism by which these drugs induce a TTP-like syndrome is not understood.

Laboratory Diagnosis

Drug-dependent antibodies capable of causing immune thrombocytopenia can often be detected by incubating normal platelets with the patient's serum in the presence and absence of the implicated drug and showing that antibody becomes bound to the target cell or glycoprotein when drug is present but not in its absence.[8,21] In patients sensitive to certain drugs such as vancomycin, quinine, and sulfamethoxazole, antibodies of this type can usually be detected and, when present, provide strong evidence that drug sensitivity is the cause of thrombocytopenia.

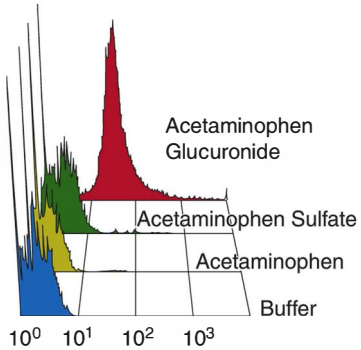

FIGURE 62.3 An antibody from a patient with acetaminophen-induced thrombocytopenia reacted with normal platelets in the presence of acetaminophen glucuronide, but not acetaminophen or acetaminophen sulfate. (From Bougie DW, Benito AI, Sanchez-Abarca LI, et al. Acute thrombocytopenia caused by sensitivity to the glucuronide conjugate of acetaminophen. *Blood* 2007;109:3608–3609.)

The most convenient, and probably the most sensitive method for antibody detection is flow cytometry (FIGURE 62.3). In general, such reactions are highly specific for the causative drug and it is usually safe to administer a pharmacologic equivalent possessing a different structure. When an antibody is not detected and the suspicion of drug-induced thrombocytopenia is very strong, a diagnostic challenge can be considered. However, it is important that very small amounts of drug, for example, 5 to 10 mg at most, be given initially and that the platelet count be carefully monitored. The potential risk of this procedure, although small, should of course be explained to the patient. If an *in vivo* challenge is to be undertaken, it should be done relatively soon after recovery of platelet levels since, although some drug-dependent antibodies persist for years, others decline and disappear over a period of several months. A recently described mouse model, in which drug-dependent platelet clearance by human antibodies can be demonstrated (FIGURE 62.2), may provide a useful surrogate for an *in vivo* challenge but requires validation in further studies.[35]

Prognosis and Treatment

For any patient suspected of having drug-induced immune thrombocytopenia, all drugs currently being taken should be discontinued and, where necessary, pharmacologic equivalents should be substituted. Patients with acute, profound thrombocytopenia and widespread petechiae and ecchymoses ("wet purpura") have a significant short-term risk of intracranial bleeding and should be treated aggressively. Whether glucocorticoids or intravenous immunoglobulin reduce the risk of serious bleeding is uncertain, but these therapies can be considered in patients with life-threatening hemorrhage. Platelet transfusions often fail to elevate the platelet count during the acute phase of thrombocytopenia, but can be given for their possible hemostatic effect. Plasma exchange has been used in severely ill patients, but whether this treatment accelerates the rise in platelet counts is unknown. Sensitivity to drugs that cause immune thrombocytopenia is usually permanent; patients should therefore be warned to avoid reexposure.

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CHAPTER

63 Inherited Thrombocytopenias

PAQUITA NURDEN AND ALAN T. NURDEN

Bleeding syndromes arising from an inherited defect of platelet production are an important group of rare diseases with a variety of causes.[1,2,3,4] Some, such as the Bernard-Soulier syndrome (BSS) and Wiskott-Aldrich syndrome (WAS), associate a low circulating platelet count with a deficiency of a known functional protein. In others, platelet dysfunction has not been shown and bleeding arises from the inability of megakaryocytes (MKs) to produce platelets in sufficient numbers to ensure hemostasis. In some, there may be associated illnesses such as an increased risk for leukemia or β-thalassemia; sometimes, developmental changes result in profound skeletal defects. Haploinsufficiency of transcription factors can lead to decreased expression of multiple platelet proteins. In many cases, a low platelet count is accompanied by changes in platelet morphology with macrothrombocytopenias and enlarged or even giant platelets in variable numbers. Gene defects have been described for the major inherited thrombocytopenias and their characterization is providing basic knowledge of how MKs develop from the pluripotent hematopoietic stem cell (HSC) under the influence of thrombopoietin (TPO). Notwithstanding, the molecular basis for many moderate congenitally low platelet counts, often of mild clinical significance, remains to be elucidated. Our review deals with the biology, genetics, and clinical management of inherited thrombocytopenias. The fundamental aspects of megakaryocytopoiesis are dealt with in Chapter 23. Most important in this group of patients is the need to ensure correct diagnosis and to avoid inappropriate therapy such as steroid treatment and splenectomy. Where possible, a phenotypic approach has been taken to classifying the disorders (Table 63.1).

GIANT PLATELET SYNDROMES AFFECTING THE GPIb/VON WILLEBRAND FACTOR AXIS

In this group of disorders, the circulating platelet count is low and is accompanied by the presence of variable numbers of large platelets. Quantitative or qualitative defects of the GPIb-IX-V complex also result in defects of the platelet–vessel wall interaction (see Chapter 65); they also result in profound changes in megakaryocytopoiesis in the bone marrow.

(i) *BSS:* First reported in 1948, BSS associates macrothrombocytopenia with decreased platelet adhesion to subendothelium caused by an absence (or nonfunctioning) of GPIbα.[3,4,5] A simple diagnostic test is that the giant platelets fail to agglutinate with ristocetin, an antibiotic that promotes the binding of von Willebrand factor (vWF) to GPIbα. The products of four separate genes (*GPIBA*, *GPIBB*, *GP9*, and *GP5*) assemble in a 2:2:2:1 ratio within maturing MK to form the GPIb-IX-V complex, stabilized through cytoplasmic domains linked to the membrane cytoskeleton via filamin A (FlnA).[6] Generally inherited as an autosomal recessive trait, mutations within *GPIBA*, *GPIBB*, and *GP9* interfere with the biosynthesis, maturation, and/or trafficking of the complex through the endoplasmic reticulum and Golgi apparatus of MKs.[7,8] Recent data suggest that bleeding results from the loss of high shear- and vWF-dependent platelet-to-platelet interactions in thrombus formation, as well as a defective platelet adhesion and the low platelet count.[9] The additional absence of GPIbα-dependent binding of P-selectin, TSP1, factor VIIa, factor XI, factor XII, αMβ2, high molecular weight kininogen, and thrombin may also contribute to the phenotype (see Chapter 26A). Early reports of an increased phosphatidylserine (PS) exposure but decreased prothrombin consumption and a decreased platelet survival in BSS remain largely unexplained. Nonetheless, BSS platelets of all sizes readily expose PS and undergo apoptotic-like changes on platelet activation.[10]

Early studies showed that mature MKs isolated from the bone marrow of BSS patients had major changes in morphology with an altered organization of internal membranes, many vacuoles, and a disparate localization of α-granules.[11] Such changes lead to decreased platelet production with the formation of large round platelets having distinctive morphologic abnormalities (FIGURE 63.1). The macrothrombocytopenia in BSS is usually moderate but can be severe in some patients and lead to extensive, occasionally life-threatening, mucocutaneous bleeding. In rare variant forms, platelets express decreased to normal amounts of nonfunctional GPIbα. Mutations resulting in variant BSS have included three missense mutations in GPIbα: R41H, L73F, and A156V (so-called Bolzano mutation).[12–14] While the first two were heterozygous with autosomal dominant pattern of inheritance, the Bolzano variant was homozygous with autosomal recessive inheritance. Hemizygous mutations in *GPIBB* can also cause BSS when associated with the DiGeorge/velocardiofacial syndrome, a developmental disorder characterized by a hemizygous microdeletion at 22q11, the site of localization of *GPIBB*.[15]

Mice lacking GPIbα or GPIbβ have allowed evaluation of megakaryocytopoiesis in BSS and have confirmed aberrant membrane development early during MK maturation leading to a reduced demarcation membrane system (DMS), impaired proplatelet formation, and aberrant microtubule coil assembly.[16–18] Studies of platelet production *in situ* revealed large platelets being released from unusually short, broad, proplatelets protruding into vascular sinuses.[17]

Table 63.1

Inherited thrombocytopenias classified by gene mutation and associated phenotype

Syndrome	Gene Mutation	Chromosomal Location/Inheritance	Associated Phenotype
BSS	*GPIBA, GPIBB, GP9*	17, 22, and 3 Autosomal recessive	Deficient GPIb-IX-V, giant platelets, vWF-dependent adhesion defect
Platelet-type vWD	*GPIBA*	17p13.2 Autosomal dominant	Blocked GPIbα (vWF), platelet anisotropy
vWD2B (some families)	*vWD*	12p13.3 Autosomal dominant	Enlarged platelets, sometimes platelet agglutinates, blocked GPIbα
Mediterranean macrothrombocytopenia	*GPIBA*, possibly others	17p13.2 Autosomal dominant	Mild disorder, enlarged platelets
GT-like	*ITGB3, ITGA2B*	17q21.31/32 Autosomal dominant or recessive	Platelet anisotropy, defective platelet aggregation
MYH9-related thrombocytopenia May-Hegglin anomaly, Fechtner, Epstein & Sebastian syndromes	*MYH9*	22q11.21 Autosomal dominant	Giant platelets, various combinations of neutrophil inclusions, sensorineural hearing loss, nephritis, cataracts
β1-tubulin-related thrombocytopenia	*TUBB1*	20q13.32 Autosomal dominant	Altered organization of β1-tubulin into microtubules, enlarged platelets
FlnA-related thrombocytopenia	*FLNA*	Xq28 Autosomal dominant	PNHs and other defects, platelet anisotropy
DiGeorge/velocardiofacial syndrome	Hemizygous microdeletion including *GPIBB*	22q11 Autosomal dominant	Facial dysmorphology, cardiac and other defects of development
WAS	*WAS*	Xp11.23	Immunodeficiency, eczema, lymphoma, small platelets, defective platelet and lymphocyte function
XLT	*WAS*	Xp11.23	Small platelets, no immune problems
GPS	*NBEAL2*	3p21.1-3p22.1 (3p21) Autosomal dominant (rare) or recessive	Myelofibrosis, enlarged platelets (no α-granules), platelet dysfunction
Familial platelet disorder/acute myelogenous leukemia	*RUNX1 (CBFA2, AML1)*	21q22.12 Autosomal dominant	Myelodysplasia, acute myeloid leukemia, platelet dysfunction
Chromosome 10/ thrombocytopenia 2	*MASTL, ANKRD26*	10p12.1 (both) Autosomal dominant	Bleeding syndrome only
CAMT	*MPL*	1p34.2 Autosomal recessive	Low MK content in marrow, early development of pancytopenia (in most cases), elevated TPO
Thrombocytopenia with radio-ulnar synostosis	*HOXA11*	7p15.2 Autosomal dominant or recessive	Fused radius, incomplete range of motion, sometimes other defects
TAR	*RBM8A* null allele + low-frequency regulatory SNP	1q21.1 Autosomal recessive	Shortened/absent radii bilaterally, cardiopathy
Paris-Trousseau syndrome	Hemizygous microdeletion including *FLI1*	11q23/24 Autosomal dominant (or isolated)	Psychomotor retardation, facial anomalies (Jacobsen syndrome), enlarged platelets with abnormal granules
GATA-1–related thrombocytopenia with dyserythropoiesis	*GATA-1*	Xp11.23 X-linked	Dyserythropoiesis ± anemia, thalassemia in some patients Platelet dysfunction, enlarged platelets with few α-granules

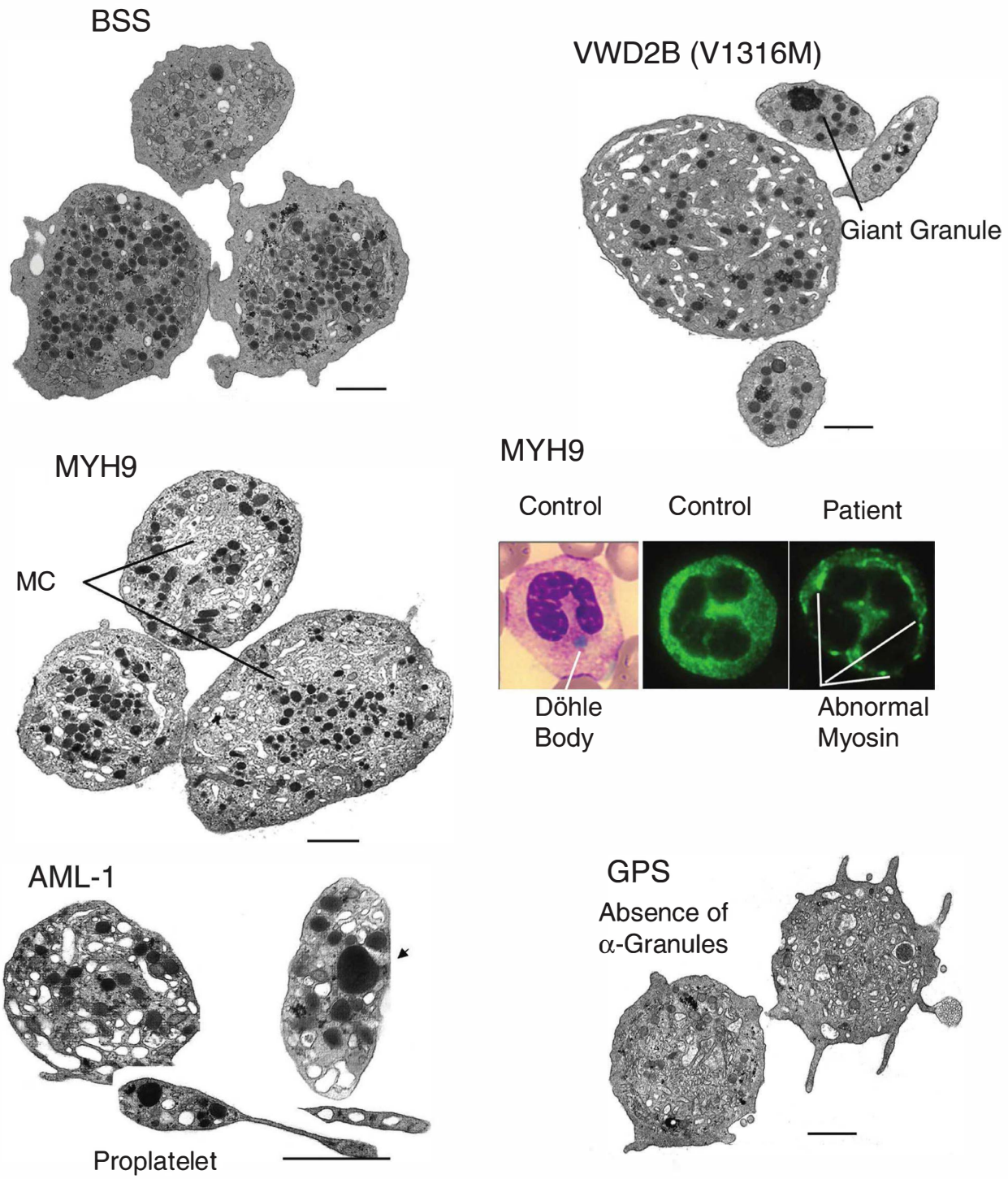

FIGURE 63.1 Composite illustration showing some of the striking ultrastructural characteristics of platelets in selected inherited thrombocytopenias. In the upper panels are shown the typical large round platelets of a patient with the BSS as seen by electron microscopy. Note the heterogeneous distribution of α-granules but the absence of large MC. Adjacent are platelets from a vWD2B patient showing size heterogeneity. An enlarged α-granule is highlighted. The middle panels first show electron microscope images of large round platelets from a patient with MYH9-related disease (May-Hegglin anomaly); note the large abnormal platelets with abundant MC. Also shown are a cytochemically stained Döhle-like inclusion and NMMHC-IIA immunoprecipitates detected by immunofluorescence in leukocytes from the same patient. In the lower panel is first shown platelet anisotropy in a patient with amegakaryocytic thrombocytopenia with predisposition to leukemia (AML-1). Platelets are immature and proplatelet fragments are present. Finally two platelets from a patient with the GPS are shown. These platelets are totally lacking α-granules. Bars = 1 μm

GPIbβ–/– MKs differentiated *in vitro* showed fewer proplatelet extensions with thicker shafts, fewer branches, and an increased diameter of terminal coiled elements that were enriched in tubulin fibers.[18] Significantly, the macrothrombocytopenia was much less severe in a novel murine model where platelets were engineered to express a GPIbα subunit in which the extracytoplasmic sequence was replaced by that of the α-subunit of the human interleukin-4 receptor; despite improvement in platelet production a severe bleeding phenotype persisted.[19,20] This suggests a role for the GPIbα cytoplasmic tail in signaling essential for normal MK maturation. Significantly, no correlations have yet been seen in BSS between phenotype (and bleeding severity) and genotype.[21]

(ii) *Mediterranean Macrothrombocytopenia*. In Mediterranean countries, patients manifesting a moderately low platelet count (70,000 to 150,000/μL), increased mean platelet volume, autosomal dominant inheritance with mild mucocutaneous or no bleeding, normal platelet function, and normal MK numbers are frequently observed. Linkage analysis and mutation screening of 12 Italian families identified a heterozygous A156 substitution in GPIbα in six with GPIb-IX levels comparable to those of BSS heterozygotes.[22] It is noteworthy that A156V is the same mutation as that causing the Bolzano variant of BSS (see above). Balduini et al.[23] cultured MKs from six patients and showed that proplatelet formation was much reduced compared to controls. Again, proplatelet tips were enlarged and α-tubulin distribution abnormal. Because the GPIbα mutation is heterozygous and occurs in the context of the wild-type protein, it must be exerting an as yet unexplained dominant negative effect, especially since obligate heterozygotes for classic BSS are largely asymptomatic.

(iii) *Platelet-type von Willebrand disease (platelet-type vWD)*. Platelet-type vWD is characterized by a gain of function phenotype with spontaneous binding of plasma vWF to platelets and increased platelet agglutination by low amounts of ristocetin in the presence of normal plasma. Platelet size can be increased in platelet-type vWD and there can be moderate thrombocytopenia. Selected heterozygous GPIbα substitutions with autosomal dominant inheritance cause platelet-type vWD, namely, G233V/S and M239V substitutions in the N-terminal domain; a 27-bp deletion in the macroglycopeptide-coding region of *GPIBA* implies that long-range conformational changes can also give rise to platelet-type vWD.[4,24,25] Mechanistically, the GPIbα mutations promote and stabilize platelet adhesion to vWF at low shear by enhancing formation and increasing longevity of the tether bond.[26] Interestingly, a knock-in mouse model with the G233V mutation not only had a phenotype mimicking the human disorder, but also showed increased bone mass.[27] In the same model, spontaneous binding of vWF blocked the capacity of platelets to bind fibrinogen when stimulated.[28] This probably contributes to the bleeding caused by an inhibited GPIbα although enhanced ADAMTS13 cleavage of the more stable platelet-bound vWF multimers may moderate the condition.[29]

(iv) *vWD2B*. Enlarged platelets with size heterogeneity (FIGURE 63.1) and thrombocytopenia can also occur in vWD, type 2B (vWD2B) where mutated plasma vWF spontaneously binds to a normal GPIbα.[30] As with platelet-type vWD, inheritance is autosomal dominant. In one such family with an R1308P vWF substitution, platelets showed signs of apoptosis and culture of CD34+ cells from the peripheral blood resulted in an increased surface expression of the mutant vWF on mature MKs that caused interactions between proplatelets.[31] This study was extended to nine patients with a total of seven different gain-of-function mutations and abnormal platelets typical of those seen in the circulation were produced *ex vivo* in MK cultures.[32] Circulating platelet clumps in rare patients with vWD2B prompted comparison with the previously described phenotype of the Montreal platelet syndrome (MPS); this was resolved when patients of the founder MPS kindred were shown to have the common vWF V1316M mutation.[33] Among the described characteristics of MPS were platelet calpain deficiency (probably due to autodegradation) and a reduced thrombin-induced aggregation; both findings are a consequence of the spontaneous binding of large vWF multimers to GPIbα. Significantly, lineage-specific mouse knock-in models of vWD2B show that abnormal plasma vWF (with a vWD2B mutation) can broadly reproduce the human vWD2B phenotype, emphasize its hemostatic variability, and suggest a possible role for ADAMTS13-dependent modulation of disease severity.[34,35]

PLATELET ANISOCYTOSIS AND THE αIIbβ3 INTEGRIN

Glanzmann thrombasthenia (GT), the principal disorder of platelet aggregation, is caused by quantitative and/or qualitative deficiencies of the integrin αIIbβ3 coded by the *ITGA2B* and *ITGB3* genes located at 17q21–23.[2,3] Although platelet count, volume, and morphology are normal in classic GT, several reports have inferred a role for αIIbβ3 in megakaryocytopoiesis because isolated point mutations in either of the *ITGA2B* and *ITGB3* genes combine an altered platelet production with selective deficiencies in platelet function.[36a,36b,37,38,39,40] This was brought to light by the discovery of R995Q/W mutations in αIIb and D723H in β3 that resulted in platelet anisotropy and thrombocytopenia.[37,38,39] R995 and D723, each located in the αIIbβ3 cytosolic tail, are thought to interact and aid in maintaining the integrin in its bent inactive conformation. Mutations weakening or abolishing this interaction increase the activation state of αIIbβ3 potentially changing the ability of MKs to interact with their environment and/or interfering with intracellular signaling pathways. Another mutation affecting platelet production is a heterozygous L718P substitution in the membrane-proximal extracellular domain of β3.[41] Finally, a genome-wide search in a large Italian family with an autosomal dominant trait combining thrombocytopenia, large platelets, defective platelet function, and moderate/severe mucocutaneous bleeding mapped the affected gene to chromosome 17q.[42] A novel heterozygous mutation (c.2134 + 1G>C) in the donor splice site of intron 13 of *ITGB3* resulted in an in-frame 120-bp deletion and loss of amino acids 647 to 686 within the extracellular membrane-proximal region of β3.

MYH9-RELATED SYNDROMES

May-Hegglin anomaly and the Fechtner, Sebastian, and Epstein syndromes constitute a group of disorders with autosomal dominant inheritance and macrothrombocytopenia; they are now generally referred to as MYH9-related disorders (Table 63.1).[1,2,3,4,43,44] Enlarged or giant platelets are a common feature (FIGURE 63.1).

Döhle-like inclusions in neutrophils, eosinophils, and some monocytes are diagnostic characteristics (FIGURE 63.1).[45] Thrombocytopenia is generally mild but can be severe; bleeding is infrequent and rarely life threatening. Other manifestations of these disorders include the progressive development of nephritis, sensorineural hearing loss, and cataracts. The Sebastian platelet syndrome associates macrothrombocytopenia with neutrophilic inclusions that differ in morphology from those in May Hegglin anomaly.[46] The Fechtner syndrome adds hearing loss, nephritis, and cataracts while in the Epstein syndrome only hearing loss and glomerulonephritis are present (Table 63.1).[47,48]

These syndromes result from heterozygous mutations in the *MYH9* gene encoding the nonmuscle myosin heavy chain IIA (NMMHC-IIA).[49–51] Class II myosins are hexameric proteins composed of two heavy chains and two pairs of light chains. A globular head binds to actin and ATP, has ATPase activity, and is required for motor activity. The C-terminal α-helical domain features a coiled-coil and a rod-like tail that allows the molecules to polymerize into bipolar filaments. NMMHC-IIA is found in platelets, monocytes, and granulocytes while its tissue distribution includes kidney, eye, and cochlea.[52] Immunofluorescence microscopy is now a diagnostic test with the mutated protein visualized in distinctive patches in granulocytes and platelets (FIGURE 63.1).[53] A wide range of heterozygous mutations have been identified, including substitutions, deletions or small insertions, frameshifts, and stop codons; these mutations mainly cluster in 9 of the 40 exons that encode the protein. The result is haploinsufficiency and/or a functionally defective protein.[54–56,57,58,59] The mutations may have a dominant effect, for example, on filament assembly; in this way they influence morphology of Döhle bodies and the nature of immunofluorescence staining (data reviewed in ref[14]). It is noteworthy that the same mutation can give rise to a variety of phenotypes; for example, a common D1424N substitution in the rod domain first described in May-Hegglin anomaly is also found in patients with various combinations of hearing loss, nephritis, and cataracts (data reviewed in ref[57]). In 20 families with the E1841K substitution, 15 had May-Hegglin anomaly and five Fechtner syndrome; in contrast, in families with R702C or R702H substitutions all developed the severe Fechtner or Epstein syndrome.[48,57] In a large family with Fechtner syndrome, only 5 out of 10 affected members showed renal lesions at the time of study.[49] Also, sequence analysis of *MYH9* in a family with nonsyndromic hereditary deafness showed that a R705H substitution cosegregated with the hearing loss phenotype, but giant platelets were not present.[60] Such findings suggest that the disease is not truly monogenic. Myosin molecules expressing mutations in the head domain had only 4% and 25% of the maximal ATPase activity and failed to translocate actin filaments in an *in vitro* motility assay.[54] *MYH9* mutations can affect myosin filament assembly or stability as well as function. *De novo* mutations may be the cause in sporadic cases.

Studies on MKs from patients in culture suggest that NMMHC-IIA negatively regulates megakaryocytopoiesis. In normal MKs, myosin light chain (MLC) kinase and Rho-associated kinase ensure the phosphorylation of MLC. Inhibition of this phosphorylation, possibly through the influence of stromal cell–derived factor-1, promotes proplatelet formation.[61] Perturbation of NMMHC-IIA levels and distribution in MYH9-related diseases potentially affects MK maturation, mobility, and the extent and timing of proplatelet production. MK-restricted *MYH9* inactivation in mice resulted in macrothrombocytopenia but conserved platelet aggregation and secretion as in the human disease. Nonetheless, bleeding times were prolonged, platelet shape change modest, and clot retraction defective; the latter linked to a defective αIIbβ3-outside-in signaling, a finding associated with an impaired thrombus formation under flow.[62] Mature MKs from these mice showed severe morphologic abnormalities with a poorly developed DMS and the absence of the peripheral zone that normally contains myosin filaments.[63] The MKs were unable to form stress fibers on adhesion to collagen. The number of cells extending proplatelets in culture was increased and the proplatelet tips were larger. The authors speculated that platelet release was premature, and that the decreased DMS contributed to a poor platelet production. Pecci et al.[64] supported premature release by showing that MKs cultured from MYH9-related disease patients lose the physiologic suppression of proplatelet formation exerted by type I collagen, an important extracellular matrix component and adhesive substrate for MKs as they migrate from the osteoblastic niche to the marrow vessels. These authors also showed a decreased branching of proplatelets and a lower number of proplatelet tips.

The basis for the phenotypic variability in MYH9-related diseases remains unclear and other genetic and/or environmental factors may intervene. A proposed explanation is that additional II-B and II-C myosin isoforms can compensate, at least in part, for the malfunction of defective *MYH9* A isoform when they are expressed in the same tissues.[53] Another gene, *FBLN1*, encoding fibulin-1, an extracellular matrix protein, has been proposed as a disease modifier.[65] Amino acid substitutions in the Ca^{2+}-ATPase-containing head domain appear more often to be associated with deafness and renal disease while those affecting the rod domain, and possibly myosin-IIA assembly, more frequently only have hematologic consequences.[57,66] But there are many exceptions. Missense mutations in the *APOL1* gene are highly associated with focal segmental glomerulosclerosis and end stage kidney disease risk previously attributed to *MYH9* SNPs in western African patients.[67] APOL1 is a neighboring gene to *MYH9* on chromosome 22. Such haplotypes should be studied in MYH9-related disease patients where renal disease develops often during adolescence.

The presence of functionally normal giant platelets has been speculated to compensate for the thrombocytopenia in MYH9 disorder in reducing bleeding risk and to also account for the risk of myocardial infarction in May-Hegglin anomaly patients.[68] Nevertheless because of the risk of hemorrhage, Pecci et al.[69] assessed the administration of eltrombopag, a new generation TPO mimetic.[70] Eltrombopag was able to increase platelet counts in MYH9-related disease and reduce bleeding over a 3-week test period. However, many patients with these disorders are asymptomatic and diagnosis may await adulthood or the occurrence of trauma-related bleeding. Often it is confused with immune thrombocytopenic purpura (ITP). Platelet transfusions are often given as a precaution prior to surgery, TPO mimetics may offer an alternative.

OTHER FAMILIAL THROMBOCYTOPENIAS WITH DEFECTS OF CYTOSKELETAL PROTEINS AND AN ALTERED PLATELET SIZE

Mutations of other genes encoding cytoskeletal proteins have resulted in the production of platelets in decreased numbers and of altered size.

(i) *Mutation of the β1-tubulin gene (TUBB1).* Microtubule assembly and function are key elements of proplatelet formation from MKs, trafficking granules and other organelles to the proplatelet tips where platelets are released by budding, and maintenance of the platelet discoid shape.[71,72] Freson et al.[73] found a heterozygous Q43P polymorphism in a highly conserved residue of β1-tubulin in 24.2% of 33 unrelated families with inherited macrothrombocytopenia of unknown cause. Electron microscopy showed enlarged spherocytic platelets with a disturbed marginal microtubule band and organelle-free zones. Decreased platelet reactivity to physiologic agonists was also noted. However the same mutation was also seen in approximately 10% of normal subjects. Kunishima et al.[74] studied a Japanese patient with the heterozygous Q43P mutation but found that it did not cause macrothrombocytopenia. Instead, these authors found a second heterozygous R318W mutation predicted to disrupt side chain interactions near the α and β intradimer interface of β1-tubulin. The authors found that R318W, but not Q43P, led to a 50% reduction in platelet β1-tubulin content. Cultured MKs from the patient showed morphologic changes consistent with a disturbed platelet production; the R318W β-tubulin was localized in punctate structures suggesting that it was forming aggregates. This unstable protein may be essential for the phenotype; while β1-tubulin (−/−) mice are thrombocytopenic and their platelets lose their discoid shape, heterozygotes do not show macrothrombocytopenia.[75]

(ii) *Mutations in the filamin A gene (FLNA).* Heterozygous mutations in the *FLNA* gene encoding the cytoskeletal protein FlnA are associated with a series of rare X-linked autosomal dominant diseases, the major feature being periventricular nodular heterotopia (PNH).[76] FlnA is the attachment site for GPIbα in the platelet cytoskeleton, helping to maintain thrombus stability at high shear.[77] Bleeding is an important feature for a cohort of patients with *FLNA* mutations as is thrombocytopenia and platelet anisotropy.[78] This was shown to be due to a defective megakaryocytopoiesis, a finding confirmed from studies on a conditional mouse model where FlnA null platelets also showed signaling defects and a reduced shear-dependent platelet accumulation on collagen.[79] Mice whose platelets possessed intermediate amounts of FlnA also showed macrothrombocytopenia.

(iii) *WAS.* This X-linked recessive disease combines thrombocytopenia and small platelets (microthrombocytopenia), recurrent infections due to immune deficiency, and eczema, autoimmune manifestations, and malignancy.[1,2,3,4,80,81] A milder form lacking the immune manifestations is known as hereditary X-linked thrombocytopenia (XLT). WAS platelets aggregate poorly and, due to their small size, have a reduced number of α-granules, δ-granules, and mitochondria. T cells, naturally occurring T regulatory cells, dendritic cells, and B lymphocytes also show defective function. The *WAS* gene is composed of 12 exons and encodes a 502–amino acid protein (WASP).[82] Genetic defects in *WAS* result either in the decreased expression of WASP or its absence. A secondary defect is abnormal expression of CD43 (sialophorin), a membrane glycoprotein of T lymphocytes. A wide range of genetic mutations occur in WAS; these are spread across the gene and result in the decreased expression or absence of WASP. For example, in a recent European demographic study, a total of 87 affected males and 48 carrier females were genotyped.[83] The most common sequence variations were missense mutations and small insertions, followed by nonsense and splice site mutations and insertions. Only one large deletion was identified. Generally, missense mutations accompanied by partially expressed and functional protein produce the milder XLT.[80,81,83] They most often affect exons 1 to 3. WASP-negative patients show a higher susceptibility to infection and eczema and have a greater tendency for malignancy. Rare mutations lead to spontaneously activated WASP with increased actin polymerizing activity and cause an X-linked form of neutropenia with variable myelodysplasia (in the absence of thrombocytopenia and T-cell immunodeficiency).[84] WASP is a multifunctional protein and is a key regulator of actin polymerization in hematopoietic cells. It is involved in signal transduction having tyrosine phosphorylation sites and adapter protein function; it acts through the Arp2/3 complex. Despite thrombocytopenia, MK numbers are normal, but the defect leads to premature proplatelet formation in the marrow where a lack of actin-rich podosomes slows MK migration to the vascular sinus.[85] Altered *in vivo* reactivity with immunoglobulin also contributes to decreased platelet survival, another characteristic of WAS. An autosomal dominant variant form of WAS has been described.[86] Although female heterozygotes for classic WAS have no clinical signs because of preferential selection of the normal nonmutated X chromosome in hematopoietic cells, X-linked WAS has been reported in girls, presumably due to coinheritance of a trait causing skewed X inactivation.[87] Infections (often bacterial) and malignancies are major causes of death in WAS and hemorrhage is also a problem. Overall, prognosis is poor and WAS remains primarily a disease of children. Antibiotic prophylaxis and IVIgG infusions can raise life expectancy while splenectomy can increase platelet numbers.[88] HSC transplantation is the optimal treatment as it remedies both immunologic as well as hematologic manifestations with a >80% survival using matched sibling donors.[89a] WAS is amenable to HSC gene therapy and genetically modified HSC have permitted its first successful use in 2 German patients with marked clinical improvement over 3 years.[89b]

GRAY PLATELET SYNDROME

Gray platelet syndrome (GPS) is usually a mild bleeding disorder with predominantly autosomal recessive inheritance and is characterized by the selective absence of α-granules and their contents.[90,91] The absence of α-granules gives the platelets a typical gray appearance on blood smears. The platelets are enlarged and often vacuolated, but not giant (FIGURE 63.1). Other clinical features are the early onset of myelofibrosis and splenomegaly. Patients with GPS can have moderate but progressive thrombocytopenia, likely due to negative regulation of megakaryocytopoiesis by spontaneously released cytokines/chemokines and other α-granule proteins. A novel recent finding is a high serum level of vitamin B_{12}.[91] Secretion-dependent platelet aggregation can be affected. In a French patient with GPS, collagen-induced platelet aggregation was accompanied by reduced GPVI expression.[92] Platelet α-granules contain biologically active proteins involved not only in hemostasis, but also in wound healing and tissue repair.[93] Pro- and antiangenic proteins are organized into different subpopulations of granules; both are affected in

GPS.[94,95] Vestigial granules may be seen and the basic molecular defect likely involves packaging or storage of proteins during α-granule biogenesis. Glycoproteins such as P-selectin, present in the α-granule membrane, are often retained in variable amounts distributed throughout the remaining membrane systems.

The genetic defect responsible for GPS has long remained an enigma. A report of X-linked GPS due to a GATA-1 Arg216Gln mutation is atypical since this and other related GATA-1 mutations also give rise to dyserythropoietic anemia, a finding not characteristic of GPS (see below).[96] Recently, genome-wide linkage analysis of a cohort of 25 GPS patients from 14 unrelated families spanning several continents localized the GPS gene(s) to a 9.4-Mb interval on 3p21.1-3p22.1 containing 197 protein-coding genes.[91] This approach was then used to make an even finer localization of the affected gene to 3p21.[97a] The affected gene has been identified as *NBEAL2* in 3 parallel studies using different molecular approaches including whole exome sequencing.[97b-d] *NBEAL2* is a member of the neurobeachin-like gene family and is likely a key element in α-granule biogenesis.

FAMILIAL THROMBOCYTOPENIA WITH A PREDISPOSITION TO ACUTE MYELOGENOUS LEUKEMIA

A large kindred with a bleeding tendency has been reported in which an autosomal dominant disorder of platelet production and function was linked to a propensity to develop myeloid leukemia.[98] The thrombocytopenia was moderate with considerable platelet size heterogeneity and structures resembling incompletely fragmented proplatelets (FIGURE 63.1). Linkage to markers on chromosome 21q identified an 880-kb interval containing the disease gene. Further analysis on this and other families confirmed the autosomal dominant pattern of inheritance and revealed heterogeneous germline nonsense and missense mutation, as well as intragenic deletions of the hematopoietic transcription factor *RUNX1 (CBFA2, AML1)* gene. Other developmental defects including mental retardation were present in some patients.[99–102] While haploinsufficiency of RUNX1 may be sufficient, a second genetic event and a biallelic alteration involving RUNX1 strongly favors disease progression to acute myelogenous leukemia (AML).[103] The latter may be aided by the presence of an expanded population of undifferentiated HSCs. RUNX1 is thought to have an important role in HSC differentiation and may also act as a tumor suppressor. The haplodeficiency and mutations interfering with DNA binding appear to lead to a decreased CFU-MK and decreased platelet production from birth. Mutated RUNX1 may heterodimerize with normal protein, leading to loss of function. Recently described interactions between RUNX1 and the GATA-1/FOG-1 and *FLI1* transcription factors could be causally related to the thrombocytopenia.[104] An altered regulatory interplay between miR-27a and *RUNX1* during megakaryocytopoiesis may also influence phenotype.[105] Impaired agonist-induced platelet aggregation, secretion, αIIbβ3 activation, and protein phosphorylation have all been described in patients with RUNX1 mutations. Recent elegant studies from Jalagadugula et al.[106] have shown that regulation of platelet MLC (MYL9) by RUNX1 has implications for both thrombocytopenia and platelet dysfunction; other platelet proteins for which gene transcription is under the control of RUNX1 include platelet 12-lipoxygenase (ALOX12), platelet protein kinase C-θ, and platelet factor-4.[107–109]

AUTOSOMAL DOMINANT THROMBOCYTOPENIA WITH PLATELETS OF NORMAL SIZE

There are rare reports of lifelong moderate to severe thrombocytopenia, but with normal sized platelets, in large kindreds with autosomal dominant inheritance. Clinical manifestations were a propensity to easy bruising and increased bleeding at times of hemostatic stress. Myeloid or erythroid cells were not affected and there was no progression to aplastic anemia. Linkage studies on large American and Italian families localized to chromosome 10.[110,111] Studies on cultured MKs from the American patients suggested a late block in terminal differentiation. A novel putative kinase, FLJ14813 (MASTL), was identified as a candidate gene mutated in the American family; a missense mutation encoding E167D segregated perfectly with the thrombocytopenia.[112] Knockdown of the MASTL kinase in zebrafish resulted in a deficiency of circulating thrombocytes as well as a decreased expression of the TPO receptor, c-Mpl, and αIIb encoding genes.[113] MASTL mutations were not found in the Italian family. Instead in this and eight other families, a series of mutations were clustered in a highly conserved 19-bp sequence located in the 5′ untranslated region of the *ANKRD26* gene (chromosome 10p12.1) encoding a proapototic protein whose function in megakaryocytopoiesis remains to be determined.[114] A mutation of human cytochrome c that enhances the intrinsic apoptotic pathway also causes thrombocytopenia with the release of "immature" platelets in a family with autosomal dominant thrombocytopenia.[115]

CONGENITAL AMEGAKARYOCYTIC THROMBOCYTOPENIA

In congenital amegakaryocytic thrombocytopenia (CAMT), severe thrombocytopenia at birth often evolves into pancytopenia and severe aplastic anemia; occasionally, developmental decay, facial malformations, and neurologic problems are also present. CAMT has autosomal recessive inheritance and is a lifelong bleeding syndrome. Affected patients are born with low numbers of MKs in their marrow. Defects in the *MPL* gene that encodes the TPO receptor means that TPO is unable to fulfill its normal thrombopoietic role.[116] The fact that neither platelets nor haematopoietic progenitor cells show reactivity to TPO means that plasma TPO levels are greatly elevated (>10-fold above normal) and this is a diagnostic feature of CAMT along with a lack of c-Mpl expression on platelets. Ballmaier et al.[117,118] highlighted heterogeneity in CAMT, distinguishing patients with a severe form and early development into pancytopenia (type I disease) from others with mild thrombocytopenia in the first year of life but which then worsens over a 10-year period with pancytopenia in later life (type II disease). Frameshift or nonsense mutations predicting complete loss of c-Mpl led to severe disease; missense mutations in patients possessing residual c-Mpl were associated with slower progression of the phenotype. Rare mutations interfere with c-Mpl function rather than expression or even block receptor trafficking.[119,120]

The absence of c-Mpl function in HSCs cells leads to defective MK colony formation. Early exhaustion of multipotential progenitors may account for the pancytopenia. Mice that are genetically null for c-Mpl are thrombocytopenic, and although they

do not develop aplasia, marrow cell assays reveal severe reduction in precursors for all hematopoietic lineages.[121] Allogeneic bone marrow transplantation is a recommended treatment for CAMT, although potential donors among relatives need to be screened for mutations that may limit MK colony formation even in the heterozygous state. For some patients with expressed but nonfunctional c-Mpl, the possibility exists that they may react with TP-mimetics while not reacting with TPO itself.[122]

There are also activating mutations in the *c-Mpl* gene that give rise to familial essential thrombocythemia.[123] One example is a heterozygous S505N substitution inherited in an autosomal dominant manner and found in the transmembrane domain of c-Mpl. The result is spontaneous intracellular signaling, activated megakaryocytopoiesis, and excessive platelet production. The JAK2 kinase is critical for phosphorylation of c-Mpl and other signaling molecules, leading to activation of the Jak/signal transducer and activator of transcription and Ras/Raf/Mek1/2-Mapk-Erk pathways. Activating mutations in JAK2 and constitutive tyrosine kinase activities give rise to elevated blood cell counts in myeloproliferative disorders such as polycythemia vera.[124]

INHERITED THROMBOCYTOPENIAS ASSOCIATED WITH DISORDERS OF DEVELOPMENT

A series of thrombocytopenic disorders have been linked with severe defects in development with or without skeletal defects.

(i) *Amegakaryocytic thrombocytopenia with radioulnar synostosis*. Rare patients with what appears to be autosomal dominant inheritance, associated bone marrow failure with radioulnar synostosis and other skeletal abnormalities.[125,126] For some children, symptomatic thrombocytopenia with bleeding requires correction by bone marrow or umbilical cord stem cell transplantation. Marrow studies show few or no MKs and pancytopenia develops in some but not all individuals. A heterozygous mutation found in exon 2 of the homeobox gene, *HOXA1*, results in a stop codon in the third helix, a domain critical for DNA binding, and leads to a truncated protein in affected individuals. Studies on mice had previously shown that targeted disruption of the *HOXA10* and *HOXA11* genes affected forearm development in the embryo but did not result in thrombocytopenia (reviewed in[1]). Further studies have suggested that the *HOXA11* mutation inhibits MK differentiation.[127] It should be emphasized that radioulnar synostosis gives rise to mildly deformed forearms rather than their absence as in thrombocytopenia with absent radii (TAR, see below). The phenotype should also be distinguished from Fanconi anemia where skeletal abnormalities often affecting forearms, a predisposition to malignancies, and progressive pancytopenia are characteristic features.

(ii) *Thrombocytopenia with absent radii*. The TAR syndrome is a rare congenital defect with thrombocytopenia (often severe) and osteodysgenesis, namely shortened or absent forearms due to bilateral radial aplasia.[128,129] The inheritance is complex, with both autosomal recessive and autosomal dominant inheritance of various penetrance being reported. Other skeletal abnormalities as well as various developmental defects (e.g., facial dysmorphism, cardiac defects) can be present. Thrombocytopenia is often most severe at birth (most affected babies show purpura), but platelet numbers increase during childhood and platelet count can be near normal in adulthood. Platelet size is normal. MKs are reduced in number and appear small on biopsy smear. Serum TPO levels are elevated. Studies of MK differentiation *in vitro* in TAR syndrome patients showed a profound defect in MK progenitors.[130,131] MK differentiation is poorly stimulated by TPO or other cytokines and this is associated with a decrease in *c-Mpl* transcripts and Mpl protein. However, screening the *c-Mpl* gene has failed to show mutations and a defect in the early events of Mpl signal transduction has been proposed to explain the disorder. Furthermore, sequencing of the *HOXA10, HOXA11*, and *HOXD11* genes of 10 TAR syndrome patients revealed no mutations (see[128]). An interstitial microdeletion involving chromosome 1q was identified in a cohort of 30 individuals with TAR,[132] but was also seen in unaffected family members. More recently, the TAR syndrome has been shown to be given in 53 of 55 cases by compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit *RBM8A*.[133]

(iii) *Paris-Trousseau syndrome*. The Paris-Trousseau syndrome is a variant of Jacobsen syndrome characterized by haplodeficiency of chromosome 11q23-24.[134–137] This can give rise to growth and psychomotor retardation, facial dysmorphism, congenital heart defects, respiratory infections, and malfunction of multiple organs. Thrombocytopenia (or pancytopenia) affects approximately 90% of patients and distinguishes the Paris-Trousseau syndrome; the low platelet count can be chronic ($<50 \times 10^9$/L) and platelet function may be affected. However, the thrombocytopenia can correct within the first 2 years of life. Paris-Trousseau syndrome is said to have autosomal dominant inheritance, although several isolated cases have been described where *de novo* chromosome breakage may have occurred. Circulating platelets are often large and have giant α-granules visible on Giemsa-stained blood smears or by electron microscopy. Two morphologically distinct populations of MKs are present; one composed of normal cells and the other of small immature cells with hypolobulated nucleus and arrested maturation. The giant α-granules are a feature of platelets rather than MKs and may represent granule fusion after platelet production. Storage pool disease may also be present.

The chromosome 11 microdeletion is of variable length and includes two ETS transcription factor genes, *ETS-1* and *FLI-1*. A decrease in the *FLI1* protein results in reduced GATA-1/FOG-1–dependent transcription of MK-specific genes[138]; affected proteins can be downregulated (e.g., GPIba). Studies in mice favor a role for *FLI1* deficiency rather than ETS1 in the pathogenesis of Paris-Trousseau syndrome.[139] Lentivirus-mediated overexpression of *FLI1* in CD34+ cells from a Paris-Trousseau syndrome patient restores megakaryocytopoiesis *in vitro*, thereby confirming that *FLI1* hemizygous deletion contributes to the dysmegakaryocytopoiesis.[140] Furthermore a half-reduction in *FLI1* gene dosage generated a subpopulation of $CD41^+$ $CD42^-$ cells completely lacking *FLI1* transcription with a block in MK differentiation and the presence of a subpopulation of micromegakaryocytes that fail to reach the platelet production stage. Defects of other genes in the deleted region including *ETS-1* may contribute to the wide-ranging Jacobsen syndrome phenotype.[135,141]

(iv) *Other syndromes.* A moderately low platelet count is a feature of Ghosal syndrome, a rare autosomal recessive disorder characterized by progressive sclerosing diaphyseal dysplasia and refractory anemia. A genome-wide search in two large inbred families from Algeria and Tunisia first mapped disease gene to chromosome 7q33-34; it was subsequently identified as *TBXAS1* encoding thromboxane synthetase.[142,143] Noonan syndrome is a common, clinically and genetically heterogeneous condition characterized by distinctive facial features, short stature, chest deformity, congenital heart disease, and other comorbidities; thrombocytopenia is variable but often present.[144] The disease gene maps to chromosome 12q.24.1 and identified mutations in the Ras/MAPK (mitogen-activated protein kinase) signal transduction pathway including PTPN11 encoding the protein tyrosine phosphatase SHP-2 account for a majority of cases. Chronic thrombocytopenia and predisposition to AML associated with growth restriction, variable developmental delay, and facial dysmorphism has been associated with microdeletions at chromosome 21q encompassing the RUNX1 gene (see Section on AML).[145]

DiGeorge/velocardiofacial syndromes can have autosomal recessive inheritance but most often arise spontaneously.[146] They are seen in approximately 1:4,000 births. The phenotype arises from a microdeletion in chromosome 22q11.2 on one of a pair of chromosomes. Phenotypic features can include conotruncal cardiac abnormalities, learning disabilities, velopharyngeal insufficiency, immunodeficiency, facial dysmorphology, and thymic hypoplasia. A haploinsufficiency of a single gene, *Tbx1* (encoding a T-box containing transcription factor) largely accounts for the phenotype.[147] Surveys of DiGeorge syndrome suggest that mild thrombocytopenia and platelets of increased size affect about 20% of patients.[146,148] The gene encoding GPIbβ is adjacent to *Tbx1* (see Section on BSS). Bleeding is not a noted characteristic in DiGeorge syndrome patients, similar to the absence of bleeding in heterozygous BSS.

X-LINKED THROMBOCYTOPENIA WITH DYSERYTHROPOIESIS WITH OR WITHOUT ANEMIA

A special category of X-linked familial thrombocytopenia segregates dyserythropoiesis with or without anemia. Thrombocytopenia ranges from moderate to severe; splenomegaly may be present and platelet aggregation is affected. The gene responsible for this combination of defects was first mapped to the X-chromosome (Xp11-12) and identified as that encoding the transcription factor GATA-1 (reviewed in[149]). GATA-1 together with its cofactor FOG-1 (friend of GATA-1) plays a key role in both erythropoiesis and megakaryocytopoiesis. GATA-1 contains two zinc fingers, the C-terminal of which accounts for sequence-specific DNA binding and the N-terminal (N-f) for both stabilization of DNA binding and for the interaction with FOG-1. GATA-1 mutations give a wide range of phenotypic features besides thrombocytopenia, including mild β-thalassemia (XLTT), neutropenia, and congenital erythropoietic porphyria (CEP) in males. Mutations that give rise to thrombocytopenia all occur in the N-f domain; they interfere with the ability of GATA-1 to bind specific DNA sequences and/or to interact with FOG-1.

A small series of families have XLT without β-thalassemia. Typically, the marrow shows structurally abnormal MKs and an abundance of large multinucleated erythroid precursors. Circulating platelets are enlarged with few α-granules and contain an abundance of membrane complexes (MC). Erythrocytes are abnormal in size and shape and anemia may vary between mild and severe. Examples of GATA-1 mutations giving rise to severe XLT include V205M, G208R, and D218Y substitutions, while G208S and D218G result in milder phenotypes (reviewed in[149]). All appear to primarily affect GATA-1–FOG-1 interactions rather than DNA binding. Semiquantitative RNA analysis has revealed a low transcription of GATA-1 target genes such as *GPIbβ* and *GP9* and a reduced transcription of late MK maturation markers. Platelet function is affected with a poor response to ristocetin. Phenotypic variability is influenced by the nature of the substituted amino acid.[150–152] Skewed X inactivation in female carriers has also been reported.

Yu et al.[153] reported a R216Q substitution in the N-terminal finger of GATA-1 in the family that originally led to the description of XLT, but where red cell abnormalities were consistent with β-thalassemia (XLTT). The same mutation was subsequently found in an Italian family.[154] These were the first reports of β-thalassemia in man caused by a mutation in an erythroid transcription factor. The mutation allows programming of erythroid differentiation, though somewhat less efficiently than wild-type protein. Generally, XLTT results from mutations that affect the DNA binding face of the N-f domain of GATA-1 with the FOG-1 interaction conserved.[149] Thrombocytopenia is less severe than in XLT; again platelets contain few α-granules and have morphologic defects (see also Section on GPS). Platelet GPIb levels were higher than in controls whereas αIIbβ3 was lower. Not least, these studies confirm that the mutation changes rather than abrogates the expression of GATA-1–dependent target genes. Studies on marrow showed that mature MKs were often small and profoundly dysmorphic while immature cells retained a capacity to proliferate.

A GATA-1 mutation (R216W) that causes CEP has been described as has the association of GATA-1 with trisomy 21 (reviewed in[149]). Overall, a defective interaction of GATA-1 with either DNA or FOG-1 can disrupt hematopoiesis and give rise to disorders sharing common themes but with diverging phenotypes. The role of GATA-1 was confirmed by transgenic mouse models, with immature low ploidy MK progenitors proliferating profusely *in vitro* as a result of a unique MK differentiation arrest, a finding associated with dyserythropoietic anemia.[155,156] Recent biologic approaches have shown that in addition to FOG-1, the NURD complex, SCL/TAL-1, the zinc finger regulators GFI1B and ZFP143, and the compressor ETO2 all interact with GATA-1 to regulate terminal MK maturation.[157]

OTHER DISORDERS

Isolated reports of thrombocytopenia of unknown origin abound; often, insufficient evidence has been obtained to allow an understanding of the molecular cause. One example is a mild bleeding diathesis associating giant platelets with abnormal surface glycoproteins and mitral valve insufficiency.[158] Even more unusual is a report of hereditary macrothrombocytopenia with the atypical expression of the principal red cell glycoprotein, glycophorin A on the giant platelets.[159] Deafness developed in later life; no genetic defect has been described, although chromosomal crossover may be a possibility. A more complete case is that reported by Willig et al.[160] concerning a young boy with

macrothrombocytopenia and mild neutropenia. Morphologic abnormalities of MKs included an abnormal fragmentation of the cytoplasm and the presence of large MC. GPIb was selectively decreased but no sequence abnormalities were found in the genes encoding GPIb-IX-V. Significantly, there was a complete lack of sialyl-Lewis-X antigen on polymorphonuclear leukocytes from the patient and the authors concluded that a posttranslational modification of membrane glycoproteins was the origin of the defect. This was subsequently confirmed with inactivation of the Golgi CMP-sialic acid transporter through the combination of a heterozygous microdeletion and a heterozygous splice site mutation in the donor splice site of intron 6 of the *SLC35A1* gene.[161] Thrombocytopenia due to a defective O-glycosylation of GPIbα and expression of terminal *N*-acetylgalactosamine residues (Tn syndrome) is associated with somatic mutations in *COSMC* (*C1GALT1-specific chaperone*).[162,163] Here platelets are thought to have a reduced lifespan due to an accelerated clearance from the circulation; giant platelets are not present.

Platelets and MKs express the Gs-coupled VPAC receptor for which the pituitary adenylyl cyclase-activating peptide and the vasointestinal peptide are agonists. Patients with trisomy 18p and PCAP overexpression have mild thrombocytopenia and a reduced MK number with VPAC negatively regulating megakaryocytopoiesis via its control of cAMP levels.[164] Regulator of G-protein signaling 2 (RGS2) negatively regulates Gs signaling by inhibiting the activation of adenylate cyclase (AC). A heterogeneous mutation (G23D) in three related patients led to an enrichment of a large RGS isoform with strong AC inhibitor activity. Neurologic deficiencies and short fingers were among associated problems. Despite the absence of thrombocytopenia, platelets were enlarged and round with abnormal α-granules suggestive of changes in megakaryocytopoiesis.[165] An interesting situation whereby platelet production is influenced by a secondary gene defect is sitosterolemia (phytosterolemia). In this metabolic disease, there is an unrestricted gut adsorption of cholesterol and plant-derived cholesterol-like molecules. The disease is linked to mutations in the *ABCG5* and *ABCG8* genes. Patients are born with a normal platelet count and size, but rapidly develop macrothrombocytopenia.[166,167] Studies on *Abcg5*(−/−) mice showed a defective MK development with a deterioration of the DMS system and decreased polyploidy.[168,169] Platelet size was increased threefold and platelets showed a decreased lifespan as well as functional defects.

DIFFERENTIAL DIAGNOSIS

When a low platelet count is unexpected, it is first necessary to exclude artifacts and blood sampling errors (Table 63.2). With electronic counters falsely low platelet counts (and elevated leukocyte counts) often occur in giant platelet syndromes; platelet size should therefore also be examined on a blood film (reviewed in[43]). So-called pseudothrombocytopenia due to platelet clumping can occur in both healthy subjects and patients and is most often encountered in blood collected into ethylenediaminetetraacetic acid (EDTA) anticoagulant. Platelets may also show EDTA-dependent attachment to leukocytes (termed *platelet rosetting* or *satellitism*). Pseudothrombocytopenia is caused by naturally occurring agglutinins binding to an epitope exposed on αIIbβ3 after Ca^{2+} chelation.[170] Although platelet counts may be restored on using citrate anticoagulant, this is not always so and heparin or hirudin-based anticoagulants should also be tested.

Table 63.2

Causes to take into account when diagnosing patients with suspected inherited thrombocytopenia

Pseudoagglutination
ITP
Childhood acute
Adult chronic
Posttransfusion purpura
Drug-induced thrombocytopenia
Heparin-induced thrombocytopenia
Anti-GPIIb-IIIa drugs
Other medicaments
Antiphospholipid syndrome (SLE)
Evans syndrome
Paroxysmal nocturnal hemoglobinuria
Gestational thrombocytopenia during pregnancy
Preeclampsia and eclampsia
HELLP syndrome
TTP-HUS (Defect of ADAMTS13)
Viral and bacterial infections (and sepsis)
Increased platelet consumption
Splenomegaly
Disseminated intravascular coagulation and thrombosis
Liver and organ failure, vitamin B_{12} or folic acid deficiency
Myelodysplastic syndromes and myeloproliferative disease
Drug-induced marrow suppression and/or chemotherapy
Cancer and hematologic malignancy (leukemia)

In one large series of patients with EDTA-dependent thrombocytopenia, a majority also had anticardiolipin antibodies, and their removal by adsorption led to a loss in platelet clumping.[171]

When thrombocytopenia is severe and/or is associated with platelet dysfunction, bleeding is most often detected at birth. Mild thrombocytopenia may yield sporadic bleeding particularly at times of haemostatic stress; sometimes there is no past history and a low platelet count will only be detected in routine testing (e.g., prior to surgery or during pregnancy). A detailed family history helps determine the inheritance pattern; a questionnaire determining the duration and severity of symptoms may be useful to determine a bleeding score.[172] As we have illustrated, the coexpression of other phenotypic abnormalities will define clinically recognized syndromes. Platelet morphologic abnormalities will aid diagnosis as will elevated plasma TPO levels. Balduini et al.[43] working on behalf of an Italian study group and Bolton-Maggs et al.[2] on behalf of British organizations have published algorithms aimed at improving diagnosis of inherited thrombocytopenias and platelet function disorders. Analysis of the genetic defect is the final step, although this may requires the involvement of specialist centers.

Above all is the need to exclude an acquired cause. Among the many causes of acquired thrombocytopenia are autoimmune

diseases, increased platelet consumption, splenomegaly, marrow suppression, or failure (Table 63.2). Neonatal alloimmune thrombocytopenia and ITP must be excluded to avoid incorrect treatment (corticosteroids or even splenectomy). In the absence of a family history, it is necessary to know if the thrombocytopenia is persistent. Viral infection is a common cause of thrombocytopenia (particularly among children), bacterial infections, and sepsis in adults while drug-induced thrombocytopenia is an increasingly recognized cause. The list of drugs giving rise to drug-dependent antibodies is ever increasing.[173] The detection of plasma or serum alloantibodies and autoantibodies requires immunologic testing often only readily available in specialized centers. But in ITP, the detection of an antibody does not guarantee that it is responsible for the thrombocytopenia while the "nondetection" of an antibody does not rule out the presence of an antibody reacting with a minor component of platelets. In fact, ITP is largely diagnosed by exclusion leaving open the possibility that patients with familial thrombocytopenias are included within the diagnostic net of ITP (see[70]).

Systemic lupus erythematosus (SLE) is an often encountered but complex disease in which the detection of antinuclear antibodies is a sensitive diagnostic feature. Thrombocytopenia can be severe and is a risk factor for mortality while other organs can be affected. To complicate matters, thrombocytopenia in SLE can also have a severe familial phenotype with genetic linkages at 1q22 and 11p13.[174] Whether this linkage involves genetic polymorphisms or mutations directly implicated in the pathogenesis of the disease is unknown. Similarly, familial acquired thrombotic purpura has been reported with ADAMTS inhibitory autoantibodies in identical twins.[175] Antibodies associated with thrombocytopenia need not necessarily be directed against platelets. Neutralizing antibodies to granulocyte-macrophage colony stimulating factor (GM-CSF) or TPO have been detected in patients with amegakaryocytopenic purpura.[176,177]

PROGNOSIS

Platelet transfusions remain the most common treatment for severe spontaneous mucocutaneous bleeding in inherited disorders of platelet production, although DDAVP has been successfully used while recombinant human factor VIIa is now also widely used.[2] Isoantibody production is a risk in BSS after transfusion. When bleeding is moderate and superficial, then local measures can be applied.[2] The use of autologous platelet-rich plasma is a viable and inexpensive option for dental surgery.[178] Pregnancy and surgery represent special problems and are reviewed elsewhere.[2] An excellent systematic review of pregnancy in BSS has recently been prepared while menorrhagia in adolescents with inherited bleeding disorders has likewise been assessed.[179,180] The long-term treatment of CAMT and other severe thrombocytopenias of defined genetic basis is HSC transplantation with a matched donor. But the genetic basis of this disease suggests that it will be an excellent candidate for gene therapy for even a small population of targeted stem cells may be able to proliferate in response to TPO and repopulate the marrow. Whereas splenectomy may be an option in WAS, its efficacy in other inherited thrombocytopenias has been questioned, although it may be preferred in XLT where HSC therapy is not an option. Multidisciplinary management is required in WAS where life expectancy is low. Management of pregnancy needs to take into account that this is an X-linked disease. As stated earlier, gene therapy is now on the horizon for this disease.[89b]

Finally, it is now recognized that mean platelet volume and platelet count are highly heritable and regulated traits in a normal population. Whole-genome expression analysis and other approaches are starting to identify novel genetic markers of these parameters.[97b,181] Quite clearly, specific genetic traits and haplotypes will influence not only counts and platelet size in a normal population, but also the extent to which individual mutations responsible for inherited disorders of platelet production and/or function influence phenotype.

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CHAPTER

64 Acquired Nonimmune Thrombocytopenia

ANDREAS GREINACHER AND THEODORE E. WARKENTIN

Acquired thrombocytopenic disorders can be divided into five categories, plus the spurious entity of pseudothrombocytopenia (Table 64.1): hemodilution, sequestration (usually due to hypersplenism), decreased platelet production, increased platelet consumption (by pathologically increased "physiologic" pathways of platelet activation and clearance), and platelet destruction (e.g., phagocytosis of antibody-coated platelets). This chapter summarizes the nonimmune differential diagnosis of acquired thrombocytopenic disorders. We also discuss thrombocytopenia in patients in intensive care units, as thrombocytopenia is very frequent in these patients, and provide an approach to separate the different causes of a low platelet count.

PERIOPERATIVE THROMBOCYTOPENIA

Early postoperative thrombocytopenia is a combined effect of hemodilution and platelet consumption. It is one of the most common events prompting consultation with a hematologist. A platelet count decline of 30% to 70% occurs universally following major surgery. Such hemodilution-associated thrombocytopenia is most prominent after cardiac surgery[1] and is roughly proportional to the amount of crystalloid, colloid, or blood products administered. Most of this platelet count decline is abrupt and occurs within a few hours following surgery. Dilutional coagulopathy generally accompanies the thrombocytopenia,[2] accounting for the minor-to-moderate increases in prothrombin time and activated partial thromboplastin time that are commonly seen immediately after surgery.

Perioperative hemodilution is accompanied by platelet consumption, an effect that correlates with the magnitude of tissue trauma. An important characteristic of the postsurgery platelet count is that it usually continues to decline over the next 1 or 2 days, with the postoperative nadir usually occurring at a median of postoperative day 2, with a range between postoperative days 1 and 4 (FIGURE 64.1A).[3,4] Subsequently, there is a rise in the platelet count to levels often two to three times the patient's preoperative baseline, peaking at approximately day 14, before it returns to baseline over the next 2 weeks (~day 28).

This platelet count profile can be explained by thrombopoietin physiology. Thrombopoietin, produced at a constant rate by the liver, is regulated by the circulating platelet mass. Platelets bind thrombopoietin, thereby decreasing the amount of circulating thrombopoietin. With perioperative platelet count declines, more thrombopoietin becomes available to stimulate megakaryocytes. However, there is a lag between megakaryocyte stimulation and release of new platelets. Studies of administering the thrombopoietin receptor analogue, romiplostim, to human volunteers showed an increased platelet count beginning after 3 to 5 days and peaking about 7 to 10 days later.[5] As platelet stimulation necessarily has an approximate 3-day lag, it would be expected that platelet counts reach a nadir as late as 3 or even 4 days after major surgery (SEE FIGURE 64.1B). This time course is the most important feature differentiating the normal platelet count decrease after major surgery from immune-mediated thrombocytopenias, especially from heparin-induced thrombocytopenia (HIT) where the platelet count decrease typically *begins* on or after day 5.[4,6]

This pattern can be changed by perioperative platelet transfusions that produce a transient increase in platelet count, with subsequently clearing of the transfused platelets over the next 2 or 3 days, resulting in a shift of the platelet count nadir to the right.

PLATELET SEQUESTRATION

Hypersplenism

Hypersplenism denotes the reduction in one or more of the peripheral blood counts due to sequestration within an enlarged spleen. Unlike postoperative hemodilution, hypersplenism is characterized by subacute or chronic, rather than acute, thrombocytopenia.

Normally, approximately one-third of the total body platelet mass resides within the spleen, even though the spleen receives only 5% of the cardiac output.[7] This discrepancy occurs because the transit time of a platelet through the spleen averages 20 minutes, much longer than the time required for a platelet to return to the heart after passing through other organs (~1 minute). In hypersplenism, the proportion of platelets within the spleen can be as high as approximately 90%.[7]

Characteristic clinical features of hypersplenism include splenomegaly (measured objectively as >11 cm by imaging studies) and mild-to-moderate pancytopenia. Often, the degree of anemia is not as marked as neutropenia and thrombocytopenia. In patients with advanced cirrhosis, thrombocytopenia is further aggravated by reduced platelet production due to decreased thrombopoietin levels[8] or the toxic effects of alcohol and/or increased platelet consumption due to enhanced thrombin generation and hyperfibrinolysis.

Table 64.2 lists various causes of splenomegaly.[9] In Western societies, cirrhosis (usually secondary to alcohol consumption or chronic viral hepatitis) is a common cause, whereas other explanations are more common elsewhere (e.g., schistosomiasis, malaria).

Treatment is usually not required, as thrombocytopenia rarely reaches clinically important levels and splenectomy is not without risk.

Table 64.1

Classification of thrombocytopenia

Six general explanations for thrombocytopenia, including pseudothrombocytopenia.

Explanation	Comment	Examples
Pseudothrombocytopenia	Antibody-mediated platelet clumping that occurs *ex vivo*, that is, spurious thrombocytopenia	EDTA-induced pseudothrombocytopenia; platelet satellitism (platelet binding to leukocytes)
Hemodilution	Abrupt platelet count fall due to administration of fluids or blood products	Early postoperative thrombocytopenia
Hypersplenism	Splenomegaly may require imaging studies to detect; concomitant leukopenia and (to lesser extent) anemia are usually present	Cirrhosis-associated portal hypertension
Decreased platelet production	Usually there is concomitant leukopenia and anemia (a notable exception is certain types of hereditary thrombocytopenia)	MDS; aplastic anemia; marrow infiltration by metastatic cancer; alcohol-induced thrombocytopenia
Platelet consumption	Accelerated platelet clearance due to pathologically increased platelet activation or other poorly defined mechanisms of increased platelet clearance	DIC; septicemia; multiorgan system failure; hemophagocytic syndrome
Platelet destruction	Accelerated platelet clearance due to pathological mechanisms that target platelets, especially due to platelet-reactive antibodies	Autoimmune thrombocytopenic purpura; HIT; drug-induced immune thrombocytopenia; TTP/HUS

EDTA, ethylenediaminetetraacetic acid; HIT, heparin-induced thrombocytopenia; TTP/HUS, thrombotic thrombocytopenic purpura/hemolytic uremic syndrome.

Hypothermia

Hypothermia can be accompanied by mild-to-moderate thrombocytopenia.[10] The thrombocytopenia is transient and corrects with rewarming. Platelet kinetic studies in cooled dogs demonstrated reversible hepatic and splenic platelet sequestration.[11] Indirect evidence suggests that decreased platelet production may also contribute.[12]

DECREASED PLATELET PRODUCTION

Thrombocytopenia often is caused by decreased platelet production, which can be divided into three categories: acquired, generalized marrow disorders; acquired, isolated megakaryocytic disorders (e.g., alcohol-associated thrombocytopenia); and hereditary thrombocytopenic disorders.

Acquired, Generalized Marrow Disorders

By far the most common cause of decreased platelet production is acquired, generalized bone marrow disease. The pathologic process usually perturbs all cell lines, and pancytopenia typically results. Occasionally, however, isolated thrombocytopenia is the first abnormality detected during the early stages of a panmyelopathy, for example, myelodysplasia (MDS).

Nonneoplastic Hypoproliferative Stem Cell Disorders—Thrombocytopenia accompanies aplastic anemia, chemotherapy-induced pancytopenia, and idiosyncratic drug-induced aplastic anemia. Sometimes, isolated thrombocytopenia is a precursor of aplastic anemia.

Paroxysmal Nocturnal Hemoglobinuria (PNH)—PNH is a stem cell defect resulting in the loss of phosphatidylinositol-anchored proteins, resulting in intravascular hemolysis due to increased susceptibility of red blood cells to complement-mediated lysis; PNH is also associated with thrombocytopenia.[13] The low platelet count is a composite of a dysplastic platelet production defect, enhanced platelet consumption due to the higher susceptibility of PNH platelets to thrombin, and enhanced platelet destruction due to increased generation of lytic C5b9 complexes on platelet membranes.[14] Patients are at increased risk for thrombotic complications, perhaps induced by the production of procoagulant microparticles from affected blood cells. Diagnosis is made by showing by flow cytometry a population of blood cells with decreased/absent phosphatidylinositol-anchored proteins. A treatment option in severe PNH is the monoclonal antibody, eculizumab.[15,16]

Myelodysplasia—Isolated, refractory thrombocytopenia is the initial presentation of MDS in 1% to 3% of patients and can be confused with immune thrombocytopenic purpura (ITP).[17] Bleeding can result from thrombocytopenia or defective platelet function or from both. Findings that aid in differentiating MDS from ITP include red cell macrocytosis, a left-shifted white cell series, leukocyte morphologic abnormalities (e.g., hypogranular neutrophils and Pelger-Huët anomaly), and hypogranular, giant platelets. Marrow examination reveals dysmegakaryocytopoiesis in half of cases. Typically, the megakaryocytes are abnormally

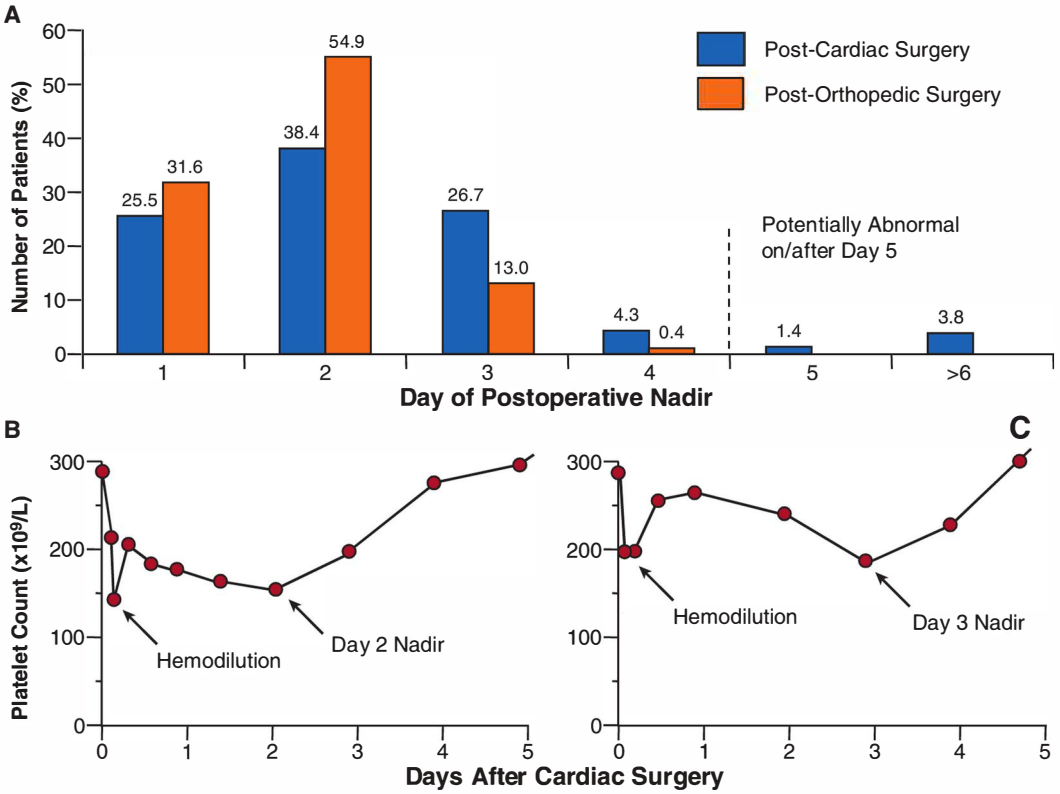

FIGURE 64.1 Early postoperative platelet count decreases. **A:** Distribution of early postoperative count nadirs. For both orthopedic and cardiac surgery patients, day 2 represents the most common day for the postoperative platelet count nadir to occur (data exclude day 0); beyond postoperative day 4, it is likely that a superimposed thrombocytopenic disorder is occurring. Orthopedic surgery data obtained from Warkentin TE, Levine MN, Hirsh J, et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N Engl J Med* 1995;332(20):1330–1335. and cardiac surgery data obtained from Greinacher A, Selleng K. Thrombocytopenia in the intensive care unit patient. *Hematology Am Soc Hematol Educ Program* 2010;2010:135–143. **B,C:** Representative postcardiac surgery platelet count decreases. Both patients illustrate early hemodilution effects (day 0) and subsequent additional early platelet count declines with nadirs of (**B**) day 2 and (**C**) day 3. Neither patient received platelet transfusions.

small, but large mononuclear and abnormal segmented megakaryocytes are found in some patients.[18] Megakaryocyte numbers are usually normal, but increased megakaryocytes (resembling ITP) or amegakaryocytic thrombocytopenia[19] have also been observed.

Neoplastic Stem Cell Disorders—Thrombocytopenia is common with primary neoplastic disorders of the bone marrow. Usually the presence of pancytopenia and circulating malignant cells points to the diagnosis; bone marrow aspirate and biopsy are confirmatory. These disorders include acute myeloid and acute lymphoid leukemia, as well as many chronic lymphoproliferative disorders, such as chronic lymphoid leukemia, hairy cell leukemia, non-Hodgkin's lymphoma, adult T-cell leukemia and lymphoma, multiple myeloma, and Waldenström's macroglobulinemia.

Infiltrative Bone Marrow Disorders—Thrombocytopenia can be secondary to infiltration of the bone marrow by nonhematologic metastatic malignancies, infectious organisms, storage-cell disorders, myelofibrosis, and osteopetrosis. When bone marrow replacement occurs from malignancies (e.g., breast cell carcinoma, prostate carcinoma), peripheral blood features include normoblasts (nucleated red cells), teardrop red cells, and leukopenia usually with leukocyte left shift. This constellation of findings is called a "myelophthisic" blood picture.[20]

Megaloblastic Anemia—Megaloblastic anemia, caused by vitamin B_{12} or folic acid deficiency, is often associated with mild thrombocytopenia. Vitamin deficiency–induced defective DNA synthesis results in megakaryocytes of low ploidy and small platelets.[21] Thus, the combination of large, heterogeneous red cells (oval macrocytes) together with small platelets (low mean platelet volume) is characteristic of megaloblastic anemia.

Isolated Thrombocytopenia Due to Drugs

Alcohol-associated thrombocytopenia is associated with heavy and prolonged alcohol consumption and can cause an acute, self-limited thrombocytopenia.[22] The platelet count increases 2 to 3 days after cessation of alcohol consumption, with "rebound" thrombocytosis. Bone marrow aspirates early in the course of the thrombocytopenia reveal reduced megakaryocyte numbers and sometimes vacuolation of normoblasts and promyelocytes.

Table 64.2

Causes of splenomegaly

Congestive Splenomegaly
Intrahepatic
Cirrhosis[a]
Extrahepatic
Portal vein obstruction
Splenic vein obstruction
Hepatic vein occlusion (Budd-Chiari syndrome)
Chronic passive congestion
Heart failure
Infections
Acute
Viral (viral hepatitis, infectious mononucleosis, cytomegalovirus infection)
Bacterial (septicemia, salmonellosis, brucellosis, splenic abscess)
Parasite (toxoplasmosis)
Subacute and chronic
Subacute bacterial endocarditis
Tuberculosis
Malaria
Kala-azar
Fungal disease
Inflammation
Felty syndrome
SLE
Serum sickness
Rheumatic fever
Sarcoidosis
Hematologic Disorders
Red cell disorders: hemolytic anemias, thalassemia, sickle cell disorders
Macrophage activation (hemophagocytic) syndrome
Neoplasia
Malignant
Myeloproliferative neoplasms
Myeloid metaplasia
Polycythemia rubra vera
Essential thrombocythemia
Chronic myeloid leukemia
Chronic lymphocytic leukemia
Hairy cell leukemia
Lymphoma
Acute leukemia
Malignant histiocytosis
Benign
Hamartoma
Hemangioma
Lymphangioma
Fibroma
Storage Diseases
Gaucher disease
Niemann-Pick disease
Miscellaneous
Amyloidosis
Cysts
Nontropical idiopathic splenomegaly (Dacie syndrome)

[a]The most common cause of hypersplenism in North America.

Interferon used for the treatment of hepatitis C causes a 10% to 50% drop in the platelet count in patients on combination therapy and 4% to 6% of patients require dose modification secondary to thrombocytopenia.[23] Interferon is thought to alter thrombopoietin production, thereby reducing platelet production. In most cases, thrombocytopenia is clinically insignificant, but reduction of the interferon dose is necessary when platelet counts decrease below 50×10^9/L and cessation of treatment is required if platelet counts decrease below 30×10^9/L.

Drugs targeting RUNX1, a transcription factor important in leukemia, have the potential to cause decreased platelet production,[24] as RUNX1 plays an important role in thrombopoiesis.[25]

Iron repletion: Mild-to-moderate thrombocytopenia can be observed also approximately 1 week after beginning iron replacement therapy[26]; this transient platelet count decline is most likely the result of a shift of stem cells to erythropoiesis.

Fatty acid–induced thrombocytopenia is most prominent in patients with adrenoleukodystrophy, who are treated with Lorenzo's oil (a mixture of glycerol trioleate and trierucate).[27] These oils change the composition of the platelet membrane, causing membrane anisotropy the production of enlarged and giant platelets, as well as platelet function defects.[28]

Viral Infections

Viral infections commonly cause thrombocytopenia, in part through decreased platelet production,[29] perhaps due to direct megakaryocyte infection as best illustrated by HIV infection.[30] In addition, viral infection can trigger platelet-reactive autoantibodies which can produce severe immune thrombocytopenia (see Chapter 61). Similarly, MMR (measles–mumps–rubella) vaccine results in acute immune thrombocytopenia beginning approximately 10 to 14 days—and up to 6 weeks—postvaccination in approximately 1/40,000 exposures.[31]

Miscellaneous Acquired Thrombocytopenia

Iron depletion—Mild-to-moderate thrombocytopenia can be observed at presentation of severe iron deficiency anemia.[32]

Acquired amegakaryocytic thrombocytopenia—Acquired amegakaryocytic thrombocytopenia is a rare disorder characterized by isolated thrombocytopenia associated with a total or marked reduction in megakaryocytes.[33,34] Sometimes, progression to aplastic anemia or MDS occurs. In other patients, there are autoimmune features (e.g., association with systemic lupus erythematosus (SLE) or a positive direct Coombs' test). Cyclic amegakaryocytic thrombocytopenia has also been observed.[35]

The pathogenesis of acquired amegakaryocytic thrombocytopenia is heterogeneous and at least three different mechanisms have been implicated: an intrinsic stem cell defect (defective growth of megakaryocyte precursors), autoantibodies directed against megakaryocyte precursors, and T-cell–mediated suppression of megakaryocyte development. Thrombopoietin levels are usually much greater than seen in patients with ITP.[36]

Treatment of these disorders have included corticosteroids, antithymocyte globulin, cyclosporine, cyclophosphamide, immunoglobulins, splenectomy, or allogenic bone marrow transplantation (BMT).[33,34]

Hereditary Thrombocytopenia

This topic is discussed in detail in Chapter 63; however, they are mentioned here because some hereditary thrombocytopenias with mild bleeding symptoms (e.g., MYH9-associated disorders) are often misconstrued to be an "acquired" disorder when previous platelet counts are not available and the associated macrothrombocytopenia suggests other diagnoses such as ITP or MDS.[37]

INCREASED PLATELET CONSUMPTION

Reduced platelet survival arising from platelet activation or accelerated platelet clearance can be represented as "consumptive" disorders. Pathologically increased thrombin generation as occurs with "disseminated intravascular coagulation (DIC)" or "consumptive coagulopathy" often produces thrombocytopenia and is discussed in Chapter 98. Decreased platelet survival (with or without overt DIC) due to enhanced macrophage-mediated platelet clearance is common in sepsis.

Septicemia

Thrombocytopenia occurs in 50% to 75% of patients with systemic bacterial or fungal infections, and in almost all patients with septic shock[38] (see Chapter 123). The thrombocytopenia is generally mild to moderate in severity and bleeding due to thrombocytopenia alone is infrequent. Thrombocytopenia in sepsis is associated with increased mortality. However, increased mortality is not attributable to thrombocytopenia-related bleeding, but rather as a marker of underlying disease severity, as exemplified by a study of post-liver transplant patients.[39]

The likelihood of laboratory evidence for DIC increases as the platelet count falls to $<50 \times 10^9$/L.[38] The mechanism of thrombocytopenia in septicemia in the absence of DIC is uncertain, but could include chemokine-induced macrophage ingestion of platelets (hemophagocytosis[40]) and platelet activation by endogenous mediators of inflammation (e.g., platelet-activating factor) or microbial products.[41] In rare situations, platelet-reactive autoantibodies have been implicated.[42] A diagnosis of sepsis-related thrombocytopenia is corroborated by a positive blood culture. Prompt recognition and treatment of the responsible infection is the most important therapy, as recovery of the platelet count tends to parallel the resolution of the infection. Prophylactic platelet transfusions are generally not required unless the platelet count falls to $<10 \times 10^9$/L, but bleeding might occur at higher platelet counts if comorbid clinical features are present (e.g., concomitant coagulopathy, an invasive procedure, renal failure). The use of heparin for patients with septic shock and DIC is controversial (see Chapter 98). However, heparin may benefit a subset of patients with clinical evidence of DIC and microvascular thrombosis (e.g., acral tissue ischemia or necrosis) if it can reduce progression of thrombosis.

Thrombocytopenia that occurs after 2 to 3 weeks of hospitalization, especially in a patient who has received antibiotics, suggests fungemia (e.g., candidemia). Worldwide, malaria is the most common parasite causing thrombocytopenia.

HIV Infection

Thrombocytopenia in patients infected with HIV poses a special diagnostic problem, because there are many potential and sometimes coexisting explanations for the thrombocytopenia.[43] These include immune-mediated platelet destruction, impaired platelet production secondary to HIV infection of megakaryocytes,[29,30] drug-induced myelosuppression (commonly implicated drugs include zidovudine, ganciclovir, and trimethoprim-sulfamethoxazole), HIV-associated thrombotic microangiopathy, hypersplenism, and marrow infiltration by tumor or opportunistic infections. Platelet kinetic studies have found a complex interaction of decreased platelet production, increased platelet destruction, and splenic platelet sequestration.[44] Immune mechanisms for platelet destruction include antibodies that cross-react with αIIbβ3 complexes ("molecular mimicry")[45] and induce platelet apoptosis,[46] as well as immune complexes containing IgM anti-idiotype antibodies (which could explain the paradox of high levels of platelet-associated IgG and IgM with low serum levels of platelet-reactive antibodies).[47] Anti-HIV chemotherapy (e.g., zidovudine, HAART) often raises the platelet count in patients with HIV-associated thrombocytopenia.[48] Most patients with HIV-associated thrombocytopenia respond to conventional treatments for ITP, including corticosteroids, splenectomy, intravenous immunoglobulin, and, particularly, anti-D.[49]

Macrophage Activation (Hemophagocytic) Syndrome

The macrophage activation (also known as the hemophagocytic) syndrome comprises a heterogeneous group of disorders characterized by fever, hepatosplenomegaly, elevated liver enzymes, cytopenias, and phagocytosis of red cells, granulocytes, and platelets by nonmalignant macrophages; hyperferritinemia, hypercytokinemia, weight loss, and sepsis-like features are characteristic, with high potential for fatal multiple organ failure or fatal complicating infections.[50,51] Table 64.3 lists various causes and diagnostic criteria.[52]

"Primary" hemophagocytic syndrome, also known as familial erythrophagocytic lymphohistiocytosis, is an autosomal-recessive

Table 64.3

Macrophage activation (hemophagocytic) syndrome: causes and diagnostic criteria

Primary
Familial
Secondary
Lymphoma (especially, certain T-cell or natural killer lymphomas)
Infection, especially, viral (e.g., Epstein-Barr virus, cytomegalovirus, HIV), bacterial (e.g., salmonella, brucellosis, mycobacteria, ehrlichiosis), or protozoal (e.g., plasmodia, babesiosis)
Immunosuppressive drugs (especially, transplant patients)
Autoimmune (especially, SLE, juvenile rheumatoid arthritis, ankylosing spondylitis)
Diagnostic criteria (five or more of the following)[a]:
Fever
Splenomegaly
Cytopenias (affecting two or more lineages in the peripheral blood): hemoglobin <90 g/L, platelets <100 × 10^9/L, neutrophils <1 × 10^9/L
Hypertriglyceridemia and/or hypofibrinogenemia triglyceride (fasting) ≥3 mmol/L (≥265 mg/dL) fibrinogen (≤1.5 g/L)
Hemophagocytic in bone marrow, spleen, or lymph nodes
Ferritin >500 μg/L
sCD25 (soluble IL-2 receptor) ≥2,400 U/mL
Low or absent NK-cell activity

[a] Adapted from Henter JI, et al. HLH-2004: diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer* 2007;48:124–131.

disorder that usually presents in infancy or early childhood. The high mortality rate associated with primary hemophagocytic lymphohistiocytosis in children warrants aggressive treatment, including antineoplastic chemotherapy and BMT.

"Secondary" hemophagocytosis affects adults and can be associated with lymphoma, infection (especially Epstein-Barr virus,[53] but also HIV[54]), immunosuppressive drugs (e.g., transplant recipients[55]), or certain autoimmune disorders.[56] Whether infection is the trigger or the consequence of macrophage activation is not always clear.

Thrombocytopenia is usually present, and may be complicated by bleeding, especially when there is concomitant hypofibrinogenemia with or without DIC. Anemia is often profound, reflecting the combination of erythrophagocytosis and the proinflammatory milieu (anemia of chronic disease).

Laboratory indicators of the macrophage activation syndrome include markedly elevated ferritin (often >10,000 μg/L),[57] soluble CD25 (interleukin-2 receptor α-chain), elevated levels of soluble CD163 (a member of the scavenger receptor cysteine-rich family of proteins, accounting for the clearance of hemoglobin–haptoglobin complexes), and morphologic evidence of hemophagocytosis.

Treatment should be directed at the underlying illness. Early administration of corticosteroids and high-dose gamma globulin appear to benefit some patients with the nonneoplastic syndrome.[58]

Envenomation

Snake bite frequently produces a profound consumptive coagulopathy that can be accompanied by thrombocytopenia.[59,60] The toxins present in snake venoms can be activators of the clotting cascade like thrombin-like enzymes (many vipers), prothrombin activators (*Echis* species, Australasian elapids), and factor X activators (Russell's viper), or they can activate platelets directly via the collagen receptor glycoprotein (GP) VI such as convulxin[61] or GPIb such as bilinexin.[62] However, unlike typical DIC, where microthrombosis and organ dysfunction are often present, venom-induced consumption coagulopathy is usually only complicated by bleeding. Nonetheless, a subgroup of patients develops a picture that resembles thrombotic microangiopathy, with renal failure, microangiopathic hemolysis, and thrombocytopenia that persists longer than the coagulopathy.[60] The most important treatment is to administer antivenom as soon as possible.

Pulmonary Embolism

Thrombocytopenia of variable severity can accompany pulmonary embolism. When the thrombocytopenia is severe, there is associated DIC.[63,64] The low platelet count presumably results from thrombin-induced platelet activation and/or platelet accretion within the thromboemboli.[65] Large thromboemboli within pulmonary vasculature may act as a reservoir for clot-bound thrombin that is relatively protected from inhibition by antithrombin-dependent inhibitors.[66] Thrombocytopenia commonly occurs with pulmonary embolism, but not with deep vein thrombosis alone.[67,68]

An important differential diagnosis in patients who develop pulmonary embolism or deep vein thrombosis associated with thrombocytopenia during treatment with heparin either in prophylactic or in therapeutic dose is HIT[68] (see Chapter 108).

Cardiovascular Disorders and Devices

Severe *congenital cyanotic heart disease* is often associated with moderate thrombocytopenia. Underlying mechanisms include a composite of increased platelet consumption due to hyperviscosity, thrombin generation due to venous stasis, and hypoxemia-related decreased platelet production. Reduction of the hematocrit to normal levels can improve hemostasis.[69]

Valvular heart disease can reduce the platelet count to some extent, especially aortic valve stenosis, most likely due to platelet activation. However, the major cause of increased bleeding in these patients is acquired von Willebrand syndrome due to shear-induced accelerated clearance of the largest von Willebrand factor multimers.[70] Endocarditis due to infected cardiac valves can cause severe thrombocytopenia through increased platelet activation, DIC, hypersplenism (subacute endocarditis), and, rarely, autoimmune mechanisms.

Artificial heart valve–associated thrombocytopenia is often caused either by infection of the valve or by a paravalvular leak produced by shear stress–induced platelet activation. However, paravalvular leaks most often produce red cell fragmentation without thrombocytopenia. One aortic bioprosthesis used in Europe is associated with a transient decrease in platelet counts in more than half the patients.[71] The platelet count decrease occurs soon after surgery, with 22% developing severe thrombocytopenia (platelet count $<30 \times 10^9$/L), but usually the platelet counts normalize within 2 to 3 weeks, without an apparent increased risk of thromboembolism or major bleeding. The responsible mechanism is unresolved.

Aortic aneurysms can cause thrombocytopenia because activation of coagulation by the abnormal vessel wall results in thrombin generation.[72] Imaging studies usually reveal adherent thrombus within the aneurysm.

Arterial Dacron vascular grafts can cause a mild decrease in platelet counts. The platelet life span improves over time, presumably because of progressive endothelialization of the implanted foreign material.[73]

Extracorporeal and intravascular devices. Extracorporeal and intravascular devices can induce thrombocytopenia for a number of reasons: their surface may be prothrombotic, causing platelet activation and thrombin generation; they may be located in areas of high shear stress, leading to shear-induced platelet activation; and they may mechanically alter platelets. The latter primarily occurs in intracardiac assist devices.

Cardiopulmonary bypass (CPB). During CPB, there is extensive contact between heparin-anticoagulated blood and the synthetic surfaces of the extracorporeal perfusion device (blood oxygenator). Generally, CPB-associated bleeding results from one or more of the following: thrombocytopenia and/or platelet dysfunction, surgical bleeding, and hyperfibrinolysis. Typically, the platelet count falls by 30% to 70% following CPB, initially due to hemodilution, aggravated by losses within the extracorporeal perfusion device, hypothermia-induced prolonged passage in the spleen, and consumption at sites of bleeding.[74] The thrombocytopenia persists for 3 to 4 days after bypass. Studies have described transient, *intrinsic* defects in platelet function. These include decreased *in vitro* platelet aggregation, decreased platelet surface membrane proteins, selective depletion of platelet α-granules,[75] and evidence of *in vivo* platelet activation and platelet vesiculation. The platelet dysfunction in heart surgery is also attributable to an *extrinsic* platelet defect resulting from thrombin inhibition by the high doses of heparin.[76] However, in most patients, the bleeding time, which rises markedly during heart surgery (>30 minutes), usually improves to <15 minutes shortly after surgery, and to normal within several hours.

Intra-aortic balloon pump. Intra-aortic balloon pumps result in a decrease of about 30% to 40% from the initial value in about half of the patients. Although thrombocytopenia may be caused directly by the device (as its removal often results in a rapid increase in platelet numbers), improved hemodynamic function may also alleviate platelet consumption.[77]

Cardiac (ventricular) assist devices. Various cardiac assist devices are designed to support left, right, or biventricular function. They differ in design (e.g., membrane pumps, roller pumps, high-speed propellors [Impella]) and flow characteristics (continuous, axial). All of these devices activate platelets and several destroy platelets mechanically as well.[78,79] Thrombocytopenia is very frequent in these patients, as are thrombotic and bleeding complications. The mechanisms by which the different devices cause platelet activation and alteration are only partly understood. Recently, acquired von Willebrand syndrome was reported with these devices.[80]

Other organ replacement devices. Hemodialysis, extracorporeal oxygenation, chronic venovenous renal replacement therapy, and plasmapheresis (with or without immunoadsorption) can all induce a mild-to-moderate decrease in platelet count.[81,82] A more pronounced decrease should prompt assessment of the anticoagulant regimen, as platelet consumption resulting from increased thrombin generation due to insufficient anticoagulation can occur. If platelet counts decrease abruptly by more than 50% within the second week after start of extracorporeal treatment devices, HIT should be considered, especially if new clots occur in the device.

Microangiopathic Hemolysis

This is characterized by anemia due to microangiopathic hemolysis (red cell fragments, increased lactate dehydrogenase, reticulocytosis) and thrombocytopenia.[83] Some disorders are well characterized, for example, thrombotic thrombocytopenic purpura (TTP) associated with autoantibodies against ADAMTS13 or hereditary deficiency of this enzyme.[84] The hemolytic–uremic syndrome (HUS, microangiopathic hemolysis and renal failure) is associated with verocytotoxin-associated diarrheal prodrome (typical HUS)[85] or hereditary abnormalities of complement (atypical HUS).[86] Others are associated with bone marrow and stem cell transplantation[87] or with medications.[88] This topic is discussed in detail in Chapter 99.

INCREASED PLATELET DESTRUCTION

Increased platelet destruction—as compared with platelet consumption—indicates reduced platelet life span caused by antibodies, such as autoantibodies, alloantibodies, or drug-dependent antibodies, which usually target platelet surface GPs. In contrast to most of the other causes of thrombocytopenia discussed above, platelet counts decrease significantly and severe thrombocytopenia ($<20 \times 10^9$/L) is often present.[89] With the exception of HIT (discussed in Chapter 108), platelets are not activated in the circulation. Mucocutaneous bleeding is the predominant complication. Immune-mediated thrombocytopenias are summarized in Table 64.4 and described in detail in Chapters 61 and 62.

SPECIALIZED TOPICS

Thrombocytopenia in the Intensive Care Unit Patient

The many comorbidities in the severely ill patient also make thrombocytopenia very common, occurring in 35% to 45% of patients in ICUs, although only 5% to 20% are severely thrombocytopenic.[4,90–95,96,97–104] About half of patients in ICUs have a low platelet count at admission ("prevalent" thrombocytopenia), the others develop thrombocytopenia during their ICU stay ("incident" thrombocytopenia).[96] This section provides a framework to differentiate among the various causes of acquired thrombocytopenia in this patient population.

Hemodilution and increased platelet consumption are very common causes of moderate thrombocytopenia after tissue

Table 64.4

Classification of immune thrombocytopenia

Autoimmune
Primary
Secondary
Rheumatic
Infectious, including HIV
Lymphoproliferative
Drugs (rare)
Alloimmune
Neonatal alloimmune thrombocytopenia
Posttransfusion purpura (PTP)
Passive alloimmune thrombocytopenia (PAT)
Transplantation-associated alloimmune thrombocytopenia
Platelet transfusion refractoriness
Drug dependent
D-ITP syndrome
HIT
GPIIb/IIIa-dependent thrombocytopenia
Drug-induced TTP
Quinine-dependent DIC

trauma, bleeding, or DIC. Severe thrombocytopenia with platelet counts <20 × 10^9/L can be due to bone marrow failure (e.g., acute leukemia), sepsis with severe coagulopathy, or immune-mediated platelet destruction. In particular, acute TTP (Chapter 99) needs to be recognized because these patients require plasma exchange. In endemic areas, malaria and viral hemorrhagic fever are important causes.

Thrombocytopenia is a marker of illness severity,[105,106] and an adverse prognostic marker predicting for prolonged ICU stay and mortality (mortality rate 31% to 46% in thrombocytopenic patients vs. 16% to 20% nonthrombocytopenic patients).[91–93,95,96,97,98,102] In contrast, it is unclear whether thrombocytopenia—other than very severe thrombocytopenia (platelet count, <10 × 10^9/L)—is a risk factor for bleeding.[107] Other factors that interfere with normal hemostasis, such as platelet function defects, hyperfibrinolysis, invasive procedures, or catheters, may be more relevant. Even if not associated with bleeding, low platelet counts often influence patient management and may prompt physicians to withhold or delay necessary invasive interventions, reduce the intensity of anticoagulation, order prophylactic platelet transfusion, or change anticoagulants inappropriately (e.g., due to unfounded fear of HIT).

When acute thrombosis complicates thrombocytopenia, one needs to consider the occurrence of severe pulmonary embolism with associated DIC,[63–65] diabetic ketoacidosis,[108] antiphospholipid syndrome (see Chapter 101),[109] and HIT (see Chapter 108).[1,6,89]

One approach to differentiating between different causes of thrombocytopenia in this setting is to consider the timing of onset of the platelet count changes (FIGURE 64.2).[4] In the vast majority of patients, the initial decrease in platelet counts within the first 2 to 4 days after trauma/surgery is transient and platelet counts reach the presurgery level at about postoperative day 5 to 7. A blunted or absent rise in platelet counts after day 4, however, is strongly associated with mortality and prolonged ICU admission and is a stronger predictor for mortality than the platelet count nadir during early ICU stay.[95,99,102,110] Prolonged thrombocytopenia is often caused by severe infection (e.g., bacterial sepsis, fungemia, endocarditis) or multiorgan failure. Moderate thrombocytopenia can be caused by sepsis alone, but when the platelets fall below 50 × 10^9/L, DIC is frequently present.[38]

A rare but important cause of severe thrombocytopenia manifesting at day 2 to 3 after surgery is *postsurgery TTP*.[111,112] Postsurgery TTP is presumably triggered by increased release of von Willebrand factor from endothelial cells as a response to major surgery on a background of low activity of ADAMTS13, either due to preexisting "compensated" anti-ADAMTS13 antibodies or due to mutations in the enzyme resulting in reduced activity. In contrast, a rapidly progressive platelet count decrease (e.g., occurring over a 1 to 3-day period) and that begins on day 5 or later (most often during the second week) strongly suggests an immunological cause (FIGURE 64.2). Relatively common examples include immune HIT (Chapter 108)[6] and drug-induced immune thrombocytopenic purpura (D-ITP), typically induced by drugs started within the last 2 weeks (Chapter 62).[113] Rare examples include αIIbβ3 inhibitor–induced thrombocytopenia of delayed onset in patients (see Chapter 62)[114] and post-transfusion purpura (PTP) (see Chapter 61).[115]

Occasionally, thrombocytopenia can begin abruptly within a few hours of the inciting event and can be due to passive alloimmune thrombocytopenia (PAT)[116] caused by transfusion of platelet-reactive alloantibodies, transfusion of bacterially contaminated blood products,[117] and rapid-onset HIT occurring in a patient with circulating HIT antibodies as a result of recent heparin exposure.[6] HIT is relatively rare in the ICU setting with an incidence of about 0.3% to 0.5%[96,118]. HIT usually features a platelet count fall >50% (from the highest value after day 4 of heparin treatment), most often to a nadir between 20 and 100 × 10^9/L, and can present with new thrombosis, typically occurring 5 to 14 days after start of prophylactic or therapeutic doses of heparin.[89] Timing of onset, the moderate nature of thrombocytopenia, and the common concurrence of thrombosis are very important factors differentiating HIT from other causes of thrombocytopenia in the ICU. Patients with early-onset thrombocytopenia or a platelet count <20 × 10^9/L usually do not have HIT.

Thrombocytopenia in Pregnancy

This topic is considered in detail in Chapter 118. Briefly, pregnancy-associated thrombocytopenia is a benign condition (gestational thrombocytopenia) that appears to be nonimmune in nature and is partly caused by dilution due to the increased plasma volume, although the exact cause is not well understood. Increased platelet consumption is the likely reason for thrombocytopenia associated with preeclampsia/eclampsia, the hemolysis, elevated liver enzymes, low platelets (HELLP) syndrome, and in coagulopathies associated with a retained fetus, placental abruption, amniotic fluid embolism, endometritis, or severe hemorrhage. Pregnancy is also associated with an increased risk for autoimmune disorders such as ITP (Chapter 61) and HUS/TTP (Chapter 99).

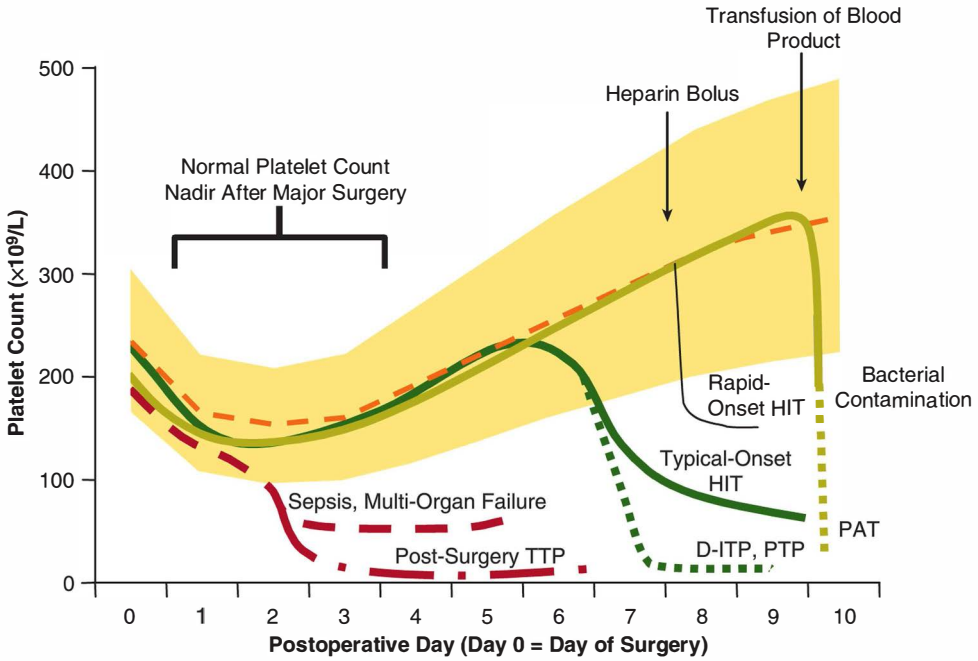

FIGURE 64.2 Timing of onset and severity of thrombocytopenia: implications for differential diagnosis. The usual postoperative platelet count nadir is seen between postoperative days 1 and 3 (inclusive). Early and progressive platelet count declines often reflect severe postoperative complications such as sepsis and multiorgan failure; severe thrombocytopenia can (rarely) indicate postsurgery thrombotic thrombocytopenic purpura (TTP). Thrombocytopenic disorders that begin approximately 1 week after surgery are often immune mediated: moderate thrombocytopenia can indicate heparin-induced thrombocytopenia (HIT), both "typical onset" and (if heparin is not being given) "delayed onset"; very severe thrombocytopenia can indicate drug-induced immune thrombocytopenic purpura (D-ITP) or (rarely) posttransfusion purpura (PTP). An abrupt decline in platelet count after receiving a heparin bolus in a patient who has received heparin within the past 7 to 100 days can indicate "rapid-onset" HIT; thrombocytopenia that begins abruptly after transfusion of a blood product can indicate sepsis from bacterial contamination or (rarely) passive alloimmune thrombocytopenia (PAT) due to transfusion of platelet-reactive alloantibodies.

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CHAPTER

65 Inherited Disorders of Platelet Function

JOEL S. BENNETT AND A. KONETI RAO

Prolongation of the bleeding time in a patient with a normal platelet count suggests the presence of either von Willebrand disease (vWD) or impaired platelet function. vWD is discussed in Chapter 52, and acquired disorders of platelet function are discussed in Chapter 66. Inherited disorders of platelet function will be considered in this chapter. For the purposes of this discussion, these disorders will be classified into disorders of platelet adhesion, aggregation, secretion, or procoagulant activity according to the predominant aspect of platelet function affected.

DISORDERS OF PLATELET ADHESION

Bernard-Soulier Syndrome (Glycoprotein Ib-IX-V Deficiency)

The Bernard-Soulier syndrome (BSS), a rare hemostatic disorder characterized by a prolonged bleeding time, very large platelets, and thrombocytopenia, was described by Bernard and Soulier[1] in 1948. Patients with BSS bleed because their platelets cannot adhere to von Willebrand factor (vWF), a consequence of deficiency or dysfunction of the platelet membrane glycoprotein (GP) Ib-IX-V (GPIb-IX-V) complex.[2–4]

Following disruption of the vascular endothelium, circulating platelets can adhere to exposed subendothelial connective tissue components such as collagen, fibronectin, and laminins. However, at the higher shear rates present in arterioles and the microcirculation, platelet adhesion requires the additional presence of vWF.[5,6] vWF is an elongated multimeric GP, each monomer of which contains two motifs that interact with receptors on platelets[7] (see Chapter 52). One motif, located in the A1 domain of vWF, mediates transient vWF binding to the GPIb-IX-V complex on unactivated platelets. The second motif, located in the C1 domain, mediates the stable interaction of vWF with the integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) on activated platelets.[8] Although vWF in plasma cannot bind spontaneously to GPIb-IX-V,[9] exposing soluble vWF to the antibiotic ristocetin or the snake venom protein botrocetin *in vitro* enables it to bind to GPIb-IX-V.[10,11] The physiologic correlates for ristocetin and botrocetin are unclear. It is possible that the A1 domain of subendothelial vWF, but not soluble vWF, has a conformation allowing it to bind to GPIb-IX-V.[12] It is also that shear stress changes the conformation of the A1 domain, GPIb-IX-V, or both, enabling vWF binding to GPIb-IX-V.[13]

GPIb-IX-V is a noncovalent transmembrane heterotrimer consisting of the heavily glycosylated 165,000 mol. wt. protein GPIb,[14] the 20,000 mol. wt. protein GPIX,[15] and the 82,000 mol. wt. protein GPV.[16] The structure of GPIb-IX-V is discussed in detail in Chapter 26A. There are approximately 25,000 copies of GPIb and GPIX on the platelet surface but only half as many copies of GPV, suggesting that individual GPIb-IX-V complexes contain two copies of GPIb, two copies of GPIX, and one copy of GPV. GPIb itself is a heterotrimer containing one copy of a 143,000 mol. wt. α subunit (GPIbα) and two disulfide linked copies of a 22,000 mol. wt. β subunit (GPIbβ).[17] GPIbα can be cleaved by trypsin or a calcium-dependent platelet protease into a soluble 135,000 mol. wt. heavily glycosylated amino (*N*)-terminal fragment glycocalicin.[18] The *N*-terminal portion of glycocalicin contains binding sites for vWF and thrombin, while its *C*-terminal portion contains a variable number of tandem 13 residue mucin-like repeats to which multiple sialic acid-rich O-linked hexasaccharides are appended.[19,20] The cytosolic portion of GPIbα binds to the *C*-terminus of filamin A (actin-binding protein),[21,22] linking GPIbα to the platelet cytoskeleton.[23] It binds to the adaptor protein 14-3-3ζ as well, linking GPIbα to platelet signaling pathways.[24–26] The cytosolic portion of GPIbβ can be phosphorylated at Ser166 by cyclic AMP-dependent protein kinase A,[27] a reaction that inhibits collagen-induced platelet actin polymerization.[28] A role for GPV in GPIb-IX-V function is unclear. Although GPV can be cleaved by thrombin,[29] this is not required for vWF binding to GPIbα, nor does it activate platelets.[30]

The *N*-terminal portion of GPIbα, as well as GPIbβ, GPIX, and GPV, contain leucine-rich repeats (LRRs) with the consensus sequence L-L–N-L–LPPGLL-G—L-.[16,31] GPIbα contains eight LRR,[9] whereas GPIbβ and GPIX contain one each and GPV contains 15. The amino acids flanking the LRR are also conserved.[31] A crystal structure for the vWF A1 domain bound to residues 1 to 290 of GPIbα revealed that LRRs 1 and 5 to 8 of GPIbα bind tightly to the A1 domain.[9] Conformational changes in a flexible loop located near the C-terminus of the GPIbα fragment, termed the "β-switch"[9] or "R-loop,"[32] may be needed for vWF binding to GPIbα. This portion of GPIbα is the site of several gain-of-function mutations responsible for platelet-type vWD.[9] The function of the LRR in GPIbβ, GPIX, and GPV is unknown, but they do not participate in intermolecular interactions as "leucine-zippers."

Biosynthetic studies using recombinant GPIb-IX-V revealed that although small amounts of GPIbα can be detected on the surface of cells expressing GPIbα and GPIbβ, GPIbα expression is much more efficient when the cells coexpress GPIbα, GPIbβ, and GPIX.[33] Thus, mutations that result in the deficient expression of either GPIbα, GPIbβ, or GPIX can produce the BSS. On the other hand, GPV is not required for GPIb-IX-V expression and why it is absent from BSS platelets is not known.[34]

Clinical Manifestations

BSS presents in infancy or childhood with ecchymoses, epistaxis, and gingival bleeding.[16] Later manifestations include menorrhagia and postpartum, gastrointestinal, and posttraumatic

hemorrhage. Hemarthroses and expanding hematomas are unusual. Although the severity of bleeding is variable and may not always correlate with the extent of thrombocytopenia and the degree of GPIb-IX-V deficiency,[35] it can be severe enough to require transfusion and suppression of menses. In a review of 59 patients, for example, there were 10 deaths.[36] In some patients with BSS, the severity of hemorrhage inexplicably declines over the course of the disease.[36]

Laboratory Findings

The bleeding time in patients with BSS is markedly prolonged to >20 minutes.[16] Most patients are thrombocytopenic to some degree, and platelet counts <20,000/μL have been reported.[37] Nonetheless, platelet counts vary within kindreds and even in individual patients. BSS platelets are large when observed on peripheral blood smears[16]; 30% to 80% have mean diameters >3.5 μm, and occasional platelets are as large as 20 to 30 μm in diameter.[38] There is contradictory evidence about the size of BSS platelets in the circulation. It has been reported that circulating BSS platelets are normal in size[39] but have increased membrane, perhaps located in the surface-connected open-canalicular system that is extruded when platelets spread during the preparation of a blood smear.[40] Others have reported that the volume of circulating BSS platelets is increased[41] and that they have a more spherical shape[41] with a more deformable membrane.[42] The molecular basis for the macrothrombocytopenia of BSS is not known. However, amelioration of the macrothrombocytopenia when an IL4-GPIbα fusion protein was expressed in a murine BSS model suggests that absence of the GPIbα cytoplasmic domain contributes to this abnormality.[43]

Erythrocytes and white blood cells are normal in BSS. Distinctive abnormalities have not been observed in marrow megakaryocytes by light microscopy,[44] although demarcation membrane system abnormalities have been observed by electron microscopy.[45] The laboratory tests that distinguish BSS from normal platelets are the failure of BSS platelets to agglutinate in the presence of ristocetin or botrocetin and the failure of vWF to restore agglutination (FIGURE 65.1).[16] Aggregation of BSS platelets in response to ADP, collagen, and epinephrine is normal.[16] However, the rate of thrombin-stimulated aggregation can be reduced.[46]

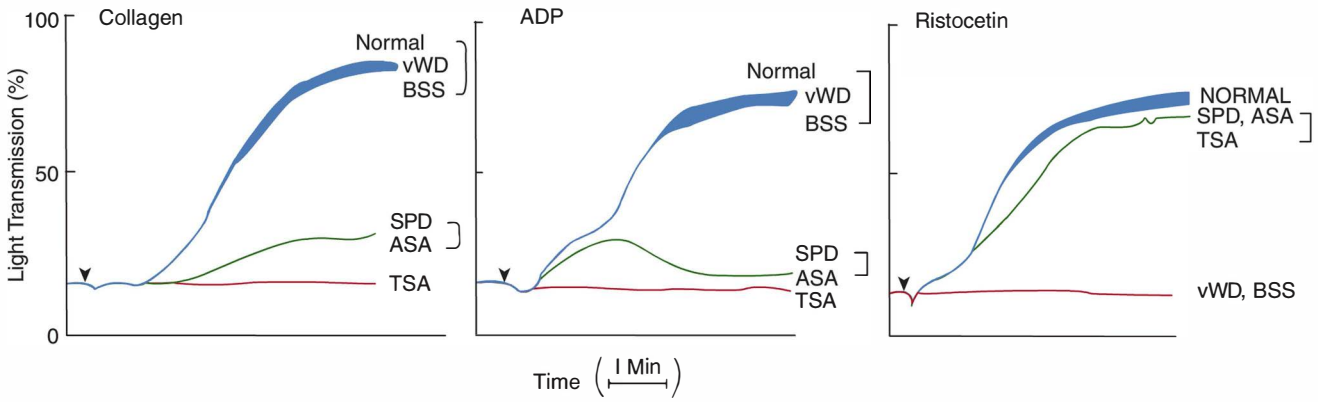

FIGURE 65.1 Platelet aggregation in various platelet disorders. Platelet aggregation can be studied *ex vivo* by a variety of techniques. Conventional turbidometric platelet aggregometry is performed in an "aggregometer" using stirred suspensions of either unwashed platelets in plasma or washed platelets in buffer. Immediately after the addition of all platelet agonists except epinephrine, there is a transient decrease in light transmittance that has been attributed to platelet shape change. This is followed by an increase in light transmission as platelet aggregates form. Two "waves" of platelet aggregation have been described. The "first wave" represents primary aggregation and is a direct consequence of agonist stimulation. The "second wave" represents secondary aggregation and is due to the formation of large irreversible aggregates when platelet secretion occurs. If the concentration of platelet agonist is sufficiently high, the first and second waves merge into a single continuous tracing. Measurements of platelet aggregation are expressed as either the extent or the rate of increase in light transmittance that occurs as platelet aggregates form.

The turbidometric aggregometer has been modified to simultaneously measure platelet aggregation and platelet dense granule secretion using the luciferin–luciferase reaction to measure secretion of dense granule ATP (lumiaggregometry). Platelet aggregation can also be measured in diluted anticoagulated whole blood based on the increase in electrical impedance (measured in ohms) that occurs as platelet aggregates accumulate on a pair of electrodes inserted into the suspension of aggregating platelets (impedance aggregometry). Impedance aggregometry tracings are similar to those obtained by the turbidometric method, except that shape change and two waves of aggregation are not seen. Lastly, because aggregometry is insensitive to the formation of small platelet aggregates, methods have been devised to measure the disappearance of single platelets immediately after platelet stimulation. Agonists are added to stirred platelet suspensions and after adding a fixative to stabilize the aggregates, their number and size are measured either visually with a hemocytometer or using an electronic particle counter or flow cytometer.

The platelet aggregation tracings shown in the figure were generated in a conventional turbidometric platelet aggregometer after the addition of collagen, ADP, and ristocetin to stirred aliquots of platelet-rich plasma. vWD, von Willebrand disease; BSS, Bernard-Soulier syndrome; SPD, storage pool disease; ASA, aspirin; TSA, Glanzmann thrombasthenia. (Adapted from Weiss HJ. Platelet physiology and abnormalities of platelet function (second of two parts). *N Engl J Med* 1975;293:580–588, with permission.)

Although the serum prothrombin time has been reported to be decreased in BSS[47] and both collagen-induced platelet procoagulant activity and factor XI binding to be absent,[48] prothrombin consumption has also been reported to be normal[49] and platelet procoagulant[49] and prothrombinase activity to be increased.[50] An unexplained decrease in thrombin-, trypsin-, and thromboxane-stimulated phospholipase C (PLC) activity has also been reported.[51] GPIb-IX-V can be a target for drug-dependent antiplatelet antibodies[52]; such antibodies fail to bind to BSS platelets.[53]

Genetics

BSS is an autosomal recessive disorder.[16] In most instances, GPIb-IX-V is not present on the surface of BSS platelets, but in a few cases, 7% to 47% residual GPIb or GPIb-IX has been detected.[54] Many, but not all, obligate BSS heterozygotes have platelets that are larger than normal and a content of GPIb intermediate between normal and affected individuals.[38,55] Consistent with its rarity, consanguinity has often been present in affected families.

The gene for GPIbα is located on chromosome 17p12-ter, the GPIbβ gene on chromosome 22q11.2, the GPIX gene on chromosome 3q21, and the GPV gene on chromosome 3q29.[16] Each gene has a compact "intron-depleted" structure; the genes for GPIbα, GPIbβ, and GPV each contain two exons and the gene for GPV contains three.[56] The open reading frame and 3′ untranslated region of each gene is encoded by a single exon except for GPIbβ where its single intron is inserted into the codon for the fourth residue of its signal peptide.[57] Like other genes expressed by megakaryocytes, the 5′ flanking region of each gene contains potential binding sites for the transcription factors GATA, Ets, and Sp-1.[58–60] In addition, polymorphic variation in the region surrounding the GPIbα translation initiation site appears to be a determinant of the level of GPIb-IX-V expression.[61]

As shown in FIGURE 65.2, at least 56 different mutations responsible for BSS have been identified in the genes for GPIbα, GPIbβ, and GPIX.[62] Most are missense mutations or nucleotide deletions and insertions resulting in frameshifts and premature stop codons. However, several unique mutations have been identified: deletion of the entire GPIbβ locus on chromosome 22q11.2, occasionally in the setting of the velocardiofacial syndrome[63,64]; a more circumscribed homozygous deletion of the *GPIBB* locus that included *SEPT5* gene resulting in the BSS, cortical dysplasia, developmental delay, and a platelet secretion defect[65]; a C to G transversion in the GATA binding site of the GPIbβ promoter resulting in decreased GPIbβ gene transcription[66]; and an Ala140Thr in the transmembrane domain of GPIX.[67,68] The latter is noteworthy because it is consistent with observations that transmembrane domain interactions

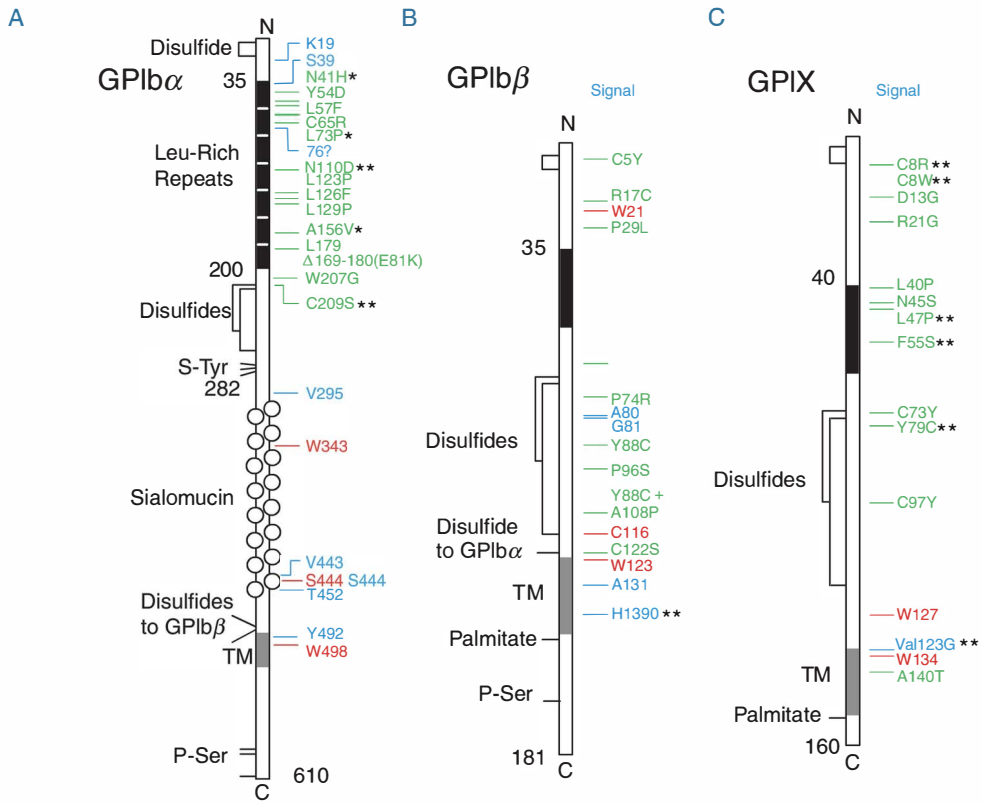

FIGURE 65.2 Mutations in the genes for GPIbα, GPIbβ, and GPIX causing the BSS mapped onto the structures of the mature proteins. *Green*, missense mutations and short deletions; *red*, nonsense mutations leading to premature stop; and *blue*, frameshift mutations leading to stop. *, autosomal dominant inheritance; **, mutations discussed in Savoia A, Pastore A, de Rocco D, et al. Clinical and genetic aspects of Bernard-Soulier syndrome: searching for genotype/phenotype correlations. *Haematologica* 2011;96:417–423. (Adapted from Berndt MC, Andrews RK. Bernard-Soulier syndrome. *Haematologica* 2011;96:355–359, with permission.)

Table 65.1

Differential diagnosis of inherited macrothrombocytopenia

Disorder	Ristocetin Response	Features	Bleeding Diathesis
BSS	No	GPIbα, GPIbβ, GPIX mutations	Yes
MHY9-related disorders	Yes	Leukocyte inclusions, deafness, nephritis, deafness, cataracts	Uncommon
Gray platelet syndrome (GPS)	Yes	Platelet α-granule deficiency	Yes
Montreal platelet syndrome	Yes	Heterozygous for V1316M mutation in exon 28 of vWF producing platelet type vWD	Yes
Mediterranean macrothrombocytopenia	Yes	Heterozygosity for BSS?	No
Mediterranean stomatocytosis/ macrothrombocytopenia	Yes	Phytosterolemia due to *ABCG 5* and *8* genes mutations	Yes
GATA-1 mutations	Decreased	X-linked inheritance, can be associated with dyserythropoiesis ± anemia	Yes
Sialyl-Lewis-S antigen deficiency	—	Progressive thrombocytopenia, hypogranular platelets, decreased GPIb, neutropenia	Yes
Paris-Trousseau syndrome	Yes	Part of Jacobson syndrome; caused by loss of the transcription factor FLI1 due to deletion of the long arm of chromosome 11	Yes
Filamin A mutations	Yes	X-linked disorder; usually associated with periventricular nodular heterotopia and the otopalatodigital syndrome	Yes
Platelet-type vWD	Yes	Hypersensitivity to ristocetin; GPIbα mutations causing spontaneous vWF binding to platelets	Yes
Type 2B vWD	Yes	Hypersensitivity to ristocetin; vWF mutations causing spontaneous vWF binding to platelets	Yes

are involved in efficient GPIb-IX-V expression[69] and with the impairment of GPIb-IX expression observed when the GPIbβ transmembrane domain was mutated *in vitro*.[70] The monoallelic GPIbα mutation Ala156Val, known as the "Bolzano mutation," has been reported to be the most common cause of inherited thrombocytopenia in Italy. It has been associated with a mild bleeding diathesis, a variable degree of thrombocytopenia, and a modest but consistent increase in platelet diameter and volume.[71] It is also noteworthy that many BSS mutations have been reported multiple times, suggesting that there may be "hot spots" for mutation in the GPIbα, GPIbβ, and GPIX genes.

Differential Diagnosis

BSS must be differentiated from other conditions that are associated with large platelets and thrombocytopenia (macrothrombocytopenia). Inherited disorders associated with macrothrombocytopenia are listed in Table 65.1 and can be readily differentiated from BSS because BSS platelets fail to agglutinate in the presence of ristocetin (FIGURE 65.1).[72] Many of these conditions are discussed in detail in Chapter 64.

Macrothrombocytopenia is a characteristic feature of mutations in the *MYH9* gene located on chromosome 22q12.3-q13.2 encoding the nonmuscle myosin heavy chain IIA (NMMHC-IIA).[73,74] Previously, these MYH9 disorders were segregated into four separate autosomal dominant inherited syndromes—the May-Hegglin anomaly and the Sebastian, Fechtner, and Epstein syndromes—based on the presence or absence of Döhle body-like leukocyte inclusions, hereditary nephritis, cataracts, and sensorineural deafness.[75] However, because each results from mutations in the *MYH9* gene, it now seems appropriate to consider them as a single entity whose variability results from the variable expression of *MHY9* mutations and whose common feature is macrothrombocytopenia. In general, the function of platelets affected by *MHY9* mutations is normal,[76–78] and if hemorrhage occurs, it is usually due to thrombocytopenia.[79]

Macrothrombocytopenia also occurs in the gray platelet syndrome (GPS), a disorder resulting from platelet α-granule deficiency[80] and due, at least in some cases, to mutations in the gene *NBEAL2* (see below).[81–83] In contrast to BSS platelets, GPS platelets are pale gray on Wright-stained blood smears and agglutinate normally in the presence of ristocetin.[84] In a reevaluation of the Montreal platelet syndrome, large platelets, thrombocytopenia, and spontaneous platelet aggregation were found to be due

to type 2B vWD caused by a heterozygous V1316M mutation in exon 28 of the vWF gene.[85,86] Mediterranean macrothrombocytopenia refers to putative differences in platelet count and size between Europeans of Northern and Mediterranean origin[87]; in Italy, this may be due to heterozygosity for BSS, in particular heterozygosity for the "Bolzano mutation" Ala156Val.[88] Mediterranean stomatocytosis/macrothrombocytopenia, the rare combination of stomatocytic hemolysis and macrothrombocytopenia, results from excess plant sterols in the blood and is caused by mutations in the *ABCG5* and *ABCG8* genes linked to phytosterolemia.[89] Several reported patients had significant bleeding histories. Sex-linked macrothrombocytopenia occurs in patients with GATA-1 transcription factor mutations,[90–92] and macrothrombocytopenia with dystrophic megakaryocytes and neutrophils lacking sialyl-Lewis-S antigen was described in an infant who died at 37 months due to hemorrhage.[93] In the Paris-Trousseau syndrome,[94] a part of the Jacobsen syndrome,[95] a deletion of the long arm of chromosome 11 at q23.3 containing the gene for the transcription factor FLI1 results in mild thrombocytopenia, subpopulations of large platelets and platelets containing giant α-granules, and the expansion of immature megakaryocyte progenitors.[96] Mutations in the filamin A gene located on chromosome Xq28 are responsible for periventricular nodular heterotopia and the otopalatodigital syndrome; macrothrombocytopenia and bleeding, misinterpreted as ITP, has been present in some patients.[97] In platelet-type vWD, mutations within the vWF binding domain of GPIbα induce spontaneous vWF binding to GPIbα and the clinical picture of type IIB vWD which itself can be associated with macrothrombocytopenia.[9,98–102] There are also anecdotal reports of thrombocytopenia and giant platelets, often associated with a mild bleeding diathesis but lacking the features of either BSS or the *MHY9* disorders.[103,104] In two other reports, macrothrombocytopenia was associated with the presence of large platelet granules whose origin was uncertain.[105,106]

Acquired BSS-like disorders can be differentiated from the congenital BSS by history. GPIb antibodies occur in some patients with idiopathic thrombocytopenic purpura[107]; whether the antibodies themselves are responsible for bleeding is not clear because of concomitant thrombocytopenia. Two patients with myelodysplasia and a BSS-like syndrome have been reported,[108,109] as has a patient with a lymphoproliferative disorder and a circulating IgG antibody that inhibited ristocetin-induced platelet agglutination, but was directed against an unidentified 210,000 mol. wt. platelet protein.[110]

Therapy

Treatment of hemorrhage in patients with the BSS usually requires platelet transfusion.[111] Hormonal control of menses has been effective in managing menorrhagia.[112] Splenectomy has been attempted to increase the platelet count[113] but has resulted in only transient increases and has not ameliorated the platelet function defect. Corticosteroids are not beneficial in this disorder. Desmopressin acetate (DDAVP) has been reported to shorten the bleeding time in some, but not all, affected individuals in whom it has been administered,[114–117] but its efficacy in patients with hemorrhage is unclear. Antifibrinolytics such as epsilon aminocaproic acid and tranexamic acid can be useful adjuncts to other therapy.[118] A number of anecdotal reports suggest that recombinant VIIa may be effective in the treatment of hemorrhage in BSS patients, although the degree of effectiveness has varied.[119–123] Lastly, bone marrow transplantation from HLA-identical donors has been performed successfully in patients with BSS.[124,125]

DISORDERS OF PLATELET AGGREGATION

Glanzmann Thrombasthenia (Integrin $\alpha_{IIb}\beta_3$ [Glycoprotein IIb-IIIa] Deficiency)

Thrombasthenia, described by Glanzmann[126] in 1918, is a rare bleeding disorder characterized by a prolonged bleeding time, a normal platelet count, and absent macroscopic platelet aggregation (see FIGURE 65.1).[127] Platelets from affected individuals are unable to aggregate because of a deficiency or dysfunction of the major platelet integrin $\alpha_{IIb}\beta_3$, also as known as GPIIb-IIIa.[128]

Platelet aggregation is essential for the formation of a hemostatic platelet plug.[129] In contrast to platelet adhesion, platelet aggregation is an active metabolic process, requiring platelet stimulation by agonists such as thrombin, collagen, and ADP and exposure of a binding site for fibrinogen or vWF on the integrin $\alpha_{IIb}\beta_3$.[130–132] $\alpha_{IIb}\beta_3$-bound fibrinogen or vWF then cross-links adjacent platelets into an occlusive plug. $\alpha_{IIb}\beta_3$, like other members of the integrin family, resides on the cell surface in an equilibrium between inactive (low affinity) and active (high affinity) conformations.[133,134] Platelet stimulation shifts $\alpha_{IIb}\beta_3$ to its active conformation, enabling it to bind ligands and mediate platelet aggregation.[134] Platelet stimulation also enables interaction of the $\alpha_{IIb}\beta_3$ cytoplasmic tails with submembranous actin filaments, providing a link between the force of cytoskeletal contraction and a fibrin clot, resulting in clot retraction.[135,136]

$\alpha_{IIb}\beta_3$ is a calcium-dependent heterodimer and dissociates into α_{IIb} and β_3 monomers in the presence of calcium chelators.[137] In rotary-shadowed electron micrographs, $\alpha_{IIb}\beta_3$ appears as a 12 × 8 nm globular head with two 18-nm stalks extending from one side.[138] When incorporated into phospholipid vesicles, the globular head extends ≈20 nm above the vesicle surface with the tips of the tails inserted into the phospholipid.[139]

α_{IIb} itself has an unreduced molecular weight of 136,000 and dissociates into a 125,000 mol. wt. heavy chain ($\alpha_{IIb}\alpha$) and 23,000 mol. wt. light chain ($\alpha_{IIb}\beta$) following disulfide bond reduction.[137] α_{IIb} is synthesized as single chain precursor (Pro-GPIIb) in the rough endoplasmic reticulum (ER)[140] where it associates with β_3. The resulting heterodimer is transported to the Golgi complex where Pro-α_{IIb} is cleaved into heavy and light chains.[141] In contrast to α_{IIb}, β_3 is a single chain protein containing 56 cysteine residues and 28 disulfide bonds[142]; its apparent molecular weight of 90,000 on unreduced sodium dodecyl sulfate gels increases to 110,000 following disulfide bond reduction.[137] The disulfide bonds in β_3 are concentrated in three regions of the extracellular portion of the molecule: a cysteine-rich, protease-resistant amino terminus (residues 1 to 62), a protease-sensitive central region (residues 101 to 422), and a disulfide-rich, protease-resistant core (residues 423 to 622).[143]

In the absence of heterodimer formation, α_{IIb} and β_3 monomers are retained in the ER and eventually degraded.[144] The factors responsible for the retention of uncomplexed monomers are unknown. However, it is clear from studies of various thrombasthenic mutants that formation of $\alpha_{IIb}\beta_3$ heterodimers alone is not sufficient to guarantee egress of $\alpha_{IIb}\beta_3$ from the ER.[144,145]

There are approximately 80,000 copies of $\alpha_{IIb}\beta_3$ on the surface of unstimulated platelets.[146] Glanzmann thrombasthenia

(GT) results when platelets lack sufficient numbers of functional $\alpha_{IIb}\beta_3$ to support platelet aggregation.[128] However, the platelets of obligate thrombasthenic heterozygotes aggregate normally, indicating that 50% of the normal amount of $\alpha_{IIb}\beta_3$ is at least sufficient to support platelet aggregation.[147]

Platelets express a second β_3-containing integrin, $\alpha_v\beta_3$, the vitronectin receptor.[148] There are 50- to 500-fold fewer copies of $\alpha_v\beta_3$ than $\alpha_{IIb}\beta_3$ on platelets.[149,150] While $\alpha_{IIb}\beta_3$ expression is restricted to cells of the megakaryocytic lineage,[151] $\alpha_v\beta_3$ is expressed by a variety of cells including endothelial cells, smooth muscle cells, and osteoclasts.[152]

Clinical Manifestations

The clinical manifestations of GT in a large cohort of patients have been described in detail.[127] GT typically presents with mucocutaneous bleeding during the neonatal period or infancy, occasionally with bleeding following circumcision. Spontaneous petechiae are uncommon and bleeding generally results from conditions that would also cause bleeding in an otherwise normal individual. Thus, epistaxis is a frequent type of bleeding, especially during childhood, as are gingival bleeding and menorrhagia. Bleeding at menarche may be severe and require transfusion. Similarly, parturition represents a severe hemorrhagic risk. Other hemorrhagic manifestations include gastrointestinal bleeding and hematuria, but hemarthroses and deep hematomas are unusual. Serious bleeding may follow trauma or surgery. However, the severity of hemorrhage in thrombasthenia is not predictable, even within single kindreds, and does not correlate with the degree of $\alpha_{IIb}\beta_3$ deficiency. The apparent decline in the clinical severity of GT with age is likely due to a decrease in the incidence of conditions such as epistaxis with aging.

Laboratory Findings

Platelet counts and platelet morphology on peripheral blood smears are normal. The bleeding time of affected individuals is markedly prolonged.[153] A diagnosis of GT is usually suspected when platelet aggregometry reveals absent agonist-stimulated platelet aggregation (see FIGURE 65.1). Platelet secretion induced by strong agonists such as thrombin is normal, but secretion in response to weak agonists such ADP and epinephrine which requires platelet aggregation does not occur. Coagulation tests, such as the prothrombin time and the partial thromboplastin time, are normal in GT, whereas clot retraction in the presence of GT platelets is absent or reduced.

In addition to their inability to aggregate, GT platelets do not spread normally on the subendothelial matrix because of an impaired ability to interact with fibronectin and vWF in the matrix.[154] The amount of fibrinogen in the α-granules of GT platelets is decreased to absent.[127] Human megakaryocytes do not synthesize fibrinogen, but rather, it is derived from plasma by an $\alpha_{IIb}\beta_3$-mediated endocytic process.[155–159] A number of biochemical reactions in platelets that require the presence of $\alpha_{IIb}\beta_3$ are impaired in GT platelets. For example, the tyrosine phosphorylation of multiple intracellular signaling proteins depends on ligand binding to $\alpha_{IIb}\beta_3$ and does not occur in GT platelets.[160] Calpain, a calcium-dependent thiol protease, is activated when stirred normal platelets are stimulated by thrombin, but not when GT platelets are treated in a similar manner.[161] The kinetics of calcium exchange in unstimulated GT platelets is inexplicably decreased[162] since it is not clear what role, if any, $\alpha_{IIb}\beta_3$ plays in calcium transport.[163,164] Clot retraction in blood containing GT platelets is either absent or reduced.[127,135]

Aspects of platelet function that do not depend on $\alpha_{IIb}\beta_3$ are normal in GT. GT platelets interact normally with collagen[153] and undergo secretion when stimulated by "strong agonists" such as thrombin.[165] Incubation of GT platelets with ristocetin induces vWF binding to GPIb-IX-V[10] and GT platelets adhere to vWF in the subendothelium.[154] GT platelets also express normal amounts of procoagulant activity following lysis.[50] However, agonist-stimulated prothrombinase activity varies and is influenced by the nature of platelet agonist.[50,166]

Genetics

The genes for α_{IIb} and β_3 are located on the long arm of chromosome 17 at q21→23[167] with the α_{IIb} gene at minimum distance of 365 kb downstream from the β_3 gene.[168] The α_{IIb} gene (*ITGA2B*) spans ≈18 kb and consists of 30 exons ranging in size from 46 to 220 bp.[169] Like the genes for other proteins expressed in megakaryocytes, its 5′ flanking region lacks TATA or CAAT boxes. Instead, it contains a linear array of regulatory elements that includes two motifs recognized by the GATA-1 transcription factor and its cofactor FOG[170,171]; sequences flanking each GATA-1 binding site are recognized by Fli-1 and perhaps other members of the Ets family of transcription factors[172–175]; and an Sp1-binding silencer element is located between the GATA-1 sites.[176] The gene for β_3 (*ITGB3*) spans 63 kb and contains 15 exons.[151,177] Like the α_{IIb} gene, the 5′ flanking region of the β_3 gene lacks TATA or CAAT boxes.[178]

GT is an autosomal recessive disorder with disease clusters in populations where consanguinity is common. It has been subclassified into three types based on the amount of $\alpha_{IIb}\beta_3$ present per platelet and the presence or absence of α-granule fibrinogen and clot retraction.[127] In type I, platelets contain <5% of the normal amount of $\alpha_{IIb}\beta_3$ and clot retraction and α-granule fibrinogen are absent. In type II, platelets contain 10% to 20% of the normal amount of $\alpha_{IIb}\beta_3$, clot retraction is decreased, and α-granule fibrinogen is present. In "variant" thrombasthenia, the platelet content of $\alpha_{IIb}\beta_3$ is ≥50% of normal, indicating that the $\alpha_{IIb}\beta_3$ abnormality is qualitative, rather than quantitative.

Because the expression of $\alpha_{IIb}\beta_3$ on the platelet surface requires the formation of correctly folded heterodimers,[179] mutations in the genes for either α_{IIb} or β_3 can produce GT. Approximately 200 different mutations responsible for GT have been identified[180] (FIGURE 65.3). The vast majority of these mutations consist of a mix of missense and nonsense mutations, nucleotide deletions and insertions, and alternative splicing, resulting in type I and type II GT. However, the most informative mutations have been qualitative $\alpha_{IIb}\beta_3$ abnormalities (variant GT) that primarily perturb $\alpha_{IIb}\beta_3$ function rather than the level of $\alpha_{IIb}\beta_3$ expression.

Mutations located in the extracellular portion of $\alpha_{IIb}\beta_3$ cause GT by either impairing fibrinogen or vWF binding to the active $\alpha_{IIb}\beta_3$ conformation or by paradoxically inducing constitutive $\alpha_{IIb}\beta_3$ activation but causing GT by concurrently decreasing the amount of $\alpha_{IIb}\beta_3$ expressed on the platelet surface. Six missense mutations (the α_{IIb} mutation Tyr143His[181] and the β_3 mutations Asp119Tyr,[182] Val193Met,[183] Arg214Trp,[184,185] Arg214Gln,[186] and Aspr217Val[187]) are located in regions implicated in ligand binding to $\alpha_{IIb}\beta_3$ and cause GT by preventing ligand binding.[188,189]

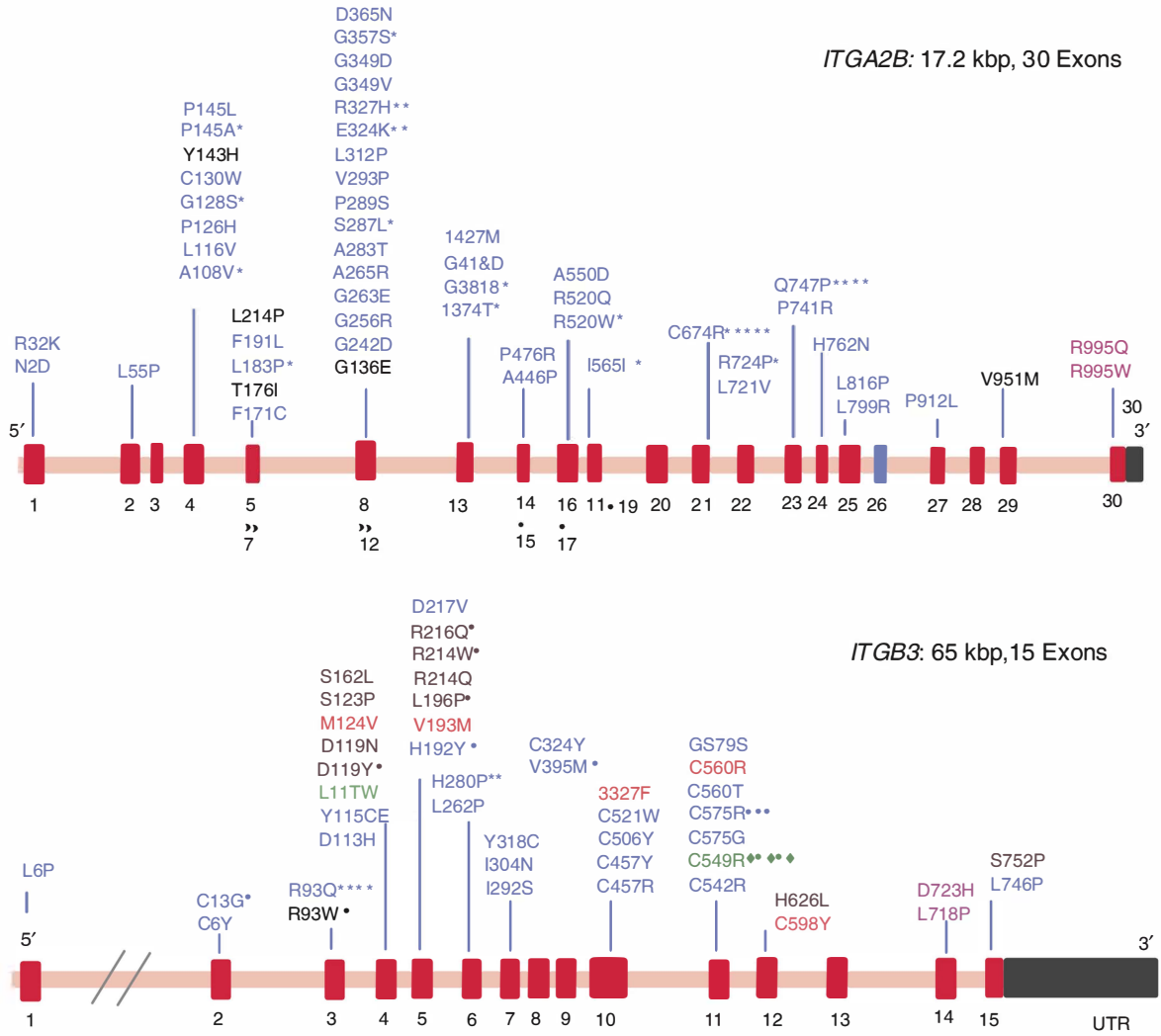

FIGURE 65.3 Missense mutations in the ITGA2B and ITGB3 genes resulting in GT. The exons in the ITGA2B and ITGB3 genes are shown schematically as *red bars*. Mutations shown in *black* are lack of function mutations, those shown in *blue* primarily impair $\alpha_{IIb}\beta_3$ expression, those in *green* are characteristic of particular ethnic groups, those in *orange* cause constitutive $\alpha_{IIb}\beta_3$ activation, and those in *rose* are associated with macrothrombocytopenia. *Asterisk* indicate the number of times the mutation has been reported. (Adapted from Nurden AT, Fiore M, Nurden P, et al. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. *Blood* 2011;118:5996–6005, with permission.)

Similarly, an insertion of two amino acids (Arg–Thr) into the Cys146–Cys167 loop of α_{IIb} produced an inactive $\alpha_{IIb}\beta_3$ heterodimer.[190] An eighth mutation, β_3 Ser123Pro, did not impair $\alpha_{IIb}\beta_3$ expression or agonist-induced ligand binding but was associated with absent ADP- and collagen-induced platelet aggregation.[191] The β_3 mutation, Leu196Pro, was identified independently in two French patients and severely restricted $\alpha_{IIb}\beta_3$ expression.[192,193] However, the residual $\alpha_{IIb}\beta_3$ that made it to the platelet surface was unable to bind ligands, presumably because the mutation prevents $\alpha_{IIb}\beta_3$ from assuming its active conformation. By contract, the β_3 mutation Cys560Arg locked $\alpha_{IIb}\beta_3$ in a high-affinity conformation, but like Leu196Pro, it likely produced a GT-phenotype because the small amount of constitutively active $\alpha_{IIb}\beta_3$ on the platelet surface was insufficient to support platelet aggregation.[194]

The extracellular stalks of $\alpha_{IIb}\beta_3$ consist of the thigh, calf-1, and calf-2 domains of α_{IIb} and the epidermal growth factor (EGF)-like domains 1 to 4 and the βTD domain of β_3.[195] The distal portions of the stalks form an interface containing interacting energetic "hot spots" that are critical for maintaining $\alpha_{IIb}\beta_3$ in a stable, inactive conformation.[196,197] Thus, it is not surprising that four mutations involving cysteine residues located at the third and fourth EGF domains of β_3 (Cys49Arg,[198] Cys560Phe,[199] Cys560Arg,[194] and Cys598Tyr[199,199a]) as well as a Gly579Ser mutation, not only resulted in type II thrombasthenia but also induced constitutive ligand-binding activity in the residual $\alpha_{IIb}\beta_3$. Similarly, a Ser527Phe mutation detected in the third β_3 EGF domain of a patient who presented with a mild bleeding diathesis was found to cause constitutive activation of recombinant $\alpha_{IIb}\beta_3$ expressed in Chinese hamster ovary cells.[200]

Naturally occurring mutations involving the β_3 cytoplasmic domain have confirmed the singular importance of this structure in regulating $\alpha_{IIb}\beta_3$ function. The first mutation identified, a missense mutation that results in the substitution of Pro for Ser at residue 752, prevents $\alpha_{IIb}\beta_3$ activation by cellular agonists,[201–203] likely because it abrogates binding of the cytoplasmic protein kindlin-3 to β_3 (see below).[204] A second missense mutation, identified separately in two unrelated patients,[205,206] converts the Arg724 codon into a stop codon, resulting in the synthesis of a truncated β_3 molecule lacking 39 *C*-terminal amino acids. As a consequence, $\alpha_{IIb}\beta_3$ is unable to transduce both "inside-out" and "outside-in signals," likely because β_3 is now unable to interact with both kindlin-3 and talin.[204] A third mutation, identified in an Palestinian Arab with GT, produces aberrant mRNA splicing at the intron 14/exon 15 junction and replacement of the β_3 sequence distal to residue 741 with 40 unrelated amino acids.[207] This results in the absence of the distal talin binding site in the β_3 cytoplasmic domain, as well the binding site for kindlin-3, and an $\alpha_{IIb}\beta_3$ extracellular domain that appears to be "locked" in its inactive conformation.[208]

Lastly, several heterozygous mutations located in the highly conserved membrane-proximal regions of the α_{IIb} and β_3 cytoplasmic domains have been associated with GT-like phenotypes and macrothrombocytopenia.[209] Thus, replacement of α_{IIb} Arg995 with either Gln[209,210] or Trp[211] and β_3 Asp723 with His[212,213] results in decreased, but not absent, platelet aggregation, an approximate 50% decrease in the total amount of platelet $\alpha_{IIb}\beta_3$ predominantly affecting the surface—rather than the granule—membrane pool, a moderate degree of macrothrombocytopenia and platelet "anisocytosis," and partial $\alpha_{IIb}\beta_3$ activation when $\alpha_{IIb}\beta_3$ is expressed in tissue culture cells. A β_3 Leu718Pro mutation produced a similar phenotype,[214] as did a two residue deletion in the βTD domain of β_3.[215] How these mutations in $\alpha_{IIb}\beta_3$ produce this spectrum of abnormalities is not clear since "classic" GT is not associated with alterations in platelet number or size, but the association of macrothrombocytopenia with BSS and the MYH9 disorders suggests that perturbed $\alpha_{IIb}\beta_3$-cytoskeleton interactions could be involved.

Differential Diagnosis

A history of lifelong bleeding, a prolonged bleeding time, and absent platelet aggregation are diagnostic of GT and differentiate it from other disorders of platelet adhesion and secretion. Rare instances of acquired GT have been reported and can be differentiated from congenital GT by history. Although autoantibodies against $\alpha_{IIb}\beta_3$ are frequently detected in patients with idiopathic thrombocytopenic purpura,[216,217] they rarely induce a GT-like state.[218–221] There are anecdotal reports of autoantibodies producing acquired GT in patients with Hodgkin and non-Hodgkin lymphoma, hairy cell leukemia, and after an allergic reaction to diclophenac.[222–224] A patient with multiple myeloma has also been reported whose $IgG_1\kappa$ paraprotein was directed against β_3 and inhibited $\alpha_{IIb}\beta_3$ function.[225] Congenital afibrinogenemia may be associated with a prolonged bleeding time and decreased *in vitro* platelet aggregation due to the absence of sufficient fibrinogen to support normal platelet aggregation.[226]

In the LAD-III/LAD-1/variant syndrome, patients manifest mucocutaneous bleeding and absent platelet aggregation, despite normal or nearly normal $\alpha_{IIb}\beta_3$ expression.[227–229] Concurrently, they suffer from recurrent and severe bacterial infections due to defective leukocyte integrin function, implying that the syndrome results from a general defect in integrin activation. Although initially attributed to defective expression of the Rap-1 activator CalDAG-GEFI,[229–231] in several kindreds,[232,233] mutations have been identified in the *KINDLIN3* gene encoding the protein kindlin-3 that binds to β_1, β_2, and β_3 integrin cytoplasmic tails and is required for agonist-stimulated integrin activation.[204,234,235]

Therapy

The mainstay of treatment of bleeding in GT remains the transfusion of normal platelets.[111] Because bleeding is a lifelong problem, use of HLA-matched platelets should be considered to lessen the chance of refractoriness to transfusion due to platelet alloimmunization. Patients with GT, especially patients with null mutations, not infrequently develop alloantibodies against normal $\alpha_{IIb}\beta_3$ after transfusion, potentially limiting the effectiveness of transfused platelets.[236–239] Protein A-Sepharose immunoadsorption has been reported to restore the efficacy of transfused platelets in this situation.[240] Oral contraceptives have been useful in controlling menorrhagia. Regular dental care is essential in minimizing gingival bleeding, and fibrinolytic inhibitors, in addition to platelet transfusions, may be useful in controlling bleeding after dental extractions. Corticosteroids are not efficacious in managing bleeding in thrombasthenic patients,[153] and there is little evidence that desmopressin (DDAVP) is useful.[114,241] Recombinant factor VIIa has been found to be efficacious for the treatment of bleeding episodes and for obtaining surgical hemostasis in patients with GT, particularly in patients who have developed alloantibodies from prior platelet transfusions.[111,242,243] The GT phenotype of several severely affected individuals has been corrected by bone marrow,[244–247] stem cell,[248,249] and unrelated donor cord blood bone marrow transplantation.[250] Lastly, platelet gene therapy has improved hemostatic function in $\alpha_{IIb}\beta_3$-deficient dogs,[251] suggesting that at some point in the future, gene therapy could be used in humans.

DISORDERS OF PLATELET SECRETION

Platelets contain four types of granules: dense granules containing ADP, ATP, calcium, serotonin, and pyrophosphate; α-granules containing a variety of proteins, some derived from the plasma, others synthesized by the megakaryocyte; lysosomes containing acid hydrolases; and microperoxisomes containing a peroxidase activity. Following platelet activation, the contents of these granules are extruded in an exocytic process known as platelet secretion or the platelet release reaction.[252] Disorder of platelet secretion are heterogeneous and are generally manifest as decreased platelet aggregation and absence of the second wave of aggregation when platelets are stimulated with weak agonists like ADP and epinephrine. They can be due to a decrease in the number of granules, a decrease in granule contents, or perturbation of the mechanism of granule secretion.

The Gray Platelet Syndrome (α-Granule Deficiency, α-Storage Pool Disease)

The gray platelet syndrome (GPS), described by Raccuglia[80] in 1971, is a rare disorder that results from the specific absence of morphologically recognizable α-granules in the platelets of affected individuals.

Biology and Molecular Aspects of the Disorder

Normal platelets contain ≈50 spherical or elongated structures termed α-granules that contain a variety of proteins, some of which are specific or relatively specific for platelets and others that are also found in plasma.[253] The former include platelet factor 4 (PF4), β-thromboglobulin (βTG), platelet-derived growth factor, and thrombospondin (TSP), and the latter include fibrinogen, vWF, albumin, coagulation factor V (FV), IgG, fibronectin, and a number of protease inhibitors.[254] The platelet-specific proteins and several of the plasma proteins such as vWF[255] are synthesized by megakaryocytes, whereas others, such as albumin and IgG, reach the α-granules via endocytosis of circulating protein.[256] The α-granule membrane contains many of the same proteins present in the platelet plasma membrane ($\alpha_{IIb}\beta_3$[257] and GPIb-IX-V[258]) and others specific for α-granules (P-selectin[259,260] and osteonectin[261]). Each can be translocated to the platelet surface following platelet activation.

Pathogenesis

GPS is markedly heterogeneous.[262] Electron micrographs of megakaryocytes and platelets from patients with this syndrome reveal absence of normal α-granules[263] and the presence of vacuoles and small α-granule precursors containing material that stains for vWF and fibrinogen.[264] Other types of platelet granules are present in normal numbers. The vacuole membranes and small α-granule precursors contain P-selectin and $\alpha_{IIb}\beta_3$ that can be translocated to the platelet surface after thrombin stimulation.[257,265] This indicates that α-granules are present in gray platelets and suggests that the abnormality responsible for GPS is an inability to target proteins to these structures,[266] accounting for the elevated plasma concentrations of PF4 and βTG and the bone marrow fibrosis observed in patients with the syndrome.[267] The abnormality responsible for GPS appears to be restricted to megakaryocytes since electron micrographs of endothelial cells from affected patients reveal typical vWF-containing Weibel-Palade bodies.[268] However, there is a report of three children with GPS whose neutrophils had a gray appearance due to a decrease in secondary granules.[269] In a second report of five affected individuals, neutrophil granularity was normal,[270] consistent with the heterogenous nature of the disorder. Two macrothrombocytopenic disorders resembling GPS have also been described. In the White platelet syndrome, autosomal dominant macrothrombocytopenia was associated with mild-to-moderate bleeding, defective platelet aggregation, incomplete granule formation, and 30% or more gray-appearing platelets on blood smears.[271] In the Medich giant platelet disorder, macrothrombocytopenia was associated with a marked decrease in platelet α-granules, but in contrast to the GPS, empty vacuoles without granule contents were not present in the platelet cytoplasm but were replaced by membranous inclusions resembling cigars or scrolls.[272]

Clinical Presentation and Laboratory Findings

Patients with GPS present with a lifelong history of mild to moderate mucocutaneous bleeding.[273] Patients also exhibit variably prolonged bleeding times, moderate thrombocytopenia, reticulin fibrosis of the bone marrow, and large platelets whose gray appearance on a Wright-stained blood smear gives the name to the disorder.[80,84] Platelet counts vary from 25,000 to 150,000/μL but generally range between 60,000 and 100,000/μL. Platelet aggregation induced by ADP, epinephrine, arachidonic acid, and the calcium ionophore A23187 has generally been normal or nearly normal,[274] but collagen-induced aggregation has been decreased to absent in some, but not all, cases, perhaps due to an associated deficiency of GPVI.[84,275,276] Responses to thrombin have also been variable, with decreased aggregation and secretion in response to low thrombin concentrations in some cases.[84,274,277,278] Gray platelets agglutinate normally when exposed to ristocetin, and the content of their dense granules is normal.[279] As expected, the PF4, βTG, fibrinogen, vWF, FV, fibronectin, and TSP content of gray platelet α-granules is markedly decreased, but surprisingly in some patients, gray platelets contain substantial quantities of albumin and IgG.[280] The PF4 and βTG concentrations in the plasma of affected individuals are normal or elevated.[84,274]

Genetics

Genetic analysis of families with GPS suggests that platelet α-granule deficiency is a heterogenous disorder. Falik-Zaccai et al.[273] reported 59 GPS patients. The presence of GPS in males and females in the same family indicated that it is an autosomal disease, but its mode of inheritance was uncertain. On the other hand, a family has been reported in which GPS was inherited in an X-linked fashion and was associated with an Arg216Gln mutation in the *GATA1* gene.[281] A disorder resembling the GPS is present in the Wistar Furth strain of rat and is inherited as an autosomal recessive.[282] Studies from three separate groups have identified mutations in the *NBEAL2* gene as the genetic cause of GPS.[81–83] *NBEAL2* encodes a protein containing a BEACH domain that is predicted to be involved in vesicular trafficking and may be critical for the development of platelet α-granules. NBEAL2 localizes to the dense tubular system in platelets.[82] It is noteworthy that silencing *nbeal2* in zebra fish abrogates thrombocyte formation.[81]

Therapy

Treatment of severe bleeding episodes in GPS may require the transfusion of normal platelets. Desmopressin (DDAVP) was found to shorten the bleeding time in one patient and was used as successful prophylaxis for a dental extraction.[283]

The Quebec Platelet Disorder (Factor V Quebec)

Two unrelated families with an autosomal dominant bleeding disorder of moderate severity have been identified in Quebec.[284–286] Their disorder is characterized by mild thrombocytopenia, defective epinephrine-induced platelet aggregation, a decrease in the amount of multiple α-granule proteins due to degradation, and bleeding unresponsive to platelet transfusion but controlled by fibrinolytic inhibitors. Initially, the disorder was attributed to a decrease in platelet factor V, hence the name factor V Quebec.[287] In contrast to GPS, the α-granules in these platelets appear morphologically normal.[285] However, the amount of the α-granule proteins factor V, multimerin, fibrinogen, vWF, fibronectin, osteonectin, and TSP is decreased, and many are substantially degraded[285] due to the enhanced expression of urokinase-type plasminogen activator (u-PA) in α-granules.[288,289] This has been attributed to an increased megakaryocyte expression of the UPA gene (*PLAU*) due to an abnormality in a *cis* regulatory element.[290,291] A patient has also been reported whose platelets contained a reduced amount of factor V but no evidence of enhanced α-granule proteolytic activity, indicating that some other process reduced α-granule factor V.[292]

Dense Granule Deficiency (δ-Storage Pool Disease)

Normal platelets contain three to six 200 to 300 nm dense or δ-granules that are intracellular storage sites for ADP, ATP, calcium, polyphosphate, and serotonin.[293] Because of their high calcium content, δ-granules can be visualized in electron micrographs of unfixed, unstained platelet whole mounts.[293] They can also be visualized by electron microscopy of thin sections of fixed platelets after staining with uranyl ions (uranaffin reaction) and by fluorescence microscopy after staining platelets with mepacrine (quinacrine).[293] The δ-granule membrane contains a 40,000 mol. wt. protein named granulophysin (CD63) that is translocated to the platelet surface following platelet activation[294] and corresponds to the melanoma-associated antigen ME491, the lysosomal membrane protein-1 (LIMP-1), and the lysosomal membrane-associated protein-3 (LAMP-3).[295] The δ-granule membrane also contains P-selectin, GPIb, and $\alpha_{IIb}\beta_3$.[296] δ-granules undergo exocytosis coincident with the second wave of platelet aggregation (see FIGURE 65.1).

Platelet δ-granules are related to a group of lysosome-related granules including melanosomes, neutrophil azurophilic granules, the lytic granules of cytotoxic T lymphocytes and natural killer cells, basophilic granules in basophils and mast cells, and class II major histocompatibility complex compartments in antigen-presenting cells.[295,297] The biogenesis of δ-granules in megakaryocytes is poorly understood. However, δ-storage pool deficiency occurs in tyrosinase-positive albinism in humans,[295] pigment dilution syndromes in mice and rats,[295] and as part of a Chediak-Higashi-like syndrome in cattle, cats, mink, foxes and killer whales,[298] implying that the biogenesis of dense granules, melanosomes, and lysosomes is related. How dense granules accumulate ATP and ADP is also not known. One candidate for mediating ATP and ADP accumulation is MRP4 (multidrug resistance protein 4, ABCC4), a nucleotide transport pump, since it colocalizes with dense granules by immunofluorescence microscopy and its distribution is altered in dense granule-deficient platelets.[299] A second candidate is the gene *Slc35d3*, a nucleotide sugar transporter, that was identified in a strain of hypopigmented ashen mice (a model of the human Griscelli syndrome[300]) that independently developed platelet dense granule deficiency.[301]

Normal platelets contain two pools of adenine nucleotides that exchange very slowly, if at all.[302] One pool is a metabolic nongranule pool in which the ratio of ATP to ADP content is 8-10:1. The second pool is present in the δ-granules and contains 65% of the platelet adenine nucleotides with an ATP to ADP ratio of 2:3. Thus, in normal platelets, the whole platelet ATP to ADP ratio is <2.5. On the other hand, serotonin is not synthesized by megakaryocytes but is taken up from the plasma and stored in the δ-granules where it is protected from platelet monoamine oxidase.[303]

Etiology and Pathogenesis

δ-storage pool disorders are heterogeneous with δ-granule deficiency as the common feature. Storage pool disease (SPD) can be subdivided into deficiency states associated with albinism and those in otherwise normal individuals.[304] SPD has been reported in patients with the Wiscott-Aldrich syndrome/X-linked thrombocytopenia,[305,306] the syndrome of thrombocytopenia with absent radii,[307] the Ehlers-Danlos syndrome,[308] and osteogenesis imperfecta.[309] When SPD is associated with albinism, as in the Hermansky-Pudlak syndrome (HPS) (oculocutaneous tyrosinase-positive albinism, platelet dense granule deficiency, ceroid-like inclusions in cells of the reticuloendothelial system, progressive pulmonary fibrosis, and granulomatous colitis)[295] and the Chediak-Higashi syndrome (CHS) (partial oculocutaneous albinism, frequent pyogenic infections, and giant lysosomal granules in cells of hematologic and nonhematologic origin),[298] there is a quantitative δ-granule deficiency. In platelets of nonalbinos, however, the number of uranaffin- and mepacrine-positive granules is normal to only slightly decreased, suggesting an inability to package δ-granule contents in these patients.[310,311] The platelet granulophysin content also parallels the number of δ-granules in storage pool-deficient patients, being low in HPS and CHS, but normal in nonalbinos.[312] The δ-granule abnormality in some nonalbino is also associated with a variable of α-granule deficiency (αδ-SPD).[313,314] The content of lysosomal acid hydrolases in SPD platelets is normal, but thrombin-induced acid hydrolase secretion may be impaired in severely affected individuals, an abnormality corrected by the addition of ADP *in vitro*.[315]

Clinical and Laboratory Findings

Patients with SPD have a mild to moderate bleeding diathesis. Platelet counts and morphology are usually normal, but bleeding times are usually, but not always, prolonged.[316] Aggregometer tracings of SPD platelets stimulated by weak agonists such as ADP and epinephrine usually lack a second wave of aggregation (see FIGURE 65.1).[317] Responses to low concentrations of collagen are also diminished to absent, but responses to high collagen concentrations may be normal or nearly normal; aggregation response to arachidonic acid also may be normal.[318] On the other hand, patients have been reported with normal aggregation studies.[316] The steady-state serotonin content of SPD platelets is decreased, but the rate of uptake of serotonin is normal[302] because without storage sites, the serotonin either leaks out of the platelet or is metabolized by platelet monoamine oxidase. In SDP, the dense granule pool of ADP no longer contributes to the whole platelet ADP content and the ATP:ADP ratio of δ-storage pool deficient platelets is 3.0 or greater, a useful diagnostic finding.[316] Flow cytometry of mepacrine-labeled platelets may be a rapid method to detect δ-storage pool-deficient platelets.[319,320]

Genetics

HPS and CHS are autosomal recessive disorders.[295,298] There are at least 9 known human HPS genes and 15 genetically distinct strains of mice with phenotypes similar to human HPS.[321,322] Nine of the mutant mouse genes are orthologs of the human HPS genes. HPS-1, the HPS prototype, occurs with a frequency of 1 in 1,800 in northwest Puerto Rico[323,324] and is one of the most common causes of oculocutaneous albinism in Japanese.[325] In addition to SPD and tyrosinase-positive albinism, patients with HPS-1 suffer from decreased visual acuity, nystagmus, granulomatous colitis, and usually fatal pulmonary fibrosis.[326,327] HPS-1 results from mutations in a gene located on human chromosome 10q23.1-23.3 that encodes an 80,000 mol. wt. protein and corresponds to the gene responsible for the mouse mutant *pale ear*.[323,328,329] HPS-1 in Puerto Rico results from 16-bp duplication in the exon 15 of *HPS-1*[330] and at least 22 additional mutations have been identified in non–Puerto Ricans.[322] The HPS-1 protein is one component of a cytosolic and membrane-bound complex (BLOC-3, biogenesis of lysosome-related organelles complex 3)

that also contains the 77,000 mol. wt. protein that is mutated in HPS-4.[321] Thus, the phenotypes of patients with HPS-1 and HPS-4 are similar.[331] The gene mutated in HPS-4 corresponds to the gene involved in the mouse mutant *light ear*.[332] HPS-2 results from mutations in *ADTB3A*, the gene encoding the β_3A subunit of the adaptor complex AP-3; mutations in this gene are also responsible for the mouse mutant *pearl*.[333] Patients with HPS-2 have phenotypes ranging from mild to severe and are distinguished from other HPS mutants by the presence of neutropenia and recurrent childhood infections.[333–335] HPS-3, a relatively mild form of HPS,[336] is the human homologue of the mouse mutant *cocoa*[337] and results from mutation of a gene located on human chromosome 3q24 encoding a 113,700 mol. wt. protein of unknown function.[338] HPS-5 has been identified in five patients who manifested hypopigmentation and platelet SPD without colitis or pulmonary fibrosis and results from mutation of a gene located on chromosome 11p14.[339] HPS-6, detected in a single patient from Belgium[340] and 20 individuals from an extended Israeli Bedouin family,[341] produces a phenotype similar to HPS-5 and results from mutation of a gene located on chromosome 10q24.1-q25.1.[341] HSP-5 and HPS-6 correspond to the mouse mutants *ruby-eye 2* and *ruby-eye*, respectively.[340] Biochemical studies indicate that HPS-3, HPS-5, and HSP-6 interact to form a multisubunit complex called BLOC-2.[342] HPS-7, identified in a Portuguese woman with mild HPS, results from a mutation in the gene for dysbindin, responsible for the mouse mutant *sandy*.[343] Dysbindin is a component of the BLOC-1 complex that also contains the proteins pallidin, muted, and cappuccino associated with variants of murine HPS.[344,345] HPS-8, identified in a large consanguineous Pakistani family, is due to a frameshift mutation in the gene *BLOC1S3*, another component of the BLOC-1 complex.[346] HPS-9, discovered in a male child of Indian ancestry who presented at birth with generalized hypopigmentation and was found to be homozygous for a mutant gene located at chromosome 15q21.1 that encodes the BLOC-1 subunit pallidin.[347] Although the function of the BLOC-1, BLOC-2, BLOC-3, and AP-3 in the biogenesis of platelet δ-granules is not known, BLOC-1 and AP-3 have been shown to physically associate and to facilitate the trafficking of melanosomal membrane protein tyrosinase-related protein 1.[348] Similarly, BLOC-1 has been found to associate with BLOC-2 where it participates in a cargo transport pathway from early endosomes to melanosomes.[348,349]

CHS results from mutations in the gene *CHS1* (*LYST*) located on human chromosome 1q42-q43.[350,351] *CHS1* is the ortholog of the murine *bg* gene, mutation of which is responsible for the beige mouse.[351] *CHS1* encodes a 430,000 mol. wt. cytosolic protein whose role in normal lysosome development is unknown.[298]

Therapy

Bleeding in patients with SPD can be controlled not only by platelet transfusion but also, in some patients, by cryoprecipitate[352] or the vasopressin analog DDAVP.[353] DDAVP avoids transfusion of blood products and may be tried as the initial therapy of choice. The precise mechanism of its beneficial effect in patients with platelet defects is uncertain. However, DDAVP releases large vWF multimers and tissue plasminogen activator from endothelial stores.[241] In several series of patients with platelet disorders, DDAVP shortened the bleeding time in 43% to 100% of patients.[353–355] In some some patients DDAVP did not shorten the bleeding.[464] This variability in response indicates that if DDAVP is to be used for surgical prophylaxis in patients with SPD, it may be prudent to assess the effect of DDAVP on the bleeding time prior to surgery.

In an unconfirmed study, prednisone at doses of 20 to 50 mg for 3 to 4 days was used to improve hemostasis in patients with inherited platelet disorders.[356] However, prednisone was ineffective in patients with GT[153] or in normal individuals given aspirin.[357] There are also reports suggesting that recombinant VIIa can be effective in managing bleeding in patients with SPD.[120,358]

HPS-1 and HPS-4 are associated with progressive pulmonary fibrosis that is generally fatal by the fourth to sixth decades of life.[359] The drug pirfenidone, an antifibrotic agent, has been reported to slow the progression pulmonary fibrosis in patients with HPS.[360] One patient was treated successfully by bilateral lung transplantation.[361]

Bone marrow transplantation has been effective in reversing the immunologic and hemostatic manifestations of CHS,[362] but surviving patients later developed neurologic deficits.[298]

Disorders of Secretion Due to Defects in Platelet Signal Transduction

Platelet signal transduction is triggered by the interaction of agonists with specific surface platelet receptors, leading to the activation of specific platelet signaling pathways. Platelet signal transduction is discussed in detail in Chapter 33. Although the prevalence of inherited abnormalities in platelet signal transduction is unknown, these patients have been informative in understanding platelet physiology. [380]

Inherited Abnormalities in Platelet Agonist Receptors

Mutations in the TXA_2 Receptor

Patients have been identified with impaired platelet responsiveness to TXA_2.[363–365] Although the responsible mutations have not been identified in all patients, Hirata et al.[366] described a homozygous Arg^{60} to Leu mutation in the first cytoplasmic loop of the thromboxane A_2 (TXA_2) receptor in two unrelated patients. These patients had a mild bleeding disorder with an autosomal dominant pattern of inheritance. Although binding of TXA_2 analogs to platelets was normal,[367,368] TXA_2-mediated GTPase activity was diminished,[368,369] suggesting a defect in TXA_2 receptor-G-protein coupling. TXA_2-stimulated platelet aggregation was also impaired in the heterozygous family members.[366] Since half the normal number of TXA_2 receptors is sufficient to stimulate irreversible platelet aggregation,[370] it is possible that this mutation had a dominant negative effect. Absent TXA_2-stimulated platelet aggregation has also been observed in patients without detectable TXA_2 receptor abnormalities.[371]

ADP Receptor Mutations

Platelets express three ADP receptors: $P2Y_1$, $P2Y_{12}$, and $P2X_1$.[372,373] ADP binding to $P2Y_1$ induces PLC activation, intracellular Ca^{2+} mobilization, and platelet shape change, while ADP binding to $P2Y_{12}$ mediates or inhibits platelet adenylyl cyclase activity. ADP-induced platelet aggregation requires concurrent activation of both $P2Y_1$ and $P2Y_{12}$. $P2X_1$ functions as an ATP- and ADP-gated cation channel.

A number of patients presenting with mild to moderate mucocutaneous bleeding and platelet phenotypes that

included blunted ADP-induced platelet aggregation, impaired suppression of PGE_1-induced elevations in cAMP, and normal ADP-stimulated shape change were found to have mutations in the gene for $P2Y_{12}$.[374–379] Three patients were homozygous for a deletion in the $P2Y_{12}$ gene, resulting in premature termination and lack of $P2Y_{12}$ protein.[374,377,380] A fourth patient was homozygous for a missense mutation in the translation initiation codon of the $P2Y_{12}$ gene,[376] and a fifth had a two nucleotide deletion in one $P2Y_{12}$ allele, resulting in a frame shift and a premature stop codon.[375,381] However, a mutation was not detected in the other $P2Y_{12}$ allele. A sixth patient was found to be a compound heterozygote with one $P2Y_{12}$ allele containing an R256N substitution in the sixth $P2Y_{12}$ transmembrane domain, and the other, a R265W substitution in its third extracellular loop.[378] It is noteworthy that a seventh patient has been described whose bleeding diathesis was also associated with a heterozygous P258T mutation in the third extracellular loop.[382] Lastly, in a screen of 92 patients with type 1 vWD, a heterozygous mutation in the second extracellular loop of $P2Y_{12}$ was detected in one patient and several family members that was associated with decreased 2MeS-ADP binding and modest defects in ADP-induced platelet aggregation,[379] suggesting that mild platelet function defects could contribute to the variable severity of vWD.

Although $P2X_1$ is not thought to directly contribute to ADP-induced platelet aggregation, a heterozygous deletion of a single leucine in a stretch of four leucine residues in the second transmembrane domain of $P2X_1$ was detected in a 6-year-old child with a history of petechiae, ecchymoses, and severe expistaxis.[383] There was isolated impairment of ADP-induced platelet aggregation, raising the possibility that the mutant protein had a dominant negative effect on $P2X_1$-mediated calcium channel activity.

Platelet Collagen Receptor Mutations

Collagen plays an essential role in platelet function as a substrate for platelet adhesion, a binding site for vWF in the extracellular matrix, and an agonist for platelet aggregation and secretion (see FIGURE 65.1). As discussed in detail in Chapters 28 and 30, there are two receptors, GPVI[384] and the integrin $\alpha_2\beta_1$,[385,386] that mediate the interaction of platelets with collagen.

Glycoprotein VI

A 62,000 mol. wt. member of the immunoglobulin gene superfamily,[387,388] GPVI is associated with the Fc receptor γ chain in the platelet plasma membrane.[389] Exposing normal platelets, but not GPVI-deficient platelets, to collagen or collagen-like peptides results in γ chain phosphorylation on tyrosine residues, the phosphorylation of the proteins SLP-76, Syk, Lyn, LAT, and phospholipase Cγ2, followed by platelet aggregation.[390,391] Murine platelets lacking either the γ chain or Syk are unable to respond to collagen.[392] The interaction of GPVI-deficient human platelets with either immobilized and soluble collagen is markedly impaired.

There are at least five reports concerning patients with congenital GPVI deficiencies.[393–397] These patients manifest mild mucocutaneous bleeding and platelets that fail to aggregate following stimulation with collagen despite the presence of normal amounts of $\alpha_2\beta_1$, but retain some ability to adhere to collagen-coated surfaces. A sixth patient had more severe bleeding, including epistaxis requiring transfusion, severe bleeding after trauma, and significant postpartum hemorrhage.[398] This patient's platelets expressed GPVI and the FcR γ chain but aggregated poorly in response to collagen, convulxin, and collagen-related peptide, as well as to heparin-induced thrombocytopenia antibodies, implying a defect in collagen-induced signaling downstream of the FcR γ chain. GPVI deficiency has also been reported in conjunction with the GPS.[276] At least six patients have been described whose plasma contained autoantibodies against GPVI that inhibited collagen-induced platelet function.[399–404] Notable features of these patients include little or no detectable GPVI on the platelet surface, absence of detectable GPVI mutations, and, in several instances, restoration of GPVI following treatment of the immunologic disease.[403,404] It is likely that absence of platelet-surface GPVI in these patients was a consequence of antibody-mediated GPVI ectodomain shedding,[405] a phenomenon previously observed in mouse models.[406] Two patients have also been described, one with myelodysplasia and a second with chronic lymphocytic leukemia, whose platelets did not respond to collagen or convulxin but contained GPVI by flow cytometry.[407]

$\alpha_2\beta_1$

The widely expressed integrin $\alpha_2\beta_1$ (GPIa-IIa, VLA-2) binds with high affinity to type I collagen, as well as to collagen types II to V and to laminins 1 and 5, depending on the cellular context.[408] It is one of nine integrins whose α subunit contains a 200 residue I-domain that is largely, if not exclusively, responsible for ligand binding.[409] Besides platelets, $\alpha_2\beta_1$ is present on endothelial cells, fibroblasts, epithelial cells, Schwann cells, and activated T cells.[410] With regard to the relative contributions of $\alpha_2\beta_1$ and GPVI to collagen-initiated platelet function, current data suggest a model in which platelet tethering initiated by platelet GPIb-IX-V binding to collagen-bound vWF enables GPVI to bind to collagen and generate "outside-in" signals. In turn, these signals increase the affinity of $\alpha_2\beta_1$ for collagen, resulting in platelet adhesion to collagen that is sufficiently firm to resist the shear stress of flowing blood.[386,411,412]

Several patients have been reported whose platelets lacked $\alpha_2\beta_1$ and failed to interact normally with collagen. Of two patients with bleeding diatheses, platelets from one contained approximately 15% to 25% of the normal amount of α_2, failed to aggregate in response to collagen, and failed to adhere to collagen under both static and flow conditions.[413,414] Platelets from the second lacked α_2 as well as the α-granule protein TSP, failed to aggregate in response to low concentrations of collagen, and adhered to, but did not spread on, collagen-coated surfaces.[415] Platelets of two families with autosomal dominant thrombocytopenia and mild cutaneous bleeding were also found to express 38% to 63% of the normal amount of $\alpha_2\beta_1$ and to adhere poorly to collagen-coated surfaces, whereas collagen-induced platelet aggregation was either normal or only slightly perturbed.[416]

There are inherited differences in the density of $\alpha_2\beta_1$ on the platelet surface resulting from polymorphisms in the coding and promoter regions of the α_2 gene.[417–419] *In vitro* studies suggest a positive correlation between the extent of $\alpha_2\beta_1$ expression and platelet adhesion to type I and type III collagen.[420] In addition, some, but not all, epidemiologic studies have suggested that increased $\alpha_2\beta_1$ expression is a risk factor for myocardial infarction and stroke in younger individuals and for diabetic retinopathy in patients with type II diabetes mellitus.[421–427]

Mutations in Platelet Adrenergic Receptors

The response of platelets to stimulation of α_2-adrenergic receptors (α_2AR) by epinephrine is variable or may even be absent in otherwise normal individuals.[428] Nonetheless, a family with a history of easy bruising and minimally prolonged bleeding times has been described in which several members had impaired platelet aggregation and secretion solely in response to epinephrine and in which the number of platelet α_2AR was decreased.[429] It is noteworthy that inhibition of adenylate cyclase by epinephrine was normal, implying that the coupling of the α_2AR to aggregation and adenylate cyclase are different. Other families with defective epinephrine-induced platelet responses have been reported,[430] but the relationship of the functional defects to bleeding is unclear.

Inherited Abnormalities in Platelet GTP-Binding Proteins

GTP-binding proteins link surface receptors to intracellular signal transduction. Thus, they are a potential locus for mutations leading to platelet dysfunction. Abnormalities involving Gαq, Gαi_2, and Gαs have been described in human platelets. Although deficiency of Gαz in humans has not been reported, Gαz-deficient mice have impaired epinephrine responses.[431]

Gαq Deficiency

Gabbeta et al.[432] described a patient with a mild bleeding disorder, impaired platelet aggregation and secretion in response to several agonists, and diminished platelet GTPase activity. Binding of ^{35}S-GTPγS to platelet membranes was diminished, a result of the selective decrease in platelet membrane Gαq with normal levels of Gαi_2, Gα_{12}, Gα_{13}, and Gαz. Responses to the thrombin receptor agonist peptides SFLLRN and GYPGKF were impaired, demonstrating that Gαq is coupled to both the PAR 1 and PAR 4 thrombin receptors. Although the Gαq coding sequence in this patient was normal, Gαq mRNA levels were decreased in platelets but not neutrophils.[433] Thus, the responsible mutation remains to be identified.

Gαs Abnormalities

Gαs activation increases platelet cAMP levels and inhibits agonist-stimulated platelet responses. Two siblings and an individual have been described with inducible Gαs hyperactivity that was manifest as a bleeding diathesis, markedly prolonged bleeding times, variable mental retardation, and skeletal malformations.[428] Platelet aggregation was reportedly normal, but the platelets had increased sensitivity to inhibition by agents that elevate cAMP levels. The Gαs gene (*GNAS1*) has two extra promoters and a first exon that can be alternatively spliced, generating differentially imprinted transcripts. Use of an upstream promoter results in the paternal expression of a transcript for an extralarge form of Gαs, XLαs. Each of the patients was heterozygous for a 36-bp insertion, as well 2-bp substitutions, in the first exon of XLαs. The insertion and basepair substitutions were also detected in 6 of 276 normal controls. When they were present in the paternal allele, the control platelets also displayed inducible Gαs hyperfunction. Nonetheless, XLαs is not activated by Gαs-coupled receptors in platelets, so the mechanism leading to excessive cAMP in patient platelets is not apparent.

Conversely, Gαs deficiency associated with abnormal imprinting of the GNAS1 region was detected in the platelets of a patient with pseudohypoparathyroidism Ib.[430] The patient had decreased cAMP formation following Gαs-coupled receptor stimulation but apparently had no hemostatic or thrombotic phenotype.

Gαi_1 Deficiency

Gαi inhibits agonist-stimulated adenylyl cyclase activity in platelets. Thus, in a patient whose platelets expressed only 25% of the normal amount of Gαi_1, there was no inhibition of forskolin-stimulated cAMP synthesis when patient platelets were stimulated by ADP, thrombin, or epinephrine.[434] By contrast, Gαq-mediated responses were normal. The abnormalities in platelet biochemistry were accompanied by clinical bleeding and abnormal platelet aggregation and dense granule secretion, although the linkage between Gαi activation and the latter responses is not clear.

Inherited Abnormalities in Signaling Intermediates

Phospholipase C Abnormalities

Agonist-stimulated PLC activation results in the generation of the signaling intermediaries inositol triphosphate ($InsP_3$) and diacylglycerol, leading to an increase in the intracellular concentration of Ca^{2+} and protein kinase C (PKC) activation, respectively. Human platelets contain at least seven PLC isozymes in the quantitative order PLC-γ2 > PLC-β2 > PLC-β3 > PLC-β1 > PLC-γ1 > PLC-δ1 > PLC-β4.[435] Study of two related patients with impaired platelet aggregation and secretion revealed decreased agonist-stimulated $InsP_3$ and diacylglycerol formation suggesting a defect in PLC activation.[436–438] Subsequent study of one of the patients revealed decreased amounts of PLC-β2 mRNA and protein in platelets, but not in neutrophils, suggesting a megakaryocyte-specific defect in PLC-β2 gene regulation.[435,439]

Presumptive abnormalities in PLC related pathways have been suggested to explain the bleeding diatheses of a number of additional patients. In seven of eight patients with abnormal platelet aggregation and secretion, receptor-mediated Ca^{2+} mobilization and/or pleckstrin phosphorylation was also abnormal, but the defects were corrected using the direct PKC activator 1,2, dioctonoyl-sn-glycerol and the calcium ionophore A23187, suggesting defective PLC activation,[440] either at the level of PLC-β2,[435,438] Gαq,[432] or PKC-θ.[441] Study of eight other patients with decreased platelet aggregation in response to weak agonists[442] revealed decreased phosphatidylinositol hydrolysis and phosphatidic acid formation[443] and, in one, decreased pleckstrin phosphorylation.[444]

There are numerous reports of other patients with mild bleeding diatheses in whom platelet phosphoinositide metabolism was impaired.[368,380,440,442–448] However, the responsible biochemical abnormalities remain to be determined.

Protein Kinase C-θ Deficiency

PKC isozymes are serine/threonine-specific kinases whose activity has been linked to $\alpha_{IIb}\beta_3$ activation and platelet secretion. PKC-θ deficiency has been described in a patient with mucocutaneous bleeding, mild thrombocytopenia, abnormal platelet aggregation and dense granule secretion, and a heterozygous mutation in the transcription factor RUNX1.[441,449]

Impaired Liberation of Arachidonic Acid from Phospholipids

The initial and rate-limiting step in TXA_2 synthesis is the hydrolysis of arachidonic acid from membrane-bound phospholipids by PLA_2. Four patients have been described in whom thrombin-stimulated arachidonic acid mobilization from phospholipids was impaired.[450] In one of the patients, membrane and cytosolic PLA_2 were normal, but agonist-induced increments in cytosolic Ca^{2+} were impaired due to $G\alpha q$ deficiency.[432,437] On the other hand, a patient with recurrent small intestinal ulceration, markedly decreased eicosanoid synthesis, and platelet dysfunction was found to have two heterozygous missense mutations in the PLA_2 coding region.[451]

Defective Platelet Cyclooxygenase and Thromboxase Synthetase Activity

Malmsten et al.[452] reported platelet cyclooxygenase (COX) deficiency in a patient with a mild bleeding disorder and impaired aggregation responses to ADP, epinephrine, collagen, and arachidonic acid but with normal responses to PGG_2. Subsequently, other patients with defects in TXA_2 synthesis were reported.[453–458] In five such patients, Roth and Machuga[457] found normal amounts of COX enzyme, implying that the defect in the enzyme was qualitative, not quantitative. Similarly, of three patients whose platelets were able to convert the endoperoxide PGH_2, but not arachidonic acid, to TXA_2, protein corresponding to COX-1 was undetectable in two, but was normal in one, indicating that the latter was a functionally abnormal molecule.[458] Two patients have been described with thromboxane synthetase deficiency.[459,460] In a third patient, thrombin-induced TXA_2 synthesis was markedly diminished in platelet-rich plasma, but there was substantial TXA_2 production when the patient platelets were suspended in buffer containing no albumin, suggestive of a partial defect in arachidonic acid metabolism.[461]

Platelet Dysfunction Associated with Transcription Factor Deficiencies

Transcription factor abnormalities may be the basis for the platelet dysfunction in some patients. RUNX1 haplodeficiency is associated with thrombocytopenia and predisposition to leukemia;[462] abnormal aggregation secretion responses, and protein phosphorylation, dense granule deficiency, and alpha-granule deficiency;[441,463,464] and deficiencies of platelet PKC-θ,[465] PF4,[466] myosin light chain,[467] and 12-lipoxygenase.[468] Numerous genes are downregulated in platelets,[469] and are direct RUNX1 targets (MYL9,[467] ALOX12,[468] PF4,[466] and PRKCQ[465]). These patients have impaired megakaryopoiesis[470] and decreased platelet thrombopoietin receptors (Mpl).[471] Patients with GATA-1 mutations may have impaired responses to collagen and ristocetin,[90] diminished platelet GS[90] and gray platelet syndrome (R216N),[281] in addition to macrothrombocytopenia.

Therapy

Platelet transfusion is the mainstay of therapy for patients with platelet secretion disorders. However, the intravenous administration of DDAVP that shortens the bleeding time in a substantial number of patients with platelet function defects is a viable alternative.[472–475] Nonetheless, it is not clear whether DDAVP improves hemostasis in the absence of an effect on the bleeding time. Responses in patients with SPD have been variable with shortening of the bleeding time in some[353,475] but not others.[472,474] In uncontrolled studies, it has been possible to manage selected patients undergoing surgical procedures with DDAVP alone.[472,474] Abnormal *in vitro* platelet function is not corrected by DDAVP.[474]

Recombinant factor VIIa has been used successfully to manage bleed in patients with SPD.[120,242,358] Other approaches include a short 3- to 4-day course of prednisone (20 to 50 mg)[356] and administration of the antifibrinolytic agents epsilon aminocaproic acid or tranexamic acid.[118]

DISORDERS OF PLATELET PROCOAGULANT ACTIVITY

The plasma membrane of activated platelets provides a procoagulant surface for the assembly of the "tenase" and "prothrombinase" complexes that activate coagulation factor X and prothrombin, respectively (see Chapter 35). Although it is uncertain whether there are specific membrane protein receptors for this activity, it clearly results from the calcium-induced exposure of anionic phospholipids on the platelet surface.[476] The phospholipid composition of the platelet plasma membrane bilayer, like that of other cells, is asymmetric with phosphatidylcholine and sphingomyelin localized predominantly in the outer membrane leaflet and phosphatidylserine and phosphatidylethanolamine concentrated in the inner leaflet.[477] This asymmetry is established by at least two enzymatic activities: a "flippase," an ATP-dependent aminophospholipid translocase[478,479] that has been identified as a P4 ATPase[480] directing the inward transport of lipids, and a "floppase," an ATP-requiring transporter encoded by the gene ABCC1 that directs the transport of choline- and amino-phospholipids to the outer leaflet.[476] Following an agonist-stimulated rise in intraplatelet $Ca,^{2+}$ membrane asymmetry is lost with exposure of anionic phospholipids on the platelet surface,[481] as well as on the surface of shed platelet membrane vesicles or microparticles (see Chapter 36).[482,483] A similar response to increased intracellular calcium has been detected in erythrocytes[484] and lymphocytes.[485] Redistribution of membrane phospholipids also accompanies apoptosis, but the development of platelet procoagulant activity and apoptotic membrane changes operate through different pathways.[480,486] The Ca^{2+}-induced movement of phospholipids between membrane leaflets in platelets and other cells appears to be mediated by a membrane-associated phospholipid scramblase activity, an essential component of which is the protein TMEM16F.[487] TMEM16F is a member of a 10-member protein family in humans whose founding member is a calcium-activated chloride channel.[488,489] The decrease in Ca^{2+} ionophore-stimulated anionic phospholipid exposure when TMEM16F expression is knocked down in Ba/F3 cells supports its role as a component of scramblase.[487]

Isolated deficiency of platelet procoagulant activity is exceedingly rare. In addition to the one well-studied patient after whom the disorder was named (Scott syndrome),[490] three additional patients have been reported.[166,491,492] A recessive disorder identical to Scott syndrome has also been detected in a pedigree of German shepherd dogs and has been localized to canine chromosome 27.[493] The index patient for Scott syndrome, Mrs. Scott, presented with a history of a spontaneous retroperitoneal hematoma and bleeding after surgery and dental procedures.[490] Prothrombin and partial

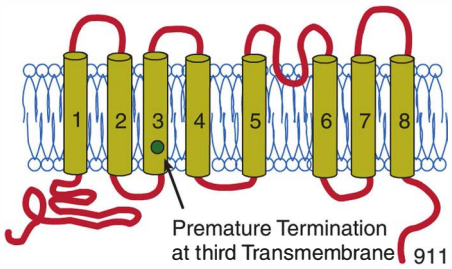

FIGURE 65.4 Schematic diagram of the TMEM16F protein showing the location of the mutation detected in the index patient with Scott syndrome. (Adapted from Suzuki J, Umeda M, Sims PJ, et al. Calcium-dependent phospholipid scrambling by TMEM16F. *Nature* 2010;468:834–838, with permission.)

thromboplastin times, bleeding times, and studies of platelet function were normal, but serum prothrombin times were short and the patient's platelets were unable to express normal procoagulant activity. Although the patient's platelets secreted normal quantities of factor Va, they expressed only 20% to 25% of the normal number of factor Xa binding sites.[494] In addition, factor X activation by factor IXa and factor VIIIa was impaired, suggesting a concomitant deficiency of factor IXa and/or factor VIIIa binding sites.[495] When her platelets were stimulated with the combination of thrombin and collagen, there was decreased exposure of anionic phospholipids on the platelet surface and decreased membrane vesiculation.[496–498] Similarly, there was decreased membrane vesiculation and procoagulant activity when the patient's erythrocytes were treated with the ionophore A23187.[484] In addition, membrane-associated phospholipid scramblase activity was impaired in the patient's platelets, erythrocytes, and lymphocytes.[477]

Recent evidence suggests that TMEM16F mutations are responsible for the Scott syndrome phenotype. A homozygous mutation at the splice acceptor site for intron 12 of the *TMEM16F* gene has been detected in DNA from Mrs. Scott.[487] This results in alternative splicing, a frame shift, and premature termination of protein translation in exon 14 (FIGURE 65.4). Analysis of genomic DNA from a second patient revealed compound heterozygosity for a mutation at the donor splice site of intron 6 and a single nucleotide insertion in exon 12 causing a frame shift and premature termination of translation.[499] Taken together, these data provide strong evidence that *TMEM16F* mutations can be causative in Scott syndrome; whether TMEM16F is the membrane scramblase itself remains to be determined.

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CHAPTER

66 Acquired Disorders of Platelet Function

A. KONETI RAO AND JOEL S. BENNETT

Platelets are essential for normal hemostasis. Thus, defects in platelet function may lead to bleeding diatheses. Platelet function defects can be inherited or acquired, with the latter being far more common. Moreover, abnormalities are often observed in multiple facets of platelet function, including adhesion, aggregation, secretion, and procoagulant activity. Although it would be preferable to classify these disorders on the basis of specific biochemical or functional defects, this is often not possible because in many instances, the basis for the defective platelet function is poorly understood. In this chapter, acquired platelet function disorders are described in relation to the disease states in which the perturbed platelet function is observed (Table 66.1). In some disease states, such as the myeloproliferative disorders, intrinsically abnormal platelets are produced by the bone marrow. In others, platelet dysfunction results from the interaction of otherwise normal platelets with exogenous factors such as drugs, artificial surfaces, toxic metabolites, or antibodies. Acquired platelet dysfunction is a prominent feature of renal failure, liver failure, and cardiopulmonary bypass. The platelet dysfunction associated with these conditions is discussed specifically in Chapters 126, 127, and 129.

Bleeding due to acquired platelet dysfunction is usually mucocutaneous in nature. However, the impact of these disorders on clinical hemostasis is often unpredictable, and in some instances, perturbed platelet function can only be detected in the laboratory. When bleeding occurs, it is usually mild to moderate, although the potential for severe life-threatening bleeding exists. Acquired platelet function disorders can prolong the cutaneous bleeding time and perturb *ex vivo* measurements of platelet aggregation. For example, aspirin ingestion impairs platelet aggregation and secretion in response to weak agonists such as ADP and epinephrine in most individuals, but only prolongs the bleeding time in half of them.[1,2] Moreover, the correlation between bleeding time prolongation and clinical bleeding is weak. This also applies to other methods used to detect abnormal platelet function, including the platelet function analyzer (PFA), the VerifyNow rapid PFA, the Impact cone and plate(let) analyzer (CPA), and flow cytometry.

CHRONIC MYELOPROLIFERATIVE DISORDERS

Bleeding and thrombosis are frequent complications of the chronic myeloproliferative disorders such as essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (MF).[3,4] Factors that contribute to these hemostatic abnormalities include the increased whole-blood viscosity present in PV, intrinsic defects in platelet function, elevated platelet counts, as well as leukocyte and/or endothelial dysfunction.

Clinical Features

Large retrospective analyses have indicated that the frequency of thrombosis consistently exceeds the frequency of hemorrhage in these disorders.[5] Nonetheless, a wide range in the probability of major thromboses has been reported: 7.6% to 29.4% and 11.2% to 38.6% for newly diagnosed ET and PV, respectively.[5] By contrast, bleeding has been reported in 3% to 18% of patients with ET and 3% to 8.1% of patients with PV.[5] Most symptomatic patients experience either bleeding or thrombosis; however, some develop both complications during the course of their disease. Bleeding usually involves the skin or mucous membranes, but may also occur after surgery or trauma. Thrombosis can involve arteries, veins, and the microvasculature and may occur in unusual locations such as abdominal wall vessels or the hepatic, portal, and mesenteric circulations.[6–9] Indeed, full-blown or latent myeloproliferative disorders account for a substantial proportion of patients with the Budd-Chiari syndrome or portal vein thrombosis.[6,10–12] Individuals with ET may experience ischemia and necrosis of the fingers and toes due to digital artery thrombosis, microvascular occlusion in the coronary circulation, and transient neurologic symptoms due to cerebrovascular occlusion.[13] Erythromelalgia, characterized by redness and burning pain in the extremities, is strongly associated with ET and PV and is thought to be due primarily to arteriolar platelet thrombi.[14,15] It is difficult to predict the risk of bleeding or thrombosis in an asymptomatic patient,[16] but in a review of 891 patients with ET as defined by the World Health Organization criteria and followed for a median time of 6.2 years, 12% experienced arterial and venous thrombosis at a rate of 1.9% patient years.[17] A similar thrombosis rate was observed in the UK MRC Primary Thrombocythemia 1 study that compared treatment of high-risk ET patients with hydroxyurea/aspirin versus anagrelide/aspirin.[18] Predictors for arterial thrombosis in the former study were age >60 years, a history of thrombosis, cardiovascular risk factors, leukocytosis, and the presence of the JAK2 V617F mutation. This analysis is consistent with previous studies also showing that vascular complications are more likely to occur in patients older than 60, in patients with other risk factors for vascular disease, and when baseline leukocytosis is present.[19–23] By contrast, only male sex predicted venous thrombosis. Surprisingly, platelet counts $>10^6/\mu L$ were associated with a significantly lower risk of arterial thrombosis in both the JAK2 V617F-positive and -negative patient populations.

A major advance in understanding the pathogenesis of the myeloproliferative disorders was the identification of gain-of-function mutations in the *JAK2* or *MPL* genes in approximately 95% of patients with PV and approximately 50% to 60% of patients with ET and MV.[24–30] While it is plausible that

Table 66.1

Disorders associated with acquired defects in platelet function

Uremia
Myeloproliferative neoplasms
Essential thrombocythemia
Polycythemia vera
Chronic myelogenous leukemia
Primary Myelofibrosis
Acute leukemias and Myelodysplastic syndromes
Dysproteinemias
Cardiopulmonary bypass
Acquired von Willebrand Disease
Acquired Storage pool deficiency
Antiplatelet antibodies
Liver disease
Drugs and other agents

gain-of-function mutations in the protein products of these genes might influence hemostatic mechanisms, including the activation state of platelets,[31–33] their precise impact on platelet function and thrombotic risk remains unclear.[5,34–36] For example, some studies concluded that the presence of the *JAK2* V617F or a high *JAK2* V617F allele burden[37,38] confers increased thrombotic risk in ET, but others have failed to show such an association.[39–42] It may be useful in the future if such correlative studies were to focus on the *JAK2* V617F allele burden in platelets and granulocytes separately.[40,43]

Abnormalities in Platelet Function and Biochemistry

Under the light or electron microscope, platelets from patients with these disorders may be larger or smaller than normal, may be abnormally shaped, and may exhibit a reduction in the number of storage granules.[44] In ET, platelet survival may be modestly reduced.[45] In addition, a number of diverse functional and biochemical abnormalities have been described. The most frequently encountered abnormality is a decrease in platelet aggregation and secretion in response to epinephrine, ADP, or collagen.[46] The defect in epinephrine-induced aggregation often includes absence of the primary wave of aggregation. This is not simply due to an elevated platelet count, because it is not encountered in reactive thrombocytosis.[47,48]

The reduced platelet aggregation and secretion has also been associated with one or more of the following: decreased agonist-induced release of arachidonic acid from membrane phospholipids[49,50]; reduced conversion of arachidonic acid to prostaglandin endoperoxides or lipoxygenase products[51]; reduced platelet responsiveness to thromboxane A2[52]; deficiency of dense- or α-granules[53,54]; deficiency of the integrin $\alpha_2\beta_1$, resulting in variable changes in platelet responsiveness to collagen[55]; and decreased numbers of α_2-adrenergic receptors.[56,57] Similarly, a reduction in platelet procoagulant activity has been reported in some patients,[58] as have specific platelet membrane abnormalities, including decreased expression and activation of the integrin $\alpha_{IIb}\beta_3$[59]; decreased amounts of the glycoprotein Ib-V-IX complex, resulting in an acquired Bernard-Soulier syndrome[60]; decreased numbers of prostaglandin PGD2 receptors[61]; increased numbers of FcγRIIa receptors[62]; an increase in CD36 expression[63–65]; and decreased expression of Mpl receptors.[66,67] By contrast, spontaneous platelet aggregation, as well as increased thromboxane biosynthesis, has been reported for platelets from patients with ET and PV.[68–70] It has also been reported that Src kinase in unstimulated PV and ET platelets is present in a preactivated state (i.e., the inhibitory residue Tyr527 is dephosphorylated perhaps due to constitutive activation of the phosphatase SHP-2), perhaps causing these platelets to be hypersensitive to agonist stimulation.[71]

It is important to recognize that none of these biochemical and functional abnormalities are unique to a particular myeloproliferative disorder, their relative frequencies vary widely in reported series, and none are predictive of either bleeding or thrombosis. Since these disorders represent clonal abnormalities of hematopoiesis, megakaryocytes likely acquire these abnormalities as they develop from a clone of abnormal progenitors.

Treatment

Therapy should be considered for symptomatic patients, for patients >60 years of age, and for individuals about to undergo surgery.[20,22,34,72,73] Treatment includes correction of polycythemia and maintenance of a normal red cell mass, which may be approximated by a hematocrit <45% in males and <42% in females,[74] as well as treatment of the underlying disorder.[34,75–78] Platelet count reduction to <400,000/mm^3 in patients with thrombocytosis, either by plateletpheresis or by cytoreductive agents, has generally been considered to be the target value associated with clinical improvement.[21,42,77,79]

Effective cytoreductive agents include the ribonuclease reductase inhibitor hydroxyurea,[80] interferon-α, and anagrelide.[20,79,81,82] In a prospective, randomized trial of 114 "high-risk" individuals with ET who were either >60 years of age or had a previous history of thrombosis, hydroxyurea significantly reduced the incidence of new thrombosis from 24% to 3.6%.[80] Anagrelide, an imidazoquinazolin derivative, is thought to decrease platelet counts by specifically impairing megakaryocyte maturation.[83] Anagrelide has essentially no effect on red and white cell counts and is not known to be leukemogenic. Nevertheless, 10% to 20% of patients experience neurologic, gastrointestinal, and cardiac side effects, in particular fluid retention, often necessitating discontinuation of the drug.[82,84,85] When hydroxyurea and anagrelide were compared head-to-head in a randomized trial of 809 patients with ET, subjects in the anagrelide group showed an increased rate of arterial thrombosis, major bleeding, and transformation to MF compared to the group treated with hydroxyurea.[18] Progression to MF despite treatment with anagrelide was also observed in a phase II study.[86] During an episode of acute bleeding in the chronic myeloproliferative disorders, 1-desamino-8-D-arginine vasopressin (DDAVP) infusion may temporarily improve hemostasis if the patient has an acquired storage pool defect or acquired von Willebrand disease (AvWD).[54,87]

Low-dose aspirin (100 to 300 mg/d) may be useful in patients with ET and thrombosis, particularly those with erythromelalgia

or with digital or cerebrovascular ischemia.[15,34,78,88,89] In a recent double-blind, placebo-controlled study of 518 patients with PV who were judged to have no contraindications to daily low-dose (100 mg) aspirin, subjects in the aspirin arm exhibited a reduced risk of nonfatal arterial and venous cardiovascular endpoints. While aspirin was well tolerated, there was no effect of aspirin on overall and cardiovascular mortality.[90] However, aspirin can exacerbate a bleeding tendency in patients with myeloproliferative disorders.[20,91]

ET and PV in pregnancy pose special challenges because of an apparent increased risk of unsuccessful pregnancy, thrombotic or bleeding complications, and potential teratogenicity of hydroxyurea.[92] In ET, the risk of first-trimester miscarriages may be higher among women with the *JAK2* V617F mutation.[93] Barbui and Finazzi[94] have recommended a risk-stratified approach to management in pregnancy in which high risk is defined as either previous major bleeding or thrombotic episodes, previous pregnancy complications, or a platelet count >1.5 million/μL. Low-risk individuals are maintained at a hematocrit <45% and receive aspirin, 100 mg/d during pregnancy and low molecular weight heparin, 4,000 U/d for 6 weeks after delivery. Interferon-α is considered rather than aspirin if there has been previous major bleeding or if platelets are >1.5 million/μL. In addition, high-risk patients are recommended to receive low molecular weight heparin throughout pregnancy. However, in a retrospective analysis of 122 consecutive pregnancies in 92 women with ET, the risk of spontaneous abortion was 2.5-fold higher than in a control population and aspirin did not affect the live birth rate, but treatment with interferon-α was associated with better outcomes.[95] Consistent with previous reports, the presence of the *JAK2* V617F mutation was again found to be an adverse prognostic factor.

ACUTE LEUKEMIAS AND MYELODYSPLASTIC SYNDROMES

Thrombocytopenia is the major cause of bleeding in acute leukemias and myelodysplastic syndromes (MDS). However, bleeding complications may occur in patients with normal platelet counts because of platelet dysfunction. Acquired platelet defects have been reported in acute myelogenous leukemia, as well as in acute lymphoblastic and myelomonoblastic leukemias and hairy cell leukemia.[96–101] The reported abnormalities in these patients include impaired platelet aggregation responses to ADP, epinephrine, and collagen and diminished nucleotide secretion, serotonin uptake and release, TXA_2 production, platelet PDGF and β-thromboglobulin levels, and platelet procoagulant activities. In patients with hairy cell leukemia, the platelet dysfunction appears to improve postsplenectomy.[102–104] Acquired forms of vWD[105] and Bernard-Soulier–like platelet defects have been described in hairy cell leukemia and juvenile MDS,[60] respectively. Decreased platelet aggregation in response to one or more agonists has been described in MDS.[106–108]

In acute leukemias and MDSs, platelets may be large and morphologically abnormal, with a balloon-like appearance. Ultrastructural studies have shown decreased microtubules, reduced number and abnormal size of dense granules, and excessive membranous systems[97–99]; megakaryocytes can have a dysplastic appearance.[97,98] Bleeding in acute leukemias and MDS usually responds to transfusion of platelets.

DYSPROTEINEMIAS

Bleeding in patients with dysproteinemias can result from multiple hemostatic abnormalities including platelet dysfunction, specific coagulation abnormalities, hyperviscosity, and amyloid deposition in vessel walls.[109,110] Qualitative platelet defects occur in 33% of patients with IgA myeloma or Waldenstrom macroglobulinemia, 15% of patients with IgG multiple myeloma, and infrequently in monoclonal gammopathy of undetermined significance. Several coagulation abnormalities have been reported in patients with dysproteinemias including factor X deficiency related to amyloidosis,[111] circulating heparin-like anticoagulants,[112] and impaired fibrin polymerization.[113] An acquired form of vWD has been described in some patients.[114–117] Platelets express Fc receptors, and it has been proposed that paraprotein binding to these receptors interferes with normal platelet membrane functions. In rare cases, paraprotein binding to platelet GPIIIa inhibited platelet aggregation or binding to GPIb interfered with its interaction of von Willebrand factor (vWF).[114–116,118]

Treatment

The platelet dysfunction in these diseases appears to correlate with serum paraprotein levels. Thus, acute bleeding episodes can be managed with plasmapheresis to lower the paraprotein levels and chronic bleeding may be controlled by chemotherapy aimed at reducing the concentration of the abnormal protein. DDAVP may be transiently effective in patients with AvWD.[114,116]

ACQUIRED vON WILLEBRAND DISEASE

AvWD is a bleeding disorder that is often unrecognized. Most patients with AvWD are older (median age 62 years) and have no previous personal or family history of a bleeding diathesis. Disorders associated with AvWD in these patients are diverse[119,120,121]; the major associations include lymphoproliferative or plasma cell proliferative disorders (50%), cardiac disease (20%), and myeloproliferative diseases (15%). AvWD has been reported in chronic lymphocytic leukemia, hairy cell leukemia, acute myeloid and lymphoblastic leukemias, and non-Hodgkin lymphoma. In patients with myeloproliferative diseases and in reactive thrombocytosis, there is a strong correlation between the plasma vWF abnormalities and elevated platelet counts.[122,123] AvWD has been observed in patients with autoimmune disorders, including systemic lupus erythematosus, scleroderma and mixed connective tissue disease, hypothyroidism, and the antiphospholipid antibody syndrome,[119,120] and following administration of ciprofloxacin,[124] valproic acid,[125] griseofulvin,[126] and the plasma expander hydroxyethyl starch.[127,128] AvWD has been reported in patients with solid tumors, most notably Wilms tumor, but case reports have noted an association with others as well (adrenocortical, lung, and gastric carcinoma).[119,129] Patients with severe aortic stenosis and congenital valvular heart disease may have excess bleeding (particularly gastrointestinal), and several reports have documented AvWD in these patients,[130–135] as well as in patient with left ventricular assist devices (LVADs).[136–138] Vincentelli et al.[131] studied 50 consecutive patients with aortic stenosis and found skin or mucosal bleeding in 21% of patients with severe aortic stenosis. Abnormal platelet function documented by the PFA-100, decreased vWF binding to collagen, and loss of the largest

vWF multimers, or a combination of these findings, has been reported in 67% to 92% of patients with severe aortic stenosis. These abnormalities were corrected following surgery.

AvWD arises from a variety of mechanisms.[119,121] Some patients (especially those with dysproteinemias or lymphoproliferative diseases) have antibodies directed against functional domains of vWF[119,121,132] that the antibodies that inhibit ristocetin cofactor activity or interfere with platelet or collagen binding. In some patients, antibodies enhances vWF clearance. Increased shear stress–induced proteolysis of vWF can produce AvWD, especially in patients with severe aortic stenosis,[131,133,134] congenital cardiac diseases,[130] and LVAD.[136–138] In some patients, cellular adsorption and removal of vWF by malignant or other cells is the responsible mechanism.[119,121] Nonimmunologic binding and precipitation of vWF has been noted following administration of hydroxyethyl starch.[127] Lastly, decreased vWF synthesis has been invoked in patients with hypothyroidism[139] and those receiving valproate.[125]

Laboratory findings in AvWD have included various combinations of a prolonged bleeding time, decreased plasma levels of vWF antigen and ristocetin cofactor activity and decreased factor VIII levels; most important is a selective reduction in large vWF multimers. Plasma levels of activated vWF, representing GPIbα binding form of vWF, are decreased in severe aortic stenosis.[134] vWF propeptide levels have been found to be elevated.

Therapy

The goals of treatment are first to raise plasma vWF levels to treat or prevent bleeding and second to address the underlying associated condition.[121] The first goal can be achieved by administration of DDAVP or factor VIII concentrates containing vWF.[119,121,132] Cryoprecipitate is also effective, but is associated with the risk of transfusion-transmitted diseases. Recombinant factor VIIa has also been used to control bleeding in patients with AvWD.[140] Several reports have found intravenous immunoglobulin to be effective, including in patients with plasma cell dyscrasias.[119,120,132,141,142] Other modalities include plasma exchange[116] and extracorporeal immunoadsorption.[143]

Treatment of the underlying disorder is an important component of managing patients with AvWD. In patients with elevated platelet counts due to myeloproliferative disorders, cytoreduction is effective in reversing the vWF abnormalities.[122,144] Remissions have occurred spontaneously or after therapy of the underlying disease, such as lymphoproliferative disease, Wilms tumor, multiple myeloma, hypothyroidism, chronic myelogenous leukemia (CML), or cardiac valve abnormalities.[119,131]

ACQUIRED STORAGE POOL DISEASE

Inherited deficiency of the platelet-dense granule pool of ATP and ADP (storage pool deficiency, SPD) is associated with impaired platelet function and a bleeding diathesis (Chapter 65). Several patients have been reported in whom SPD appears to have been acquired as a result of *in vivo* activation and release of platelet-dense granule contents or the production of abnormal platelets by the bone marrow. The presence of antiplatelet antibodies has been associated with acquired SPD.[145,146] It has also been reported in patients with disseminated intravascular coagulation, hemolytic-uremic syndrome, renal transplant rejection, multiple congenital cavernous hemangiomas, myeloproliferative diseases, chronic granulocytic leukemia, hairy cell leukemia, and acute nonlymphocytic leukemia.[50,98,147–152] Depletion of platelet granule contents has also been reported in patients with severe valvular disease and with Dacron aortic grafts, in patients undergoing cardiopulmonary bypass, and in platelet concentrates stored for transfusion.[153–156]

ANTIPLATELET ANTIBODIES AND PLATELET DYSFUNCTION

Antibody binding to platelets can lead to accelerated destruction, cell lysis, platelet aggregation and secretion, and expression of platelet procoagulant activity. Antibody binding can also impair platelet function by inhibiting platelet activation or by binding to surface glycoproteins. The overall impact may be thrombocytopenia and/or impaired platelet function, as noted in a wide range of autoimmune disorders, including primary immune thrombocytopenic purpura (ITP), collagen vascular diseases, and AIDS. In addition, these antibodies inhibit megakaryocytopoiesis and proplatelet formation.[157,158] Patients with ITP have decreased platelet survival with increased platelet-associated antibodies demonstrable in most patients. It has been generally considered that the circulating platelets in these patients are young and have enhanced function; for example, bleeding times have been reported to be disproportionately shortened in relation to the platelet counts in patients with ITP compared with those with regenerative thrombocytopenia.[159] However, the function of platelets in patients with ITP may also be impaired, even when platelet counts are adequate. In one study, defective *in vitro* platelet aggregation was observed in 9 of 11 patients with chronic ITP and normal platelet counts.[160] Serum globulin fractions from some of these patients were found to inhibit the aggregation responses of normal platelets to collagen and ADP. In another study, IgG and F(ab')2 fragments from some ITP patients inhibited responses of normal platelets.[161] Impaired platelet aggregation responses to agonists (ADP, epinephrine, and collagen) with and without prolonged bleeding times have been reported in ITP and in disorders such as systemic lupus erythematosus and Graves disease, diseases in which platelet-associated antibodies have been detected.[145,146,162–168]

Multiple mechanisms have been reported that produce antibody-induced platelet dysfunction. In some patients, the antibodies are directed against specific platelet surface membrane glycoproteins (GPIb,[169,170] $\alpha_{IIb}\beta_3$,[171,172] GPIa-IIa,[173,174] and GPVI[175,176]) or glycosphingolipids.[177,178] In others, antibody specifically blocked platelet aggregation induced by collagen through interaction with GPVI[175] or GPIa/IIa.[173,174] In one report, an anti-GPVI antibody induced selective clearance of the GPVI/FcR gamma chain complex from the platelet surface.[176] Antibodies against GPIb-IX and $\alpha_{IIb}\beta_3$ have been detected in 5% to 29% and 10% to 75% of ITP patients, respectively.[169,171,179] Rarely, these antibodies induce acquired forms of the Bernard-Soulier syndrome and Glanzmann thrombasthenia. One study noted that the severity of thrombocytopenia was not related to the glycoprotein specificity of the autoantibody.[180] Most of the platelet antibodies in ITP have been IgG, with IgM and IgA occurring less frequently.[181] Other functional defects in ITP platelets include impaired platelet arachidonate metabolism, as reflected by increased lipoxygenase activity and decreased cyclooxygenase (COX) products (TXA_2 and hydroxyheptadecatrienoic acid),[165] and deficiency of platelet granule contents.[145,146,182]

Table 66.2

Drugs that affect platelet function

COX Inhibitors
Aspirin
Nonsteroidal anti-inflammatory agents (indomethacin, phenylbutazone, ibuprofen, sulfinpyrazone, sulindac, meclofenamic acid, naproxen, diflunisal, piroxicam, tolmetin, and zomepirac)
ADP Receptor Antagonists
Thienopyridines: ticlopidine, clopidogrel, and prasugrel
Ticagrelor
GPIIb-IIIa Antagonists
Abciximab, tirofiban, eptifibatide
Drugs that Increase Platelet Cyclic AMP or Cyclic GMP
Prostaglandin I_2 and analogs
Phosphodiesterase inhibitors (dipyridamole, cilostazol, caffeine, theophylline, and aminophylline)
Nitric Oxide and Nitric Oxide donors
Antimicrobials
Penicillins
Cephalosporins
Nitrofurantoin
Hydroxychloroquine
Miconazole
Cardiovascular Drugs
β-adrenergic blockers (propranolol)
Vasodilators (nitroprusside and nitroglycerin)
Diuretics (furosemide)
Calcium channel blockers
Quinidine
Angiotensin-converting enzyme inhibitors
Anticoagulants
Heparin
Thrombolytic Agents
Streptokinase, tissue plasminogen activator, urokinase
Psychotropics and Anesthetics
Tricyclic antidepressants (imipramine, amitriptyline, and nortriptyline)
Phenothiazines (chlorpromazine, promethazine, and trifluoperazine)
Selective serotonin release inhibitors (fluoxetine, paroxetine, etc.)
General anesthesia (halothane)
Chemotherapeutic Agents
Mithramycin
1,3-bis (2-chloroethyl)-1-nitrosurea
Daunorubicin
Dasatinib
Miscellaneous Agents
Dextrans and hydroxyethyl starch
Lipid-lowering agents (clofibrate and halofenate)
e-Aminocaproic acid
Antihistamines
Ethanol
Vitamin E
Radiographic contrast agents
Food items and food supplements (ω-3 fatty acids, vitamin E, chocolates, onions, garlic, ginger, cumin, turmeric [curcumin], clove, black tree fungus, and Ginkgo)

LIVER DISEASE

The hemostatic defect in chronic liver disease is complex and results from deficiencies of blood coagulation factors and their inhibitors, disseminated intravascular coagulation, decreased fibrinolytic inhibitors and increased fibrinolysis, thrombocytopenia, and qualitative platelet defects (see Chapter 126). The overall contribution of platelet dysfunction to the hemostatic defect in severe liver disease is unknown; the major contributors are the changes in blood coagulation. Platelet abnormalities noted in patients with chronic liver disease of various etiologies include prolongation of the bleeding time, diminished platelet survival, and reduced platelet aggregation, platelet adhesiveness, and platelet procoagulant activities.[183–188] In some studies, platelet aggregation responses were normal.[189] Platelet TXA_2 production has been reported to be decreased, associated with a defect in the release of free arachidonic acid from phospholipids and in phosphatidic acid formation.[187,188,190] The platelet dysfunction in severe liver disease has been attributed to elevated plasma fibrin degradation products,[183,185] abnormal high-density lipoproteins,[191] and ethanol.[192,193] Therapy for the hemostatic defect in severe liver disease involves administration of products to correct the coagulopathy. Platelet transfusions are indicated in patients with serious bleeding and severe thrombocytopenia. Intravenous and subcutaneous DDAVP shortens the bleeding time of patients with cirrhosis[194–196] and may be useful in the treatment of some patients (Chapter 60).

MISCELLANEOUS CONDITIONS WITH IMPAIRED PLATELET FUNCTION

Platelet function abnormalities have been described in severe vitamin B_{12} deficiency,[197] eosinophilia[198–200], atopia,[201] asthma and hay fever,[201,202] and Bartter syndrome.[203] The clinical importance of these observations is uncertain.

DRUGS THAT INHIBIT PLATELET FUNCTION

Many drugs affect platelet function (Table 66.2), and the clinical relevance is the potential for either spontaneous or provoked

bleeding. However, for several drugs, the antiplatelet effects have been established largely *in vitro* and the clinical relevance of these findings is not clear. The impact on hemostasis is unknown for many drugs, even when they have been shown to decrease platelet responses *ex vivo*. Moreover, the impact of combined effects of multiple drugs, each with a mild effect on platelet function , is unknown even though this is a clinically relevant issue.

Aspirin (see Chapter 34)

Because of its widespread use, aspirin is an important cause of platelet dysfunction. Aspirin irreversibly acetylates and inactivates platelet COX-1 and inhibits endoperoxide (PGG_2 and PGH_2) and TXA_2 synthesis.[204] Much of the effect of orally administered aspirin on platelet COX occurs in the presystemic (portal) circulation.[205] As little as a single 80 mg tablet administered daily completely blocks platelet TXA_2 production.[206] The efficacy of aspirin in the primary and secondary prevention of arterial vascular events is well established.[204] Its effects as an antithrombotic agent and in impairing primary hemostasis are both related to the inhibition of platelet TXA_2 production, although aspirin has other effects, including acetylation of plasma proteins (prothrombin and fibrinogen).[204] In otherwise normal individuals, the overall result of aspirin ingestion is a mild impairment of hemostasis. Aspirin prolongs the bleeding time in many but not all normal individuals; this effect is influenced by the direction of the forearm incision and the venostasis applied during the procedure.[207–209] Aspirin ingestion results in inhibition of platelet aggregation and secretion following stimulation with weak agonists such as ADP, epinephrine, and low concentrations of collagen and thrombin, and in particular, when platelets are stimulated by arachidonic acid. Closure times in the PFA-100 are often prolonged using the cartridge coated with collagen and epinephrine but not collagen and ADP.[210] The bleeding risk with aspirin is dose related.[204,211] In a meta-analysis of 192,036 patients from 31 clinical trials, the risk of hemorrhage was lowest with aspirin dose <100 mg compared to higher doses.[211] The overall risk of a major bleed with aspirin is <1% per year.[204] Evidence for aspirin's adverse effect on hemostasis, particularly the risk of major extracranial and intracranial hemorrhages, comes from the larger aspirin trials. Several observations point to the clinical significance of the aspirin-induced platelet defect. Ingestion of aspirin during pregnancy has been reported to result in excessive bleeding at delivery in both the neonate and the mother.[212–214] In the Physicians' Health Study, 22,071 healthy, male physicians were randomized to receive placebo or aspirin (325 mg every other day); over a 5-year follow-up there was a significantly higher incidence of easy bruising, mucosal bleeding (including gastrointestinal bleeding), and blood transfusion requirements.[215] The rate of hemorrhagic stroke was higher with aspirin (0.04% per year) compared to placebo (0.02% per year, $P = 0.06$).[215] In the Nurses' Health Study cohort of 79,000 women (34 to 59 years of age), high frequency use of aspirin (>15 tablets per week) was associated with increased risk of subarachnoid hemorrhage, particularly among older or hypertensive women.[216] Preoperative aspirin increases blood loss, transfusions, and repeated operations in patients undergoing cardiac surgery.[217,218,219] The increased bleeding appears with aspirin doses >325 mg/d.[218] However, other studies have suggested that preoperative aspirin improves graft potency and survival.[218,220] Firm recommendations as to when to discontinue aspirin prior to coronary artery surgery (CABG) remain to be established. The American College of Cardiology and American Heart Association guidelines recommend that aspirin be stopped 7 to 10 days before CABG, while the American College of Chest Physicians recommends continuing aspirin up to and beyond the time of surgery.[219]

Gastrointestinal bleeding is a major adverse effect of aspirin.[204,215,221,222,223] Aspirin-induced gastrointestinal toxicity, but not its antithrombotic effect, is dose related in the range 30 to 1,300 mg daily.[204,221] However, even at low doses (30 to 50 mg daily), aspirin can cause serious gastrointestinal bleeding. The overall relative risks of gastrointestinal complications with the use of nonsteroidal anti-inflammatory drugs (NSAIDs including aspirin) have been estimated to be between 3.0 and 5.0 as compared to nonusers.[223]

Individuals with underlying hemostatic defects such as vWD, hemophilia, or mild platelet function defects, and those on oral anticoagulant therapy, may develop considerable postoperative or even spontaneous bleeding with concomitant aspirin therapy. In these patients, aspirin ingestion often leads to a striking prolongation of the bleeding time and an increase in bleeding manifestations and should be avoided. In otherwise normal subjects who are on aspirin and need to undergo elective surgery, aspirin should be discontinued 7 to 10 days prior to the procedure. If excessive perioperative hemorrhage is encountered, it is generally responsive to platelet transfusions. Moreover, DDAVP infusion shortens the aspirin-induced prolongation in the bleeding time.[224] In the context of spinal or epidural anesthesia, a large prospective study in 924 patients undergoing orthopedic procedures indicates that preoperative aspirin therapy is not a significant risk factor for development of neurologic dysfunction from spinal hematoma.[225]

Nonsteroidal Anti-Inflammatory Drugs

Platelet function is inhibited by NSAIDs that inhibit COX-1 and may prolong the bleeding time. These drugs include indomethacin, ibuprofen, sulfinpyrazone, diclofenac, meclofenamic acid, phenylbutazone, sulindac, naproxen, mefenamic acid, piroxicam, tolmetin, and diflunisal.[226–235] COX-1 inhibition by these agents is generally short-lived and reversible. For example, the effect of indomethacin is not detectable after 6 hours.[228] In contrast, the effect of piroxicam may last for several days because of its long half-life.[236] Exposure to NSAIDs other than aspirin is also recognized to increase the risk of upper GI bleeding and/or perforation. However, different NSAIDs vary in this effect.[222,223] In a meta-analysis,[222] the relative risk was lowest for ibuprofen and diclofenac; intermediate for aspirin, indomethacin, naproxen, and sulindac; and higher for azapropazone, tolmetin, ketoprofen, and piroxicam. Ibuprofen has been administered to hemophiliacs without a major increase in bleeding,[237] although enhanced bleeding was noted in HIV-infected hemophilic patients receiving ibuprofen and zidovudine.[238] These findings may be relevant to the choice of the drug for use in patients with impaired hemostasis. Concomitant administration of ibuprofen and aspirin antagonizes the irreversible COX inhibition of aspirin and may impact its antithrombotic efficacy.[239] Like aspirin, most of these NSAIDs inhibit both forms of cyclooxygenase (COX -1 and -2).[240] The selective COX-2 inhibitors, celecoxib, rofecoxib, and valdecoxib, do not have an antiplatelet activity.

Acetaminophen does not inhibit or impair platelet function at levels attained *in vivo*, although in some subjects, it may inhibit *in vitro* platelet responses as a weak COX inhibitor[241] and may even have anti-COX-2 activity.[242]

ADP Receptor Antagonists (see Chapter 111)

The thienopyridine derivatives, ticlopidine, clopidogrel, and prasugrel, inhibit platelet function by inhibiting ADP binding to the $P2Y_{12}$ receptor.[204,243,244,245,246,247] In patients treated with a thienopyridine, platelet aggregation responses to several agonists, including ADP, collagen, epinephrine, and thrombin, are inhibited to various extents depending on agonist concentrations. All three thienopyridines mediate their effects through active metabolites generated *in vivo* by cytochrome P450 in the liver. With repeated twice-daily dosing, plasma ticlopidine levels increase approximately threefold over 2 to 3 weeks because of drug accumulation; the elimination half-life is up to 96 hours after 14 days of repeated dosing. Clopidogrel differs from ticlopidine in pharmacokinetics. It is rapidly metabolized, and the main carboxylic derivative has a plasma elimination half-life of approximately 8 hours. With repeated daily dosing (50 to 100 mg) in healthy volunteers, ADP-induced platelet aggregation is inhibited from the second day, reaching a steady state after 4 to 7 days.[204] After a single dose of 400 mg clopidogrel, platelet aggregation is inhibited at 2 hours and inhibition persists for up to 48 hours. In patients receiving continuous therapy platelet inhibition persists for 7 to 10 days after these agents are discontinued. Both ticlopidine and clopidogrel prolong the bleeding time by 1.5- to twofold over control, although more striking prolongation has also been noted.[244,248,249] PFA-100 closure times are insensitive to therapy with these agents.[210] Because of prolonged pharmacologic activity, ticlopidine and clopidogrel must be discontinued for 7 to 10 days prior to elective surgery. Both drugs have been associated with thrombotic thrombocytopenic purpura.[250–252] Prasugrel, a newer thienopyridine prodrug, is a more potent and consistent inhibitor of platelet function than clopidogrel.[245,246,247] In the Triton-TIMI38 trial, prasugrel was superior to clopidogrel in acute coronary syndromes, but was associated with increased bleeding.[253]

Ticagrelor is a nonthienopyridine $P2Y_{12}$ antagonist. Unlike thienopyridines, it is not a prodrug and is a direct and reversible $P2Y_{12}$ inhibitor.[245,246] It is a more rapid and more potent inhibitor of platelets than clopidogrel.

GPIIb-IIIa Antagonists

Platelet activation results in a conformational change in the integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa), leading to fibrinogen binding to $\alpha_{IIb}\beta_3$ and platelet aggregation. Accordingly, $\alpha_{IIb}\beta_3$ antagonists inhibit platelet aggregation.[204,245] This class of drugs includes abciximab (Reopro), a humanized chimeric monoclonal antibody against the GPIIb-IIIa receptor; eptifibatide (Integrilin), a synthetic cyclic heptapeptide containing the KGD sequence; and tirofiban (Aggrastat), a nonpeptide arginine-glycine-aspartic acid (RGD) mimetic. These drugs are administered intravenously and are used in acute coronary syndromes and in the context of percutaneous coronary interventions. They are potent inhibitors of platelet aggregation in response to all platelet agonists, and they prolong the bleeding time.[204] As would be expected, bleeding is a complication of using these drugs. Acute thrombocytopenia occurring within 24 hours has been described with all three GPIIb-IIIa antagonists; it is mediated by naturally occurring antibodies and may occur even at the first exposure to the drug.[254]

Drugs that Increase Platelet Cyclic AMP or Cyclic GMP

Drugs that increase intracellular cAMP levels by activating adenylyl cyclase (e.g., PGI_2, PGE_1, PGD_2) or inhibiting the cyclic nucleotide phosphodiesterases inhibit platelet responses. Intravenous infusion of prostacyclin (PGI_2) and its stable analogs prolong the bleeding time.[255,256] Dipyridamole, a weak phosphodiesterase inhibitor, blocks adenosine uptake and does not inhibit platelet aggregation in response to collagen, epinephrine, and ADP at usual doses. It has a synergistic effect with aspirin in prolonging the shortened platelet survival in thromboembolic disorders.[257] The dipyridamole formulation currently used combines an extended-release form of dipyridamole with low-dose aspirin.[204] Cilostazol is a phosphodiesterase-3 inhibitor that suppresses platelet aggregation and is used for therapy of peripheral arterial disease.[245,258,259] It does not prolong the bleeding time.[249] Other weak phosphodiesterase inhibitors such as caffeine, theophylline, and aminophylline inhibit ADP-induced platelet aggregation *in vitro*, but this appears to have no clinical significance.[260]

Sildenafil (Viagra), a selective phosphodiesterase-5 inhibitor, increases platelet cyclic GMP levels, but does not have a direct effect on platelet function, although it potentiates the *in vitro* antiaggregatory effect of sodium nitroprusside[261] and may inhibit ADP-induced activation of $\alpha_{IIb}\beta_3$.[262] Some reports have associated sildenafil with epistaxis and hemorrhoidal and variceal bleeding. Nitric oxide (NO) and NO donors inhibit platelet activation and responses by elevating cyclic cGMP levels.[263–267] Elevated cGMP inhibits platelet cAMP phosphodiesterase, thereby elevating cAMP. NO administration is associated with a prolonged bleeding time.[263,265]

Antimicrobial Agents

β-lactam antibiotics, including penicillins and cephalosporins, inhibit platelet aggregation responses, and some induce a bleeding diathesis when given in high doses. These include carbenicillin, penicillin G, ticarcillin, ampicillin, nafcillin, cloxacillin, mezlocillin, oxacillin, and piperacillin.[268–278] The effects on platelet function are dose dependent, taking about 2 to 3 days to manifest and 3 to 10 days to abate after drug discontinuation.[268,273,274,277,278] Penicillins lacking an α-carboxy group (mezlocillin, piperacillin, and apalcillin) appear to adversely affect platelet function less often than carboxypenicillins or moxalactam.[277] Cephalosporins may also impair platelet function.[277,279,280] Moxalactam has been reported to induce platelet dysfunction associated with prolonged bleeding times and clinical hemorrhage.[277,280] However, other third-generation cephalosporins appear to show little effect on platelet function.[277] Some β-lactam antibiotics (i.e., moxalactam, cefamandole, cefoperazone, and cefotetan) may induce hypoprothrombinemia and thereby contribute to hemorrhage.[277,280] These compounds contain an N-methylthiotetrazole side chain, which inhibits vitamin K epoxide reductase, thus interfering with the carboxylation of the vitamin K–dependent coagulation factors.

Several generalizations may be made regarding the impact of β-lactam antibiotics on platelets. First, the effects on platelet

function and hemostasis are dose- and time-dependent, with effects discernible over several days.[268,273,274,277,278] Second, the patients at particular risk of bleeding appear to be those with concurrent illnesses including sepsis, malnourishment, thrombocytopenia, and malignancy. Third, the platelet inhibitory effect of β-lactam antibiotics is influenced by plasma albumin; binding of the antibiotic to platelets and the impact on platelet responses are inversely related to albumin concentration.[281] Last, with some cases, bleeding is related to a concomitant inhibition of synthesis of vitamin K–dependent coagulation factors.[277,279,280]

Multiple mechanisms have been invoked to explain platelet inhibition induced by β-lactam antibiotics. Some of the drugs inhibit platelet aggregation and secretion, as well as platelet adherence to subendothelial structures and collagen-coated surfaces.[272] However, these effects were often observed only *in vitro* and at concentrations well above those attained *in vivo*. Short-term *in vitro* exposure of platelets to penicillin has been reported to result in impaired aggregation responses and decreased binding of agonists to specific receptors (ADP, vWF, and α_2-adrenergic) on platelets.[282,283] Although the latter effect of penicillins was found to be rapidly reversible,[282,283] the platelet inhibitory effect of β-lactam antibiotics appears to persist for several days after the drugs are discontinued, indicating that other mechanisms underlie the sustained effect. Other reported effects of β-lactam antibiotics include inhibition of intracellular signaling events, calcium mobilization,[284] and TXA_2 synthesis,[283,284] and alterations in activation-induced changes in $\alpha_{IIb}\beta_3$ and GPIb-IX.[285]

The context in which the bleeding events are encountered in patients on antibiotics precludes clear definition of the role played by the antimicrobials because of the simultaneous presence of thrombocytopenia, disseminated intravascular coagulation, infection, and/or vitamin K deficiency. Discontinuation of a specifically indicated antibiotic may not be possible or necessary. Supportive measure using blood products and other interventions (correcting metabolic abnormalities, vitamin K deficiency) are indicated in the overall management.

Other antimicrobials shown to inhibit platelet function include nitrofurantoin,[286] hydroxychloroquine,[287] and miconazole.[288]

Cardiovascular Drugs

Relatively high concentrations of propranolol *in vitro* inhibit the aggregation responses of normal platelets to ADP and epinephrine.[289–291] At lower concentrations, propranolol inhibits secondary aggregation and secretion in patients whose platelets are susceptible to a low threshold concentration of agonists, but not uniformly in all patients taking the drug.[289,292] At conventional doses, propranolol inhibits and normalizes the increased sensitivity to agonists of platelets from patients with angina pectoris.[293] The mechanism by which propranolol alters platelet behavior is probably unrelated to its β-adrenergic blocking effects and may be due to an effect on platelet membranes.[289,292] Propranolol inhibits the release of free arachidonic acid from phospholipids and TXA2 production during platelet activation.[294,295] Overall, propranolol is a weak inhibitor of platelet function and does not influence the bleeding time. Other β-blockers (pindolol, atenolol, metoprolol, and carvedilol) have also been reported to inhibit platelet responses when studied *in vitro*.[290,296] Other cardiovascular drugs known to inhibit platelet responses include furosemide,[297] nitroprusside,[298–301] nitroglycerin and isosorbide dinitrate,[298,302] and quinidine.[303,304] Although calcium channel blockers (nifedipine, verapamil, and diltiazem) inhibit platelet responses, this is only seen *in vitro* at drug concentrations higher than those achieved during therapy.[305–309] These agents inhibit platelet α_2-adrenergic receptors.[307,308]

The angiotensin-converting enzyme (ACE) inhibitors captopril,[310,311] perindopril,[312] and fosinopril,[313] but not quinapril,[312] enalapril,[314] and lisinopril,[315] have been reported to inhibit platelet aggregation in human studies.[316] Captopril[311,314] and fosinopril[314] decrease TXA_2 production. Interestingly, reduced circulating levels of plasma fibrinogen, factor XII, factor XI, tissue plasminogen activator, and plasminogen activator inhibitor-I have been observed with the use of ACE inhibitors.[316]

Anticoagulants and Thrombolytic Agents

Heparin–platelet interactions are clinically relevant, particularly because of the paradoxical antibody-mediated thrombocytopenia and thrombosis. Heparin binds to platelets[317] and enhances aggregation responses to platelet agonists *in vitro*.[318] On the other hand, a bolus injection of heparin into normal subjects prolongs the bleeding time[319] and heparin inhibits vWF-dependent platelet responses *in vitro* and *ex vivo*.[320] The impact of these disparate effects on hemostatic mechanisms during heparin therapy is complex and unclear. Interestingly, protamine sulfate, widely used to neutralize heparin, also inhibits thrombin-induced platelet responses *in vitro*.[321] The major complication following the administration of thrombolytic agents (streptokinase, urokinase, and recombinant tissue plasminogen activator [rt-PA]) is hemorrhage. This is a consequence of the effect of plasmin on the plasma coagulation system and platelets, but predominantly results from the dissolution of clots providing hemostasis at the site of vascular injury. The bleeding time in patients receiving rt-PA may be prolonged.[322] *In vitro*, plasmin induces several effects in platelets: initial platelet activation followed by inhibition; cleavage of membrane glycoproteins; inhibition of TXA_2 production; and disaggregation of platelet clumps.[323–327] Plasmin-induced platelet aggregation has been attributed to cleavage of protease-activated receptor-4.[328] Urinary metabolites of TXA_2 are increased during thrombolytic therapy with rt-PA or streptokinase, indicating *in vivo* activation of platelets.[329,330]

Psychotropic Drugs and Anesthetics

This heterogeneous group of drugs is generally thought to stabilize biological membranes by increasing their fluidity. Tricyclic antidepressants (imipramine, amitriptyline, and nortriptyline) inhibit platelet aggregation and secretion responses to ADP, epinephrine, and collagen.[331,332] Platelets incorporate serotonin into their dense granule by uptake from plasma. Selective serotonin reuptake inhibitors (SSRIs, e.g., fluoxetine [Prozac], paroxetine [Paxil]) are widely used antidepressants that cause an acquired but reversible serotonin deficiency in platelet-dense granules; this may be related to an apparent benefit of SSRIs in coronary artery disease.[333] Case reports indicate that fluoxetine can prolong the bleeding time, impair platelet aggregation, and cause excessive bruising.[334,335] Paroxetine prolongs the PFA-100 closure time and decreases thrombin-induced platelet surface expression of CD63.[336] As a group, SSRIs inhibit platelet function[337] and are associated with increased risk of GI bleeding, transfusions

during orthopedic surgery, and hospital admissions for bleeding manifestations.[338,339] A retrospective study found an increased risk of bleeding in patients taking an SSRI together with aspirin or aspirin plus clopidogrel compared to the antiplatelet regimen alone.[340] Phenothiazines (chlorpromazine, promethazine, and trifluoperazine) inhibit platelet responses to stimulation *in vitro* and perhaps *in vivo*.[341–345] Trifluoperazine may impair platelet responses by inhibiting calmodulin-dependent processes in platelets.[344] Halothane has been reported to increase the bleeding times during general anesthesia[346,347] but probably without effect on surgical hemostasis. Thiopental appears to inhibit platelet responses in surgery patients.[348,349]

Miscellaneous Drugs

Dextrans are branched polysaccharides of 40,000 or 70,000 kDa and have been used as antithrombotic agents. They prolong the bleeding time after infusion and inhibit platelet–factor 3 availability, glass bead retention, aggregation, and secretion.[350–355] Their effects on the bleeding time and platelet inhibition are most pronounced 4 to 8 hours after transfusion.[353] Dextran-induced platelet dysfunction has been attributed to their adsorption to platelets and to a decrease in plasma factor VIII-vWF.[354,356] It is unclear if their use, even in the perioperative state, leads to excessive hemorrhage.[357] In one multicenter study in surgical patients, prophylaxis with dextran-70 was associated with less bleeding than low-dose heparin.[358] Hydroxyethyl starch, another volume expander, may enhance bleeding and prolong the bleeding time,[359–361] as well as cause AvWD.[127,128]

Other drugs that impair platelet function include lipid-lowering agents (clofibrate and halofenate)[362,363]; chemotherapeutic agents such as daunomycin, mithramycin, and 1,3-bis (2-chloroethyl)-1-nitrosurea (BCNU)[364–369]; and antihistamines.[370,371] Dasatinib, a tyrosine kinase inhibitor used for the treatment of CML, has been associated with clinical bleeding symptoms[372]; it inhibits human platelet responses to collagen more effectively than responses to ADP and thrombin *in vitro* and following administration to mice.[373] The fibrinolytic inhibitor epsilon aminocaproic acid (EACA, Amicar) can prolong the bleeding time at the generally administered dose.[374] Ethanol inhibits platelet responses *in vitro*.[375–377] However, it does not prolong the bleeding time by itself. Nonetheless, acute ingestion of 50 g of ethanol potentiates the aspirin-induced prolongation of the bleeding time even in otherwise normal subjects.[378] Other agents reported to inhibit platelet function include radiographic contrast agents[379–381] (although some contrast agents activate platelets[382]), vitamin E,[383,384] fish oil with ω-3 fatty acids,[385,386], and food items and their extracts such as onions,[387–389] garlic,[389,390] ginger,[391] cumin,[392] turmeric,[392–394] clove,[395] flavanol-rich cocoa beverage and chocolates,[396–398] Chinese food, and black tree fungus.[399,400] Inhibition of eicosanoid synthesis appears to be the mechanism of action for several of the spices, although they have other effects, such as lowering of plasma lipids. For most of these agents, there is little evidence that they impair clinical hemostasis.

Herbal medicines, such as Ginkgo (*Ginkgo biloba*) and garlic (*Allium sativum*), inhibit platelet function and have been associated with increased bleeding, particularly when administered concomitantly with other medicines (warfarin and aspirin).[401] With the increasing use of herbal medicines and food supplements, their role and interaction with pharmaceutical drugs needs to be considered in the evaluation of patients with unexplained bleeding.

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CHAPTER

67 Laboratory Markers of Platelet Activation

PAUL HARRISON AND ALAN D. MICHELSON

Prevention of platelet activation in the intact circulation is normally controlled by the antithrombotic nature of the endothelial cell surface, which exhibits a number of effective mechanisms for inhibiting platelets. Under normal conditions, platelets are therefore unable to form stable contacts on the vessel wall. Platelet adhesion, however, can occur upon vessel wall damage, on diseased/activated endothelial cells, or at sites of disturbed flow (e.g., atherosclerotic plaques). Exposure of the subendothelial matrix (e.g., collagen) initiates rapid platelet activation, which is then further amplified by the release of platelet-derived adenosine diphosphate (ADP), thromboxane, and serotonin, resulting in further platelet activation and recruitment of other platelets in the vicinity to rapidly form a hemostatic plug. The high shear stresses associated with mature atherosclerotic lesions can also initiate platelet activation.

Platelet activation results in a complex series of changes including a physical redistribution of receptors, changes in the molecular conformation of receptors, secretion of granule contents, cleavage and release of various glycoproteins (the "sheddome"), generation of thromboxane, development of a procoagulant surface, generation of platelet-derived microparticles, and formation of both platelet–platelet and leukocyte–platelet aggregates. All of these changes can potentially be used as markers of platelet activation (Table 67.1). Although platelet activation is usually initiated locally at the site of formation of the hemostatic plug or thrombus, it is possible to measure the consequences of these local events in the systemic circulation—in part because thrombi are very dynamic with transient adhesion, "rolling," and embolization of platelets often occurring. Also, in thrombotic disease, platelets may be "primed" or more reactive to agonists in the circulation or conversely "exhausted" (due to excessive activation) and less activatable *ex vivo*. This can be augmented by a genetic predisposition causing a preexisting reactive phenotype. However, individuals may also acquire a more reactive phenotype due to many environmental phenotypic factors including diet, lifestyle, ageing, various diseases, etc. Therefore, it is now possible to utilize a number of sensitive tools to measure inappropriate platelet activation and/or reactivity in health and disease and also to monitor various forms of antiplatelet therapy to potentially modify treatment and prevention strategies.

Whole blood flow cytometry[1] is now regarded as the research method of choice for the measurement of all these changes, except the secretion of soluble molecules and shed glycoproteins, which are now usually measured by ELISA. Whole blood flow cytometry has many advantages: (a) minuscule volumes (~5 µL) of blood can be used; (b) platelets are directly analyzed in their physiologic milieu of whole blood; (c) the minimal manipulation of samples prevents artifactual *in vitro* activation and potential loss of platelet subpopulations; (d) both the activation state of circulating platelets and the reactivity of circulating platelets can be determined; and (e) a spectrum of different activation-dependent changes can be determined.

METHODS AVAILABLE TO MEASURE PLATELET ACTIVATION

Biochemical Markers

Thromboxane generation provides one of the most important amplification loops in platelet biochemistry. This is exemplified by the widespread use of aspirin to target cyclooxygenase (COX)-1, resulting in irreversible inhibition of thromboxane A_2 generation for the lifespan of the platelet and partially explains why aspirin is such an effective antithrombotic therapy. Serum, plasma, and urinary assays for thromboxane B_2 (a stable metabolite of thromboxane A_2) and other metabolites have utility in the measurement of platelet activation and for monitoring aspirin therapy.[2,3,4] Modern assays are ELISA based and are normally performed on fresh serum samples that have been generated *ex vivo* under standard conditions of incubation (e.g., 1 hour at 37°C). The assays provide sensitive, specific, and relatively simple measurements of COX-1 inhibition, and samples can be stored and batched for analysis. Samples from aspirin-treated patients are unable to generate thromboxane. It is recommended that for monitoring aspirin it is important to measure the consequences of inhibiting the biochemical target of the drug, so these assays provide a simple means for monitoring compliance and effectiveness of aspirin therapy.[5] More recently, ELISAs of 11-dehydro thromboxane B_2 in stabilized and stored urine samples have also become popular and commercialized (AspirinWorks) and provide a simple noninvasive measurement of thromboxane generation that seems to correlate with aspirin efficacy.[4,5,6] The latter assays have the disadvantage that they are also more nonspecific to the platelet and can measure other sources of thromboxane generation.[7]

Another biochemical marker, the measurement of the phosphorylation status of vasodilator-stimulated phosphoprotein (VASP) by flow cytometry, is discussed in section II.C.5 below.

Soluble Markers

α-Granular Proteins

Providing there is a sufficient stimulus platelets secrete the contents of their granules into the plasma. Platelet α-granules contain a number of growth factors, clotting factors, and adhesion molecules, thus providing a way to release high concentrations of important mediators of inflammation, chemotaxis,

Table 67.1

Summary of laboratory markers of platelet activation

Biochemical markers
• Thromboxane (serum, urine)
• VASP
Soluble markers
• α-Granule releasates
• Dense granule releasates
• Platelet "sheddome"
Changes in platelet surface glycoproteins
Platelet surface exposure of granule membrane proteins
Platelet–platelet aggregates
Leukocyte–platelet aggregates
Platelet surface PS
Platelet-derived microparticles

and hemostasis within the local environment of the growing platelet thrombus. As some of these markers are also soluble and specific to the megakaryocyte–platelet lineage, their measurement provides a way to systemically and specifically measure the degree of platelet activation occurring in various disease states. Platelet-derived α-granular markers are now usually measured by ELISAs that have superseded radioimmunoassays. Of these molecules, the most commonly and traditionally measured markers of platelet activation are the α-granular soluble constituents, β-thromboglobulin (β-TG),[8,9] and platelet factor 4 (PF4).[8,9] More recently, some α-granular membrane proteins have been found to be expressed on the surface of activated platelets where they are rapidly cleaved by proteases and are shed into the plasma. These include soluble P-selectin[10,11] and soluble CD40 ligand (sCD40L)[12] (see the platelet "sheddome" section below). However, as a result of the obligatory plasma separation procedures, all of these assays are vulnerable to artifactual *in vitro* platelet activation.[8,9]

Dense Granular Proteins

Platelet-dense granules contain a unique pool of molecules (e.g., serotonin [5-HT], nucleotides, polyphosphate [Poly-P]) that can also provide a sensitive measurement of platelet activation. Lumiaggregometry can be used to measure the *ex vivo* release of adenosine triphosphate (ATP) from dense granules during the aggregation response to an exogenous agonist.[13] Levels of platelet-released ATP and ADP can also be measured by luminometry within standardized platelet-rich plasma preparations that have been activated and lysed.[14] These measurements are useful for the diagnosis of storage and release disorders. Circulating serotonin is actively taken up by platelets and packaged into the dense granules. Some *in vitro* platelet activation assays utilize the uptake of radio-labeled serotonin and measure its release after stimulation with different agonists. Dense granular Poly-P has recently been shown to be an endogenous activator of factor XII, a component of the intrinsic clotting cascade and proinflammatory pathways.[15,16] However, as none of these substances are exclusively synthesized and stored within platelets, their measurement within *ex vivo* blood samples is not as common as with some of the more specific platelet α-granular-derived or shed glycoproteins.

The Platelet "Sheddome"

It has become increasingly apparent that proteolysis of a large number of different platelet surface and granular membrane proteins during or after platelet activation results in the generation of a growing list of potentially useful plasma markers of platelet activation. These protein fragments have been given the collective term the platelet "sheddome" and are released from the platelet surface by proteinases such as ADAM17 or ADAM10.[17] Shedding tends to be slower than platelet activation and is a selective process rather than a wholesale loss of all surface glycoproteins. Among those proteins shed are glycoprotein (GP) Ib (glycocalicin), GPVI, GPV, P-selectin, endothelial cell–specific adhesion molecule, junctional adhesion molecule-A, semaphorin 4D, and CD40L.[17] Many of the shed protein fragments may play roles in platelet and vascular biology, although the full implications of this shedding process are still largely unknown. Nevertheless, measurement of components of the sheddome may prove to be useful as sensitive and specific biomarkers of platelet activation *in vivo*. For example, soluble GPVI is an excellent candidate for a specific biomarker of platelet activation as it is found in the plasma at relatively low levels in healthy subjects but is elevated in patients with thrombotic risk, heparin-induced thrombocytopenia, and immune thrombocytopenic purpura.[18,19] Exposure of platelets *in vitro* to high shear stress also results in increased soluble GPVI levels. Further work is required to determine the clinical potential of this unique marker. Platelet surface GPV is also cleaved by thrombin,[20–22] releasing a soluble form of GPV (sGPV) into plasma, which may serve as another biomarker of platelet activation.[23] A number of the shed glycoproteins (e.g., CD40L and P-selectin) are also present in α-granule membranes and are only detectable on the surface of activated platelets, where they can be cleaved and shed by the action of proteases. Release of sCD40L from the platelet surface is the predominant source of sCD40L.[24,25] sCD40L is thought to be prothrombotic by stabilizing $\alpha_{IIb}\beta_3$-dependent arterial thrombosis[26] but can also be proinflammatory[27,28] (although not all studies agree on this latter point[24]). sCD40L levels have been shown to be elevated in patients with acute myocardial infarction and other acute coronary syndromes. P-selectin (CD62P) is also externalized from the α-granules during platelet activation and is gradually cleaved and shed. Increased levels of soluble P-selectin have been found in many diseases and may predict major adverse cardiovascular events.[11]

An important practical point is that many published patient studies of shed glycoprotein levels were performed using serum. However, blood clotting results from thrombin generation and it is accompanied by platelet activation and the *ex vivo* release of large amounts of these markers into the serum. Therefore, accurate measurement of *in vivo* circulating molecules released from platelets requires assays performed using plasma rather than serum.[29] Platelets also have to be carefully removed during plasma preparation, without any significant activation; anticoagulants (e.g., citrate, theophylline, adenosine, and dipyridamole [CTAD]) containing some platelet inhibitory agents have

been traditionally used for measurement of PF4 and β-TG to help minimize any additional *ex vivo* platelet activation. Soluble P-selectin in plasma may also be of endothelial cell origin although it is thought that this is a predominant marker of platelet activation.[10]

Flow Cytometric Measurement of Platelet Activation

Introduction

Flow cytometry enables the rapid measurement of characteristics of a large number of individual cells.[30] For flow cytometric analysis, cells in suspension are fluorescently labeled, typically with a fluorescently conjugated monoclonal antibody. In the flow cytometer, the suspended cells pass through a focused laser beam at a rate of 1,000 to 10,000 cells per minute. After the laser light activates the fluorophore at the excitation wavelength, detectors process the emitted fluorescence and light scattering properties of each cell.[30] The intensity of the emitted light is directly proportional to the antigen density or the characteristics of the cell being measured.

Clinical studies utilizing flow cytometric assays of washed platelets or platelet-rich plasma are, like other assays of platelet function, potentially susceptible to artifactual *in vitro* platelet activation during obligatory separation procedures. The introduction of whole blood flow cytometry by Shattil et al.[31] was therefore a major step toward the application of flow cytometry to clinical settings.

A typical schema of sample preparation for whole blood flow cytometry is shown in FIGURE 67.1. The anticoagulant is usually buffered trisodium citrate (105 to 109 mmol/L or 129 mmol/L). Samples are then diluted (typically 1:10) in the presence of antibodies or agonists. The purpose of the dilution is to minimize the formation of platelet aggregates (see below). A minimum of two monoclonal antibodies is used, each conjugated with a different fluorophore. A wide variety of fluorophores are available for antibody conjugation (e.g., phycoerythrin, fluorescein, peridinin chlorophyll protein [PerCP], phycoerythrin-Cy5, phycoerythrin-Texas Red [Red-670], allophycocyanin [APC]). The "test" antibody (the monoclonal antibody recognizing the antigen to be measured) is added at a saturating concentration. The "platelet identifier" monoclonal antibody (e.g., GPIb, integrin α_{IIb}, or integrin β_3 specific) is added at a near saturating concentration. Physiologic agonists can be used in the assay, including thrombin, thrombin receptor-activating peptides (TRAPs), ADP, collagen, collagen-related peptide (CRP), the complement fraction C5b-9, and thromboxane A_2 analogs. Nonphysiologic agonists include phorbol myristate acetate and the calcium ionophore A23187. Samples are stabilized by fixation, typically with final concentrations of 0.2% to 1% formaldehyde or paraformaldehyde. Antibodies can be added after fixation, provided fixation does not interfere with antibody binding[32] (see below). Samples are then analyzed in a flow cytometer. After identification of platelets both by their characteristic light scatter and by the "platelet identifier" antibody, binding of the test monoclonal antibody is determined by analyzing 5,000 to 10,000 individual platelets. During *ex vivo* activation and labeling, the formation of platelet aggregates is minimized by using very gentle mixing (tapping of the tube) and dilution to minimize collisions between platelets.

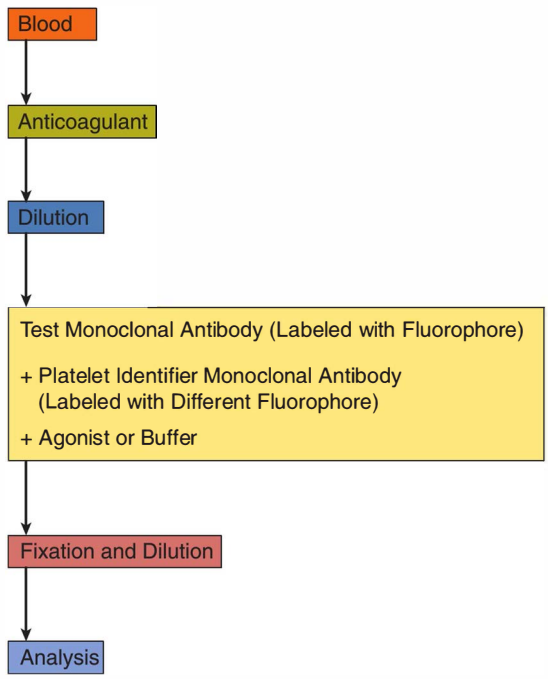

FIGURE 67.1 A typical schema of sample preparation for analysis of platelets by whole blood flow cytometry.

For specific protocols of whole blood flow cytometric assay of platelet function, the reader is referred to various references.[33,34] Laboratory markers of platelet activation include activation-dependent conformational changes in integrin $\alpha_{IIb}\beta_3$ (aka GPIIb-IIIa, CD41/CD61), exposure of granule membrane proteins, platelet surface binding of secreted platelet proteins, and development of a procoagulant surface (Table 67.2). The two most widely studied types of activation-dependent monoclonal antibodies are those directed against conformational changes in $\alpha_{IIb}\beta_3$ and those directed against granule membrane proteins.

Changes in Platelet Surface Glycoproteins

The integrin $\alpha_{IIb}\beta_3$ is a receptor for fibrinogen and von Willebrand factor (vWF) that is essential for platelet aggregation.[35] Whereas most monoclonal antibodies directed against $\alpha_{IIb}\beta_3$ bind to resting platelets, the monoclonal antibody PAC1 is directed against the fibrinogen-binding site that is exposed on $\alpha_{IIb}\beta_3$ by platelet activation (Table 67.1).[36] Thus, PAC1 only binds to activated platelets, not to resting platelets. Other $\alpha_{IIb}\beta_3$-specific activation-dependent monoclonal antibodies are directed against either ligand-induced conformational changes in $\alpha_{IIb}\beta_3$ (ligand-induced binding sites [LIBS])[37] or receptor-induced conformational changes in the bound ligand (fibrinogen) (receptor-induced binding sites [RIBS])[38] (Table 67.1). Rather than integrin $\alpha_{IIb}\beta_3$-specific monoclonal antibodies, fluorescein-conjugated fibrinogen can also be used in flow cytometric assays to detect the activated form of platelet surface $\alpha_{IIb}\beta_3$,[39,40] but the concentration of unlabeled plasma fibrinogen and unlabeled fibrinogen released from platelet α-granules must also be considered in these assays. $\alpha_{IIb}\beta_3$ is expressed at high copy number on the resting platelet surface, but as this protein is also distributed throughout the open canalicular system (OCS) and α-granular membranes, a significant increase in surface protein

Table 67.2

Activation-dependent monoclonal antibodies, that is, antibodies that bind to activated but not resting platelets

Activation-dependent Surface Change	Prototypic Antibodies	References
Conformational Changes		
Activation-induced conformational change in integrin $\alpha_{IIb}\beta_3$, resulting in exposure of the fibrinogen-binding site	PAC1	36
LIBS on integrin $\alpha_{IIb}\beta_3$	PM 1.1., LIBS1, LIBS6	37,73,219
RIBS on bound fibrinogen	2G5, 9F9, F26	38,117,220
Activation-induced conformational change in integrin $\alpha_2\beta_1$	IAC-1	221
Exposure of Granule Membrane Proteins		
P-selectin (α-granules)	S12, AC1.2	222,223
GMP-33 (α-granules)	RUU-SP 1.77	224,225
CD63 (lysosomes)	CLB-gran/12	226
LAMP-1 (lysosomes)	H5G11	227
LAMP-2 (lysosomes)	H4B4	228
CD40L	TRAP1	92
Lectin-like oxidized LDL receptor (LOX-1)	JTX68	229
Platelet Surface Binding of Secreted Platelet Proteins		
Thrombospondin	P8, TSP-1	230,231
Multimerin	JS-1	232,233
Development of a Procoagulant Surface		
Factor V/Va binding	V237	60
Factor X/Xa binding	5224	61
Factor VIII binding	1B3	62
PS exposure	Annexin-V or lactadherin	59,64

copy density can also be detected on activated platelets. This is normally reflected as an increase in mean fluorescence intensity (MFI) with most fluorescent antibodies against this receptor. Although the vWF receptor GPIb is also expressed at high copy density on resting platelets, changes also occur after activation. As the protein can be internalized into the OCS and/or cleaved to form soluble glycocalicin, significant downregulation in the copy density of this receptor can occur with a decrease in MFI, although the percentage of positive platelets remains 100%.[41,42]

Granule Membrane Markers

The most widely studied types of activation-dependent monoclonal antibodies directed against granule membrane proteins are P-selectin (CD62P) specific. P-selectin is a component of the α-granule membrane of resting platelets that is only expressed on the platelet surface membrane after α-granule secretion. Therefore, a P-selectin-specific monoclonal antibody only binds to degranulated platelets, not to resting platelets. The activation-dependent increase in platelet surface P-selectin is not reversible over time *in vitro*.[43,44] However, *in vivo* circulating degranulated platelets rapidly lose their surface P-selectin, although they continue to circulate and function.[45,46] Platelet surface P-selectin is therefore not an ideal marker for the detection of circulating degranulated platelets, unless (a) the blood sample is drawn immediately distal to the site of platelet activation, (b) the blood sample is drawn within 5 minutes of the activating stimulus, or (c) there is continuous activation of platelets. The length of time that other activation-dependent markers remain on the platelet surface *in vivo* has not been definitively determined, but it is becoming clear that some of these are also shed from the surface (see the platelet "sheddome" section above). CD63 is a membrane protein of dense granules and lysosomes and, like P-selectin, it is only expressed upon the surface of activated platelets. However, unlike P-selectin, this glycoprotein is not prone to proteolysis and may provide a more stable marker of platelet activation.

Leukocyte–Platelet Aggregates

P-selectin mediates the initial adhesion of activated platelets to monocytes and neutrophils via the PSGL-1 counter-receptor on the leukocyte surface.[47] Monocyte–platelet and

neutrophil–platelet aggregates are readily and easily identified by whole blood flow cytometry.[34,48] These interactions are important in cardiovascular disease and many other clinical disorders in which platelets are activated (see section on platelet activation in clinical disorders below).[49,50,51]

Tracking autologous infused biotinylated platelets in baboons by three-color whole blood flow cytometry directly demonstrated *in vivo* that (a) platelets degranulated by thrombin very rapidly (within 1 minute) form circulating aggregates with monocytes and neutrophils (FIGURE 67.2, upper panel); (b) the percent of monocytes with adherent infused platelets is greater than the percent of neutrophils with adherent infused platelets (FIGURE 67.2, upper panel); and (c) the *in vivo* half-life of detectable circulating monocyte–platelet aggregates (~30 minutes) is longer than both the *in vivo* half-life of neutrophil–platelet aggregates (~5 minutes) and the previously reported[46] rapid loss of surface P-selectin from nonaggregated infused platelets (FIGURE 67.2, upper panel).[48]

These findings suggest that measurement of circulating monocyte–platelet aggregates may be a more sensitive indicator of *in vivo* platelet activation than either circulating neutrophil–platelet aggregates or circulating P-selectin-positive nonaggregated platelets. Indeed it has been demonstrated that after percutaneous coronary intervention (PCI), there is an increased number of circulating monocyte–platelet (and, to a lesser extent, neutrophil–platelet) aggregates, but not P-selectin-positive platelets, in peripheral blood.[48] Similarly in patients presenting to an emergency department with chest pain, those with acute myocardial infarction had more circulating monocyte–platelet aggregates than patients without acute myocardial infarction and normal controls. Furthermore, circulating P-selectin-positive platelets were not increased in patients with chest pain with or without acute myocardial infarction.[48]

Taken together, these studies indicate that circulating monocyte platelet aggregates are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin.

Vasodilator-Stimulated Phosphoprotein

Measurement of VASP phosphorylation by flow cytometry can be performed by using a specific monoclonal antibody for VASP and fixed and permeabilized platelets in whole blood.[52] VASP is an intracellular platelet protein which is not phosphorylated under basal conditions. VASP phosphorylation is regulated by cAMP, and the assay is based upon stimulation/activation of *ex vivo* blood samples with prostaglandin E_1 (PGE_1) in the presence

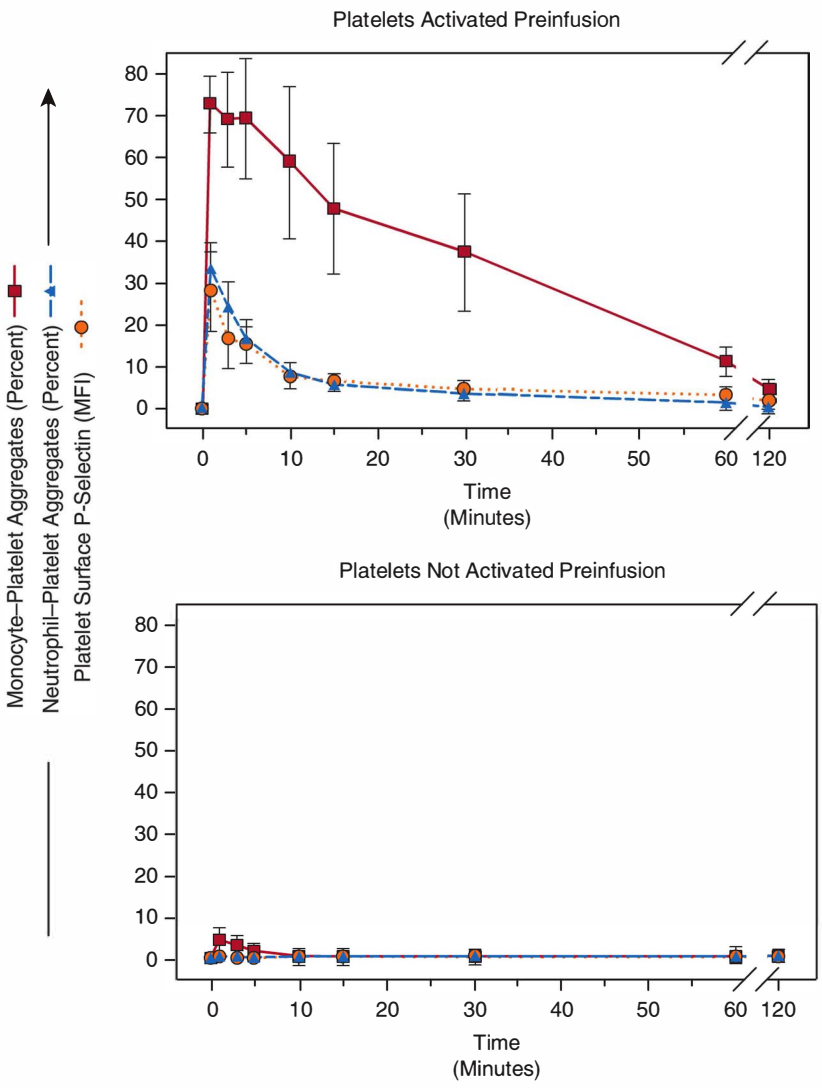

FIGURE 67.2 *In vivo* tracking of platelets. Baboons were infused with autologous, biotinylated platelets that were **(upper panel)** or were not **(lower panel)** thrombin-activated preinfusion. Surface P-selectin on the infused platelets and participation of the infused platelets in circulating monocyte–platelet and neutrophil–platelet aggregates was determined by three-color whole blood flow cytometric analysis of peripheral blood samples drawn at the indicated time points. The "0" time point refers to blood samples taken immediately preinfusion. Platelet surface P-selectin is expressed as mean fluorescence intensity (MFI), as a percentage of the fluorescence of a preinfusion maximally activated (10 U/mL) thrombin control sample. Monocyte–platelet and neutrophil–platelet aggregates are expressed as the percent of all monocytes and neutrophils with adherent infused platelets. Data are mean ± SEM. (Reproduced from Michelson AD, Barnard MR, Krueger LA, et al. Circulating monocyte-platelet aggregates are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. *Circulation* 2001;104:1533–1537, with permission.)

and absence of ADP. PGE_1 and ADP (acting via $P2Y_{12}$) have opposite effects on VASP with either promotion or inhibition of phosphorylation respectively. Therefore the nonphosphorylated VASP correlates with the activity of $P2Y_{12}$. Interference with the activity of this receptor by the active metabolites of thienopyridines (e.g., clopidogrel or prasugrel) or direct antagonists (e.g., ticagrelor or cangrelor) results in persistent VASP phosphorylation induced by PGE_1 even when the platelets are stimulated with ADP. By comparing the level of VASP phosphorylation in whole blood samples stimulated with either PGE_1 or a combination of PGE_1 and ADP, it is possible to derive a platelet reactivity index (%).[53] The assay provides the most direct and specific measurement of the activity of $P2Y_{12}$ and can be used to monitor the efficacy of antiplatelet drugs that target this receptor.[54,55] Accordingly, the VASP phosphorylation assay has been used to monitor the effects of clopidogrel in humans, and an impaired restoration of VASP phosphorylation by clopidogrel has been shown to be associated with subacute stent thrombosis following PCI.[54,55] Further work is required to determine whether this indicates that a high level of receptor blockade is required to prevent stent thrombosis, particularly as the assay has been reported to be insensitive to low levels of $P2Y_{12}$ inhibition.

Phosphatidylserine

The distribution of phospholipids in the plasma membrane of resting platelets is asymmetric with the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) sequestered in the inner leaflet. The sustained increases in cytosolic calcium elevation that accompanies platelet activation by strong agonists result in the activation of scramblase,[56] and PS then provides a surface upon which the tenase and prothrombinase complexes can assemble, resulting in a burst of thrombin generation. It is possible to measure the extent of PS exposure by flow cytometry either *ex vivo* or *in vitro* after activation by using probes that bind to PS. Annexin-V is a high-affinity calcium-dependent probe for detecting PS and has been extensively used in platelet studies.[57] Recently, the calcium-independent probe lactadherin has also been utilized and shown to be more sensitive at detecting low levels of PS exposure.[58,59] Other probes based upon various clotting factors including monoclonal antibodies against factor V or factor VIII or directly labeled proteins have also been used.[60–62]

Platelet-Derived Microparticles

The sustained cytosolic calcium elevation that results in PS exposure also causes membrane blebbing and the release of microparticles, some of which are PS positive.[63] As determined by flow cytometry, *in vitro* activation of platelets by agonists such as C5b-9, collagen/thrombin, CRP, and the calcium ionophore A23187 in the presence of extracellular calcium ions results in the formation and release of platelet-derived microparticles. The microparticles are between 100 nm and <1 μm in size as determined by low forward-angle light scatter and binding of a platelet-specific monoclonal antibody and may be procoagulant as determined by binding of monoclonal antibodies to activated factors V or VIII or direct detection using the high-affinity probes annexin-V or lactadherin.[60,62,64] Procoagulant platelet-derived microparticles play an important role in the assembly of the "tenase" and "prothrombinase" components of the coagulation system *in vivo* as exemplified by the very rare inherited platelet disorder "Scott syndrome" where the patients present with a bleeding diathesis and cannot expose PS on cell surfaces or generate microparticles.[65] There are many methods available for measuring microparticles including ELISA, thrombin generation, and procoagulant assays but flow cytometry is regarded as the method of choice. Many flow cytometric methods for the direct detection of procoagulant platelet-derived microparticles in whole blood have been reported.[66,67,68,69] However, many flow cytometers are limited in their ability to detect particles below approximately 300 to 500 nm. The use of standardization beads (e.g., Megamix, Biocytex) facilitates optimal resolution of labeled microparticles from electronic noise and improves both within- and between-laboratory standardization.[68] Platelets can also release exosomes that are true nanoparticles formed within the α-granules from multivesicular bodies.[70] Platelet and other cellular derived microparticles are elevated in plasma in a wide range of disease including many thrombotic disorders where platelets are activated and thus may be novel disease biomarkers.[71]

Methodologic Issues

Preanalytical Considerations: Blood Drawing, Sample Handling, and Processing

It is imperative that any *ex vivo* artifacts are prevented during the processing and preparation of samples for the measurement of platelet activation markers. Some markers are sensitive to adrenergic stimulation and exercise, so subjects are often rested for at least 10 minutes prior to venipuncture. Platelet activation and aggregate formation can be minimized in the preparation of platelets for whole blood flow cytometry by a combination of the following methods: (a) preparing reagents in advance and avoiding delays in procedure; (b) using a light tourniquet and a needle not narrower than 21 gauge to collect blood; (c) smooth, easy flow from the blood draw; (d) discarding the first few milliliters of blood; (e) using polypropylene (or siliconized glass) tubes or syringes; (f) immediate mixing with the anticoagulant; (g) no washing, centrifugation, gel filtration, vortexing, or stirring steps; (h) reducing the platelet count by dilution of the samples; (i) if thrombin is the agonist, including the synthetic tetrapeptide Gly–Pro–Arg–Pro (GPRP) in the assay (see below); (j) mixing gently after addition of agonist, then incubating undisturbed; (k) fixation (see use of fixatives section below). RGD-containing peptides have also been used to prevent platelet aggregates,[72] but these peptides may interfere with the binding of detecting antibodies such as PAC1, and they can result in exposure of LIBS.[37,73]

Each sample should be monitored for evidence of platelet aggregation ("smearing" of the platelets into the upper right quadrant of the log side [orthogonal] light scatter vs. log forward light scatter histogram). After discarding the first few milliliters, blood should be collected into buffered 3.2% (105 to 109 mmol/L) trisodium citrate Vacutainer tubes (Becton Dickinson, Rutherford, NJ), which does not result in significant platelet activation.[74] However, each laboratory should determine whether their method of collection, including the drawing of samples through PCI and other catheters, results in artifactual *in vitro* platelet activation, as determined by the binding of activation-dependent monoclonal antibodies. To measure platelet activation markers, blood should be kept at room temperature and rapidly processed as *ex vivo* changes in various markers can occur rather quickly (e.g., 10 minutes).

Although the weak calcium chelator sodium citrate is the most commonly used anticoagulant, other anticoagulants have

been successfully used, for example, corn trypsin inhibitor (an inhibitor of activated factor XII).[75] The strong calcium chelator ethylenediaminetetraacetic acid (EDTA) should be avoided, because it can dissociate $\alpha_{IIb}\beta_3$. Nonchelating anticoagulants like PPACK (a direct thrombin inhibitor) or hirudin may be preferable for the monitoring of GPIIb-IIIa antagonist therapy.[76] Citrate has also recently been shown to interfere with *in vitro* but not *ex vivo* measurement of $P2Y_{12}$ inhibition by low concentrations of cangrelor and the prasugrel metabolite.[77] Another alternative anticoagulant available is EDTA-CTAD.[78] Platelet count, mean platelet volume (MPV), number of platelet clumps, mean platelet component, numbers of P-selectin-positive platelets, and platelet–leukocyte aggregates have all been shown to be stable for 360 minutes in blood samples kept at 4°C.

Flow Cytometric Methods

Use of Agonists in Whole Blood Assays

In the absence of an exogenous platelet agonist, whole blood flow cytometry can determine the activation state of circulating platelets, as judged by the binding of an activation-dependent monoclonal antibody. In addition to this assessment of platelet function *in vivo*, inclusion of an exogenous agonist in the assay enables analysis of the reactivity of circulating platelets *in vitro*. In the latter application, whole blood flow cytometry is a physiologic assay of platelet function in that an agonist results in a specific functional response by the platelets: a change in the surface expression of a physiologic receptor (or other antigen or bound ligand), as determined by a change in the binding of a monoclonal antibody. In addition, as discussed below, whole blood flow cytometric enumeration of monocyte–platelet aggregates and procoagulant platelet-derived microparticles are also sensitive markers of *in vivo* platelet activation. A large number of useful reagents now exist for the *in vitro* activation of platelets. These include ADP, thrombin, TRAPs, collagen and CRP, various snake venoms (e.g., convulxin), U46619, arachidonic acid, platelet activating factor (PAF), and the calcium ionophore A23187. It is possible to construct dose–response curves for each agonist to study their influence on different activation markers. One important consideration is to ensure that the agonists do not interfere with the eventual analysis of the platelets in diluted whole blood samples. Platelet activation by thrombin, a physiologically important platelet activator,[79–81] can be directly measured in whole blood in the presence of the synthetic peptide GPRP.[42,82,83] In the absence of GPRP, addition of thrombin to whole blood results in a fibrin clot. Furthermore, thrombin is a potent inducer of platelet-to-platelet aggregation, precluding analysis by flow cytometry of activation-dependent changes in individual platelets. However, GPRP inhibits both fibrin polymerization and, to a lesser extent, platelet-to-platelet aggregation, without affecting thrombin-induced platelet activation.[42,82,83] An alternative to the use of thrombin and GPRP in a whole blood flow cytometric assay is the use of TRAPs. There are now two TRAP agonists for the protease-activated thrombin receptors 1 and 4 (PAR1 and PAR4).[84] TRAP peptides directly activate platelets in whole blood without producing a fibrin clot.[85] However, the use of each TRAP individually may not mimic all aspects of thrombin-induced platelet activation, because PAR1 and PAR4 are not the only platelet receptors for thrombin.[86] CRP is a very useful reagent for specifically stimulating the GPVI activation pathway and provides a soluble, easy-to-use, reliable, and standardized alternative to collagen which can often be insoluble and prone to considerable variations between different preparations.

Detection of the Activation-Dependent Decrease in the Platelet Surface Expression of the GPIb-IX-V Complex

Whole blood flow cytometric assays frequently employ a GPIb- or GPIX-specific monoclonal antibody to identify platelets. Because GPIb and GPIX are not present on any circulating blood cell except platelets,[87,88] the activation-induced decrease in the expression of GPIb/IX on the platelet surface[41,42,89] generally does not result in fluorescence below the threshold used to distinguish platelets from other cells and debris.[31,83] Thus, no platelet subpopulations are excluded. A method of avoiding activation-induced decrease in binding of a GPIb-specific monoclonal antibody is to add a direct conjugate of the GPIb-specific antibody before addition of agonist ([90] and see below).

To specifically analyze the activation-induced decrease in the expression of the GPIb/IX complex on the platelet surface in whole blood, an α_{IIb}- or β_3-specific monoclonal antibody can be employed as the platelet-identifying reagent.[74]

There is an important methodologic point that needs to be emphasized with regard to the flow cytometric detection of the activation-induced decrease in the platelet surface expression of GPIb/IX.[90] If a fluorescein isothiocyanate (FITC)-conjugated GPIb/IX-specific test monoclonal antibody is added prior to the platelet agonist (as in the typical schema shown in FIGURE 67.1), the activation-induced redistribution of GPIb/IX to the surface-connected canalicular system[91] will not result in a significant decrease in platelet fluorescence because a flow cytometer can detect FITC fluorescence regardless whether the conjugated antibody is on the surface or within the interior of the platelet. Therefore, in flow cytometric assays examining platelet surface GPIb/IX modulation, GPIb/IX-specific antibodies that are directly conjugated (e.g., with FITC) must be added to the assay *after* the addition of the agonist.

Use of Fixatives

Sample fixation is very advantageous in a clinical setting where there may not be immediate access to a flow cytometer. Fixation prevents subsequent artifactual *in vitro* platelet activation. For most antibodies, the "antibody labeling before fixation" method described above, and shown in FIGURE 67.1, results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of fixation.[42] A "fixation before antibody labeling" method also results in no significant differences in fluorescence intensity between samples analyzed immediately and samples analyzed within 24 hours of antibody labeling.[74] However, fixation is an important variable to be controlled for, especially in a "fixation before antibody labeling" method, because the binding of activation-dependent monoclonal antibodies to fixed platelets is often decreased compared to unfixed platelets.[32] Furthermore, the binding of some antibodies further decreases after fixation in a time-dependent manner. Therefore, the optimal fixation method for each new monoclonal antibody must therefore be defined by each laboratory.

A compelling argument in favor of immediate sample fixation is that activation-dependent changes are often time dependent, at least *in vitro*. For example, the platelet surface expression of the GPIb/IX/V complex decreases within 30 seconds of platelet activation, reaching a nadir at approximately 5 minutes, but over the next approximately 45 minutes, surface expression returns to normal.[43,85] The activation-dependent increase in the platelet surface expression of integrin $\alpha_{IIb}\beta_3$ and CD40 ligand (CD40L, CD154) is also reversible with time.[44,92] In contrast, although circulating degranulated platelets rapidly lose surface P-selectin *in vivo*,[46] the activation-dependent increase in platelet surface P-selectin is not reversible over time *in vitro*.[43,44]

A new closed system fixative (Platelet Solutions) is now available that stabilizes P-selectin measurements in whole blood samples for up to 9 days.[93] This facilitates standardized flow cytometry testing of remotely collected samples sent to a central testing facility.

Choice of Antibodies

Because exposure of different antigens reflects different aspects of platelet activation, it may be preferable to use a panel of monoclonal antibodies. Monoclonal antibodies are preferable to polyclonal antibodies in whole blood flow cytometry, because they (a) more reliably saturate all specific epitopes and (b) produce less nonspecific binding. Platelet-specific monoclonal antibodies are available from a wide variety of commercial sources and can often be purchased conjugated to fluorescein, biotin, phycoerythrin, PerCP, APC, or a tandem conjugate (e.g., phycoerythrin-Cy5 or RED-670). Alternatively, unlabeled antibodies can be FITC conjugated by the method of Rinderknecht[94] or more easily and rapidly using commercially available kits (Boehringer Mannheim, Indianapolis, IN; Molecular Probes, Eugene; and Sigma-Aldrich, St. Louis, MO). Antibodies can be biotinylated as described by Shattil et al.[31] or by following the manufacturer's directions. The use of antibodies that are directly conjugated with fluorescein, phycoerythrin, PerCP, APC, or tandem conjugates eliminates the requirement for the addition of secondary antibodies, thereby avoiding time-consuming additional incubations and washing procedures which, in unfixed samples, may result in artifactual *in vitro* platelet activation. Furthermore, the use of secondary antibodies is likely to result in increased background fluorescence and decreased assay sensitivity. Finally, the use of directly conjugated antibodies allows multiple color analysis with, for example, a number of differently conjugated murine antibodies.

F_{ab} fragments of monoclonal antibodies can be used to avoid F_c-mediated binding and the F_c-induced platelet activation that has been reported with some monoclonal antibodies.[95] However, the use of F_{ab} fragments is usually unnecessary, provided: (a) the absence of antibody-induced binding of other activation-dependent monoclonal antibodies is demonstrated in control samples, or the problem is avoided by fixation before test antibody binding; (b) the F_c-mediated and nonspecific binding obtained from parallel samples with isotypic species-specific immunoglobulin is subtracted from the binding of the test antibody, or (c) the FcγRIIa receptor-specific monoclonal antibody IV.3, immunoglobulins, or F_c fragments are used as blocking reagents.

Saturating concentrations of each antibody must be specifically determined by each laboratory. Concentrations are typically between 0.25 and 20 μg/mL. In addition, when more than one monoclonal antibody is used in the same assay (as is standard in whole blood flow cytometry), it is necessary to determine that the antibodies do not compete for binding to the platelet surface.

Platelets can be detected in whole blood by light scatter only. However, some of the particles falling within the light scatter gate for platelets may not bind any platelet-specific monoclonal antibody. Therefore, it is recommended that a minimum two-color/two-antibody technique be used for whole blood flow cytometry: one monoclonal antibody to identify a particle as a platelet; another monoclonal antibody to quantify the expression of the glycoprotein of interest.

Reporting of Results

Antibody binding can be expressed as MFI or as the percent of platelets staining positive for a particular antibody (based on a positive analysis region placed just to the right of the negative isotype control fluorescence histogram). Depending on the experimental conditions and the nature of the antigen being measured, either MFI or percent positive platelets may be more relevant. Unlike the MFI method, the "percent positive platelets" method is independent of variations in signal amplification (e.g., as a result of changes in photomultiplier tube [PMT] voltage or gain), because the negative control signal increases in proportion with the test sample. The "percent positive platelets" method may detect subpopulations of platelets arising from a local *in vivo* insult. However, it is important to realize that "antibody-positive" platelets may have very little antigen expressed on their surface. For example, in a given clinical setting, the data may be reported as 20% circulating activated platelets, based on P-selectin positivity. However, if each P-selectin-positive platelet expresses only 10% of maximal platelet surface P-selectin, then the overall average increase in platelet surface P-selectin is only 2%. If the goal is to determine the total amount of platelet surface antigen, MFI is therefore the preferred method of data presentation. For activation-dependent antibodies, inclusion of a control sample maximally activated by thrombin, TRAP, or phorbol myristate acetate assists in the quantification of the amount of surface antigen per platelet. The activation-dependent decrease in platelet surface GPIb/IX/V[42,85] should be quantified by MFI rather than by the "percent positive platelets" method, because the decrease in platelet surface GPIb/IX/V on each platelet is usually insufficient to result in a "negative" platelet.

Although standard flow cytometry does not produce a measure of the absolute number of binding sites, Shattil et al.[31] and Johnston et al.[96] used monoclonal antibodies double labeled with ^{125}I and biotin to demonstrate a direct linear relationship between the number of antibody binding sites per platelet as determined by ^{125}I-labeled and (after incubation with phycoerythrin–streptavidin) fluorescent-labeled antibody. Once this relationship is known for a given monoclonal antibody, it is possible to use subsequent batches of the biotinylated or FITC-conjugated antibody for binding site quantitation, provided that the molar ratio of fluorescein to antibody is known.

Commercial kits (e.g., Quantum, Sigma-Aldrich, and Biocytex) containing a set of calibrated fluorescent standards and software can be used to determine molecules of equivalent soluble fluorochrome (MESF). Use of these standards allows (a) quantitation of the fluorescence intensity of samples in terms of MESF, (b) determination of the fluorescence threshold of the instrument, (c) determination of the linearity and stability of the instrument, and, most importantly, (d) data comparison

over time and between different instruments and laboratories.[97] Flow cytometric methods are now available for quantitation of the number of antibodies bound per cell (e.g., Quantum Simply Cellular Microbeads Kit, Sigma-Aldrich; Platelet Calibrator & Platelet Gp Screen, BioCytex, Marseille, France). The lower limit of detection of antibody binding by flow cytometry is approximately 500 antibody molecules per platelet.

PLATELET ACTIVATION IN CLINICAL DISORDERS

Acute Coronary Syndromes

Platelets play an important role in the pathogenesis of coronary artery syndromes including unstable angina and acute myocardial infarction.[98,99] Angiography[100] and angioscopy[101] during acute coronary ischemic syndromes frequently reveal platelet-rich intracoronary arterial thrombi.[102] Whole blood flow cytometric studies have demonstrated circulating activated platelets in patients with stable angina, unstable angina, and acute myocardial infarction.[50,103–106] In addition, as determined by activation-dependent monoclonal antibodies, PCI results in platelet activation in coronary sinus blood.[107,108] Flow cytometrically detected exposure of LIBS is strongly associated with the development and progression of heart transplant vasculopathy.[109]

Circulating leukocyte–platelet aggregates are increased in stable coronary artery disease,[49,50] unstable angina,[103] acute myocardial infarction,[110,111] and cardiopulmonary bypass.[112] Circulating leukocyte–platelet aggregates also increase after PCI and to a greater extent in patients experiencing late clinical events.[113] As discussed above, circulating monocyte–platelet aggregates (but not neutrophil–platelet aggregates) are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin in the clinical settings of stable coronary artery disease,[50] PCI,[48] and acute myocardial infarction.[48] Furthermore, circulating monocyte–platelet aggregates are an early marker of acute myocardial infarction.[114]

Platelet-derived microparticles are also increased in acute coronary syndromes[115] and after cardiopulmonary bypass.[116,117]

Elevated plasma levels of sCD40L have been demonstrated in unstable angina,[118] PCI,[118] and cardiopulmonary bypass.[119] GPIIb-IIIa antagonists inhibit the release of sCD40L from platelets both *in vitro*[120,121] and *in vivo*.[122] Measurement of sCD40L in the first 12 hours after the onset of ischemic symptoms in patients with unstable angina identifies a subgroup of patients that has a much greater clinical benefit from abciximab treatment.[123] High plasma concentrations of sCD40L may be associated with increased cardiovascular risk in apparently healthy women.[124]

Elevated plasma levels of sGPV have also been demonstrated in patients with coronary artery disease.[23]

In large clinical trials, antiplatelet agents have been shown to reduce the incidence of cardiovascular ischemic events, supporting the concept of an association between platelet activation and cardiovascular ischemia.[125] Furthermore, some investigators have found biochemical markers of platelet activation to be elevated during episodes of acute cardiac ischemia,[2,126] which may identify patients who could benefit from additional antiplatelet therapy.[126] Flow cytometric analysis of platelet activation-dependent markers can be used to determine optimal antiplatelet therapy in clinical settings, for example, in acute coronary syndromes[127,128] and after coronary stenting.[129,130] It is well known that thrombotic events still occur in coronary artery disease patients receiving aspirin therapy. Although there has been much interest in the phenomenon of aspirin "resistance," recent flow cytometric studies in 700 aspirin-treated patients using arachidonic acid stimulation of platelet activation markers (e.g., P-selectin and platelet–leukocyte aggregates) suggest that aspirin underdosing and noncompliance occurs in only a small minority of patients, and underlying platelet reactivity via a COX-independent pathway induced by ADP stimulation is responsible for residual arachidonic acid activation.[6] However, arachidonic acid-induced COX-1-dependent platelet activation measured by flow cytometry was not associated with poor clinical outcomes up to 2 years in contrast to a COX-1-independent assay.[131]

Flow cytometric analysis of platelet activation markers before PCI can predict an increased risk of acute and subacute ischemic events after PCI.[132–135] Clopidogrel is a widely used antithrombotic prodrug that inhibits the platelet ADP receptor $P2Y_{12}$. It is now clear that about 30% of whites cannot efficiently metabolize the drug and are at risk of increased thrombosis after PCI. There are a number of assays available for monitoring clopidogrel efficacy including flow cytometric measurement of VASP and measurement of various activation markers (e.g., P-selectin, platelet–monocyte complexes, and activated $\alpha_{IIb}\beta_3$) after *ex vivo* stimulation with ADP.[53] VASP offers the only relatively direct measurement of $P2Y_{12}$ blockade and has proven to be useful for identifying poor clopidogrel responders.[136] Despite evidence that decreased metabolism can result in decreased clopidogrel responsiveness, various flow cytometric studies suggest that preexistent variability in platelet ADP responsiveness can also partially account for the variability in clopidogrel response.[137] Therefore, posttreatment platelet reactivity may be a better predictor of ischemic events than measurement of clopidogrel nonresponsiveness. Flow cytometry has also been used to determine whether discontinuation of clopidogrel results in rebound platelet hyperreactivity and increased thrombotic risk.[138] A substudy of the TRITON-TIMI 38 trial also demonstrated that prasugrel results in greater inhibition of ADP-mediated platelet function than clopidogrel when measured by VASP.[139]

It is intriguing that platelet hyperreactivity also generalizes to multiple forms of stimulation including submaximal doses of epinephrine as measured by activation (surface P-selectin and PAC1 binding), aggregation (to multiple agonists ADP, arachidonic acid, collagen, etc.), and shear stress assays (e.g., PFA-100 and CPA).[140] Recent evidence suggest that platelet activation responses vary widely between individuals but are consistent within individuals with strong heritability.[141] Some, but not all, studies suggest that the Pl^{A2} polymorphism of GPIIIa is associated with ischemic coronary syndromes.[142] Indeed, flow cytometry has been used to demonstrate that (a) Pl^{A2}-positive platelets can display a lower threshold for activation and (b) platelets heterozygous for Pl^{A} alleles may show increased sensitivity to antiplatelet drugs.[143] The genetic basis of interindividual variability in platelet responsiveness has been recently explored in detail using flow cytometric measurement of consistent *ex vivo* responses to CRP and ADP in 500 healthy Northern European subjects. This resulted in the identification of 17 novel associations with platelet function, which accounted for about 46% of the variation in response.[144] Further studies in this cohort have identified novel proteins that regulate the platelet response including a novel variant on chromosome 7 that associates with MPV, platelet count, and function.[145,146]

Cerebrovascular Ischemia

Increased numbers of circulating P-selectin-positive, CD63-positive, activated $\alpha_{IIb}\beta_3$-positive platelets, monocyte–platelet complexes and platelet-derived microparticles have been reported in acute cerebrovascular ischemia.[147–155] Evidence of platelet activation can still be detected 3 months after the acute event, suggesting the possibility of an underlying prothrombotic state.[149,151,153] Furthermore, increased expression of surface P-selectin on platelets is a risk factor for silent cerebral infarction in patients with atrial fibrillation.[156] Flow cytometric measurements can also be used to monitor platelet activation markers in stroke patients receiving antiplatelet therapy.[157–160]

Platelet-derived microparticles are increased after transient ischemic attacks.[117,161] Increased platelet-derived microparticles and procoagulant activity occur in symptomatic patients with prosthetic heart valves and provide a potential pathophysiologic explanation of cerebrovascular events in this patient group.[162]

Peripheral Vascular Disease

Circulating activated platelets and platelet hyperreactivity (as determined by P-selectin expression, platelet aggregates, and platelet-derived microparticle formation) are increased in patients with peripheral arterial occlusive disease compared with healthy volunteers.[163,164] In addition, plasma levels of sCD40L[165] and sGPV[23] are elevated in peripheral arterial occlusive disease. Interestingly, platelet reactivity measured by ADP stimulation of P-selectin expression seems to be greater in patients who have more extensive distribution of atherosclerotic vascular disease than in those who have coronary artery disease and diabetes mellitus.[166] Soluble P-selectin and increased levels of platelet microparticles but not sCD40L are also associated with disease severity.[167,168] As the level of platelet activation seems to correlate with the severity of peripheral arterial disease, this suggests that new strategies for platelet inhibition are required in these patients.[169]

With regard to peripheral venous disease, Peyton et al.[51] demonstrated an increased number of circulating monocyte–platelet aggregates in patients with chronic venous stasis ulceration as compared to control individuals without venous disease. These changes were present not only in blood drawn from the lower extremity veins of affected individuals, but also in blood drawn from arm veins, suggesting that the changes are systemic rather than localized to the lower extremities.[51] Powell et al.[170] further characterized these findings as being related to the presence of chronic venous disease rather than the presence of venous ulceration since increased numbers of monocyte–platelet aggregates were noted in patients with all classes of venous disease, not just in patients with deep-venous valvular insufficiency. Furthermore, increased levels of monocyte–platelet aggregates were even observed in patients with only superficial venous stasis disease manifested by the presence of varicose veins. Even more intriguing is the fact that the number of monocyte–platelet aggregates remains elevated 6 weeks after total correction of the venous insufficiency by stripping of the abnormal veins, resulting in normal venous physiology as documented by postoperative duplex scanning.[171] This suggests an underlying predisposition to the development of chronic venous disease in these patients, perhaps mediated by monocyte–platelet interactions.

OTHER CLINICAL DISORDERS ASSOCIATED WITH PLATELET HYPERREACTIVITY AND/OR CIRCULATING ACTIVATED PLATELETS

Other conditions in which whole blood flow cytometric measurement of platelet hyperreactivity, circulating activated platelets, and/or circulating leukocyte–platelet aggregates may prove useful include diabetes mellitus,[172,173] the metabolic syndrome,[174] preeclampsia,[175,176] cystic fibrosis,[177] migraine,[178] placental insufficiency,[179] nephrotic syndrome,[180] inflammatory bowel disease,[181] inflammatory skin diseases,[182,183] hemodialysis,[184] glomerulonephritis,[185] sickle cell disease,[186] thalassemia,[187] systemic inflammatory response syndrome,[188] septic multiple organ dysfunction syndrome,[189,190] antiphospholipid syndrome,[191] systemic lupus erythematosus,[191] rheumatoid arthritis,[191] myeloproliferative disorders,[192,193] and Alzheimer's disease.[194] High levels of circulating monocyte–platelet aggregates can predict rejection episodes after orthotopic liver transplantation.[195] Uremic patients with thrombotic events have higher numbers of circulating platelet-derived microparticles than those without thrombotic events.[196]

QUALITY CONTROL OF STORED PLATELET CONCENTRATES IN THE BLOOD BANK

Many studies have provided evidence of significant changes in platelet activation markers associated with the "platelet storage lesion" in stored platelet concentrates.[197] As shown by flow cytometric measurements of P-selectin, CD63, PS, microparticle generation, and $\alpha_{IIb}\beta_3$, platelets stored in the blood bank prior to transfusion display time-dependent platelet activation and/or apoptosis.[198–203] These changes also seem to correlate with modifications in platelet morphology (decrease in swirling), leakage of lactate dehydrogenase, and release of soluble markers such as β-TG and P-selectin.[197,198] The expression of P-selectin has been proposed as a potentially useful quality control measurement, based on correlations between the platelet surface expression of P-selectin in stored blood bank platelets and (a) posttransfusion platelet counts,[204] (b) platelet survival determined by In^{111},[199,205] and (c) clearance of P-selectin-positive platelets from the recipient.[199] It has been recently proposed that a novel objective light scattering measurement of platelet aggregate and microparticle generation in response to temperature change could provide a simple way to assess platelet quality without the need for cytometry.[206,207,208] Preliminary evidence suggests that this measurement does indeed correlate with the *in vivo* clinical efficacy of platelet concentrates and could be potentially used as a simple quality assurance test by blood banks.[209] It has also been demonstrated that apheresis donors with the highest platelet reactivity as measured by flow cytometry produce concentrates with increased levels of platelet activation.[210] A planned clinical trial will reveal whether these platelets are more efficacious *in vivo*.

Platelet Hyporeactivity

Although there are currently few published studies in this area, whole blood flow cytometry could be useful in the clinical assessment of platelet hyporeactivity. For example, compared to adults, the platelets of very low-birth-weight preterm neonates are markedly hyporeactive to thrombin, ADP/epinephrine, and thromboxane A_2, as determined by flow cytometric

detection of (a) the exposure of the fibrinogen-binding site on integrin $\alpha_{IIb}\beta_3$, (b) fibrinogen binding, (c) the increase in platelet surface P-selectin, and (d) the decrease in platelet surface GPIb.[211–213] This hyporeactivity may contribute to the propensity of very low-birth-weight neonates to intraventricular hemorrhage.[214,215] Prediction of hemorrhage in severe thrombocytopenia is usually based on the platelet count. However, low levels of platelet P-selectin expression have been reported to be a prognostic marker for hemorrhage in acute myeloid leukemia.[216] This flow cytometric methodology has obvious advantages over other platelet function tests that require a near normal platelet counts and cannot be applied to patients with severe thrombocytopenia.

SUMMARY AND FUTURE PERSPECTIVES

The measurement of platelet activation has become established as an invaluable and reliable research tool for assessing *in vitro*, *in vivo*, and *ex vivo* platelet function in many clinical and research laboratories worldwide.[1] Flow cytometry has proven to be an excellent and sensitive method to measure platelet activation and, providing that careful quality control procedures are followed, it can be used to determine either the *in vivo* level and/or the *ex vivo* function of circulating platelets in a wide range of various clinical disorders. This, coupled with the discovery that platelets can release a much larger number of soluble proteins (the "platelet sheddome") than previously thought, may eventually translate into a large library of simple laboratory tests that may help to guide therapy for patients at high risk of bleeding or thrombosis.[17] Recent examples include the measurement of VASP and the remote collection of stabilized blood samples (for P-selectin measurement in a central laboratory) to monitor anti-$P2Y_{12}$ receptor therapy and the development of a new test of platelet quality to potentially ensure the *in vivo* efficacy of platelet transfusions.[93,136,209] With the complete definition of platelet genome and transcriptome, many more activation markers are likely to be discovered. Flow cytometry of classical activation markers after platelet stimulation has proven to be an important platelet function test in various genome-wide association studies and helped to identify significant genes and proteins associated with either platelet hyper- or hypofunction.[144,145] Understanding the genetic contribution to platelet function may elucidate the risks and possible benefits associated with specific genotypes and improve antiplatelet therapy in high-risk individuals. With advances in automation and flow cytometry technology, it is now possible to devise multicolor flow cytometry tests that measure a combination of platelet activation markers and to fully automate sample preparation and processing using 96- to 384-well plates. Such assays may prove useful for many types of large-scale studies to screen for and identify novel antiplatelet drugs and to study platelet function and *ex vivo* platelet activation in large numbers of samples simultaneously stimulated with multiple agonists. Some assays are now also fully automatable within full-blood counter instruments that also measure fluorescence, light scatter, and impedance parameters simultaneously.[217,218]

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Full Reference Lists can be found online

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CHAPTER 68

Overview of Primary Disorders of Blood Vessels

WILLIAM C. AIRD

The cardiovascular system consists of a closed system or arteries, arterioles, capillaries, venules, and veins, in addition to an open system of lymphatic vessels, which connect to the blood circulation by way of the veins. Each blood vessel type, in turn, is comprised of cellular and noncellular components, the nature of which differs not only between blood vessel types (e.g., arteries and veins) but also between different organs (e.g., capillaries in the heart and brain). Blood vessels are highly dynamic entities; they participate in many different functions, including the control of vasomotor tone and maintenance of blood pressure, the selective permeability of cells and solutes between blood and underlying tissue, innate and acquired immunity, regeneration and repair, and the regulation of hemostasis. Thus, vascular pathophysiology may involve not only distinct vascular beds but also selected functions. The vascular wall is integrally linked to hemostasis and thrombosis, and the theme of vascular disease is weaved throughout all sections of this volume. In the current section, we have chosen to cover a few additional examples of such disorders.

Vascular disorders may be classified in a number of different ways. For example, they may be categorized according to whether they are congenital or acquired. Among the more prominent congenital disorders are connective tissue disorders, arteriovenous malformations, hereditary hemorrhagic telangiectasia, and cerebral cavernous malformations. As discussed in Chapters 69 to 71, recent studies have shed fascinating light on the molecular mechanisms underlying these defects. Vascular disease may be classified according to whether it involves primarily veins, arteries, small blood vessels, or lymphatics. Atherosclerosis is so common in the Western world that is often viewed synonymously with arterial disease. However, there are many other forms of arterial pathology, including arteriosclerosis, aneurysm formation (Chapter 72), and vasculitis (which is covered under the section on complex diseases). The most recognizable disorders associated with veins are superficial and deep thrombosis and varicose veins. Venous thromboembolism is discussed in detail in other chapters in the current volume. Another approach to vascular disease is to consider it from the perspective of the organ or vascular bed affected. For example, pulmonary vascular disorders, including pulmonary hypertension and diseases associated with the bronchial circulation, are considered quite separately from disorders of the kidney vasculature (e.g., renal artery stenosis and glomerulonephritis). Chapter 73 showcases how a specialist from a single discipline (neurology) approaches distinct vascular disease phenotypes in their organ of interest (subarachnoid hemorrhage and intracranial bleeding). Chapter 74 provides another example of how one approaches vascular disease from the perspective of a single organ, namely, the eye. In reading this chapter, we gain an appreciation for the complexity of the eye vasculature and the breadth of disorders that affect it. Indeed, in perusing the chapters in this section, it becomes clear that subspecialization in medicine, though a necessary development over the past 50 years, has limited our capacity to fully grasp the vasculature—warts and all—as an organizing principle in health and disease.

CHAPTER

69 Disorders of Connective Tissue and Extracellular Matrix

FRANSISKA MALFAIT AND ANNE DE PAEPE

THE CONNECTIVE TISSUE AND THE EXTRACELLULAR MATRIX

The connective tissue is the most abundant tissue throughout the body and serves both as a structural support and as a metabolic reservoir for tissue interactions. During recent years, the historical view on connective tissue as solely a supportive structure has been abandoned. It has become evident that the connective tissue actively participates in several important physiologic processes, including cell expression patterns and development, proliferation, differentiation, cell migration, and apoptosis, mainly acting through various cell signaling pathways. Three main connective tissues can be recognized: the loose or soft connective tissues (that embed organs and organ parts), the hard connective tissues (cartilage and bone), and blood. They consist of an extracellular matrix (ECM) and metabolically active cells that synthesize and maintain the ECM. Embryologically, connective tissue develops mainly from the mesoderm, the middle layer of the three-layered embryo. Mesodermal cells develop a loosely organized embryonic connective tissue, called mesenchyme, and differentiate further in specific cell types, including fibroblasts/fibrocytes, chondroblasts/chondrocytes, and osteoblasts/osteocytes. These cells synthesize and maintain the ECM in, respectively, the loose connective tissues, cartilage, and bone, according to the tissue's specific needs. The ECM consists of four main components: elastic fibers and microfibrils, collagen fibers, proteoglycans, and glycoproteins.

Elastic fibers provide elasticity and resilience to all deformable tissues. They are abundantly present in the aorta, arterial vessels, lung, and, to a lesser degree, skin. They are composed of two ultrastructurally distinguishable components: an amorphous component, "elastin", which contributes to the elasticity of the fiber, and a surrounding sheet of *microfibrils*. The microfibrillar component of the elastic fibers consists of fibrillin-rich microfibrils. Fibrillins are highly homologous glycoproteins, of which five isoforms have been described with the same overall arrangement of repetitive domains. Fibrillin-rich microfibrils interact with many other nonfibrillar components, which can be integral parts of the microfibrils or may associate with them. These microfibrils have a structural role and are functionally important in different processes such as microfibril assembly, elastogenesis, interactions with other ECM proteins, anchoring microfibrils to basement membranes and cell surfaces, and growth factor sequestration. For many of these molecules, however, the exact spatiotemporal relation and function still need to be addressed. Examples of nonfibrillar components include microfibril-associated glycoproteins, fibulins (a seven-member family of extracellular glycoproteins), and the latent transforming growth factor-beta–binding proteins (LTBPs), which are important players in the bioavailability of transforming growth factor-beta (TGF-β) in the ECM.

Collagens are the most abundant proteins in the body. They are trimeric proteins that are characterized by the presence of triple-helical domains. To date, 43 collagen genes have been described, the products of which combine to form at least 28 different collagen molecules. Most collagens form supramolecular assemblies, such as fibrils and networks, and the collagen superfamily has been divided in several subfamilies on the basis of their genomic structures, their assembly, and other structural features. The fibril-forming or fibrillar collagens, which include the collagen types I, II, III, V and XI, represent the most widespread and abundant class of collagens, and defects in these proteins are found in different heritable disorders of connective tissue (HCTD), such as osteogenesis imperfecta (OI), (type I collagen), Stickler syndrome (type II and XI collagen), and the Ehlers-Danlos syndromes (EDS) (type I, III, and V collagen). Fibrillar collagens are observed in tissues as long, highly ordered fibrils with a characteristic banding pattern. Type I collagen, a heterotrimer of two α_1-chains and one α_2-chain, is the major collagen type in the body and has a widespread tissue distribution. Type II and type XI collagens are predominantly found in cartilage. Type III collagen, a homotrimer consisting of three identical α_1-chains, is an essential component of stretchable tissues such as the blood vessel walls, the gastrointestinal tract, the uterus, and the skin. Type V collagen is a minor fibrillar collagen that coassembles with type I collagen, and acts as a regulator of collagen fibril diameter through the retention of a noncollagenous amino-terminal domain of the proα_1(V) collagen chain.[1] Each fibrillar collagen has a central uninterrupted triple-helical domain with short nonhelical domains at the carboxy- and amino-terminal ends. The presence of glycine, the smallest amino acid, in every third position of each chain is a prerequisite for the formation of a stable collagen helix. The biosynthesis of fibrillar collagens in the fibroblast is a complex process and starts with the synthesis of soluble precursor molecules, procollagens. These contain globular amino- and carboxy-terminal propeptide extensions, called the *N*- and the *C*-propeptide. Intracellular association of three proα chains occurs through interaction and disulphide bonding at the *C*-propeptide. In this way, correct alignment of the growing polypeptide chain is obtained as required for formation and propagation of the triple helix from the *C*- to the *N*-terminal end of the molecule. During helix propagation, the proα chains undergo extensive enzymatic modifications (e.g., hydroxylation of prolyl and lysyl residues), which cease when the helix is formed. Mature triple-helical procollagen molecules are secreted into the extracellular

environment where they are converted to collagen by enzymatic removal of the *N*- and the *C*-propeptides. Individual collagen molecules spontaneously assemble in a nonenzymatic process to form fibrils and fibers, which are stabilized by covalent cross-linking.

Proteoglycans consist of a core protein to which short oligosaccharides or longer chains of glycosaminoglycans (GAGs) are covalently attached. There is a large variety of proteoglycans based on the types and lengths of GAGs as well as the sequence and length of the protein core. Chondroitin sulfate, keratan sulfate, heparan sulfate, and dermatan sulfate are four different forms of GAGs that consist of repeating disaccharide units. The sulfates that are present on the GAGs create a highly negatively charged environment that is hydrophilic. In addition to water, proteoglycans also bind cationic proteins, such as growth factors, resulting in a mechanism by which tissues can store growth factors in the ECM.

A large group of noncollagenous matrix *glycoproteins* make up a substantial part of the ECM. Important members of this group include thrombospondins (TSP), fibronectin, tenascins, laminin, and osteopontin. As "ground substance" of the ECM, they display a variety of functions in tissue morphogenesis and remodeling, including cell adhesion, cell cycle regulation, cell–matrix interactions, and interactions with growth factors and various other matrix molecules.

HERITABLE DISORDERS OF CONNECTIVE TISSUE

The Ehlers-Danlos Syndrome

Epidemiology and Criteria for Diagnosis

EDS comprise a clinically and genetically heterogeneous group of HCTD, of which the principal clinical features are due to varying degrees of tissue fragility of the soft connective issues, including the skin, ligaments, blood vessels, and internal organs. The prevalence of EDS is estimated to be approximately 1 in 5,000 births, with no racial predisposition.[2] However, the incidence rises with increased physician awareness. The first attempts to classify EDS resulted in the Berlin nosology in 1986, in which 10 subtypes were recognized.[3] Elucidation of the molecular basis of several types of EDS resulted in revision of this classification, established in 1997 as the Villefranche nosology[4] that recognizes six subtypes, based on clinical characteristics, mode of inheritance, and the nature of the underlying biochemical and molecular defect(s). For each subtype, major and minor clinical diagnostic criteria were defined. Several EDS subtypes are caused by mutations in the genes for collagen type I (arthrochalasis type), type III (vascular type), or type V (classic type), or in the genes involved in the processing of type I collagen (kyphoscoliosis and dermatosparaxis type) (Table 69.1). In terms of management and counseling, it is very important to establish the correct subtype of EDS. Over the past few years, however, it has become clear that the Villefranche classification remains insufficient and that many patients present with overlapping forms of EDS, which cannot be classified unambiguously into one of the six recognized subtypes. Recent insights into the molecular and biochemical basis of some EDS variants call for a refinement of the Villefranche classification, which will likely improve genetic counseling of affected families.[5]

Clinical Characteristics

Cutaneous Manifestations

Cutaneous hyperextensibility (FIGURE 69.1) is one of the cardinal features of EDS in general, except for the vascular subtype. Skin extends easily and snaps back after release (unlike the lax and redundant skin observed in the cutis laxa (CL) syndromes). The skin is typically very smooth and velvety to the touch. It is also very fragile, as manifested by splitting of the dermis following relatively minor trauma. Wounds take a longer time to heal, and stretching of scars after apparently successful primary wound healing is characteristic. Scars become wide, with a "cigarette paper"–like or papyraceous appearance (FIGURE 69.2). Other dermatologic features in EDS include molluscoid pseudotumors, subcutaneous spheroids, and piezogenic papules. Less frequent cutaneous manifestations include acrocyanosis, chilblains, and, rarely, elastosis perforans serpiginosa.

Musculoskeletal Manifestations

Joint hypermobility (FIGURE 69.3) should be assessed using the Beighton scale (Table 69.2), the most widely accepted grading system for the objective semiquantification of joint hypermobility.[6] It may lead to major articular complications, such as habitual subluxation and dislocation of the joints most often affecting shoulder, patella, digits, hip, radius, and clavicles. When they occur, these dislocations usually resolve spontaneously or are easily managed by the patient. Many individuals with EDS suffer from chronic joint and limb pain, despite normal skeletal radiographs. Other problems related to the joint hypermobility are joint instability, foot deformities such as congenital clubfoot or pes planus, temporomandibular joint dysfunction, joint effusions, and osteoarthritis. At birth, uni- or bilateral dislocation of the hip may be present.

Bruising and Bleeding

The bleeding tendency primarily manifests itself as easy bruisability, bleeding of gums, prolonged bleeding after dental or surgical procedures, and prolonged menstrual episodes. Easy bruising is, to a variable degree, a common complaint in all subtypes of EDS. This bleeding diathesis is explained by an abnormal capillary structure with deficiency of normal perivascular collagen, resulting in poor support of cutaneous blood vessels that rupture when subjected to shearing forces. Laboratory investigation of platelet aggregation, clotting factors, and bleeding time in patients with EDS is usually normal. The Rumpel-Leede (or Hess) test may be positive, indicating capillary fragility.[7,8] To perform this test, pressure is applied to the forearm with a blood pressure cuff inflated to between systolic and diastolic blood pressure for 10 minutes. After removing the cuff, the number of petechiae in a 5-cm diameter circle of the area under pressure is counted. Normally, <15 petechiae are seen. Fifteen or more petechiae indicate capillary fragility. In children with EDS, excessive bruising is often the presenting complaint to the pediatrician. If pronounced, it can cause suspicion of a hematologic disorder, a malignancy, or child abuse. Careful evaluation of the medical and family history, and rigorous clinical examination with special attention to skin features that are characteristic for EDS, are mandatory to distinguish between a HCTD and other causes of bruising.

Spontaneous arterial rupture leading to life-threatening complications is a hallmark of the vascular subtype of EDS. It may also occur in the rare individual with a severe form of the classic or the kyphoscoliotic type of EDS. Rupture of middle-sized

Table 69.1
The Villefranche nosology for EDS

	Clinical Manifestations				
Type	**Major Criteria**	**Minor Criteria**	**IP**	**Protein**	**Gene**
Classic (gravis type I, mitis type II)	• Skin hyperextensibility • Widened atrophic scarring • Joint hypermobility	• Easy bruising • Smooth and velvety skin • Molluscoid pseudotumors • Subcutaneous spheroids • Muscular hypotonia • Complications of joint hypermobility • Surgical complications • Positive family history	AD	Type V procollagen	*COL5A1* *COL5A2*
Hypermobility (type III)	• Generalized joint hypermobility • Mild skin involvement	• Recurring joint dislocations • Chronic joint pain • Positive family history	AD	mostly unknown Tenascin-X (5%–10%)	mostly unknown *TNX-B*
Vascular (type IV)	• Excessive bruising • Thin, translucent skin • Arterial/intestinal/ • Uterine fragility or rupture • Characteristic facial appearance	• Acrogeria • Early-onset varicose veins • Hypermobility of small joints • Tendon and muscle rupture • Arteriovenous or carotid-cavernous sinus fistula • Pneumo(hemo)thorax • Positive family history, sudden death in close relative(s)	AD	Type III procollagen	*COL3A1*
Kyphoscoliotic (type VI)	• Severe muscular hypotonia at birth • Generalized joint laxity • Kyphoscoliosis at birth • Scleral fragility and rupture of the globe	• Tissue fragility, including atrophic scars • Easy bruising • Arterial rupture • Marfanoid habitus • Microcornea • Osteopenia	AR	Lysyl hydroxylase-1	*PLOD1*
Arthrochalasis (type VIIA&B)	• Severe generalized joint hypermobility with recurrent subluxations • Congenital bilateral hip dislocation	• Skin hyperextensibility • Tissue fragility, including atrophic scars • Easy bruising • Muscular hypotonia • Kyphoscoliois • Mild osteopenia	AD	Type I procollagen	*COL1A1* *COL1A2*
Dermatosparaxis (type VIIC)	• Severe skin fragility • Sagging, redundant skin • Excessive bruising	• Soft, doughy skin texture • Premature rupture of membranes • Large herniae	AR	Procollagen-*N*-proteinase	*ADAMTS-2*

IP, inheritance pattern; AD, autosomal dominant; AR, autosomal recessive.
Modified from Beighton P, De Paepe A, Steinmann B, et al. Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers- Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). *Am J Med Genet* 1998;77(1):31–37.

FIGURE 69.1 Skin hyperextensibility in a patient with classic EDS.

arteries has also been observed in individuals with a classic/vascular EDS-overlap phenotype, which results from mutations causing specific arginine-to-cysteine substitutions in *COL1A1*, the gene encoding the proα_1(I) chain of type I collagen.[9]

Neuromuscular Involvement

Mild to moderate neuromuscular involvement is common in various types of EDS. Primary muscular hypotonia may cause delayed motor development and problems with ambulation. Muscle weakness, muscle cramps, musculoskeletal pain, and fatigue are frequent complaints. Muscle rupture can occur, especially in the vascular subtype of EDS.[10] Cerebrospinal fluid leak has rarely been reported to cause postural hypotension and headache in individuals with classic EDS.[11]

FIGURE 69.2 Atrophic scarring on the knees and shins in a patient with classic EDS.

FIGURE 69.3 Severe joint hyperlaxity in a patient with EDS arthrochalasis type.

Cardiovascular Manifestations

Structural cardiac malformations are uncommon in EDS. Mitral valve prolapse (MVP) and, less frequently, tricuspid valve prolapse may occur. Stringent criteria should be used for the diagnosis of MVP. Severe cardiac valvular anomalies necessitating surgery at young adult age are observed in a rare autosomal recessive form of EDS, caused by mutations that lead to a total absence of the α_2(I)-procollagen chain.[12,13]

Manifestations of Generalized Tissue Fragility

Manifestations of tissue extensibility and fragility may be observed in multiple organs. Patients with EDS may suffer from repetitive hernia, such as inguinal, umbilical, hiatal, or incisional hernia. In early childhood, recurrent rectal prolapse may be observed. Pregnancy-related manifestations include increased risk for extension of episiotomy incisions, tearing of the perineal skin, and prolapse of the uterus, and/or the bladder may occur after delivery. Prematurity, mostly due to premature rupture of the membranes, occurs more often than normal, especially in more severe forms of EDS and if the fetus is affected. Because of hypotonia, breech presentation is more frequent if the baby is affected and may lead to dislocation of the hips or shoulder of the newborn.

Vascular Ehlers-Danlos Syndrome

The vascular type of EDS is an autosomal dominant disorder that is caused by defects in the proα_1(III) chain of type III collagen, encoded by the *COL3A1* gene. Of all EDS subtypes, it has the worst prognosis because of the risk of potentially fatal vascular and intestinal complications.[14,15] Unlike other types of EDS, the skin is not hyperextensible, but rather thin and translucent, showing a visible venous pattern over the chest, abdomen, and extremities.[2,16] Excessive wrinkling and thinness of the skin over hands and feet may produce an old-looking appearance, referred to as "acrogeria."

Excessive bruising is the most common sign and is often the presenting complaint. Other early manifestations include premature rupture of the membranes, congenital clubfoot or congenital

Table 69.2

Beighton's criteria for joint hypermobility

Joint/Finding	Negative	Unilateral	Bilateral
Passive dorsiflexion of the fifth finger >90 degrees	0	1	2
Passive flexion of thumbs to the forearm	0	1	2
Hyperextension of the elbows beyond 10 degrees	0	1	2
Hyperextension of the knees beyond 10 degrees	0	1	2
Forward flexion of the trunk with knees fully extended and palms resting on the floor	0	Present = 1	
A total score of at least 5/9 defines hypermobility			

Modified from Beighton P, Solomon L, Soskolne CL. Articular mobility in an African population. *Ann Rheum Dis* 1973;32(5):413–418.

hip dislocation, inguinal hernia, pneumothorax, and recurrent joint dislocation or subluxation.[17] Patients with vascular EDS often display a characteristic facial appearance, with prominent eyes (due to lack of subcutaneous adipose tissue around the eyes), a thin, pinched nose and small lips, hollow cheeks, and lobeless ears. Hypermobility is usually limited to the small joints of the hands.

The generalized vascular fragility largely dominates the clinical picture. Apart from excessive bruising and bleeding, it may cause precocious and severe varicosities and arterial rupture, potentially resulting in sudden death, usually in the third or the fourth decade of life. The vascular fragility affects large as well as small blood vessels, and bleeding may occur at every possible site in the body. The most common location of arterial bleeding is the abdominal cavity due to rupture of medium-sized arteries, such as the renal or splenic arteries, rather than of the aorta itself. Acute myocardial infarction due to coronary dissection or rupture is a rare complication. Some affected individuals may harbor predisposing lesions such as aneurysms or arteriovenous fistulae, but in other patients ruptures occur at locations that appear completely normal by angiography.[2] Besides the vascular ruptures, dangerous internal complications such as spontaneous rupture of the bowel (usually the colon, sometimes the intestine), the gravid uterus, and hemorrhagic pneumothorax may occur.[14,18–21]

Although uncommon, vascular EDS is a cause of stroke in young adults. The mean age of intracranial aneurismal rupture, spontaneous carotid–cavernous sinus fistula, and cervical artery aneurysm is 28 years.[22] Obstetrical complications are frequent and include vascular, intestinal, or uterine rupture, vaginal lacerations, prolapse of uterus and bladder, and premature delivery because of cervical insufficiency or fragility of the membranes. Patients with vascular EDS who are pregnant should be enrolled in a high-risk obstetrical program.

The clinical appearance of patients with vascular EDS may deviate from the typical picture, and especially the facial and cutaneous features, such as the acrogeria, may be very subtle or even absent. In the absence of a positive family history or a major vascular or intestinal complication, clinical diagnosis is difficult, especially in children.

From Genotype to Phenotype

EDS is extremely heterogeneous not only at the clinical but also at the molecular level. Abnormalities in the expression or structure of the fibrillar collagen types I, III, and V, as well as enzymatic abnormalities in the posttranslational modification and processing of these collagens, and more recently also in molecules involved in intracellular trafficking and extracellular organization of these molecules, have been identified in a number of EDS subtypes. Studies suggest that approximately 50% of individuals with classic EDS have an identifiable mutation in the *COL5A1* or *COL5A2* gene, the genes encoding the α_1- and the α_2-chain of type V collagen, respectively.[23] However, since no prospective molecular studies of *COL5A1* and *COL5A2* have been performed in a clinically well-defined patient group, this number may underestimate the real proportion of classic EDS patients harboring a mutation in one of these genes.[24]

The vascular type of EDS results from mutations in the *COL3A1* gene, encoding the proα_1 chain of type III collagen. A wide spectrum of *COL3A1* mutations has been identified, the majority of which are point mutations leading to substitutions for the obligatory glycine in the triple-helical region of the collagen molecule. Other types of mutations include splice-site mutations, partial gene deletions, and, rarely, mutations resulting in *COL3A1* haplo-insufficiency.[2,25] Parental mosaicism for *COL3A1* mutations has been documented in vascular EDS[26–29] and may explain unexpected recurrences in families in which a "new" dominant mutation has been identified.

Whereas defects in the genes encoding type I collagen generally result in different forms of OI, a subset of mutations in the *COL1A1* or the *COL1A2* gene result in specific subtypes of EDS. Mutations that cause loss of the *N*-proteinase cleavage site of either the proα_1(I) or the proα_2(I) collagen chain give rise to the arthrochalasia type of EDS. Mutations residing in the most amino-terminal part of the collagen type I triple helix result in a distinct EDS/OI overlap phenotype with variable joint hypermobility and bone fragility.[30] In addition, some of these patients display increased vascular fragility with bleeding diathesis (personal observation). These mutations have been shown to interfere with removal of the *N*-terminal propeptide, even though the *N*-proteinase cleavage site remains intact.[31] Specific arginine-to-cysteine (R-to-C) substitutions at different positions of the α_1(I) helical domain have been associated with a range of EDS phenotypes, including a classic EDS-like phenotype in childhood, and a vascular EDS-like phenotype with increased risk for spontaneous arterial rupture in young adulthood.[9,32,33] Finally, mutations leading to

total absence of the proα_2(I) chain, due to a homozygous or compound heterozygous *COL1A2*-mutation, can be associated with severe cardiac valvular anomalies necessitating surgery at young adult age.[12,13]

Homozygous or compound heterozygous mutations in genes encoding enzymes involved in collagen biosynthesis have been documented in several autosomal recessive forms of EDS. Homozygous mutations in *PLOD1* (procollagen-lysine, 2-oxoglutarate 5-dioxygenase or lysyl hydroxylase-1), the gene encoding lysyl hydroxylase-1, have been identified in patients with the kyphoscoliotic type of EDS.[34] Lysyl hydroxylase-1 is required for the hydroxylation of specific lysine residues to hydroxylysines, which act as precursors for the cross-linking process that is essential for the tensile strength of collagen. A deficient activity of procollagen-*N*-proteinase due to homozygous and compound heterozygous mutations in the *ADAMTS-2* gene is responsible for the dermatosparaxis type of EDS.[35]

Recently, several new EDS variants have been characterized, implicating genetic defects in molecules involved in processes as diverse as intracellular trafficking, enzymatic modification of ECM proteins, and cell–matrix interactions (Table 69.3). Total absence of tenascin-X (*TNXB* gene), a large ECM glycoprotein that plays a role in the maturation and the maintenance of the dermal collagen and elastin network, results in a classic EDS-like phenotype with extensive bruising, skin and joint hypermobility, and occasionally increased laxity of the genitourinary tract causing uterine and vaginal prolapse, increased risk for postpartum hemorrhage, progressive muscle weakness, and distal joint contractures.[36,37,38] Deficiency of SLC39A13 (*SLC39A13* gene), an intracellular zinc transporter, results in spondylocheirodysplastic EDS[39], and deficiency of Ras- and Rab interactor 2 (*RIN2*), which is involved in Rab5-mediated early endosomal trafficking, results in an EDS-like "RIN2 syndrome."[40,41] Deficiency of galactosyltransferase-I (*β4GALT7*) and of dermatan-4-sulfotransferase 1 (*CHST14*), two enzymes involved in dermatan sulfate biosynthesis, causes the progeroid and the musculocontractural subtype of EDS, respectively.[42,43,44] Furthermore, a zinc-finger protein (*ZNF469*), the exact function of which remains currently unknown, has been shown to be associated with brittle cornea syndrome, a condition with substantial phenotypical overlap with EDS kyphoscoliotic type,

Table 69.3

Newly identified subtypes of EDS, which are not present in the current Villefranche nosology, associated genes, and discriminating features

Gene	Protein	Disease Name	Discriminating Features	IP
COL1A1	Type I collagen (α_1(I) chain)	—	Aneurysm/dissection of middle-sized arteries	AD
COL1A2	Type I collagen (α_2(I) chain)	Cardiac valvular EDS	Cardiac valvular disease	AR
TNX-B	Tenascin-X	—	Skin hyperextensibility and easy bruising without atrophic scarring, laxity of GU tract, progressive muscle weakness, distal joint contractures	AR
B4GALT	Galactosyltransferase-I	Progeroid form of EDS	Progeroid appearance, radioulnar synostosis, short clavicle	AR
CHST14	dermatan-4-sulfotransferase 1	Musculocontractural EDS	Joint contractures, clubfeet, progressive muscle weakness, tubular fingers, multisystemic involvement	AR
ZNF469	ZNF 469	Brittle cornea syndrome	Brittle cornea, blue sclerae	AR
SLC39A13		Spondylocheirodysplastic EDS	Tubular fingers, distal joint contractures, platyspondyly, metaphyseal broadening	AR
RIN2	RIN2	MACS syndrome or RIN2 syndrome	Progressive craniofacial dysmorphism, progressive scoliosis, sparse hair/alopecia, macrocephaly, skin hyperextensibility/CL	AR
?	?	Periodontotic EDS	Severe early-onset periodontal disease, chronically inflamed pretibial plaques	AD
FLNA[93]	Filamin A	EDS with periventricular nodular heterotopia	Periventricular nodular heterotopia	X-linked dominant

IP, inheritance pattern; AD, autosomal dominant; AR, autosomal recessive; GU, genitourinary; MACS, macrocephaly, alopecia, cutis laxa, scoliosis

that is primarily characterized by fragility of the cornea, leading to corneal rupture.[45]

Diagnosis

The evaluation of the patient in whom EDS is suspected should start with a detailed clinical and family history and a thorough physical examination. Laboratory investigation of platelet aggregation, clotting factors, and bleeding time in patients with EDS is usually normal.

In certain EDS subtypes, ultrastructural, biochemical, and molecular analyses can be very helpful to confirm the diagnosis. To this end, a skin biopsy is required to obtain cultured skin fibroblasts.

Ultrastructural examination of the skin, performed by electron microscopy, usually reveals abnormalities of collagen fibrillogenesis. These include irregular, disrupted collagen fibrils ("collagen flowers") and variability in the diameter of the individual collagen fibrils. However, these abnormalities are found in several EDS variants and do usually not allow to discriminate between individual EDS subtypes, and cannot be used as a diagnostic test. Only in the dermatosparaxis subtype of EDS, ultrastructural examination of the skin is diagnostic, showing a pathognomonic "hieroglyphic" aspect of the collagen fibril architecture.[46,47]

Biochemical study of the collagen types I, III, and V includes SDS polyacrylamide gel electrophoresis of radiolabeled collagens extracted from the cultured fibroblasts, and can detect abnormalities of these proteins, regardless of the underlying cause. In the vascular subtype of EDS, biochemical analysis of type III procollagen is diagnostic in more than 95% of all patients. It allows to detect quantitative (reduced amounts of type III collagen) and/or qualitative (abnormal type III collagen with altered electrophoretic mobility) abnormalities of type III collagen. Biochemical analysis can also be helpful in the diagnosis of the arthrochalasis, kyphoscoliosis, dermatosparaxis, and some other rare subtypes of EDS, where distinct abnormal migration patterns of type I (pro) collagen chains are observed. For the majority of patients with the classic type of EDS, on the other hand, analysis of type V collagen is an ineffective method for routine diagnostic evaluation, since the chains of type V collagen are synthesized by fibroblasts at a low rate, causing poorly reproducible quantification and evaluation of alterations in electrophoretic mobility of this collagen.[23]

Molecular investigations usually start from genomic DNA and messenger RNA, extracted from cultured dermal fibroblasts. For the classic subtype of EDS, the majority of the mutations result in a nonfunctional *COL5A1* allele.[23] As a first step therefore, a *COL5A1* "null"-allele test is performed, using polymorphic markers in the expressed region of the genomic DNA, to determine if both *COL5A1* alleles have stable transcripts. This test determines if the individual is heterozygous for one of several *COL5A1* polymorphic exonic markers in genomic DNA and then establishes at the cDNA level whether both alleles are expressed. If only one of the two *COL5A1* alleles is present in cDNA, it is assumed that the absent allele is "null." Since it examines both genomic DNA and cDNA, *COL5A1* "null"-allele testing requires cultured skin fibroblasts. It does not identify mutations within the *COL5A1* gene.

Sequence analysis of the different genes involved in the various subtypes of EDS can be performed either on genomic DNA or cDNA. Once the mutation is known, its presence or absence can easily be verified on genomic DNA obtained from leucocytes from the patient and from relatives.

Prenatal diagnosis for pregnancies at increased risk is possible by analysis of DNA extracted from fetal cells obtained by amniocentesis usually performed at about 15 to 18 weeks' gestation or chorionic villus sampling at about 10 to 12 weeks gestation. The disease-causing allele of an affected family member must be identified before prenatal testing can be performed. Requests for prenatal testing for conditions that (like classic EDS) do not affect intellect or life span are not common. Differences in perspective may exist among medical professionals and within families regarding the use of prenatal testing, particularly if the testing is being considered for the purpose of pregnancy termination rather than early diagnosis. Although most centers would consider decisions about prenatal testing to be the choice of the parents, discussion of these issues is appropriate. Preimplantation genetic diagnosis may be available for families in which the disease-causing mutation has been identified in an affected family member.

Guidelines for Management

As EDS affects many systems in the body, different medical specialists need to be involved in the management, depending on the extent and severity of the symptoms of the clinical phenotype. Each patient presents with a particular set of clinical problems, requiring a treatment plan that is tailored to his or her specific needs. Coordination of the multidisciplinary approach to ensure that all areas of the syndrome are covered is preferably done by a clinical geneticist, who will also provide genetic counseling. No causal therapy is available for EDS; however, a series of "preventive" guidelines are applicable to all forms of EDS. The key aspects of management include cardiovascular workup, physiotherapy, pain management, and psychological follow-up.

A baseline echocardiogram should be performed in all patients with EDS in order to evaluate aortic root diameter, as adjusted for age and body surface area, and to evaluate cardiac valvular abnormalities. Because longitudinal data on progression of aortic dilation are not available, specific recommendations for follow-up in individuals with a normal aortic diameter do not exist. However, if no abnormalities are found on echocardiogram in an adult, a follow-up echocardiogram is probably not necessary. For children and adolescents, it is reasonable to repeat the echocardiogram, approximately every 3 years until adulthood. Annual echocardiograms are warranted only if an abnormality such as aortic dilatation or MVP is present.

Children with pronounced skin fragility should wear protective pads or bandages over the forehead, knees, and shins in order to avoid skin lacerations. Dermal wounds should be closed without tension, preferably in two layers. Deep stitches should be applied generously. Cutaneous stitches should be left in place twice as long as usual, and additional fixation of adjacent skin with adhesive tape can help to prevent stretching of the scar.

Patients with pronounced bruising are advised to avoid contact sports and heavy exercise. Protective pads and bandages can also be useful in the prevention of bruises and hematomas. Drugs that interfere with platelet function, including aspirin (acetylsalicylic acid), dipyridamole, clopidogrel, and nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen and diclofenac should be avoided where possible. Paracetamol (acetaminophen) and cyclooxygenase-1-sparing NSAIDs, such as celecoxib, do not influence hemostasis and can be

considered safe. By definition, anticoagulant drugs will increase the bleeding tendency, and should also be restricted to the minimum especially in the vascular subtype of EDS. These include the traditional oral vitamin K antagonists (coumarins) such as acenocoumarol, fenprocoumon and warfarin, heparin and low molecular weight heparins, and the newer oral thrombin inhibitors and pentasaccharides. There are currently no guidelines regarding the administration of anticoagulant drugs for the prophylaxis of deep venous thrombosis or pulmonary embolism in vascular EDS patients. Standard guidelines should be followed, but the treating physician needs to be aware of an increased risk for dramatic complications during anticoagulant treatment. Supplementation of ascorbic acid, a cofactor for cross-linking of collagen fibrils, can ameliorate the tendency toward bruising in some patients. The vasopressin analogue desmopressin acetate, 1-desamino-8-D-arginine vasopressin has been reported to reduce a bleeding tendency temporarily in subjects undergoing a dental or surgical procedure.[48] A case report describes the successful use of recombinant factor VIIa in a patient with vascular type EDS, in whom continued bleeding was successfully stopped after intravenously administered recombinant factor VIIa.[49]

In patients with hypotonia and delayed motor development, a physiotherapy program is important. Non–weight-bearing muscular exercise, such as swimming, is useful to promote muscular development and coordination. Sports with heavy joint strain, such as contact sports, are discouraged. Besides prescription of physiotherapy, the rheumatologist and/or physical therapist may also provide assisting devices such as braces, ring splints, and soft collar necks, while the occupational therapist may provide tools that make the living and working environment more user-friendly for the patient. Many patients will have undergone one or more orthopedic procedures prior to the diagnosis of EDS, such as arthroplasty and capsulorrhaphy. The degree of joint stabilization, pain reduction, and duration of improvement are usually far less in patients with EDS than in those without this disorder. It is often appropriate to delay surgery in EDS patients in favor of physical therapy and bracing.

Pain management is also very important. Pain medication should be tailored to the individual's subjective symptoms. Cognitive behavioral therapy can be beneficial in patients with hypermobility and chronic pain. Finally, psychological follow-up designed to explore coping strategies and to recognize and treat depression is of utmost importance.

For the vascular type of EDS, some prophylactic measures are of special importance.

Invasive vascular procedures to determine sources of bleeding such as arteriography and catheterization should be avoided because of the risk of vascular ruptures, which cause significant morbidity and may have fatal outcome.[50,51] They should rather be replaced by ultrasonography and/or subtraction angiography. Surgical interventions are generally discouraged because of increased vascular fragility, and conservative therapy is recommended. When surgery is required for the treatment of arterial or bowel complications or other health problems, thorough investigation of platelet function and clotting is appropriate, as affected persons are already subject to bleeding from ruptured vessels or organs and an intrinsic clotting defect (unrelated to EDS) may complicate clinical outcome. Surgical exploration and intervention should be minimized and manipulation of vascular and other tissues should be done with extreme care.[15]

Recently, a multicenter randomized trial showed that celiprolol, a long-acting β_1-antagonist with partial β_2-agonist properties, decreased the incidence of arterial rupture or dissection by three times in patients with the clinical diagnosis of vascular EDS. This study represents a substantial breakthrough in the evidence-based management of the syndrome.[52]

Pregnancy for women with the vascular type of EDS is a high-risk venture. The risk of maternal death is as high as 12% from uterine rupture or peripartum arterial rupture.[14] On the other hand, some women with vascular EDS have had one or several successful pregnancies prior to recognition of their underlying condition. It is prudent to follow pregnant women with the vascular type of EDS in a high-risk obstetrical program. It is not clear whether elective caesarean section is preferred to vaginal delivery.

The Marfan Syndrome

More than a century ago, the French pediatrician Antoine-Bernard Marfan described a 5-year-old girl, Gabrielle, with long slender digits, long-bone overgrowth, and muscle hypoplasia.[53] Clinical research further delineated this pleiotropic condition, known as the Marfan syndrome (MFS), as a systemic connective tissue disorder with severe manifestations mainly involving the cardiovascular, ocular, and skeletal systems and necessitating a multidisciplinary approach for diagnosis and management.

The estimated prevalence of this rather common disease is 1 in 5,000, affecting both sexes equally.[54] The disease demonstrates autosomal-dominant inheritance with high penetrance and marked inter- and intrafamilial variability. International criteria for diagnosis have been established and were recently refined in the revised Ghent nosology.[55] These criteria put much weight on the two cardinal features of MFS, namely, aortic root aneurysm/dissection and ectopia lentis (displacement of the eye's crystalline lens). In the absence of any family history, the presence of these two cardinal features is sufficient for the unequivocal diagnosis of MFS, irrespective of systemic involvement. In the absence of one of these two clinical manifestations, the presence of a bonafide *FBN1* mutation or a combination of systemic manifestations is required. For the latter, a new scoring system has been designed in which a score of seven or more indicates systemic involvement compatible with MFS (Table 69.4).

Clinical Characteristics

Cardiovascular System

A key diagnostic criterion for MFS is aortic root aneurysm or dissection. These cardiovascular manifestations are life threatening, with the majority of aortic dissections/ruptures occurring in early adult life if left untreated. The onset and growth rate of the aortic dilatation vary highly between individuals. Timely recognition and appropriate medical and surgical management of the disease have increased the mean survival age to 72 years.[56] Aortic dilatation is generally greatest at the sinuses of Valsalva. Aortic root measurements should be performed parallel to the plane of the aortic valve and perpendicular to the axis of the blood flow. The largest correctly measured root diameter obtained from at least three transthoracic images should be corrected for age and body size, and interpreted as a Z-score.[55] Aortic root dilatation is present when the Z-score is ≥2. MVP is also a common finding in MFS. There are no special criteria for diagnosing MVP in MFS and standard practices

Table 69.4

Scoring of systemic features for MFS

Clinical Sign	Points
Wrist and thumb sign	3
Wrist or thumb sign	1
Pectus carinatum deformity	2
Pectus excavatum or chest asymmetry	1
Hindfoot deformity	2
Plain pes planus	1
Pneumothorax	2
Dural ectasia	2
Protrusio acetabuli	2
Reduced US/LS and increased arm/height and no severe scoliosis	1
Scoliosis or thoracolumbar kyphosis	1
Reduced elbow extension	1
Facial features (3/5) (dolichocephaly, enophthalmos, downslanting palpebral fissures, malar hypoplasia, retrognathia	1
Skin striae	1
Myopia >3 diopters	1
MVP	1
Maximum total: 20	
Score ≥7 indicates systemic involvement	

Modified from Loeys BL, Dietz HC, Braverman AC, et al. The revised Ghent nosology for the Marfan syndrome. *J Med Genet* 2010;47(7):476–485.

should be applied. Patients with MFS can develop aortic enlargement or dissection at segments distant from the aortic root. The frequency of this finding (particularly at the proximal descending thoracic aorta and in the abdomen) appears to be increasing with the prolonged survival due to improved management of disease at the aortic root. Descending aortic aneurysm/dissection is rare in the absence of aortic root enlargement.

Ocular System

The most prominent ocular features of MFS are lens dislocation (FIGURE 69.4) and myopia. Ectopia lentis reflects failure of the ciliary zonules, and the diagnosis is based on slit-lamp examination after maximal dilatation of the pupil. Myopia is very common in MFS, is usually early in onset, and can progress rapidly.

Musculoskeletal System

Long-bone overgrowth contributes to disproportionately long limbs and anterior chest deformities due to rib overgrowth. Pectus carinatum is believed to be more specific for MFS than pectus excavatum. Other major manifestations include arachnodactyly, limited extension of the elbows, scoliosis or spondylolisthesis, protrusio acetabuli (detected by X-ray), and hindfoot valgus in combination with forefoot abduction and

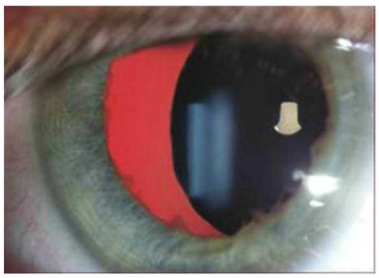

FIGURE 69.4 Ectopia lentis in a patient with MFS.

lowering of the midfoot. Arachnodactyly requires a positive thumb sign (when the entire distal phalanx of the fully adducted thumb extends beyond the ulnar border of the palm) and a positive wrist sign (when the tip of the thumb covers the entire fingernail of the fifth finger when wrapped around the contralateral wrist). Patients may present with typical facial characteristics including downslanting palpebral fissures, enophthalmia, retrognathia, and a highly arched palate with dental crowding. Joint hypermobility may predispose to ligamentous injury, dislocations, chronic joint pain, and premature osteoarthritis. Muscle hypoplasia and myalgia are often underrecognized and may result in fatigue and spinal pain.

Dura Mater

Dural ectasia (a gradual stretching and weakening of the dura mater) is a common observation, with a high prevalence in MFS patients. For the detection of dural ectasia, no preferred method (CT or MRI) or uniformly accepted cutoffs are available. It is a sensitive but not a specific sign of MFS, as it is commonly seen in other conditions, such as the Loeys-Dietz syndrome (LDS), or the EDS. It occasionally causes discomfort such as low-back pain, headaches, and irradiating leg pains.[57]

Skin/Pulmonary System

Cutaneous manifestations in MFS include striae atrophicae, which are not associated with weight changes or pregnancy, and which are present on uncommon locations such as the mid-back, lumbar region, upper arm, axillary region, or thigh. The most important pulmonary feature is spontaneously occurring pneumothorax.

Differential Diagnosis

The differential diagnosis of MFS is extensive, comprising other connective tissue disorders and metabolic diseases with skeletal, ocular, and/or cardiovascular involvement (Table 69.5).

Molecular Pathogenesis

MFS is caused by mutation in the *FBN1* gene, encoding fibrillin-1. Thus far, over 1,000 different mutations have been identified, dispersed all over the *FBN1* gene and often family specific. Until recently, it was believed that structural deficiency of the fibrillin-1 protein was the most important explanation for the weak connective tissues in MFS, a hypothesis based mainly on the microscopic observations showing rarefaction of elastic fibers. While this helped to explain the aortic pathology and ectopia lentis, it did not reconcile the observation of other clinical features such as long-bone overgrowth, thickening of

Table 69.5

Differential diagnosis of MFS, associated genes, and discriminating clinical features

Differential Diagnosis	Gene	Discriminating Features
MFS	*FBN1*	Aortic root dilatation, presence of ectopia lentis, systemic features (Table 69.4)
Loeys-Dietz syndrome (LDS)	*TGBR1/2*	Bifid uvula/cleft palate, arterial tortuosity, hypertelorism, diffuse aortic and arterial aneurysms, craniosynostosis, clubfoot, cervical spine instability, thin and velvety skin, easy bruising
Shprintzen-Goldberg syndrome	*FBN1* and other	Craniosynostosis, mental retardation, hypertelorism, proptosis
Congenital contractural arachnodactyly (CCA)	*FBN2*	Crumpled ears, contractures
Weill-Marchesani syndrome	*FBN1* and *ADAMTS 10*	Microspherophakia, brachydactyly, joint stiffness, short stature
Ectopia lentis syndrome	*FBN1, LTBP2, ADAMTS4*	Lack of aortic root dilatation
Homocystinuria	*CBS*	Thrombosis, mental retardation
Familial thoracic aortic aneurysm syndrome (FTAA)	*TGFBR1/2*	Lack of marfanoid skeletal features
	ACTA2	Levido reticularis, iris flocculi, CVA
	SMAD3	Osteoarthritis, arterial tortuosity, soft skin
	MLCK	Gastrointestinal abnormalities
FTAA with BAV		Lack of marfanoid skeletal features, levido reticularis, iris flocculi
FTAA with PDA	*MYH11*	Lack of marfanoid skeletal features, levido reticularis, iris flocculi
Arterial tortuosity syndrome (ATS)	*SLC2A10*	Generalized arterial toruosity, arterial stenosis, facial dysmorphism
EDS (vascular, valvular, kyphoscoliotic type)	*COL3A1, COL1A2, PLOD1*	Middle-sized artery aneurysm, severe valvular insufficiency, translucent skin, dystrophic scars, facial characteristics
cutis laxa (CL) syndromes	*ELN* (AD)	CL with variable involvement of internal organs (lung, aorta), association with BAV
	FBLN4 (AR)	CL, emphysema, arterial tortuosity, aortic aneurysm, joint laxity, pectus excavatum, diaphragmatic hernia, bone fragility

BAV: bicuspid aortic valve, PDA: patent ductus arteriosus, CVA: cerebro-vascular accident.

the cardiac valves, or muscle hypoplasia. The study of fibrillin-1 mutant mouse lines that recapitulate human MFS has recently challenged this "mechanistic" view and show that structural fibrillin-1 deficiency leads to activation of the sequestered or latent cytokine TGF-β.[58,59] Dysregulation of TGF-β signaling, one of the major biologic pathways, has been shown to play a pivotal role in the development of aortic aneurysms, emphysema, and the muscle hypoplasia seen in MFS, and progression of aortic root enlargement can be effectively blocked with TGF-β antibodies.[58,60,61] In mouse models for MFS Losartan, an angiotensin II type 1 receptor blocker has been shown to stop aortic growth and reconstitute elastic fiber conservation.[61] Losartan directly blocks the effect of TGF-β, diminishes the expression of TGF-β receptors, and inhibits the activity of TSP, an ECM enzyme that enables the release of TGF-β.[62,63] This changing concept of MFS, from a syndrome characterized as a structural connective tissue disorder to a condition manifesting also perturbed cytokine signaling, has opened new and unexpected targets for drug treatment.

Guidelines for Management

Diagnosis and management require a multidisciplinary approach by geneticists, cardiologists, orthopedic surgeons, and ophthalmologists with experience in this field.[63] Cardiovascular follow-up should include yearly evaluation with echocardiography or CT/MRI angiography when visibility of the aortic root and

ascending aorta is limited. Evaluation frequency should be tailored according to aortic dimensions, rate of aortic growth, and family history. More frequent imaging should be performed if the aortic diameter is approaching the threshold for surgery (≥4.5 cm in adults, less well defined in children) or shows rapid growth (≥0.5 cm/y) or when there are concerns regarding heart or valve function.[55] Beta-adrenergic blockade, titrated to physiologic response, is a standard treatment to slow aortic growth, mainly by virtue of its antihypertensive and negative inotropic effects.[64] If beta-blockade is contraindicated (asthma, Raynaud phenomenon, psoriasis, depression, fatigue), calcium antagonists or angiotensin converting enzyme inhibitors may be used although no randomized studies currently exist. New therapeutic strategies, based upon the physiopathology in MFS, propose the angiotensin receptor blocker Losartan as an antihypertensive agent attenuating TGF-β signaling. Encouraging results were observed in a pilot experience in children with severe MFS, and several multicenter trials of Losartan versus or on top of beta-blockade in MFS are currently under way.[65–67]

Surgical repair of the aorta is indicated once the maximal diameter exceeds 5 cm in adults, when the rate of aortic growth exceeds >0.5 to 10 mm/y, or in the presence of significant aortic regurgitation. In cases where aortic valve function is preserved, the preferred technique is the valve-sparing procedure avoiding lifelong anticoagulant therapy, especially in females in their fertile period. Although the superiority of this technique in comparison with the composite graft has not definitely been established, the 10-year experience is very promising.[68] After surgical repair of the aortic root, imaging of the whole aorta is warranted for timely detection of aortic graft pseudoaneurysms, more distally occurring aneurysms, and coronary artery aneurysms.[63]

Pregnancy in women with MFS is associated with an increased cardiovascular risk, with the majority of aortic complications occurring in the third trimester or in the early postpartum period. The risk for aortic root complications is increased when the aortic root diameter is above 4 cm at the start of the pregnancy.[63] Therefore, pregnancy should be followed intensively, with special care in the peripartum period, through a high-risk obstetric clinic.

Orthopedic complications, mainly those involving deformities of the anterior chest and vertebral column, should be followed carefully, especially during and just after puberty. Since extreme pectus deformity has little impact on pulmonary function, surgery is considered as a cosmetic issue. Correction, preferably using the minimally invasive Nuss procedure, should not be done before the age of 11 years[69], as earlier intervention might lead to recurrent deformity due to continued rib growth. In contrast, severe (kypho-) scoliosis has a major impact on quality of life, needing surgical stabilization as bracing often remains inadequate.[70] Protrusio acetabulae is often asymptomatic in young adults, and the benefit of surgery is questioned.[71] Hormone therapy is indicated if predicted final height is unacceptable.[72]

Diffraction anomalies due to lens subluxation, flat cornea, or myopia can easily be corrected by eyeglasses or contact lenses. Lens extraction for manifest ectopia lentis or cataract severely increases the preexisting risk for retinal detachment and glaucoma, which causes significant visual impairment. Yearly examination by an experienced ophthalmologist is highly recommended for every Marfan patient.

Finally, lifestyle modifications may promote cardiovascular and psychosocial health, and prevent or relieve many locomotor inconveniences including fatigue, myalgia, osteoarthritis, and chronic joint pain. Patients should avoid competitive sport, straining, or isometric exercise as these increase blood pressure and cause considerable dynamic stress on the aortic root. Additionally, contact sports should be avoided as these may precipitate aortic dissection.[73]

Prenatal diagnosis for pregnancies at increased risk is possible by analysis of DNA extracted from fetal cells obtained by amniocentesis usually performed at about 15 to 18 weeks gestation or chorionic villus sampling at about 10 to 12 weeks gestation. The disease-causing allele of an affected family member must be identified before prenatal testing can be performed. Preimplantation genetic diagnosis may be available for families in which the disease-causing mutation has been identified in an affected family member.

Other HCTD that Can be Associated with Aortic Aneurysms

The Loeys-Dietz Syndrome

The LDS is an autosomal dominant aortic aneurysm syndrome characterized by a triad of hypertelorism, bifid uvula or cleft palate, and arterial tortuosity with ascending aortic aneurysm/dissection (FIGURE 69.5 shows bifid uvula). LDS is caused by heterozygous mutations in the TGF-β receptor 1- and TGF-β receptor 2-encoding genes *TGFBR1* and *TGFBR2,* resulting in perturbation of TGF-β signaling.[74] There is significant clinical overlap between LDS and MFS. However, LDS patients rarely satisfy diagnostic criteria for MFS. The main differences are the absence of long-bone overgrowth and lens dislocation, and the presence of multiple other findings, including the dysmorphic features, craniosynostosis, Chiari malformation, clubfeet, patent ductus arteriosus (PDA), and aneurysms and dissections throughout the arterial tree. In contrast to this typical presentation, which was later coined as LDS type I, some patients show less craniofacial abnormalities but more prominent joint and skin manifestations, reminiscent of vascular EDS. This subset of patients (referred to as LDS type II) is characterized by easy bruising, a velvety translucent skin, widened atrophic scars, uterine rupture, severe peripartal bleeding, and arterial aneurysm/dissection throughout the arterial circulation.

The natural history of LDS type I and II is far more aggressive than MFS or even vascular EDS, with a mean age of death of 26 years. Aortic dissections occur in young childhood and/or at smaller aortic dimensions, and the incidence of pregnancy-related complications is high.[75] However, the amenability to

FIGURE 69.5 Bifid uvula in a patient with LDS.

early and aggressive surgical intervention clearly distinguishes these patients from individuals with vascular EDS.

Arterial Tortuosity Syndrome

Arterial tortuosity syndrome (ATS) is a rare autosomal recessive condition characterized by severe tortuosity, stenosis, and aneurysms of the large and middle-size arteries. Focal stenosis of the pulmonary and large systemic arteries can be associated. Patients usually present with characteristic dysmorphic features including elongated face, blepharophimosis and downslanting palpebral fissures, a beaked nose, highly arched palate, and micrognathia. Other connective tissue manifestations comprise a soft, hyperextensible skin and skeletal abnormalities such as arachnodactyly, pectus deformity, joint laxity, and contractures. Patients are prone to vascular complications including dissections and ischemic events. Although initially the prognosis was reported to be poor with mortality rates up to 40% before the age of 5 years,[76] more recent clinical studies and case reports have illustrated that the prognosis is better than previously thought, and that both aortic aneurysms and pulmonary hypertension due to peripheral stenosis are amenable to surgery. However, stroke remains a significant risk and progressive aortic root dilatation may occur.[77]

ATS is caused by mutations in *SLC2A10*, encoding a facilitative glucose transporter, GLUT10. Immunohistochemistry data suggest that the transporter resides on the nuclear membrane. Furthermore, similarly as in the clinically related LDS, vascular smooth muscle cells of ATS patients show enhanced TGF-β signaling.[78]

Cutis Laxa

The term cutis laxa, describing a clinical feature, is used as a common denominator for a heterogeneous group of rare connective tissue disorders, characterized by an increased number of inelastic skinfolds. Both hereditary and acquired forms can be distinguished. Acquired forms appear secondary to infections, administration of medications or as a paraneoplastic feature. Historically, classification of the hereditary forms is based on clinical grounds and mode of inheritance.

The X-linked recessive form, also known as occipital horn syndrome (OHS), includes hyperlaxity of skin and joints, bladder diverticulae, inguinal hernias, vascular tortuosity, and skeletal manifestations including the pathognomonic occipital horns (distinctive wedge-shaped calcifications at the sites of attachment of the trapezius muscle and the sternocleidomastoid muscle to the occipital bone).[79] OHS is caused by mutations in the copper-transporting ATPase gene (*ATP7A*), and affected individuals have low serum copper and ceruloplasmin concentrations.[80,81]

Autosomal dominant cutis laxa (ADCL) presents with generalized lax skinfolds resulting in a prematurely aged appearance. It is generally considered a mild condition without severe involvement of internal organs, although aortic and pulmonary abnormalities have been reported occasionally. In ADCL, heterozygous mutations are found in the elastin (*ELN*) and rarely in the fibulin-5 (*FBLN5*) genes.[82,83] Autosomal recessive cutis laxa (ARCL) comprises at least three subtypes, with typically severe emphysema, diverticulosis, disastrous vascular lesions, and a short life expectancy in ARCL type 1; developmental delay, microcephaly, and skeletal abnormalities in ARCL type 2; and corneal clouding, mental retardation, and athetosis in ARCL type 3, also known as de Barsy syndrome.

In ARCL type 1, homozygous or compound heterozygous mutations in *FBLN5*, *FBLN4* and *LTBP4* have been described.[84–86] Patients with *FBLN5* mutations encounter pulmonary emphysema as the most prominent systemic feature, while *FBLN4* mutations cause severe arterial tortuosity, aneurysm formation, and usually mild CL. Like in MFS, LDS and ATS immunostaining of aortic and lung tissue of patients with *FBLN4* mutations showed evidence of enhanced TGF-β signaling, revealing that this is a mayor player in the pathogenesis of aortic dilatation.[87] Mutations in *LTBP4* have recently been reported in a newly described type 1 ARCL constellation, eponymously named Urban-Rifkin-Davis syndrome, which includes craniofacial dysmorphism, short stature, severe bronchopulmonary dysplasia, emphysema, and marked genitourinary and gastrointestinal diverticulosis.[86]

For ARCL type 2, mutations in *ATP6V0A2* or *PYCR1* have been identified. This subgroup is characterized by CL, distinct facial features, skeletal abnormalities, and variable neurologic involvement. Mutations in *ATP6V0A2* and *PYCR1* were also found in some cases of wrinkly skin syndrome and gerodermia osteodysplasticum, two entities that share common features with ARCL type 2.[88,89] In rare sporadic cases, mutations in the gene *ALDH18A1* were identified in patients with CL, severe mental retardation, skeletal defects, and cataract.[90] Nonetheless, many cases of ARCL still remain molecularly unsolved.

Pseudoxanthoma Elasticum

Pseudoxanthoma elasticum (PXE) is a HCTD characterized by progressive calcification of elastic fibers in the skin, retina, and the cardiovascular system. In general, the earliest clinical manifestations are dermal lesions that present as small yellowish papules primarily at flexural sites, such as the axillae, antecubital fossae, neck, and groin. These papules tend to coalesce into larger plaques, rendering the skin inelastic and redundant. Laxity of the affected skin causes the appearance of premature aging and may have an extensive cosmetic impact. The characteristic ophthalmologic finding in PXE is the presence of angioid streaks in the retina resulting from breaks in the calcified elastic lamina of the Bruch's membrane, which may trigger neovascularization and bleeding, and result in loss of visual acuity and central blindness. Cardiovascular complications arise from the progressive calcification of the elastin-rich arterial blood vessels. Clinical sequelae include intermittent claudication, arteriosclerosis that may lead to hypertension, internal bleeding from the gastric arterial blood vessels, and myocardial infarction at a relatively young age. The diagnosis of PXE is confirmed by histopathologic evaluation of dermal lesions using von Kossa and Verhoeff-van Gieson stains that demonstrate mineralization of elastic fibers. PXE is inherited in an autosomal recessive pattern, and mutations in the ATP-binding cassette (ABC) subfamily C member 6 (*ABCC6*) gene are the cause of PXE. The gene codes for the ABCC6 protein, also known as multidrug resistance–associated protein 6, which is expressed predominantly in the liver and kidneys and, to a lesser extent, in tissues clinically affected by PXE.[91] The precise physiologic function of ABCC6 and what molecule(s) this transmembrane protein transports *in vivo* are currently unknown. To date, close to 300 distinct *ABCC6* mutations associated with PXE have been disclosed in various populations.

While patients with PXE rarely have a bleeding diathesis, a recently described *pseudoxanthoma-like* condition was shown to be associated with a deficiency of the vitamin K–dependent

clotting factors.[92] Affected patients present clinical overlap with classic PXE, with manifestations of yellowish papules as observed in classic PXE; however, the severity of the skin symptoms, with spreading toward the trunk and limbs and the occurrence of thick, leathery skinfolds is also reminiscent of ADCL syndrome. Affected individuals may show a bleeding tendency, including epistaxis, gingival bleeding, vaginal or postpartum hemorrhage, and hematemesis. This condition is caused by specific mutations in the *GGCX* gene encoding a vitamin K–dependent carboxylase that is essential for γ-carboxylation of clotting factors, enabling them to attach to the phospholipid bilayer of membranes as a prerequisite for blood coagulation.

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CHAPTER

70 Hereditary Hemorrhagic Telangiectasia

MICHELLE LETARTE AND CLAIRE L. SHOVLIN

Hereditary hemorrhagic telangiectasia (HHT), also known as Rendu-Osler-Weber syndrome, was first recognized as a genetic disorder in the 19th century. HHT is inherited as an autosomal dominant trait, and its prevalence is now estimated at 1 in 5,000 people. Arteriovenous malformations (AVMs), representing direct connections between arteries and veins, are the distinguishing features of HHT. Small AVMs, or telangiectasia, give rise to frequent nosebleeds in most patients and to gastrointestinal (GI) bleeds in older individuals; they can also be present on lips, tongue, ears, and fingers and increase with age. Larger AVMs are found in internal organs, primarily in lungs, liver, and brain in a large number of patients, and can give rise to serious complications. Two genes are responsible for more than 80% of cases of HHT: *endoglin* (*ENG*) for HHT1 (OMIM #187300) and *ACVRL1 (activin receptor–like kinase 1,* or *ALK1)* for HHT2 (OMIM #600376). A third gene, *SMAD4* is mutated in 2% to 3% of HHT cases, co-presenting with juvenile polyposis (JP), a condition referred to as JPHT, OMIM #175050. These three genes code for members of the transforming growth factor-beta (TGF-β) superfamily involved in regulating multiple cellular functions. Endoglin and ALK1 are transmembrane proteins primarily expressed in the vascular endothelium, where they mediate the effects of specific TGF-β superfamily growth factors on endothelial function and vasomotor tone. Reduced functional expression of these specialized receptors leads to dysregulation of pathways essential for vascular homeostasis and the generation of AVMs.

CLINICAL MANIFESTATIONS AND DIAGNOSIS OF HHT

Clinical Manifestations of HHT

The spectrum of HHT encompasses multiple organ systems and within these, numerous forms of disease. Patients presenting to hematologists usually have iron deficiency anemia due to sustained and repeated nasal and/or GI bleeding, and are recognized by characteristic mucocutaneous telangiectasia (FIGURE 70.1A). The majority of HHT patients are also affected by pulmonary (~50%, FIGURE 70.1B) and/or hepatic (30% to 70%) AVMs, with cerebral and pancreatic AVMs affecting up to 20%.[1,2] These AVMs are characteristically silent unless detected by screening protocols. Non telangiectatic/AVM pathology in HHT includes pulmonary arterial hypertension[3] and pulmonary hypertension in the context of high-output cardiac failure secondary to hepatic AVMs,[2] JP,[4] and a prothrombotic state associated with elevated plasma levels of factor VIII.[5] Reduction in life expectancy has been observed in younger individuals.[6a,6b]

The spectrum of diseases associated with HHT has been reviewed recently.[2] In addition to age-related increases in the number of telangiectasia,[2,7] there are specific times in patients' lives when their HHT pathologies become more hazardous, the most important of which is pregnancy that results in a 1.0% risk of maternal death per pregnancy (due to pulmonary AVM [PAVM] hemorrhage, cerebral hemorrhage, and thrombotic complications), with all maternal deaths occurring in women previously considered well.[8a]

One of the emerging themes in HHT is the occurrence of pathologic thromboemboli in a condition that was formerly considered a purely hemorrhagic disorder.[8b] HHT patients are not protected from general venous thromboembolism (VTE) risks, and thromboembolic strokes affect a high proportion of HHT patients with PAVMs. Provision of anticoagulant/antiplatelet therapies in the setting of chronic HHT hemorrhage may therefore be required.

Diagnosis of HHT

The international consensus diagnostic criteria for HHT are nosebleeds, mucocutaneous telangiectasia, visceral involvement, and a family history[9] (Table 70.1). Anemia is not a criterion.[9] An individual has a clinical diagnosis of "definite HHT" if three criteria are present; "suspected HHT" if two are present; and "unlikely HHT" if only one is present.[9]

Clinical diagnosis is more difficult in younger individuals and those who are paucisymptomatic. Clinical diagnosis may be assisted by detection of visceral involvement either for management of clinical problems or as a result of screening protocols (see below). Outside of HHT centers, it is critical that non-HHT specialists be aware of potential pitfalls in diagnoses, particularly in inappropriate designation of a visceral vascular malformation that is not HHT associated as a diagnostic criterion (Table 70.1). It is also important that when patients receive a diagnosis of visceral AVMs, that they receive appropriate information about recommended management to reduce potential complications that extend far beyond hemorrhagic considerations: provision of such information may be challenging outside of specialized HHT units.

After passing through a clinical diagnostic screen, many patients will be left with a diagnosis of suspected or unlikely HHT. If the HHT mutation has been identified in their family, genetic testing can confirm or refute the diagnosis for the individual. Where a clinical diagnosis of HHT has been made, DNA testing is not required to "confirm" the diagnosis.

If a mutation is not found for a family where individuals have definite clinical HHT, the absence of a detected mutation should not affect the clinical diagnosis. Mutations are not found in approximately 15% to 20% of HHT families.[10–12] In addition, an estimated 10% to 20% of families have genetic variants of

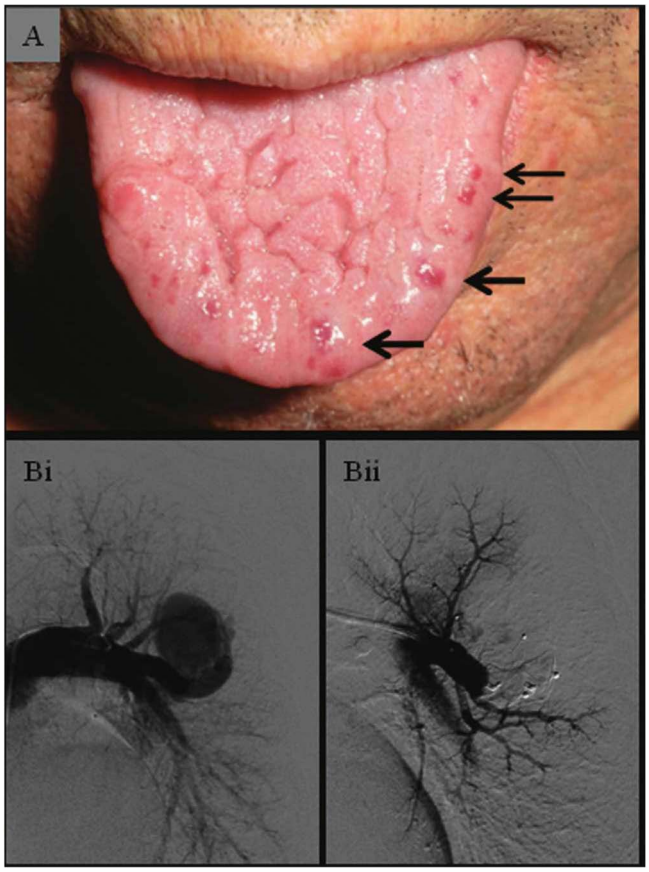

FIGURE 70.1 Clinical HHT. **A:** Tongue demonstrating typical florid telangiectasia. Note that these lesions increase with age and are often subtle or absent in younger individuals. **B(i):** Selective left pulmonary angiograms demonstrating large left lower lobe PAVM pretreatment, when it was associated with severe hypoxemia (SaO_2 85% at rest) and normal pulmonary artery pressure (27/9, mean 16 mm Hg). **B(ii):** Following embolization of separate feeding arteries using four Amplatzer plugs, the SaO_2 rose to 95% at rest, but the pulmonary artery pressure was unchanged (28/8, mean 15 mm Hg). *Angiograms courtesy of Dr James Jackson.*

uncertain significance, where a single-nucleotide change predicts an amino acid substitution that is not certain to sufficiently modify protein function to cause disease. Such "missense" changes have the potential to lead to misdiagnosis, as evidenced by families in which the alternate "real" mutation is also identified.[13,14]

Organ-Specific Screening Issues in HHT

Symptomatic patients will usually first undergo investigations performed by the relevant specialists. From a hematologic perspective, patients are often referred with iron deficiency anemia due to recurrent nasal and/or GI bleeding. In addition, a major component of management for the individual with suspected or proven HHT is screening, that is the investigation for a manifestation of HHT for which they were not previously aware, in order to improve their long-term health.

The international guidelines recommend clinical screens for pulmonary, cerebral, and hepatic AVMs, and genetic tests.[15] It is particularly in this context that overall management within, or in association with an HHT center, is recommended.[15] As discussed recently,[2] there are complex issues involved in screening for certain manifestations of HHT, particularly cerebral AVMs in which investigations and treatments carry substantial risks, and hepatic AVMs for which no treatments are currently recommended for asymptomatic patients. Assumptions that screening tests will be negative are misguided, and detailed prescreening discussions are advised.

Pulmonary

PAVM screening is recommended for all patients with possible or confirmed HHT, based on evidence of long-term efficacy of PAVM embolization resulting in improved oxygenation[15] and more recent evidence that PAVM embolization reduces stroke risk.[16] For sufficiently sensitive screening tests, the choice lies between thoracic CT scans and contrast echocardiography (CE), each of which, in dedicated HHT screening units, provides very high sensitivity as a first-line screen. The international guidelines committee recommended CE as the initial test, with 96% agreement,[15] but this may be reevaluated as the latest data manuscript calls into question the cost effectiveness of CE as the majority of contrast echocardiograms in HHT1 patients are positive, so the need for a subsequent CT scan may not be reduced.[17a] Conversely, in less experienced centers, if a negative echocardiographic study may be used to withhold a CT scan for an HHT patient undergoing PAVM screening, it is essential that methodologic reasons for a possible false negative are not overlooked.[17b] Where CT scans are ordered for PAVM screening, the rationale should be discussed with local radiologists, as use of standard protocols (CT-pulmonary angiogram and high-resolution CT scan) may miss clinically important PAVMs. This is because CT-pulmonary angiogram protocols do not image the lower part of the lungs, while high-resolution CT scans have substantial gaps between the thin slices imaged, and both can miss PAVMs.

Screening for pulmonary hypertension is not generally recommended, but for HHT patients whose impaired exercise tolerance and dyspnea appear to be out of proportion to the level of their anemia, consideration of this possible pathology is appropriate. First-line screening is by echocardiography.

Cerebral

The international guidelines recommend screening adults and children with possible or definite HHT by magnetic resonance imaging, followed by referral to centers with neurovascular expertise for invasive imaging.[15] This recommendation was made while recognizing the lack of evidence of treatment for asymptomatic individuals, and the risks of screening, stated as a 0.5% risk of permanent stroke per diagnostic angiogram.[15] However, the interpretation of the limited evidence base in HHT (as in the general population) differs between health care systems. In some countries therefore, screening is only recommended for patients who are considered at higher risk of cerebral hemorrhage, for example, in the setting of neurologic symptoms, or a family history of cerebral hemorrhage.

Hepatic

The guidelines recommend hepatic AVM screening using Doppler studies to assist diagnosis when there are fewer than three diagnostic criteria, and clinical genetics is unhelpful.[15]

Table 70.1

HHT diagnostic criteria

Curaçao Criterion	Diagnostic Issues	Specificity for HHT
1. Epistaxes (nosebleeds)	"Occur spontaneously on more than one occasion"[9]	Low[127] though likely to be enhanced if of higher severity[100]
2. Mucocutaneous telangiectasia	"Multiple, at characteristic sites—lips, oral cavity, fingers, nose"[9]	Not to be confused with actinic changes; spider naevi; Campbell de Morgan spots; or other cutaneous vascular lesions.[98,128]
3. Visceral lesions		
• *GI telangiectasia*	At endoscopy. With or without bleeding	Potential confusion with von Willebrands Disease, or other forms of angiodysplasia
• *Pulmonary AVM*	Diagnosis usually by CT or CE	Single PAVM may occur sporadically, and 8% of controls have a positive contrast echo[129] but HHT accounted for >90% of PAVM diagnoses in one center[130]
• *Hepatic AVM*		Full discussion of diagnostic issues in[131]
• *Cerebral AVM*		Does not include cavernous malformations or aneurysms (see[21,132] for spectrum)
• *Spinal AVM*		Spectrum of non-HHT disease discussed[133]
4. Family history	"A first degree relative with HHT according to these criteria"[9]	

New data suggest that where screening investigations have already identified abnormal liver function tests in an HHT family, Doppler studies may also be of value to predict a group with more severe disease who may require different follow-up regimes.[18]

Gastrointestinal

HHT patients with a *SMAD4* mutation, or a family history of GI cancer, require endoscopic investigations and management according to standard guidance for GI cancer screening in the non-HHT population.[19]

Specialized Screening Circumstances

Pregnancy

HHT centers have recommended for many years PAVM screening and treatment before pregnancy. Radiation assessment studies indicated that embolization may be undertaken during pregnancy,[20] and routine embolization in the later stages of pregnancy is performed in some centers where prepregnancy treatment was not feasible.[15] Spinal AVM screens form part of HHT pregnancy management in many units in order to facilitate the use of epidural analgesia if required during labor.[8a] No means of distinguishing a group of HHT women more likely to have significant pregnancy-related complications has been identified, and there are no data that specific screening or treatment modalities reduce pregnancy-associated risks. Importantly, prior awareness of the HHT diagnosis improves the outcome for women experiencing a life-threatening event, most likely by enhanced patients' awareness of significant symptoms, and obstetric preplanning in the event of major complications.[8a]

Children

The issues regarding screening of children are also controversial, as evidenced by the low rate of agreement for dedicated pediatric guideline recommendations.[15] There are clearly tragic cases of HHT-related deaths and disability in youngsters. Children with symptoms require specialist investigation and treatment guided by knowledge of HHT pathology. The most concerning pathology in childhood is a particular type of rare cerebrospinal arteriovenous fistula that appears to have a very high risk of hemorrhage, and high treatment-associated risks.[21] Current North American practice includes screening for these lesions from the age of 6 months, and repeating screens to capture AVMs that develop later in childhood.[21] Pediatric screening remains the matter of intense discussion in other health care systems.[2]

GENETICS OF HHT AND MOLECULAR DIAGNOSIS

HHT1 and HHT2

Mutations

ENG and *ACVRL1* mutations are responsible for >80% of cases of HHT. *ENG*, located on chromosome 9q34 and coding for the protein endoglin, was the first gene identified as mutated in HHT, resulting in HHT type 1 (HHT1).[22] *ACVRL1*, located on chromosome 12q31 encoding the ALK1 receptor, was then reported as mutated in HHT type 2 (HHT2).[23,24] Clinically, it is not possible to determine if a patient has HHT1 or HHT2, as there is extensive phenotypic heterogeneity among patients, even within the same family. A molecular diagnosis was developed and is now available in several countries, generally in association with

an HHT center, allowing for genetic counseling of the families, coordinated with patient care by expert physicians.

Results of research studies and diagnostic laboratories have led to the identification of hundreds of mutations in HHT patients.[10,25–35] The HHT mutation database (http://www.hht-mutation.org) currently lists 311 *ENG* mutations and 249 *ACVRL1* mutations. The *ENG* gene has 15 exons (including exons 9a and 9b), and mutations have been reported in all exons but 14, the latter coding for the intracellular region. All types of mutations are present in the *ENG* gene, with deletions and insertions (from single nucleotides to multiple exons, inframe, or causing a frameshift) accounting for the largest proportion. Single-nucleotide substitutions including missense, splice site, and nonsense mutations are also well represented (FIGURE 70.2). The *ACVRL1* gene has 10 exons, and the mutation frequency is highest in exons 3, 7, and 8. Single-nucleotide variants account for the majority with missense mutations being more numerous than stop codon and splice site mutations. Deletions and insertions are less frequent than in the *ENG* gene (FIGURE 70.2). Most mutations are unique but some have been reported in several families, such as in arginine codons 144, 374, 411, and 479 of *ACVRL1*. Therefore, mutation analysis requires the complete sequencing of both genes as well as the analysis of exon copy number.

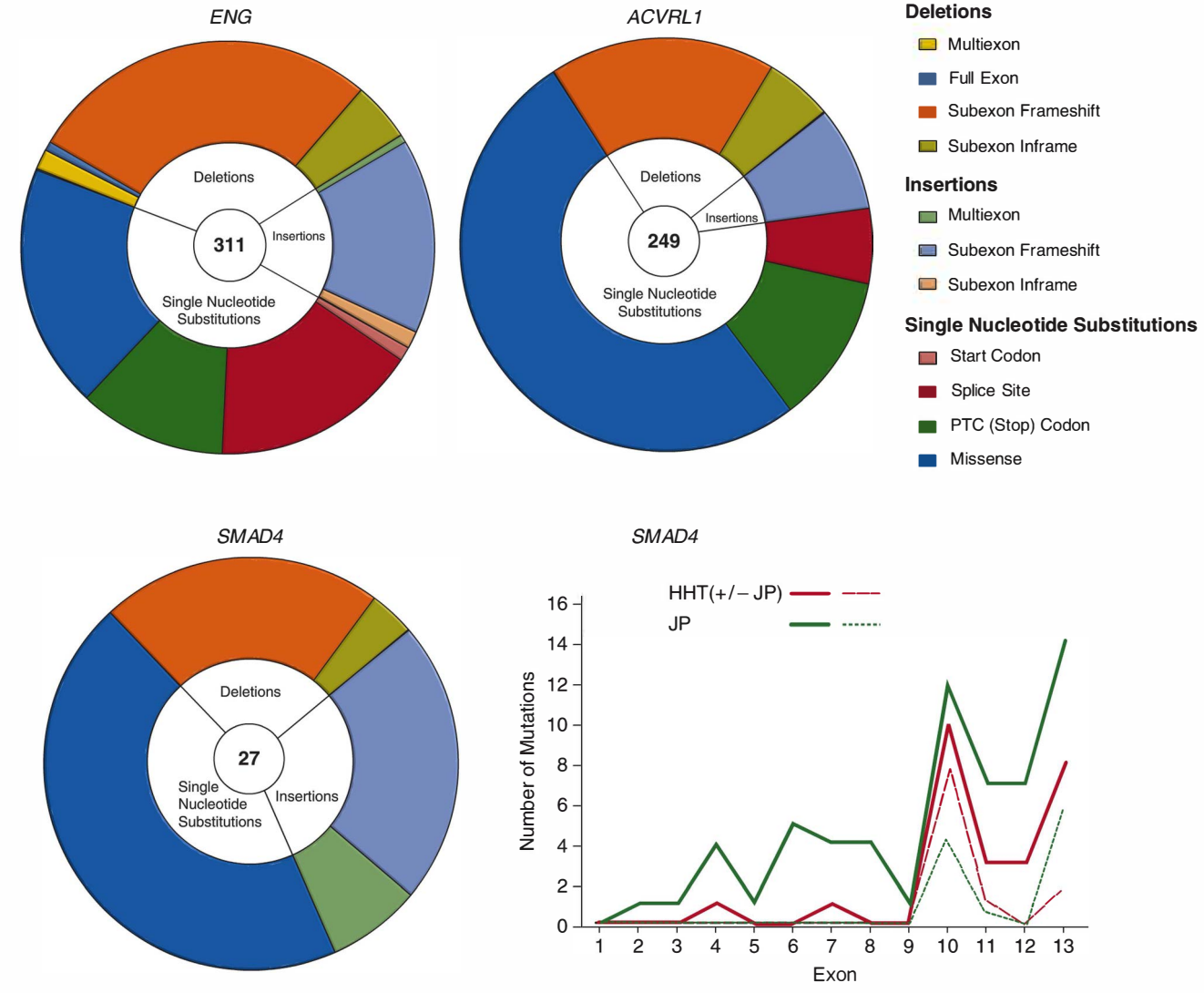

FIGURE 70.2 HHT mutations. Distribution of different types of mutations in *ENG*, *ACVRL1*, and *SMAD4* genes. PTC: premature termination codon generated directly, or by frameshift mutations. Note the higher proportion of missense single-nucleotide substitutions in *ACVRL1*. The graph illustrates the distribution of *SMAD4* mutations reported for HHT patients (*red lines*) and JP alone (*green lines*). Whereas for *ENG* and *ACVRL1*, mutations occur throughout the respective genes,[96] note the limited and highly comparable *SMAD4* mutational distribution for the two pathologies, for all mutations (*solid lines*), and missense substitutions affecting exons 10 to 13 (*dotted lines*). (Data derived from hht.mutation.org and Wooderchak WL, Zacary S, Crockett DK, et al. Repository of SMAD4 mutations: reference for genotype/phenotype correlation. *J Data Mining Genom Proteomics* 2010;1:101.) Ref.[126].

Mechanism of Disease

The underlying mechanism of HHT1 and HHT2 is haploinsufficiency, characterized by a lack of expression of the nonfunctional allele, as determined by protein quantification using metabolic labeling.[36,37] For example, in individuals with a molecular diagnosis of HHT1, the median level of endoglin was close to 50%, representing the normal allele only (in umbilical vein endothelial cells, median 45%, $n = 30$ newborns; peripheral blood–activated monocytes, median 48%, $n = 109$ patients).[31] In many cases, the absent protein will reflect absence of mRNA bearing the mutation due to nonsense-mediated decay of transcripts with premature termination codons.[38,39] The haploinsufficiency model is also supported by the distribution of mutations throughout both genes, deletions of the whole allele, and severity of the clinical phenotype not correlating with the type of mutation.

Genotype/Phenotype Correlations

Phenotypic studies in large groups of patients with identified HHT1 and HHT2 mutations indicate that the gene, rather than a particular mutation, influences the clinical phenotype.[10,40,41,42–44] The major findings in the multiple studies are that PAVMs are more frequent in HHT1 (ranging from 49% to 75%) than HHT2 patients (5% to 48%). Cerebral AVMs, despite an overall much lower occurrence, are also higher in HHT1 (8% to 20%) than HHT2 patients (0% to 2%). On the contrary, hepatic involvement, assessed by the presence of asymptomatic AVMs, is much higher in HHT2 than HHT1 patients.

JPHT

Mutations in the *SMAD4* gene, located on chromosome 18q21, are present in a subgroup of HHT patients (2% to 3%). Patients with *SMAD4* mutations, including those with HHT, are susceptible to JP.[4,45,46] Both JP and HHT are inherited as autosomal dominant traits with nonoverlapping clinical features, JP predisposing to GI tumors. *SMAD4* mutations associated with JPHT had been found mostly in the last four exons.[4,46] However, analysis of a larger number of patients has revealed mutations in other regions (FIGURE 70.2).[47] Thus, any JP patient with a *SMAD4* mutation is at risk for visceral manifestations of HHT and any HHT patient with such a mutation is at risk for early-onset GI cancer. Therefore, all patients with a *SMAD4* mutation should be monitored clinically for both HHT and JP.

HHT3

The large cohorts of patients analyzed for *ENG*, *ACVRL1*, and *SMAD4* mutations (including a search for large deletions and insertions not detected by sequencing) reveal up to 20% of patients with a convincing HHT clinical diagnosis have no identifiable mutation. This may be due in part to intronic mutations not resolved by current mutation detection strategies but also to additional genes that could be mutated in small subsets of HHT patients. Two loci have been reported: HHT3 on chromosome 5q31[48] and HHT4 on chromosome 7p14.[49] The HHT3 locus was identified in a single family with classical HHT, including PAVMs. The interval on chromosome 5 has recently been refined to 26 genes (including 10 pseudogenes) with fibroblast growth factor-1 and PPP2R2B at the centromeric and telomeric ends, respectively.[50] Both of these genes are relevant to angiogenesis and TGF-β signaling and remain candidates for HHT3; three genes in the interval are associated with known genetic disorders not related to HHT, leaving 11 other genes with no association with genetic disorders and no involvement in angiogenesis or TGF-β signaling.

HHT4

The locus for HHT4 maps to a 7-Mb region on the short arm of chromosome 7. It was identified in a four-generation family where PAVMs were detected in 50% of the 16 clinically affected individuals, who had fewer nosebleeds and telangiectasia than classical HHT patients.[49] This region covers 47 known genes, including seven implicated in vasculogenesis. However, DNA sequencing in the affected patients failed to reveal mutations in these genes. A strong candidate remains BMPER, expressed in endothelial cell precursors and which regulates duration and signaling by bone morphogenetic proteins (BMPs), having both pro- and anti-BMP activities.[51,52]

The identification of additional families with HHT3 and HHT4 should help in narrowing the loci and identify the relevant genes, their role in endothelial function, and their relationship to endoglin and ALK1.

MOLECULAR BASIS OF HHT

Endoglin and ALK1 Are Essential for Development of the Vascular System as Revealed by Mouse Models of Disease

Genetically modified mice that lack either endoglin or ALK1 are embryonic lethal at midgestation and display abnormalities in blood vessel development and maturation. *Eng* null mice die with numerous lesions in the cardiovascular tree including incomplete yolk sac vessel branching and remodeling, vessel dilation, hemorrhage, and abnormal cardiac morphogenesis.[53,54,55] *Alk1* null mice show severe vascular abnormalities such as hyperdilation of large vessels, excessive fusion of capillaries and of arteries and veins, and reduced recruitment of smooth muscle cells.[56,57] Endothelial-specific deletion of *Eng* or *Alk1* leads to AVM-like structures in several organs; furthermore, tissue wounding promoted the formation of AVMs in these *Alk1* mice.[58] Conditional knockdown of *Eng* led to the formation of AVMs in the retina, which were amplified by applying an angiogenic stimulus.[59] These findings support the concept that in HHT, expression of a single *ENG* or *ALK1* allele causes endothelial dysfunction that predisposes to AVM development upon exposure to a second hit such as wounding, increased blood flow, or angiogenic stimulus.

Heterozygous mouse models of disease have been generated. The $Eng^{+/-}$ mouse, particularly in early backcrosses, showed HHT-like clinical manifestations including nose and ear bleeds, hepatic and GI telangiectasia, stroke, and even cerebral AVMs.[60–62] Signs of HHT also develop in aged $Alk1^{+/-}$ mice.[63] In keeping with the notion of a second hit, injection of adenovirus-derived vascular endothelial growth factor (VEGF) in the brain of $Eng^{+/-}$ or $Alk1^{+/-}$ mice led to capillary dysplasia, but had no effect in control mice.[64,65] Furthermore, increasing cerebral blood flow using vasodilators had a synergistic effect on the VEGF stimulus, further promoting capillary dysplasia in genetically predisposed mice.[65]

Role of ALK1 and Endoglin in Mediating TGF-β Superfamily Signaling: Implications for HHT

There are numerous ligands of the TGF-β superfamily. In terms of HHT, the most relevant ligands are TGF-β1, TGF-β3, and BMP9. Therefore, our discussion is restricted to these growth factors and their receptors in endothelial cells. In most cell types, TGF-β mediates its effects through plasma membrane serine threonine kinase receptors type I (ALK5) and type II (TβRII) and the downstream canonical Smad pathway (FIGURE 70.3). TGF-β binds to the constitutively active TβRII, which phosphorylates ALK5 and leads to activation of Smad2 or Smad3. These so-called receptor Smads then recruit the common Smad4 and shuffle to the nucleus where they act as transcription factors to regulate expression of multiple genes. TGF-β generally inhibits endothelial cell proliferation and migration and regulates extracellular matrix synthesis, thereby maintaining quiescence and integrity of the vascular endothelium, via the ALK5 pathway.[66] Endothelial cells also express another TGF-β type I receptor, ALK1, which acts through a different set of receptor Smads, namely, Smads 1 and 5. These activated Smads also associate with the common Smad4 and shuffle to the nucleus where they regulate a different set of genes (FIGURE 70.3).

ALK1

The role of ALK1 in TGF-β superfamily signaling is still somewhat controversial. When first discovered, it was proposed as a receptor for TGF-β1.[67,68] ALK1 has been implicated in endothelial cell proliferation and migration and secondary formation of new blood vessels. In addition to angiogenesis, ALK1 also promotes vessel maturation via recruitment of pericytes and smooth muscle cells.[56,57,69] Two recent papers suggest that ALK1 stabilizes new blood vessels in human xenograft tumors implanted in mice and that targeting ALK1 reduces tumor growth and potentiates other antiangiogenic therapies.[70,71] Therefore, ALK1 and ALK5 have opposite effects on angiogenesis, and to attribute them to TGF-β1 responses, one has to argue that they are mediated by a different Smad pathway or that ALK1 directly inhibits the ALK5 pathway[66] (FIGURE 70.3). It was also reported that ALK5 is required for efficient TGF-β signaling via ALK1 and that the ratio of these two receptors on endothelial cells may determine whether TGF-β maintains quiescence or stimulates angiogenesis.[72] However, comparing the phenotypes of mice in which ALK1, ALK5, or TβR2 genes were conditionally deleted in the vascular endothelium revealed that only ALK1 deletion led to the formation of AVM-like structures.[73] These data suggest that ALK5 and TβR2 are not required for ALK1 signaling pertinent to the pathogenesis of HHT and that TGF-β signaling may be less relevant for this disease than previously thought.[73]

The observation that deficiency of TβR2 does not phenocopy that of ALK1 suggests that a non-TGF-β-ligand signals through ALK1 and/or that ALK1 interacts with another co-receptor. Indeed, recent studies have identified circulating BMP9 as a true physiologic ligand for ALK1,[74,75] raising the possibility that ALK1 haploinsufficiency as observed in HHT2, may be associated with defective BMP9 signaling. ALK1 binds BMP9 with high affinity, in the context of the BMP type II receptor (BMPRII) or activin type IIA receptor (ActRIIA), and triggers Smad1 and

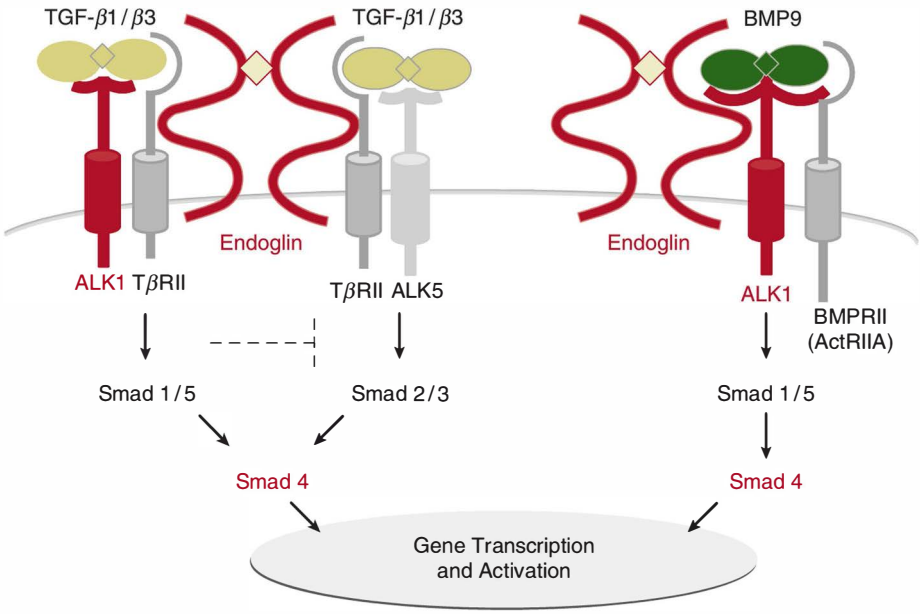

FIGURE 70.3 Role of ALK1 and endoglin in signaling for TGF-β and BMP9. In endothelial cells, TGF-β binds to TβRII, which then phosphorylates either ALK5 (the main type I receptor for this ligand) or ALK1 leading to Smad2,3 and Smad1,5 phosphorylation, respectively. These receptor Smads then interact with the common Smad4 and shuffle to the nucleus where they regulate gene transcription leading to multiple cellular responses. The ALK1 pathway is thought to inhibit the ALK5 pathway. Endoglin binds TGF-β1 and TGF-β3 isoforms in association with TβRII, thereby modulating responses. BMP9 binds ALK1 with high affinity, and its receptor complex includes either BMPRII or ActRIIA. Endoglin likely interacts more strongly with BMP9; it also binds ALK1. The BMP9 pathway is probably the pathway most affected in HHT.

Smad5 phosphorylation and transcriptional activation of several downstream genes[74–76] (FIGURE 70.3).

Endoglin

Endoglin (also referred to as CD105) is an integral transmembrane glycoprotein that was first identified in childhood ALL.[77] It was subsequently shown to be expressed in endothelial cells,[78,79] where it serves as an auxiliary receptor, or coreceptor, for TGF-β1 and TGF-β3,[80] as well as other members of the TGF-β superfamily.[81] It does not bind these growth factors on its own but via interaction with the ligand-binding receptor, and modulates multiple cellular responses. It interacts with TβRII, the receptor for TGF-β1 and TGF-β3, thereby affecting ALK5 signaling and Smad2,3 downstream pathways (FIGURE 70.3). It also forms complexes with ALK1 and promotes its signaling via the Smad1,5 pathways.[82,83]

Interestingly, when endoglin is transiently overexpressed, it can bind BMP9 in the absence of ALK1 and potentiate BMP9 responses.[75,76] Endoglin associates with ALK1 and BMP9 and mediates signaling in the presence of a type II receptor (either BMPRII or ActRIIA) (FIGURE 70.3). The observation that ALK1 and endoglin both interact with BMP9 with an apparent affinity higher than for TGF-β implicates a defective BMP9 pathway in HHT.

Endoglin has been studied more extensively in the context of its modulation of TGF-β-dependent cellular responses, as recently reviewed.[84] It generally opposes the TGF-β-induced inhibition of cell proliferation and adhesion, and the increased expression of extracellular matrix proteins. As recently reviewed,[85,86] endoglin plays an important role in angiogenesis, vascular development, and repair, and is implicated in several disorders, including HHT and preeclampsia. Endoglin is generally more abundant in proliferating endothelial cells and used as a functional marker of angiogenesis in human tumors, its level on the microvascular endothelium often correlating with a poor prognosis.[87]

A role for endoglin in the regulation of vascular tone has been identified.[88,89] Endoglin is localized to caveolae, where it binds endothelial nitric oxide (NO) synthase (eNOS). It acts as a scaffold protein, bringing the chaperone Hsp90 in close proximity with eNOS (FIGURE 70.4).[89] More recently, we have shown that ALK1 also interacts with eNOS.[90] TGF-β can increase eNOS expression[91,92] and stimulate dephosphorylation of eNOS at a specific site important for enzyme activation, leading to increased NO production.[90,93] We observed that in endoglin-deficient endothelial cells, the eNOS activation complex is impaired, producing superoxide instead of NO, due to an uncoupled state of the enzyme (FIGURE 70.4).[89,90] An eNOS-dependent rise in superoxide is also observed in the lungs of adult *Eng*$^{+/-}$ and *Alk1*$^{+/-}$ mice but not in 3-week-old mice.[90,94,95] *Eng*$^{+/-}$ mice also display an eNOS-dependent impairment in the myogenic response of resistance arteries.[89] Interestingly, treatment of *Eng*$^{+/-}$ and *Alk1*$^{+/-}$ mice with the antioxidant Tempol from the age of 3 weeks prevents the abnormal remodeling of the lung vasculature observed in the adult heterozygous mice.[90,95]

Thus, it appears that a major role of endoglin and ALK1 is to bring together TGF-β superfamily complexes and eNOS activation complex. Failure to do so leads to impaired vascular tone perhaps in response to an increase in blood flow, or an angiogenic stimulus, causing abnormally dilated capillaries, and eventually AVMs.

TREATMENT OF HHT

In HHT, management practice is guided by International Guidelines based on systematic assessments of HHT publications up to October 2006.[15] Thirty-three recommendations were

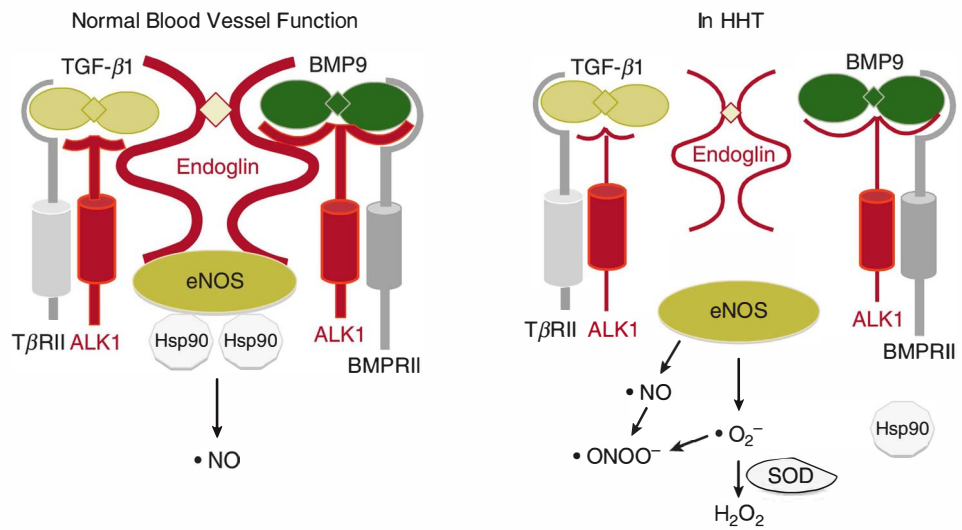

FIGURE 70.4 Model of the interaction of TGF-β and BPM9 receptor complexes with the eNOS activation complex in endothelial cells under normal conditions and in HHT. Endoglin and ALK1 can both associate with eNOS, in the caveolae of the endothelial cell and stabilize the enzyme during its activation.[89,90] In doing so, they can bring the TGF-β1 or BMP9 receptor complexes to eNOS and regulate NO production. Under normal circumstances, eNOS produces NO, which stimulates vasodilation. In HHT1 and HHT2, less endoglin and ALK1 (shown by *thinner molecules*) destabilize the eNOS complex, which generates reactive oxygen species that lead to vessel damage.

made based on a review process involving multiple experts.[15] The 2007 to 2010 data manuscripts and their recommendations are discussed in recent review articles,[96,97] but formal guidelines incorporating the post-2006 evidence base are not yet available.

Hemorrhage

The abnormal HHT vessels in HHT are prone to bleeding because of their inadequate blood vessel wall structures and high perfusion pressures.[2,98] At least one-third of HHT patients have iron deficiency anemia, with the proportion of patients on iron treatments increasing with age.[2]

Nosebleeds

Nosebleeds are a more common cause of blood loss in HHT than GI bleeding, but may not be readily stated by the patient as a clinical problem. Recommended strategies for acute management of nosebleeds include simple first aid and packing.[15] For recurrent nosebleeds, nasal humidification regimens and topical lubricants are recommended first line.[15] Dedicated expert ENT evaluation is recommended for second-line treatment (laser therapy), and third-line treatments such as septal dermatoplasty or Young's procedure.[15] The invasiveness of treatment should be proportionate to the severity of nosebleeds, with transfusion dependence suggested as a cutoff to indicate patients for whom third-line therapies may be appropriate.[99] A validated epistaxis severity score has been developed and found to be a significant predictor of invasiveness of therapy for nosebleeds.[100] Trials of topical and systemic treatments are currently recruiting (http://clinicaltrials.gov)

Gastrointestinal Bleeding

GI investigations are recommended if anemia is out of proportion to nosebleeds severity.[15] Numerous GI telangiectasias are identified in HHT patients, and evidence of bleeding (visible bleeding or associated thrombus) may help guide decisions regarding ablation treatments to specific lesions. HHT patients require more endoscopic treatments than non-HHT patients,[101,102] but the recent guidelines, recognizing procedural risks and limitations, cautioned against multiple procedures.[15]

Other Factors Contributing to Iron Deficiency Anemia

When evaluating patients with iron deficiency anemia, it is also important to consider non-HHT causes of blood losses such as menstruation and coincident (rare) coagulation disorders, as well as iron dietary intake. Patients should seek to meet more than the recommended daily allowance of dietary iron, in addition to using iron replacement strategies, transfusions if required, and specific HHT treatments outlined above. There are settings in which anticoagulants (and/or antiplatelet agents) are required in HHT [8b,15] in order to prevent major ischemic or thromboembolic sequelae. Prophylactic dose anticoagulation, for example, is required during high-risk periods for VTE, particularly for HHT patients hospitalized with PAVM-induced brain abscess.[5] Where a VTE occurs, treatment dose warfarin and heparin can be given, and in our experience, anticoagulation is tolerated surprisingly well by many patients.[2]

Arteriovenous Malformations

Ablative treatments for AVMs are beyond the scope of this chapter, but are mentioned briefly for completeness. PAVMs of any size amenable to embolization should be considered for this procedure,[15] as PAVMs below the former cutoff size of 3 mm have been associated with brain abscess and stroke.[16] The Amplatzer devices are now most commonly employed as they have more favorable profiles than the embolization coils and balloons utilized in earlier series.[103,104] Antibiotic prophylaxis is still recommended despite changes in recommendations for cardiac patients at risk of endocarditis.[105] Avoidance of intravenous air and scuba diving has also been recommended.[15] Cerebral AVM management involves complex decision making in selecting the most appropriate strategy for each patient, ranging from no therapy, through embolization,[21] microsurgery,[106] and stereotactic radiotherapy.[107] Multimodality treatments are often utilized.[108]

Hepatic AVMs that are asymptomatic are not treated, but due to the risk of catastrophic hemorrhage from hepatic AVM puncture, liver biopsies should be avoided in people with suspected or proven HHT.[15,109] For hepatic AVM patients who become symptomatic and fail to respond to intensive medical treatments, liver transplantation is the treatment of choice, with no role for hepatic artery embolization unless supported by a transplant program.[15] Excellent 10-year results are reported by the European liver transplant registry.[110]

General HHT Vascular Targeting Strategies

Hormonal Therapies

To date, the only randomized control trial (RCT) to prove efficacy in treatment of HHT bleeding relates to use of hormonal manipulations. The first RCT reported the use of high-dose estrogen–progesterone,[111] but it is poorly tolerated particularly in men, and there are concerns about thrombotic side effects. More recently, a double-blind, placebo-controlled trial of the antiestrogen tamoxifen[112] demonstrated a significant reduction in the frequency of epistaxis in the treated group, accompanied in many cases by either a rise in hemoglobin or reduction in transfusion requirements. The selective estrogen receptor modulator raloxifene[113] introduced to reduce the risks of tamoxifen side effects, particularly uterine hyperplasia, has recently been approved as an orphan drug for HHT. The mechanisms by which hormonal therapies act are incompletely understood. They are likely to include prothrombogenic effects (noting that pathologic thromboemboli are a recognized side effect of all such agents[114]) but there may also be direct modulation of endothelial cell and vascular function.[113]

Antifibrinolytics and Prothrombotic Agents

Therapeutic manipulation of coagulation and fibrinolytic pathways[115–118] has not yet been supported by data from RCTs. Recognition that venous thromboses occur in HHT, associated in many cases with coincidental inheritance of prothrombotic genetic variants such as FV Leiden, has raised concern regarding thrombophilic risk with these agents.[5] Routine measurement of FVIII, FV Leiden, and other thrombophilic markers in HHT patient assessments may assist individualized risk–benefit considerations before prothrombogenic systemic treatments are given.[5,96]

Antioxidants

There are recent uncontrolled short-term data demonstrating efficacy from oral *N*-acetylcysteine in a modest series of 43 HHT patients.[119] This specific drug is not currently licensed in many

countries but in general, antioxidants have favorable side effect profiles during long-term use.

Thalidomide

Thalidomide was considered in HHT following a chance observation in a patient undergoing treatment for cancer.[120] While efficacy in the clinical setting has not been substantiated (case reports[121] and small series,[122] have been published, but no RCTs), recent data highlight the potential for this type of drug to rescue the murine HHT phenotype.[122] Thalidomide appears to target mural cell recruitment to angiogenic sprouts, by increasing expression of platelet-derived growth factor (PDGF)-B at the endothelial tip cell, facilitating recruitment of pericytes that express PDGF receptor, increasing pericyte proliferation, and normalizing excessive sprouting in mice with HHT.[122]

Bevacizumab

Bevacizumab a recombinant full-length humanized antibody active against all isoforms of VEGF-A was also considered for HHT patients based on a chance observation in a patient with HHT undergoing treatment for malignancy.[123] A widely cited brief report of a patient whose hepatic AVMs initially responded[124a] has led to an encouraging trial reporting benefit in France in the setting of end-stage hepatic AVMs,[124b] though caution regarding side effects have been expressed,[125] and the results of RCTs are awaited. Topical approaches are being used to reduce the serious side effects associated with systemic treatments.

CONCLUSIONS

HHT is a vascular disorder affecting many organs, caused mostly by mutations in *ENG* and *ACVRL1* genes, coding for receptors of the TGF-β superfamily, and predisposing HHT patients to telangiectasia and AVMs and their associated clinical manifestations. A second hit in the form of an angiogenic stimulus such as VEGF, an increase in blood flow, or wounding is likely necessary to precipitate the vascular lesions. Although TGF-β plays an important role in regulating vascular proliferation, migration, and remodeling, we believe that BMP9, the physiologic ligand for ALK1, which also interacts with endoglin, is more relevant to HHT. Future studies will no doubt focus on this ligand and its role in endothelial function and vascular disorders. An emerging role of ALK1 and endoglin is their interaction with the eNOS activation complex, which implies that specific ligands of the TGF-β superfamily regulate NO production and vascular tone and that oxidative stress contributes to HHT.

The primary role of a hematologist reviewing a patient with known or suspected HHT is usually optimizing management of iron deficiency anemia, particularly for patients who are transfusion dependent, and/or under consideration for anticoagulant and antiplatelet therapies. Recognition of the crucial roles of ENT surgeons and gastroenterologists needs to be supplemented with knowledge of the emerging disease-modifying pharmaceutical agents. Clinical diagnostic and management pathways extend far beyond this particular specialty. Screening patients and their family members for other aspects of HHT may appear feasible, since the required investigative modalities are available in most institutions. The challenges arise, however, not with the ordering of these tests, but with the ability to provide informed pre- and post-test counseling in a field where evidence is still emerging. It is for this reason that referral to dedicated HHT centers is recommended.

Nevertheless, a striking observation for this disorder that so commonly presents with a chronic hemorrhagic state is that by 2012, only one of the 35 HHT Centers of Excellence was run by a hematologist. Since hematologic expertise is so critical for the long-term management of these complex patients and families, it is hoped that the specialty's interest in the wider aspects of HHT will increase.

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CHAPTER

71

Arteriovenous Malformations and Hemangiomas and Cerebral Cavernous Malformations

KEVIN J. WHITEHEAD, DOUGLAS A. MARCHUK, AND MARK L. KAHN

The cardiovascular system is organized into a hierarchical, branching arrangement by which blood is distributed to, and collected from, organs through arteries, arterioles, capillaries, venules, and veins.[1] This arrangement is not merely an elegant example of biologic patterning; it is also essential for physiologic control of blood flow throughout the body. Loss of the normal vascular architecture may result in localized or generalized vascular malformations in humans.[2] Vascular malformations are descriptively categorized on the basis of the type of vessel that appears to be involved, for example, arterial, venous, or capillary. Most common vascular malformations, for example, arteriovenous malformations (AVMs) in which vascular connections link arteries and veins without an intervening capillary bed, arise spontaneously in affected individuals, but others arise in a heritable pattern that indicates contribution by germline mutations (e.g., cerebral cavernous malformation [CCM] and hereditary hemorrhagic telangiectasia [HHT], discussed further below).

Although many vascular malformations are not symptomatic, vascular malformations constitute an important component of pediatric and adult vascular disease. These vascular structures are fragile and frequently cause clinical disease due to hemorrhage in vulnerable sites such as the central nervous system (CNS) or the gastrointestinal (GI) tract. AVMs also come to clinical attention due to the cardiovascular effects of blood shunting such as high-output congestive heart failure. Since vascular malformations frequently arise in the skin, they are also an important component of dermatologic disease. The treatment of vascular malformations has improved in the past two decades but remains restricted to surgical and interventional vascular approaches to either remove them or block blood flow through them.

The lack of molecular pharmacologic therapies for vascular malformations reflects the fact that until very recently the pathogenesis of such lesions was virtually unknown. However, in the past decade, genetic studies of inherited human vascular diseases have intersected with developmental studies using model vertebrate organisms such as fish and mice to provide new molecular insights into the molecular and genetic basis for vascular malformations. These studies have revealed that many human vascular malformations arise because of loss of regulatory signaling pathways that participate in the development of the blood and lymphatic vascular systems. They have also produced useful animal models of human vascular malformations. Thus, there is hope that the new knowledge and biologic tools will enable the development of pharmacologic approaches to treat human vascular malformations. This chapter focuses on two common inherited vascular malformations, CCM and HHT (FIGURE 71.1).

CEREBRAL CAVERNOUS MALFORMATIONS

Clinical Presentation and Treatment

CCMs are thin-walled, dilated vascular malformations that present primarily in the CNS in early to mid adulthood.[3,4] The prevalence of CCM in the general population is relatively high, 0.1% to 0.5%, making this malformation a relatively common condition. While the clinical symptoms associated with CCMs vary with the size and location of the malformation, seizures, headache, and stroke with associated neurologic deficits are the most common. CCMs are diagnosed by MRI, and more sophisticated imaging technologies have revealed that a majority of these lesions are clinically silent. Although the number of longitudinal studies is limited, existing studies indicate that in the case of familial CCM, both the number and size of CCMs increase over time.[5] Presently, the treatment of CCMs is neurosurgical resection of symptomatic lesions. Histologic analysis of resected CCMs reveals vascular lesions with a thin endothelial lining that by transmission electron microscopy lack the typical number and quality of tight junctions between endothelial cells,[6] an ultrastructural feature that has been valuable for comparison to animal models.

Genetics of CCM

Approximately one third of CCMs arise in patients with a family history of this disease.[4] Familial CCMs are transmitted in an autosomal dominant pattern and are both more numerous and symptomatic than nonfamilial CCMs. Positional cloning studies have identified germline heterozygous loss-of-function mutations in three genes: KRIT1 (aka CCM1),[7–9] CCM2,[10,11] and PDCD10 (aka CCM3)[12] that encode intracellular adaptor proteins without enzymatic activity. The fact that CCMs arise in older individuals who are heterozygous for a loss of function allele has led to the hypothesis that these lesions are created by a "second hit" that confers a complete loss of function in the causal cell type. Surgically resected lesions from individuals harboring germline CCM mutations have revealed second site mutations in the remaining wild-type copy of the relevant CCM gene, lending support to the two-hit mutation mechanism for CCM pathogenesis.[13–15] Recent studies of mouse models also

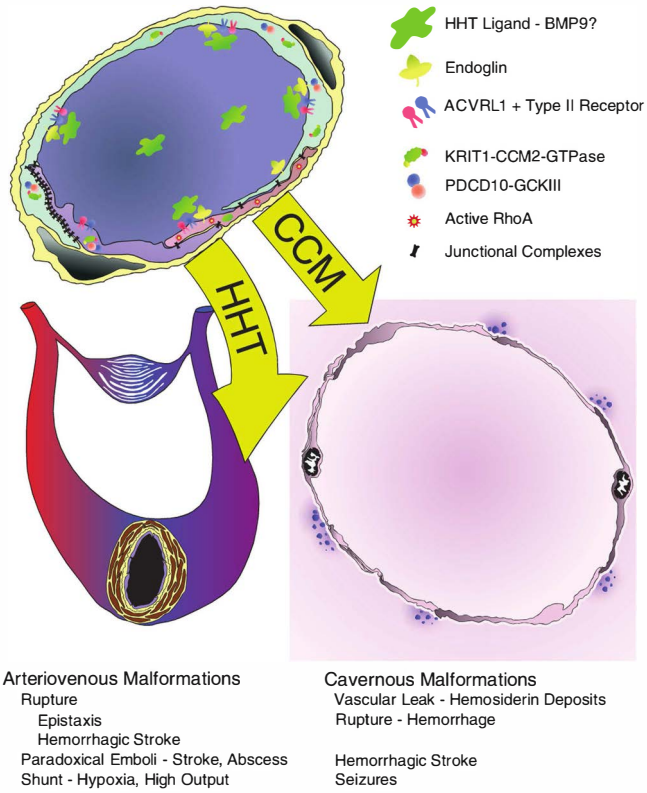

FIGURE 71.1 Pathogenesis of HHT and CCM vascular malformations. The endothelial cell signaling pathways that are affected in the pathogenesis of HHT and CCM are shown above. The resulting vascular malformations and their clinical manifestations are shown below.

support the two-hit mechanism and implicate the endothelial cell as the causal cell type.

Biology of the CCM Signaling Pathway

Genetic studies of mice and zebrafish have revealed that the CCM proteins function in a novel signaling pathway that is required for vertebrate cardiovascular development.[16,17–20,21–25,26] Despite the vascular phenotypes exhibited by deficient animals and humans, expression studies in mice have revealed that CCM1, CCM2, and CCM3 are broadly expressed genes and are particularly highly expressed in the CNS, a finding that has led to the hypothesis that this pathway regulates vascular function indirectly through signaling in neural cell types. Mice constitutively deficient in the CCM proteins die soon after the heart and vessels are formed due to defects in the formation of early vascular structures such as the branchial arch arteries that connect the heart to the aorta in the early embryo, so that these animals do not have circulating blood. Deficient zebrafish embryos exhibit similar branchial arch arterial defects, as well as a "dilated heart" phenotype conferred by deficiency of the type I transmembrane protein heart of glass (HEG).[23] Finally, biochemical studies have established that HEG, CCM1, CCM2, and CCM3 form a molecular signaling complex in which the HEG cytoplasmic tail binds CCM1, the CCM2 phosphotyrosine binding domain binds CCM1, and CCM3 binds CCM2 through an as yet unidentified mechanism.[23,27–29]

Electron microscopy of human CCMs, as well as studies in cultured endothelial cells and deficient mice and zebrafish suggest that one important endpoint of CCM signaling is positive regulation of endothelial cell–cell junctions.[6,21,23,25,30,31] However, precisely how CCM signaling regulates endothelial junctions is not completely understood. Two effectors of CCM signaling are RAP1 and RHO, proteins known to regulate cell junctions. RAP1 binds to the CCM1 FERM (4.1 protein, Ezrin, Radixin, and Moesin) domain and it has been proposed that CCM signaling is linked to RAP1 activity and localization in endothelial cells.[30,32] Several groups have demonstrated that loss of CCM proteins results in elevated levels of RHO-GTP and loosening of cellular junctions in cultured endothelial cells.[21,25,31,33,34] Thus, negative regulation of RHO may be a mechanism by which the CCM pathway regulates cell junctions. Biochemical studies and genetic studies in zebrafish have identified the GCK-III family of serine/threonine kinases as potential CCM3 binding and signaling partners *in vivo* that may regulate RHO and endothelial cell junctions.[19,25] In addition to regulating cell junctions, the CCM pathway may also maintain endothelial cell polarity and the formation of endothelial tubes.[35] Thus, the available evidence strongly supports a role for CCM signaling in positively regulating endothelial cell junctions and hint at other roles that may be connected to vessel formation and function.

CCM Pathogenesis: Insights from Animal Models

Sequence conservation and observations that zebrafish and mice lacking CCM proteins exhibit similar vascular phenotypes have suggested that the function of this pathway might be sufficiently conserved to permit in-depth investigation of human disease using genetically modified animals. Indeed, genetic studies using animal models have played a pivotal role in elucidating both the function of the CCM signaling proteins *in vivo* and the pathogenesis of human CCMs. Analysis of deficient zebrafish and mice have revealed that CCM signaling is required in vertebrates for early cardiovascular development,[16,17,23,25] a result that suggested a direct role in cardiovascular cell types such as endothelial cells. However, none of the CCM disease genes are highly or specifically expressed in cardiovascular cell types. Therefore, an early question was whether CCM proteins are required in vascular cell types such as endothelial cells or pericytes, or whether vascular CCMs arise due to loss of CCM signaling in adjacent tissues such as neural cells. Recent studies of mice in which CCM2 and CCM3 were conditionally deleted in neural, endothelial, and smooth muscle cells revealed that the defects in cardiovascular development observed in globally deficient animals arose because endothelial cells require these proteins.[21,24] These studies, as well as genetic studies of endothelial cells isolated from human CCMs, strongly suggest that human CCMs arise due to loss of this pathway in endothelial cells. The recent generation of mice carrying conditional CCM alleles has allowed this hypothesis to be directly tested using transgenic animals in which Cre recombinase can be inducibly expressed in endothelial cells and other cell types. However, results of these studies are controversial. It has recently been reported that deletion of CCM2 in endothelial cells after birth does indeed result in the formation of CCMs in the brain and retina,[22] providing strong evidence that human CCMs arise from loss of CCM function in endothelial

cells. However, deletion of CCM3 in neuroglia has also been reported to result in the formation of CCM in mice.[36] At this time, the weight of evidence favors an endothelial cell mechanism, but further studies addressing the cell specificity of CCM protein deletion in these studies will be required to definitively answer this question.

CCM Disease Mechanism: Future Directions and Unanswered Questions

Although research over the past decade has provided important insights into the genetic and molecular basis of human CCMs, it now seems likely that CCMs arise from loss of a previously unrecognized endothelial signaling pathway (SEE FIGURE 71.1). Nonetheless, numerous questions regarding the pathway and disease pathogenesis remain to be addressed. Loss of function phenotypes in mice and men reveals a remarkable conservation of function for CCM signaling, but why vascular malformations arise specifically in the CNS after deletion of endothelial CCM2 in all tissues in animal models and after stochastic endothelial loss of CCM proteins in humans is not clear. It seems likely that the CNS environment is one in which CCM signaling must be maintained at a higher level relative to other vascular beds. This could be related to maintenance of the blood–brain barrier and a role for CCM signaling in regulating endothelial cell junctions, but experiments to address this hypothesis have not yet been performed.

A clearer understanding of the molecular components of the CCM signaling pathway is also needed. Genetic and biochemical studies place the HEG receptor in the same pathway as CCM1, CCM2, and CCM3,[23] but HEG mutations have not been identified as a cause of human CCM. The identity of the HEG ligand will provide a major clue to the regulation of this pathway and its biologic function. Similarly, the nature of the downstream CCM signals required to prevent vascular malformations remain to be defined. Recent studies implicate the GCK-III family of serine/threonine kinases as downstream effectors of CCM signaling,[25] but their role in disease pathogenesis, like that of HEG, remains untested. Studies also implicate RAP1[30,32] and MEKK3[37,38] as CCM signaling effectors, but the role of these proteins in disease pathogenesis is not yet clear.

A major goal of future studies will be to use recently generated mouse models to better define the role of molecular CCM pathway components in disease pathogenesis, and to test potential therapeutic strategies. Potential therapeutic agents such as statins and fasudil that alter the levels of RHO-GTP can now be tested in animal models of CCM disease.[31] Moving from basic mechanisms of disease to the development of novel human therapies with these animal models will be very exciting and hopefully establish an important new paradigm for understanding and treating vascular diseases.

HEREDITARY HEMORRHAGIC TELANGIECTASIA

Clinical Presentation and Treatment

HHT is a syndrome characterized by hemorrhage-prone vascular AVMs that was recognized by the French physician, Henri Rendu,[39] as distinct from hemophilia in 1896. Sir William Osler and Frederick Parkes Weber then defined the clinical syndrome in the early 1900s,[40,41] accounting for the eponym, Osler-Weber-Rendu disease. The hallmark of HHT is telangiectasias representing abnormal connections between arterioles and venules that bypass intervening capillary beds and appear as small erythematous macules that blanch upon pressure as flow in the dilated channel is transiently occluded. Larger arteriovenous connections also occur with important clinical consequences. A diagnosis of HHT can be made on clinical grounds by the presence of at least three of the "Curaçao criteria": (a) a history of HHT in a first degree relative, (b) recurrent spontaneous epistaxis, (c) skin telangiectasias in typical locations, or (d) AVMs in viscera.[42] Using these criteria, HHT prevalence has been estimated at 1 in 5 to 10,000, with considerable geographic variation.[43] Unlike patients with CCM, the majority of patients with HHT are symptomatic. Spontaneous epistaxis results from telangiectasias of the nasal mucosa and is nearly universal, affecting over 90% of patients by age 30.[44,45] Telangiectasias of the fingers, lips, and oral mucosa are also extremely common (over 90% by age 30[46]), but rarely result in hemorrhage or symptoms. The consequences of visceral AVMs depend upon the affected vascular bed, with brain, lung, liver, and the GI tract as the most common clinically important sites. Brain AVMs are high-flow vascular lesions that are prone to rupture, resulting in hemorrhagic stroke and are found in approximately 10% of patients.[45,47] Lung AVMs are found in approximately 50% of patients[45,47] and result in shunting of deoxygenated blood directly into the systemic circulation, causing systemic hypoxemia and dyspnea as well as stroke due to paradoxical emboli. Like shunting in cyanotic congenital heart disease, there is a risk of systemic infections such as cerebral abscess as a result of bypassing the filtering effects of the pulmonary microvasculature. Hepatic AVMs are often clinically silent, but occasionally cause symptoms by significantly increasing the demand for cardiac output, resulting in high-output cardiac failure.[48] AVMs rarely occur in other vascular beds (GI tract, spinal cord) and screening is not generally performed unless the patient is symptomatic. A small number of patients with classic findings for HHT have an overlap syndrome with features of juvenile polyposis and a high risk for gastrointestinal malignancies.[49]

Genetics of HHT

The majority of patients with a clinical diagnosis of HHT have loss of function mutations in either the transforming growth factor-β (TGF-β) binding protein Endoglin,[50] or the type I TGF-β receptor activin receptor-like kinase-1 (ACVRL1 or ALK1,[51]). Patients with HHT and juvenile polyposis, as well as some patients with clinical features of only HHT, have mutations in the downstream TGF-β effector SMAD4 (also known as MADH4[49,52]). Two other loci on chromosome 5 and chromosome 7 are linked to HHT as well.[53,54] Disease is observed with high penetrance in patients heterozygous for a culprit mutation.

Biology of the HHT Signaling Pathway

The three known HHT genes all relate to the TGF-β superfamily of signaling proteins. This signaling pathway consists of families of ligands including TGF-β, activins, and bone morphogenetic proteins (BMPs). Ligand binding to a heteromeric

complex of type I and type II receptors leads to phosphorylation of type I receptors by the constitutively active type II receptors, recruitment and phosphorylation of the receptor-regulated SMAD proteins (R-SMAD), and downstream signaling. The type I receptor ACVRL1 phosphorylates R-SMADs −1, −5, and −8 that associate with SMAD4 and translocate to the nucleus to regulate transcription of target genes.[55] The downstream signal is potentiated by the activity of coreceptors such as Endoglin or betaglycan,[56] sometimes referred to as type III receptors. Recent work has suggested that BMP9 is a likely ligand for ACVRL1 present in physiologically relevant levels in human serum.[57,58]

HHT Pathogenesis: Insights from Animal Models

Animal studies demonstrate that *Endoglin, ACVRL1*, and *SMAD4* are all required for embryonic development.[59–64] Mice lacking *Endoglin* or *Acvrl1* die in midgestation at comparable time points and with similar defects. Both genes are required for establishing correct arteriovenous identity of the developing endothelial network in embryos.[60,65] The loss of either gene results in AVMs that profoundly disrupt circulation and perfusion. Studies of a zebrafish mutant for *acvrl1* confirm a role in preventing AVM formation, and suggest that AVMs develop from persistence of normally transient arteriovenous connections that form during angiogenesis.[66] An even earlier role for *SMAD4* in development is apparent from studies of gene-targeted mice that die very early in gestation and prior to the process of gastrulation.[64] Heterozygous mice that are the genetic equivalent of human HHT develop subtle features of HHT at a low frequency.[61,67] Careful study of these animals demonstrates that both genetic and environmental factors impact AVM formation. In mice lacking *Endoglin*, vascular malformations and hemorrhage can be observed, but are dependent upon the background strain.[68]

Mice with conditional alleles for *Endoglin* and *Acvrl1* allow for the inducible complete (homozygous) inactivation of the targeted gene after critical developmental angiogenesis has occurred. AVMs, hemorrhage, and death result from the complete loss of *Acvrl1* induced in adulthood.[69] AVMs also form in mature mice with complete loss of Endoglin in the endothelium.[70] The retinal AVMs that develop in this model differ from embryonic AVMs by expressing primarily venous markers in the smooth muscle-surrounded, enlarged vessels.[70]

As with familial CCM, the inherited mutant allele is present in all cells and all vascular beds, yet in both conditions the disease affects a characteristic subset of tissues. However, unlike CCM, protein expression is not absent in the endothelial cells of the HHT vascular lesions and loss of heterozygosity is not thought to be necessary for the creation of AVMs.[71] Telangiectasias are rarely present at birth, but develop with age. Most are found on the fingertips, lips, tongue, and sun-exposed skin of the face (such as the ears) in addition to the nasal mucosa. On the hands, the frequency of lesions tends to be higher on the dominant side. This characteristic distribution of telangiectasias suggests that trauma and subsequent vascular growth and repair is an important disease modifier, a hypothesis that is supported by studies in animal models.[69] Angiogenesis associated with inflammation may be a related physiologic stimulus of vascular malformation.[70]

HHT Disease Mechanism: Future Directions and Unanswered Questions

Angiogenesis is thought to underlie AVM development in patients with HHT. Increased levels of vascular endothelial growth factor (VEGF) have been observed in patient serum[72,73] and anecdotal reports of temporary improvement in epistaxis from patients who received bevacizumab, a monoclonal antibody directed against VEGF, have increased interest in antiangiogenic therapies for HHT.[74] Numerous case reports provide further anecdotal support for such antiangiogenic therapy,[75–80] and argue for a more systematic approach to testing such treatment using properly designed clinical trials. As with CCM, the availability of animal models of HHT is expected to facilitate preclinical studies of potential therapeutic agents. A preliminary series of studies have recently suggested that thalidomide might also carry promise in HHT as an antiangiogenic therapy.[81]

Despite its wide biologic use, the TGF-β family and its downstream signaling effectors may also prove to be tractable targets for HHT therapy. In addition, a number of genes and pathways downstream of this signaling pathway have been implicated in HHT animal and cellular models.[82,83] Whether such downstream pathways are central to disease pathogenesis or are merely epiphenomena, remains to be investigated using animal models. Although we assume that a common signaling pathway is disrupted in HHT (SEE FIGURE 71.1), there are important genotype–phenotype differences between these patients with different mutations, pointing to subtle but clinically relevant differences in the quantity or quality of downstream signaling alterations. While the association of juvenile polyposis and premature GI malignancy with SMAD4 mutations is an obvious example, the tissue distribution of AVMs differs significantly between Endoglin (more likely to have pulmonary AVM) and ACVRL1 (more likely to have liver AVM) families.[47] Targeted therapies that account for gene-specific unique mechanisms are an important clinical goal that is tied to a better understanding of the genetic and molecular basis of vascular malformations.

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CHAPTER

72 Aneurysms (Large and Small Arteries)

CARLOS E. SANCHEZ, SANGITA KAPUR, AND NEAL L. WEINTRAUB

Aneurysms of the aorta and its branch vessels are a major health care concern; more than 15,000 patients die each year in the United States from aortic aneurysms, and the incidence and prevalence continue to rise.[1] Unfortunately, most aneurysms are asymptomatic, and their presentation is often acute with catastrophic complications.[2]

As the largest blood vessel of the body, the aorta serves as a conduit to deliver oxygenated blood from the left ventricle to the systemic circulation and all organs and tissues. The normal human adult aortic wall is composed of three layers. The intima is the innermost layer and consists of the endothelium residing on a basement membrane with subendothelial connective tissue; the media is a thick layer composed of layers of elastic fibers arranged concentrically with interposed smooth muscle cells bounded by an internal and external elastic lamina; and the outer layer, the adventitia, is composed of a loose collagen network containing the vasa vasorum and nerves. The aorta is divided into thoracic and abdominal portions defined by the location above or below the diaphragm. The thoracic aorta is subsequently divided into four parts: the aortic root, which consists of the aortic valve annulus, aortic valve cusps, and the sinuses of Valsalva; the ascending aorta, which begins at the sinotubular junction and extends to the brachiocephalic artery; the aortic arch, which begins at the origin of the brachiocephalic artery and gives rise to the neck and head artery branches; and the descending aorta, which begins at the isthmus between the left subclavian artery and the ligamentum arteriosum and ends at the diaphragm. Below the diaphragm, the abdominal aorta continues caudally, giving rise to mesenteric and renal arteries and ending at the bifurcation of the common iliac arteries. The abdominal aorta is divided into suprarenal and infrarenal portions based on the location relative to the takeoff of the renal arteries.

The normal size of the aorta varies by anatomical location, age, gender, body surface area, and imaging modality. On a study based on helical computed tomography (CT) in patients without cardiovascular disease, the mean thoracic aortic diameters were 2.98 cm at the aortic valve sinus, 3.09 cm at the ascending aorta proximal to the innominate artery, and 2.43 cm at the descending thoracic aorta at the level of the diaphragm. In general, the normal abdominal aorta exhibits a diameter of approximately 2 cm.[3] Aneurysms can develop in all portions of the aorta and in smaller arteries. The purpose of this chapter is to summarize the key aspects of aneurysm disease of the aorta and its major branches, focusing on pathogenesis, diagnosis, and management.

ABDOMINAL AORTIC ANEURYSM

Abdominal aortic aneurysm (AAA) is considered to be present when the anteroposterior diameter of the aorta is ≥3.0 cm[4] or when the vessel wall exceeds the normal diameter by 50%.[5] The infrarenal aorta is the most common site of AAA; population-based screening programs have demonstrated an incidence of up to 9% in asymptomatic adults.[6] The incidence and prevalence of AAA is on the rise, and whether this is related to the implementation of ultrasound screening programs, widespread use of CT, an aging population, or a true increase in the disease incidence is unclear. Despite advances in the treatment options for aneurysms, rupture continues to be the most widely recognized and feared complication with an overall mortality rate of up to 90%.[7]

Risk Factors and Pathophysiology

Multiple risk factors have been recognized to be associated with AAA. Smoking is strongly associated with the development of AAA; in particular, the duration of smoking, and not the number of cigarettes smoked per day, has been associated with the increased risk.[8] In addition, males exhibit a prevalence of AAA that is five- to sixfold greater than females.[9] The risk of rupture in women, however, is significantly higher than in men, and aneurysm rupture occurs at smaller aortic diameters in women.[10] The prevalence of AAA increases with age, with the highest rate in patients older than 65 years. Hypertension and having a first-degree relative with AAA have also been associated with increased risk for AAA. Family history of AAA also carries a greater risk of rupture, and aneurysms tend to occur at a younger age.[11]

Atherosclerosis is closely linked to the pathophysiology of AAA. Although AAA frequently occurs in patients with atherosclerosis, and both diseases share many of the same risk factors, they exhibit distinct pathologic features and anatomic predilection. The hallmark pathologic feature of atherosclerosis is foam cell formation, whereas aneurysms are typified by intense matrix degradation, inflammation, oxidative stress, and apoptosis of smooth muscle cells. Additionally, atherosclerotic lesions are predominantly intimal in location, whereas the media and adventitia are primarily involved in AAA. The media and adventitial layers contain large amounts of matrix structural proteins, particularly elastin and collagen, which impart strength to resist aortic expansion and wall tension.[6] Degradation of the extracellular matrix proteins weakens the aortic wall with subsequent remodeling and dilatation. Once the aorta dilates and an

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aneurysm is formed, tension in the aortic wall increases progressively based on Laplace law (tension is proportional to the product of pressure and radius), causing the aneurysm to enter a progressive and sustaining loop of enlargement.

One of the defining features of AAA is inflammation.[11] The inflammatory infiltrate is most prominent in the media and adventitia of the aortic wall and is composed predominately of macrophages and T lymphocytes, although neutrophils, B lymphocytes, NK cells, and mast cells are also present.[12] Activated macrophages are a major source of matrix metalloproteinases (MMPs), a group of structurally related, zinc-dependent endopeptidases that are also synthesized by vascular wall cells, particularly smooth muscle cells. In addition, macrophages secrete serine proteases and cysteine proteases that work in concert with MMPs to degrade structural proteins, thereby playing a key role in aneurysm formation.[13,14] MMPs are secreted as inactive zymogens (pro-MMPs) that require activation extracellularly. Among the numerous classes of proteases that can lead to tissue destruction, MMP-2 and MMP-9 have in particular been implicated in the pathogenesis of AAA. Deletion of either MMP-2 or MMP-9 prevented AAA formation in a mouse model.[15] Also, MMP-9 was reported to be the most abundant protease secreted by human AAA tissue explants *in vitro*.[16] Additionally, MMP-9 transcript levels were found to be higher in aneurysms measuring 5.0 to 6.9 cm compared to aneurysms measuring 3.0 to 4.9 cm.[17] In a study in ruptured AAA, MMP-8 and MMP-9 levels were increased at the aneurysm rupture site compared to the adjacent unruptured wall, suggesting that extracellular matrix breakdown may be the final mechanism causing AAA rupture.[18] Notably, systemic inhibitors of MMPs abrogated AAA in mouse models and are under study in humans with AAA.[13,19,20]

Lymphocytes, particularly $CD4^+$ T-helper cells, have a unique activated memory phenotype and produce proinflammatory cytokines (e.g., interleukin-6, tumor necrosis factor-alpha, interferon-gamma, and osteopontin), leading to further recruitment of immune cells and induction of apoptotic cell death. Apoptosis of smooth muscle cells within the aneurysmal wall is thought to contribute to aneurysm formation and progression, since smooth muscle cells are largely responsible for the production of elastin and collagen. The degradation of aortic extracellular matrix proteins by proteases, together with a reduced capacity to synthesize new matrix proteins due to smooth muscle cell apoptosis, may thus lead to impaired repair of the aortic wall resulting in weakening and dilatation.[21]

Oxidative stress induces MMP activation and smooth muscle cell apoptosis and is increased in the setting of AAA (reviewed in ref. [21]). A study of aortic tissue in patients undergoing surgical repair demonstrated that levels of reactive oxygen species (ROS) are increased locally in AAA tissue as compared with adjacent nonaneurysmal aorta, in part through the activation of NADPH oxidase, leading to marked increases in oxidative stress.[22] A subsequent study in mice demonstrated that deletion of $p47^{phox}$, a cytosolic subunit of NADPH oxidase, reduced aortic oxidative stress, macrophage infiltration, and MMP-2 activity and markedly attenuated AAA formation.[23] ROS also stimulate the secretion of cyclophilin A from vascular smooth muscle cells. Cyclophilin A, a member of the immunophilin family of proteins and a target of the immunosuppressive drug cyclosporine, binds to the highly glycosylated protein CD147 (also known as extracellular MMP inducer) on smooth muscle cells and induces its translocation to the cell surface, where it plays a critical role in stimulating MMP activity and matrix degradation. Absence of cyclophilin A reduced aortic inflammation, oxidative stress, and matrix degradation and prevented AAA formation and rupture in a mouse model.[24] An example of the typical pathologic features of human AAA is shown in FIGURE 72.1.

Mural thrombus is frequently present lining the walls of AAA and can be both prognostically and clinically significant. Although AAA size is the most commonly used determinant to predict the risk of rupture, some studies suggest that the presence of intramural thrombus portends a higher AAA expansion rate and risk of rupture.[25,26] The presence of a mural thrombus in AAA has been associated with a thinner arterial wall, lower density of smooth muscle cells in the media, and heightened inflammation and elastolysis in the aneurysm wall.[27] This might be caused by the increased production of proteases originating from neutrophils and other circulating cells trapped within the mural thrombus in the aneurysmal wall.[28] While intramural thrombus size and growth has been suggested to be prognostically significant in asymptomatic patients with aneurysms ≤5.5 cm in size, larger studies are needed to confirm this association before it is incorporated in the decision making of timing of aneurysm repair.

Diagnosis

AAAs are frequently asymptomatic and commonly detected as an incidental finding on imaging studies or, in the present era, through AAA screening programs. Multiple imaging modalities are currently available for diagnosing and monitoring AAA with excellent sensitivity and specificity. The indication of each type of imaging modality for AAA is determined by availability, cost/benefit ratio, and technical considerations. Ultrasonography is the preferred modality for screening AAAs, with the advantage that it is relatively inexpensive, noninvasive, and it does not require administration of a contrast agent or expose patients to radiation. The evaluation of AAA with ultrasound involves measuring the anteroposterior, longitudinal, and transverse dimensions of the aorta. The sensitivity, specificity, and accuracy to detect and quantify infrarenal AAA approaches 100%.[29] Limitations of this imaging technique are that it is technician dependent, difficult to implement in morbidly obese patients, associated with significant interobserver variability, and usually not able to visualize aneurysm extension to major branches (i.e., iliac arteries), which limits its utility for planning operative repair.

The use of routine ultrasound screening for AAA in asymptomatic patients is a controversial topic. The U.S. Preventive Service Task Force (USPSTF), in its 1996 report, found insufficient evidence to recommend routine AAA screening in asymptomatic adults. Since then, numerous randomized, controlled trials evaluating population-based screening for AAA have been published. The largest trial, the Multicenter Aneurysm Screening Study, randomized 67,800 patients aged 65 to 74 years to an invitation for abdominal ultrasound screening or to an uninvited control group. After 4 years of follow-up, the AAA-related mortality in ultrasound-screened patients was reduced by 42% compared to uninvited controls.[30] The trial also provided firm estimates of the cost-effectiveness of a screening program. At 4 years, the cost was 57,000 dollars per quality-adjusted life year, and when extrapolated to 10 years, the cost was estimated at 12,500 dollars. The authors concluded that at

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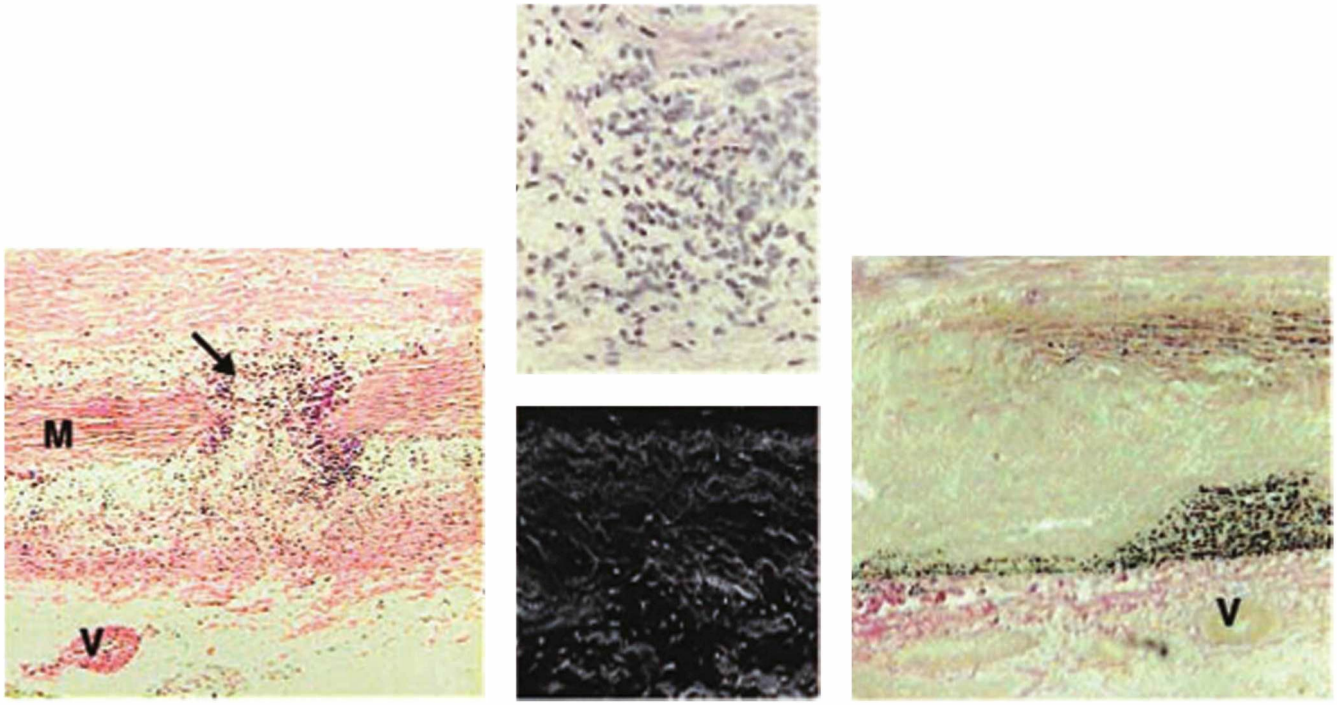

FIGURE 72.1 Inflammatory infiltrate (*left* and *upper middle panels*), focal oxidative stress (*lower middle panel*), and disruption of medial smooth muscle cells (*left panel*, *arrow*) and elastin (*right panel*) in human AAA. M indicates medial layer; V indicates vasa vasorum within the adventitial layer. (Reprinted from Miller FJ, Sharp WJ, Fang X, et al. Oxidative stress in human abdominal aortic aneurysms: a potential mediator of aneurismal remodeling. *Arterioscler Thromb Vasc Biol* 2002;22:560–565, with permission.)

4 years, the cost-effectiveness of screening for AAA is acceptable according to the current health services threshold.[31] Based on these data, and three other population-based studies, the USPSTF revisited the benefits and harms of population-based AAA screening in 2005, concluding that for men aged 65 to 75 years, an invitation to attend AAA screening reduces AAA-related mortality.[32] Unfortunately, these data do not specifically address AAA screening in women, who were underrepresented in the trials. The use of screening ultrasound for AAA in the United States is currently recommended for men aged 65 to 75 years who have smoked, and for both men and women in this age group with a family history of AAA.

CT is a well-established and widely used method for the diagnosis of AAA. The advent of multidetector CT, with its ability to perform volumetric imaging with isotropic voxels and a spatial resolution <1 mm, makes it an excellent modality for the diagnosis and follow-up surveillance of AAA. The multiplanar imaging ability of CT renders it an extremely accurate technique to serially evaluate changes in aneurysm size over time. In addition, CT permits better visualization of suprarenal aneurysms and improved assessment of the renal, mesenteric, and iliac arteries through axial images and three dimensional (3-D) reconstruction displays, thereby providing a complete description of the abdominal and pelvic vasculature prior to repair (FIGURE 72.2). An important use of CT is to measure the infrarenal neck of the aneurysm, the length of which is extremely important for endograft fixation during endovascular AAA repair. The infrarenal neck of the aneurysm is defined as the distance along the aorta from the renal arteries to the proximal origin of the aneurysm. Disadvantages of CT are its cost compared to ultrasound, exposure to radiation (particularly with repeated scans), and the use of intravenous contrast.

Noncontrast or contrast-enhanced magnetic resonance angiography (MRA) also clearly demonstrates the aortic anatomy, providing similar information to that of the CT scan. MRA is highly sensitive and specific at diagnosing and quantifying aortic diameter in AAA. In addition, MRA delineates the entire aorta and thus defines the anatomic relationship between the aorta and its principal branches for preoperative aortic aneurysm assessment. Advantages of MRA over CT scan in patients who need serial monitoring of aortic size are the absence of ionizing radiation and iodinated contrast; however, MRA is much more expensive and less widely available. Moreover, some patients cannot tolerate MRA due to implanted metal devices and/or claustrophobia and longer imaging times.

Management

Surveillance and Timing of Repair

Aneurysm size remains the single-most important predictor for aneurysm rupture and the main indication for repair in asymptomatic patients with AAA. The size of the aneurysm is directly related to the risk of rupture; however, the exact size upon which surgical intervention is justified remains a topic of debate. The optimal management and timing of surgical repair of AAA was addressed by two large randomized trials, the U.K. Small Aneurysm Trial and Aneurysm Detection and Management (ADAM) Veterans Affairs Cooperative Study. Both

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FIGURE 72.2 CTA volume rendered **(A)** and maximum-intensity projection (MIP) images **(B)** demonstrating a fusiform bilobed AAA with atherosclerotic calcifications (*arrow*).

trials randomized patients with small aortic aneurysm diameters (4.0 to 5.5 cm in U.K. Small Aneurysm Trial and 4.0 to 5.4 cm in ADAM Study) to undergo early elective surgery or follow-up surveillance. Collectively, there was no difference in survival between the early surgery group and the surveillance group after approximately 5 years of follow-up. The U.K. Small Aneurysm Trial extended their follow-up to 8 years, showing a slightly lower total mortality in the early surgery group. However, their 30-day surgical mortality rate was 5.8% compared to 2.1% in the ADAM trial.[33,34] The U.K. Small Aneurysm Trial also reported an annual risk of AAA rupture of 1.5% or less for aneurysms smaller than 5.0 cm in diameter and 6.5% for aneurysms 5.0 to 5.9 cm in diameter. Above 6 cm, the risk of AAA rupture rises sharply, with an annual risk of rupture of up to 33% in aneurysms 7.0 cm in diameter or larger.[10,35] These trials demonstrated that in patients with asymptomatic AAA, follow-up surveillance and deferment of repair until aneurysm diameter exceeds 5.5 cm is safe and associated with a very low rate of rupture. An important limitation is that female population was underrepresented in these trials; consequently, recommendations made for men may not apply stringently to women. Currently, the threshold for repair recommended by professional vascular surgery societies in women with AAA is 4.5 to 5.0 cm, based on the recognition that aneurysms tend to rupture at smaller sizes in women than in men.[36]

Rapid expansion rate is another important determinant that can also predict aneurysm rupture, with large aneurysms typically expanding more rapidly than small aneurysms in accordance with Laplace law. This hampers the exact prediction of aneurysm growth rate, even within the same individual over time. Based on multiple reports, however, the average annual expansion rates for AAA are 0.1 to 0.5 cm for aneurysms measuring <4.0 cm at the time of discovery, 0.4 to 0.5 cm for aneurysms 4.0 to 6.0 cm, and up to 0.7 to 0.8 cm for larger aneurysms.[37] In general, follow-up surveillance imaging is recommended every 12 months for individuals with AAA measuring 3.5 to 4.4 cm in diameter and every 6 months for those with an AAA measuring 4.5 to 5.4 cm in diameter, with a recommendation for elective aneurysm repair when diameter reaches 5.5 cm or increases by more than 0.5 cm within a given year.[38]

Endovascular versus Surgical Repair

Endovascular abdominal aortic aneurysm repair emerged almost two decades ago as an alternative approach to AAA repair in individuals not deemed to be surgical candidates. Endovascular aneurysm repair (EVAR) now has become the treatment of choice to repair infrarenal AAA, accounting for more than half of all AAA repairs in the United States.[39] The technique involves the use of a percutaneous stent graft, placed under fluoroscopy guidance to cover the aneurysm, excluding it from the circulation. It usually requires sufficient proximal and distal nonaneurysmal segments for adequate attachment of the stent struts to prevent distal migration. It is important to note that AAA endovascular repair has certain anatomical requirements for procedural success, and not all patients with AAA have suitable anatomy.[40] The implantation success rate, however, has approached 100% in recent years. Nevertheless, a common adverse effect continues to be the leakage of blood into the aneurysm, also called endoleak. Although they may spontaneously resolve, persistent endoleaks may lead to continual aneurysm expansion or rupture, requiring an additional intervention to repair either by endovascular implantation of a graft extension cuff or, less commonly, by conversion to open surgical repair.

Two randomized, prospective trials comparing AAA endovascular repair versus open surgical repair in individuals deemed healthy enough to undergo surgery have addressed the short- to late-term outcomes of EVAR. In the short-term outcomes, the Endovascular Aneurysm Repair trial 1 (EVAR-1), and the Dutch Randomized Endovascular Aneurysm Management (DREAM) trial, showed an almost identical improved 30-day mortality rate after EVAR compared to open surgical repair: 1.7% versus 4.7%, respectively, in the EVAR-1 trial and 1.2% versus 4.6% in the DREAM trial, respectively. Mid- and long-term data, however, have failed to demonstrate an all-cause mortality benefit, with overall survival rates converging after 2 years for both groups.[41,42] The role of endovascular repair in

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patients considered at high risk for complications or death with open repair has also been investigated in the United Kingdom Endovascular Repair-2 (EVAR-2) trial. The study randomized patients to elective endovascular AAA repair or no repair, with both groups equally treated medically. The study found no benefit of endovascular repair in patients who are deemed high risk for open aneurysm repair, even at long-term follow-up.[43] Therefore, the optimal indications for endovascular repair remain to be precisely defined. Patient preference is very important when making treatment decisions, and patients should be well informed about the pros and cons of each approach.

Medical Management

The cornerstone of medical therapy is risk factor modification. Current tobacco use has shown to be strongly associated with a significant increase in aneurysm growth rate. Hence, smoking cessation should be strongly encouraged in all patients with AAA and is likely the single-most important therapeutic intervention. Hypertension should also be controlled. Several studies have suggested that beta-blockers and angiotensin-converting enzyme inhibitors have a beneficial effect on aneurysm progression, but these data have been insufficient to make strong recommendations for their specific use to prevent aneurysm expansion.[44] Patients with AAA frequently have concomitant coronary artery disease, and beta-blockers are generally recommended for patients undergoing vascular surgery to reduce perioperative cardiac risk. Despite no clear relationship between cholesterol levels and AAA expansion rate, statin therapy has been shown in small studies to reduce infrarenal aneurysm growth rate and to reduce aortic MMP-9, which plays an important role in aneurysm formation and expansion.[45,46] In a recent study, the effects of statins on aneurysmal growth was examined in 150 patients with AAA over a median period of 3 years. Statin users averaged 2.0 mm/y aneurysm growth rate compared to 3.6 mm/y in nonusers.[47] A recent meta-analysis also suggested that statin therapy is associated with a lower rate of expansion in patients with small AAA.[48] Although these results are encouraging, larger trials are needed to confirm efficacy in order for statin therapy to be recommended for the treatment of AAA, in the absence of conventional indications.

Inflammatory AAA

Inflammatory AAA (IAAA) has been described as a variant of AAA defined by the presence of a thickened aneurysm wall, marked perianeurysmal and retroperitoneal fibrosis, and dense adhesions of adjacent abdominal organs.[49] IAAA account for 3% to 10% of AAA with a male-to-female predilection reported of up to 30:1 and with an age of occurrence 5 to 10 years younger than the mean age of patients with atherosclerotic aneurysms.[50] Unlike atherosclerotic AAA, symptoms of IAAA are common and highly suggestive of inflammation. Patients usually present with the triad of abdominal or back pain, weight loss, and an elevated sedimentation rate.[51] As with all AAA, inflammatory cell infiltrate is the pathophysiologic hallmark, but with a more pronounced inflammatory response in IAAA. Histologic features include internal elastic lamina disruption and loss of smooth muscle and elastic fibers of the media along with inflammatory infiltrates.[49] The exact etiology is unclear, but it has been hypothesized that enhanced inflammation might be related to infections such as Chlamydia and cytomegalovirus.[52,53] The rupture rate for IAAA has been suggested to be lower than noninflammatory AAA. CT scan is the gold standard diagnostic tool for IAAA, characterized by a thick inflammatory soft tissue rind around the aorta and demonstration of perianeurysmal fat stranding indicative of inflammation, which distinguishes IAAA from the more common atherosclerotic AAA.[51] More recently, increased metabolism in the AAA wall has been visualized by 18F-fluorodeoxyglucose (FDG) positron emission tomography (PET)/CT. FDG uptake has been shown to correlate with inflammation, histopathologic characteristics of aneurysm wall instability, and clinical symptoms and may contribute in the near future to improved prediction of AAA rupture risk.[54] Surgical repair is the treatment of choice for IAAA, although EVAR has emerged as an option. Findings at operation include a thickened aortic wall and a shiny, white periaortic and retroperitoneal inflammatory reaction involving adjacent structures.[51]

Aortic Dissection

Aortic dissection is a tear of the intima layer with bleeding into the wall of the aorta resulting in separation of the layers and formation of a new lumen (false lumen). In 90% of patients, the dissection plane tracks within the media layer. Population-based studies have shown that the incidence of acute aortic dissection ranges from 2 to 3.5 cases per 100,000 person-years, twice that of AAA rupture.[55] The most common predisposing factor for acute aortic dissection is arterial hypertension, with a strong male predominance. Other predisposing factors include genetic conditions such as Marfan syndrome or Loeys-Dietz syndrome, nonsyndromic familial disorders, previous aneurysms, atherosclerosis, and inflammatory diseases. Underlying genetic disorders are particularly common in patients younger than 40 years of age who suffer aortic dissection, and only a small minority of these patients have a history of aortic aneurysm prior to dissection.[56] Thus, in most patients, aortic dissection represents a syndrome distinct from aortic aneurysm. For a comprehensive review on aortic dissection, please refer to ACC/AHA guidelines for the diagnosis and management of patients with thoracic aortic disease.[57]

THORACIC AORTIC ANEURYSMS

Thoracic aortic aneurysm (TAA) is typically an indolent process, usually asymptomatic, and often diagnosed by an imaging modality performed for other indications. Hence, the true incidence of the disease is difficult to estimate, yet available data suggest it is increasing.[58] TAA involve different segments of the aorta and are usually classified based on the affected anatomical segment. The ascending aorta and/or aortic root is involved in 50% of TAA, followed by the descending aorta distal to the left subclavian artery in 40%, and the aortic arch in 10%.[59] TAA which extend below the diaphragm to the abdominal aorta are referred to as thoracoabdominal aneurysms, which constitute 10% of TAA.

Etiology and Pathophysiology

Classically, the hallmark pathologic feature of TAA is a degenerative process termed cystic medial necrosis, or more accurately, medial degeneration. The term cystic medial necrosis has fallen out of favor because histologically, there is neither necrosis nor cyst formation. In general, medial degeneration is characterized by disruption of the extracellular matrix proteins, apoptotic loss of

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vascular smooth muscle cells, and increased accumulation of proteoglycans. Inflammatory infiltration and upregulation of MMP in the aortic wall has been documented in TAA but is less pronounced than is typically seen in AAA.[60] Moreover, atherosclerotic lesion formation, which is common in AAA, is rare in TAA and extremely uncommon in cases involving the ascending aorta.[61,62]

The basis for pathologic differences of aortic histology in the thoracic versus abdominal aorta is not entirely clear. Historically, it was thought that the differences in atherosclerotic lesion formation between the atherosclerotic prone abdominal aorta and the atherosclerotic resistant thoracic aorta were due to turbulent hemodynamic flow patterns in the abdominal aorta.[63,64] However, this theory was dispelled by experimental studies demonstrating that transplanting abdominal aortic segments to the thoracic position, or thoracic aortic segments to the abdominal position, did not modulate atherosclerosis susceptibility.[64] The presence of medial degeneration without associated atherosclerosis in the thoracic aorta suggests that intrinsic abnormalities of smooth muscle cells may play a role, perhaps related to their unique embryologic origin. In this regard, smooth muscle cells in the ascending aorta arise from neural ectoderm-derived cells in the cardiac neural crest, whereas smooth muscle cells in the abdominal aorta originate from paraxial mesoderm-derived progenitors in somites.[63] Recent studies have elucidated fundamental differences in tissue growth factor beta (TGFβ) signaling in smooth muscle cells that may be pertinent to aneurysm susceptibility in thoracic versus abdominal aorta.[65] Under identical conditions, TGFβ-1 strongly induced cell proliferation, plasminogen activator inhibitor-1 expression, and DNA synthesis in neural crest–derived smooth muscle cells, whereas the same concentrations of TGFβ-1 inhibited these responses in mesoderm-derived smooth muscle cells. This may be pertinent to clinical and experimental observations, suggesting that TGFβ signaling differentially regulates TAA and AAA.[66,67]

Multiple underlying conditions have been associated with TAA, including congenital, hereditary, and acquired causes. Congenital causes include bicuspid aortic valve (BAV) disease and hereditary syndromic disorders such as Marfan syndrome, Loeys-Dietz syndrome, and Ehlers-Danlos syndrome. Although any segment of the aorta may become involved with these disorders, they typically involve the aortic root and ascending aorta. Aortic root aneurysms can cause aortic insufficiency from aortic valve annular dilatation, a condition commonly referred as *annuloaortic ectasia*[68] (FIGURE 72.3A,B). Hypertension is an important factor related to TAA. High blood pressure dilates and elongates the aorta and can hasten the process of medial degeneration that leads to weakening of the aortic wall. Other acquired disorders linked to TAA include infections such as syphilis and inflammatory diseases including giant cell arteritis (GCA) and Takayasu arteritis. An inflammatory variant has also been described in the ascending and descending thoracic aorta. Although inflammatory TAA is even more uncommon than its counterpart in the abdominal aorta, clinically and pathologically they behave the same and share similar radiologic features.

Marfan Syndrome

Marfan syndrome is an autosomal dominant disorder of the connective tissue with a high penetrance but variable expression. The cause is a mutation in the *FBN1* gene that encodes fibrillin-1, a large extracellular matrix glycoprotein that is the major element of microfibrils, which form the elastic fibers in conjunction with tropoelastin.[69] Fragmentation of elastic fibers in the aortic media causes the histologic changes of medial degeneration, which is the main pathologic feature of TAA. A second locus was recently identified linked to mutations in the gene encoding the transforming growth factor-beta type II receptor (*TGFBR2*).[70] Mutations in *TGFBR2* can also be found in patients with different syndromes, including the Loeys-Dietz syndrome.[71] The diagnosis of Marfan syndrome is based on a combination of clinical criteria of typical phenotypic manifestations in the cardiovascular, ocular, and skeletal systems.[72] The most specific manifestations of Marfan syndrome are aortic dilatation/dissection (FIGURE 72.3C,D), ectopia lentis, and dural ectasia, with almost every patient having aortic disease at some point in their lifetime. In fact, patients who present with untreated ascending aortic aneurysm or aortic dissection account for most of the mortality of this syndrome.

Loeys-Dietz Syndrome

Loeys-Dietz syndrome is a rare autosomal dominant genetic disorder that shares various clinical manifestations with Marfan syndrome. Loeys-Dietz syndrome has been associated with mutations in the genes encoding the transforming growth factor beta receptors I and II (*TGFBR1* and *TGFBR2*). The syndrome is characterized clinically by the triad of arterial tortuosity and aneurysms, widely spaced eyes (hypertelorism) and a bifid uvula, cleft palate, or both.[71] The diagnosis is confirmed through genetic testing analysis for *TGFBR1* or *TGFBR2* mutations. Almost all affected patients have aortic root aneurysms that lead to aortic dissection at an early age and at aortic diameters <5.0 cm. Molecular characterization of 52 families with Loeys-Dietz syndrome found that the median survival age was 37 years, compared to 48 years in patients with vascular Ehlers-Danlos syndrome and 70 years in patients with Marfan syndrome.[73]

Vascular Ehlers-Danlos Syndrome

Also called Ehlers-Danlos syndrome type IV, this is an autosomal dominant disorder characterized by thin, translucent skin; easy bruising, characteristic facial appearance (thin lips, small chin, thin nose, and large eyes); and rupture of arteries, intestine, and uterus. Patients most commonly present with vascular rupture/dissection or perforation from the gastrointestinal tract. Arterial rupture may be preceded by aneurysm or dissection, but it may also occur spontaneously. Vascular complications are the most catastrophic complications, reducing median age of survival to only 48 years.[74] Mutation in *COL3A1* is the only genetic abnormality thus far linked to vascular Ehlers-Danlos. The diagnosis is based on clinical findings and confirmed by abnormal type III procollagen synthesis and/or molecular genetics testing.[74]

Bicuspid Aortic Valve Disease

BAV is one of the most common forms of congenital heart disease, affecting 1% to 2% of the adult population. BAV can follow an autosomal dominant pattern of inheritance and is associated with TAA formation; medial degeneration appears to be the underlying mechanism of TAA in patients with BAV. No

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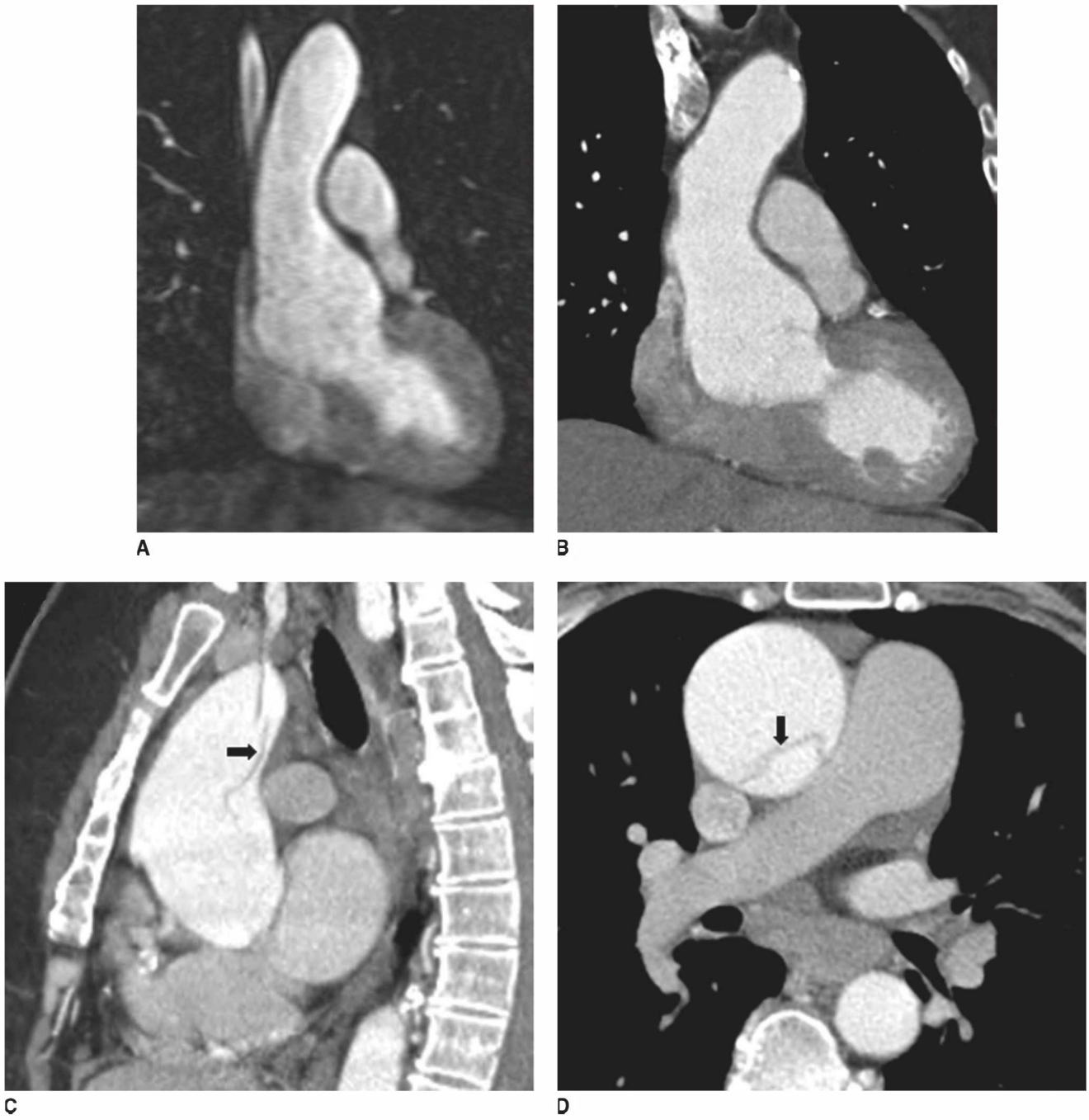

FIGURE 72.3 Contrast-enhanced MRA coronal acquisition **(A)** and CTA coronal reconstruction **(B)** demonstrating annuloaortic ectasia in a Marfan syndrome patient. Sagittal **(C)** and axial **(D)** contrast enhanced multidetector computed tomography images demonstrate dissection flap separating the two lumina in an ascending TAA (*arrows*).

single gene mutation has been conclusively linked to BAV and TAA, although mutations in *NOTCH1* have been implicated.[75] Aortic dilation is often observed in individuals with BAV who lack aortic valve stenosis or insufficiency, implying that perturbed aortic flow is not responsible for the aortic pathology.[76] BAV disease has been associated with fibrillin-1 deficiency in the aortic media and increased MMP-2 activity independent of the presence or absence of aortic valve dysfunction, suggesting that congenital fibrillin-1 deficiency may play a role in aortic dilatation in patients with BAV.[77] About 40% of patients with BAV are affected by TAA, with the ascending aorta involved in 44% of cases, followed by aortic sinuses and sinotubular junction in 20% and 15% of cases, respectively.[78] Other aortic abnormalities associated with BAV include aortic dissection, aortic rupture, and coarctation of the aorta. It is important to point out that patients who have undergone repair of aortic coarctation may develop aortic aneurysms adjacent to the surgical site; serial imaging studies are indicated to address this possibility.[79]

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Table 72.1

Genetic map for familial forms of thoracic aortic aneurysm

Chromosome	Gene	Protein	Frequency of Familial nonsyndromic (%)
10q23-24	ACTA2	Smooth muscle alpha actin 2	~14
9q33-34	TGFBR1	Transforming growth factor beta receptor 1	<1
3p24-25	TGFBR2	Transforming growth factor beta receptor 2	~3
16p12-13	MYH11	Smooth muscle myosin heavy chain 11	~1
15q13-14	Unidentified	Unidentified	~10–30
11q23-24	Unidentified	Unidentified	~<5
15q24-26	Unidentified	Unidentified	~10–20

Modified from Tromp G, Kuivaniemi H, Hinterseher I, et al. Novel genetic mechanisms for aortic aneurysms. *Curr Atheroscler Rep* 2010;12:259–266.

Familial TAA

Familial cases of TAA usually occur as a solitary manifestation not associated with other genetic syndromes or sporadic causes of aneurysms. They follow a familial pattern of inheritance, typically autosomal dominant or X-linked.[80] A recent analysis of more than 500 patients with nonsyndromic TAA demonstrated that about 20% exhibited a pattern of familial clustering.[81] Six different genetic loci have been identified in studies of familial TAA, with four genes found to harbor mutations linked to TAA[82] (Table 72.1). The most commonly identified mutation is in the *ACTA2* gene that encodes for smooth muscle alpha actin 2, found in 14% of unrelated families with TAA. This mutation has an autosomal dominant transmission pattern and can present with nonthoracic aneurysms, livedo reticularis, iris flocculi, and patent ductus arteriosus in addition to TAA.[83] The other three genes that have been identified are rare and encode for smooth muscle myosin heavy chain 11 (*MYH11*), *TGFBR1*, and *TGFBR2*. Mutations in the last two genes have also been linked to Loeys-Dietz syndrome, as previously discussed.[73]

Medial degeneration was considered the hallmark pathologic feature of Marfan syndrome; however, familial and syndromic disorders of TAA are also associated with similar pathology. The cascade of events that terminates in medial degeneration and aortic wall dilatation is not fully understood. Previous studies have shown the presence of inflammatory infiltrates, composed primarily of T cells and macrophages, in the aortic wall of TAA. Interestingly, this inflammatory component has been identified in patients with familial and syndromic TAA, as well as in nongenetic sporadic cases.[84] Furthermore, increased expression of MMP, in particular MMP-2 and MMP-9, has been consistently detected in the media of TAA associated with various disorders.[85–87]

Diagnosis

Most TAA are silent and are discovered incidentally. Symptoms, when present, usually result from a vascular complication or aneurysm expansion compressing an adjacent organ or bony structure. Similar to AAA, TAA can be diagnosed by multiple imaging modalities. Transthoracic echocardiogram (TTE) is an excellent modality to evaluate the aortic root important in patients with Marfan syndrome, who predominantly have dilatation of the proximal aorta. However, TTE can only image the first few centimeters of the aorta and may miss aneurysms in the mid and distal ascending aorta, as well as the descending aorta. Therefore, TTE should not be used to diagnose TAA in diseases other than Marfan syndrome. Transesophageal echocardiogram (TEE) allows clear visualization of the entire thoracic aorta, with exception of a blind spot in the ascending aorta close to the right main stem bronchus. However, TEE is an invasive procedure that can lead to significant complications, particularly in patients with compromised airway or respiratory function, and in those with esophageal disease. Also, accurate measurement of aortic diameter by TEE is challenging in the presence of aortic tortuosity. While TEE is a useful diagnostic tool for aortic dissection, it is not recommended to serially measure aortic diameters in patients with TAA.

Multidetector computed tomography angiography (CTA) is an excellent imaging modality to evaluate TAA (FIGURE 72.4A). Advantages of multidetector CTA include high spatial resolution of 0.5 to 0.75 mm and the ability to display the 3-D structure of the entire aorta, which makes it ideal for serial evaluation of TAA. Moreover, CTA can rapidly image the entire aorta and surrounding anatomical structures, including branch vessels, which is important in critically ill patients. CTA with prospective ECG gating, which limits pulsation artifact from cardiac motion, can accurately evaluate the aortic root and ascending aorta. Disadvantages of CT include the risk of contrast-induced nephropathy and the radiation dose, especially in patients who need follow-up surveillance over time.

MRA of the aorta also offers excellent visualization of the entire aorta and permits accurate measurement of aortic diameter, aneurysm extension, and its relation to aortic branches (FIGURE 72.4B). Advantages over CTA include the lack of ionizing radiation, which facilitates serial follow-up, particularly in younger patients. ECG-gated MRA can also evaluate aneurysms of the aortic root. When the aortic root is affected, a functional study can be performed with cine-MRI to evaluate the aortic valve for associated abnormalities. As previously mentioned, MRA requires a longer duration of scanning time, which limits its application in critically ill patients.

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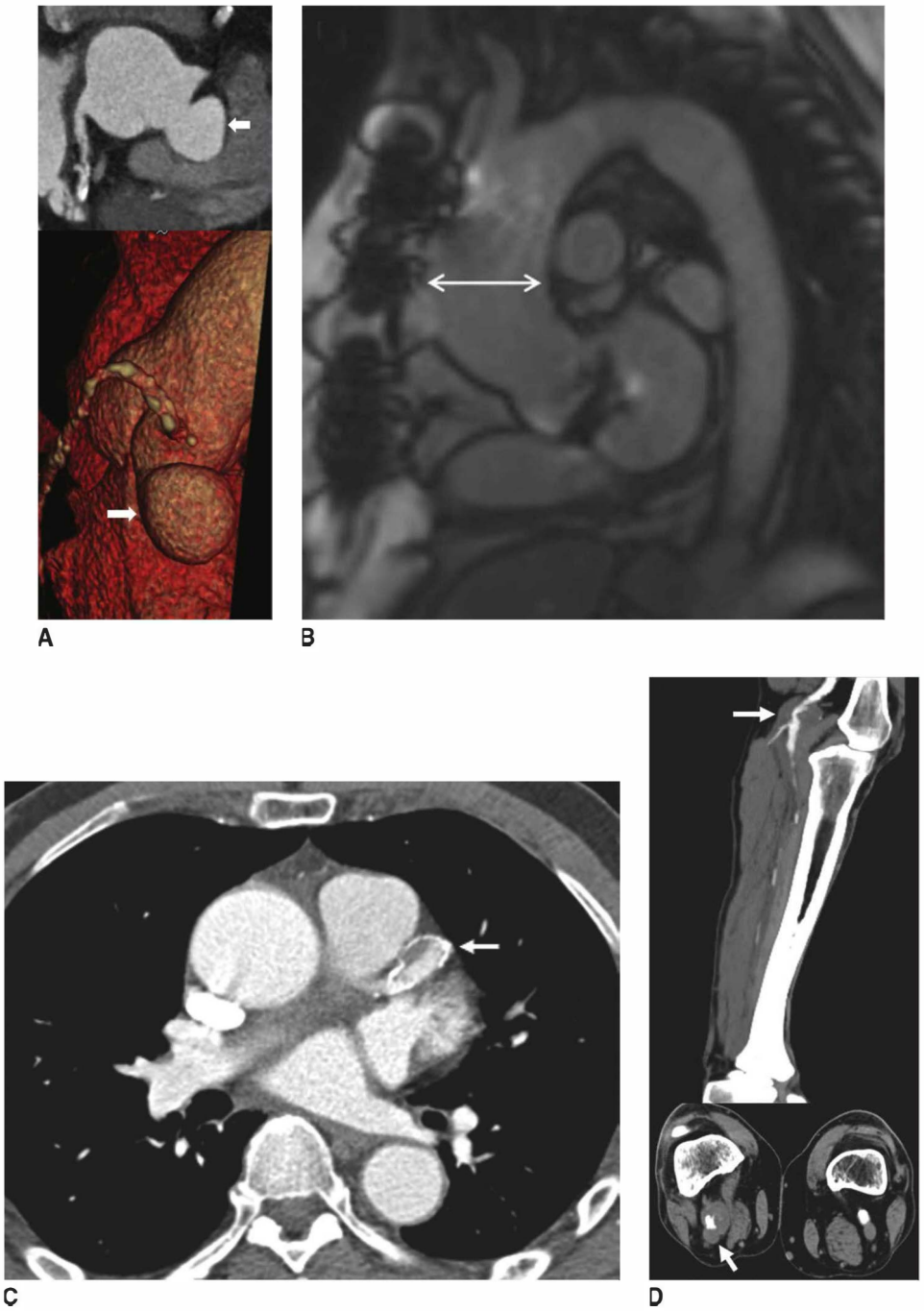

FIGURE 72.4 **A**: MIP **(top)** and volume rendered image **(bottom)** from a gated cardiac CTA demonstrating a 3 cm aneurysm of the right sinus of Valsalva (*arrows*) without mass effect on the adjacent right coronary artery. **B**: White blood non-contrast MRA Fast Imaging Employing Steady State Acquisition (FIESTA) of an ascending TAA in the sagittal view (*double arrow*). **C**: Contrast-enhanced axial CTA image demonstrating atherosclerotic aneurysm (*arrow*) of the proximal left anterior descending coronary artery with intramural thrombus and calcified plaque. **D**: Axial **(bottom)** and reconstructed sagittal MIP **(top)** from a contrast-enhanced CTA demonstrating a PAA with mural thrombus (*arrows*).

Management

Surveillance and Timing of Repair

The growth rate of TAA not associated with syndromes or other disorders is typically low, with a mean rate of 0.1 cm/y; the descending aorta expands faster at 0.19 cm/y compared to the ascending aorta at 0.07 cm/y. When rapid growth is reported, it usually reflects a measurement error, unless dissection is present.[58] Studies of the natural history of TAA have defined criteria when dissection and rupture are likely to occur, thus guiding the timing of repair. Surgical intervention in asymptomatic patients

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with aneurysms of the ascending thoracic aorta, aortic sinus, and descending thoracic aorta is indicated when the diameter is 5.5 cm or greater or a growth rate of more than 0.5 cm/y is documented in an aneurysm <5.5 cm in diameter. Although symptoms are rare with TAA, when present, repair is indicated regardless of size. In patients diagnosed with TAA who do not have indications for repair, follow-up surveillance with an imaging modality is recommended at 6 months and annually thereafter if stability is documented.[57]

In patients with Marfan syndrome, BAV, or a syndromic or familial form of thoracic aneurysm, the risk of rupture and dissection is higher at smaller diameters; thus repair is generally recommended when aortic diameter reaches 5.0 cm in size or earlier if rapid growth (>0.5 cm/y) is detected, if there is a family history of aortic dissection at an aortic diameter of <5.0 cm, or in the presence of significant aortic regurgitation.[88]

Patients with Loeys-Dietz syndrome should undergo aortic imaging examination at initial diagnosis and every 6 months thereafter; additionally, they should undergo yearly evaluation with MRI from the cerebral vasculature to the pelvis circulation. Prophylactic surgical repair of the aortic root/ascending aorta should be considered once the maximal diameter reaches 4.0 cm or greater.[57]

Surgical repair in patients with vascular Ehlers-Danlos can be challenging due to the extreme tissue fragility with poor wound healing and high risk of bleeding which may complicate the procedure. Therefore, it is important for the surgeon to know the diagnosis before the surgical procedure to optimize clinical outcomes by employing the least complicated technique for repair coupled with delicate handling of vascular tissues.[89] The role for prophylactic surgical repair of aneurysms in this syndrome has not been established, in part because of the heterogeneous spectrum of the disease, which ranges from excessive to near-normal tissue fragility. Noninvasive vascular imaging is the preferred method to evaluate the aorta and vascular structures due to the risk of major complications with invasive angiography.[74]

When the primary indication for surgery is aortic valve replacement, repair of the ascending aorta is recommended if the diameter is 4.5 cm or greater. Patients with BAV should undergo serial imaging to evaluate the size and shape of the aortic root and ascending aorta. It is important to visualize the ascending aorta distal to the sinotubular junction as aortic dilatation is common in this location. When treating patients with BAV, clinicians should be aware of the ongoing risk of aneurysm formation/aortic root dilatation even after surgical valve replacement, which necessitates the need for continued surveillance over time.[90]

Endovascular Repair

An alternative approach to surgical management of TAA is thoracic endovascular aortic repair (TEVAR), which currently is only approved for the management of descending TAA. Advantages over open surgical repair include avoidance of thoracotomy and aortic cross clamping, reduced morbidity associated with open surgery, and shorter length of hospital stay. Current indications for endovascular repair in asymptomatic patients with descending TAA are the same as for open surgical repair. It is important to recognize that not every patient is candidate for TEVAR. Patients with short aneurysm necks, aneurysms too wide to contain available devices, or poor vascular access are considered unsuitable for TEVAR. Although early effectiveness and high technical implantation success rate was demonstrated in a multicenter prospective nonrandomized phase II study,[91] long-term durability is largely unknown. In addition, prospective randomized trials have not been performed to compare endovascular versus open repair. Therefore, while TEVAR is a relatively new and appealing approach for the treatment of descending TAA, the decision to employ this approach must balance patient factors with the anticipated risks and benefits. For example, in young patients with descending TAA who are good candidates for open repair, the immediate benefits of stent grafting must be judiciously balanced against the proven efficacy and durability of open repair. On the other hand, in older patients with multiple comorbid conditions who are at increased risk for open repair, the immediate benefits of endovascular therapy likely overshadow concerns about its long-term durability.

Medical Management

Risk reduction measures, including hypertension control, lipid management, and smoking cessation, should be recommended for all patients with TAA, both before and after repair. Beta blockade has been shown to reduce the rate of thoracic aortic dilatation and aneurysm expansion in patients with Marfan syndrome and is currently recommended for all patients with this disease process who have TAA. These data were derived from an open-label study that randomized 70 patients with Marfan syndrome to propranolol versus placebo, which demonstrated reduced rate of aortic dilatation and risk of aortic dissection with propranolol treatment over a 10-year follow-up period.[92] Angiotensin II-receptor blockade (ARB) has also been demonstrated to reduce aneurysm expansion in animal models of Marfan syndrome.[93] More recently, in a small cohort study of pediatric patients with Marfan syndrome, the use of ARB slowed the rate of aortic root dilatation.[94] Further clinical trials are needed to determine the efficacy of ARB and beta blockade in patients with Marfan syndrome and TAA. Currently, there are no data to support the use of statins to prevent expansion in TAA; however, it is logical to use statins in patients with concurrent aortic atherosclerosis detected on imaging studies.

MYCOTIC ANEURYSMS AND OTHER INFECTIOUS DISEASES

Infection of the aorta, or infectious aortitis, is a rare cause of aortic aneurysms. The term *infected aneurysm* is now more commonly used than *mycotic aneurysm* because most of the infectious agents are nonfungal. Infected aneurysms occur through several mechanisms, including direct spread from contiguous structures (mediastinitis), septic embolization to the aorta (bacterial endocarditis), hematogenous dissemination (sepsis), or blood-borne vasa vasorum infection (syphilis).[95] Infection of the aorta is more common in regions of preexisting aortic wall disease, including aneurysms, extensive atherosclerosis, aortic trauma, or previous surgical sites. Bacteria such as *Staphylococcus aureus*, *Salmonella*, and *Clostridium* species are most commonly isolated. Although rarely seen at the present time, *Treponema pallidum* can cause syphilitic aortitis in 10% of patients with untreated tertiary syphilis, which most commonly occurs 10 to 25 years after the initial spirochete infection. The ascending thoracic aorta and aortic arch are most commonly involved. Other organisms, including fungi and mycobacteria,

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occasionally cause infected aneurysms, most often in immunologically compromised patients. Prompt surgical management with extensive debridement, along with antibiotic therapy, is recommended in patients with infected aneurysms. There are no data addressing the optimal duration of antibiotic therapy following surgical treatment for infected aneurysms.[95]

ANEURYSMS ASSOCIATED WITH SYSTEMIC INFLAMMATORY DISORDERS

Numerous systemic inflammatory disorders may cause inflammation of the aorta resulting in aneurysm, dissection, or rupture. It is very important to recognize these diseases given the benefit of immunosuppressive therapy to suppress the inflammatory cascade. GCA, also known as temporal arteritis, is a vasculitic syndrome that affects large- to medium-sized arteries, primarily in the elderly. It is estimated that GCA involves the aorta in up to 18% of cases, and patients with GCA are 17 times more likely to develop TAA and twice as likely to develop AAA as compared with age- and sex-matched patients without GCA.[96] The histopathologic process of GCA is manifested by granuloma formation in the aortic wall. This process is mediated by $CD4^+$ T cells and macrophages, which produce cytokines and MMPs. Although hyperplasia of the intima leading to arterial narrowing and ischemia is a more common presentation, GCA can affect the entire aorta and cause aneurysm formation many years after the diagnosis is originally made.[97] Patients with GCA have a significantly elevated erythrocyte sedimentation rate, and proof of giant cells by biopsy is diagnostic. Initial evaluation of patients with GCA should include CTA or MRI of the thoracic aorta and its branches to evaluate the possibility of aneurysms or occlusive disease. Imaging by PET with 18-fluoro-deoxyglucose (FDG) and CT allows the assessment of disease activity, vessel morphology, and localized inflammation in the same study.[98] Therapy is aimed at the inflammatory state with high-dose corticosteroids.

Takayasu arteritis is a large-vessel inflammatory vasculopathy that primarily affects the aorta and its major branches. Patients are usually females in their 2nd or 3rd decade of life. Arterial occlusion is more common than dilatation, and narrowing of the left subclavian artery is present in 90% of patients, making the left radial pulse weak or absent, hence the term *pulseless disease*.[99] The pathophysiology is poorly understood; however inflammatory mononuclear cells, primarily T cells, infiltrate the blood vessel wall through the vasa vasorum and invade the intima.[99] The inflammatory process may cause destruction or fibrotic repair of the aorta, leading to aneurysm formation or stenosis, respectively. Aneurysms are more common in the aortic root or arch, followed by the abdominal and thoracic aorta.[100] Initial evaluation of Takayasu arteritis should include assessment of the thoracic aorta and its branches with either MRI or CT scan. MRI is a good diagnostic tool to image aortic wall thickening, and MRA is useful to image the luminal appearance of the aorta and its branches. As in GCA, PET-FDG is a new imaging technique used in large-vessel vasculitis to detect increased aortic wall metabolism suggestive of inflammation and can be used in conjunction with CT to localize the disease process.[98] Initial treatment is directed to reduce inflammation with high-dose corticosteroids and other immunosuppressive agents as second line therapy.

Several other systemic inflammatory diseases, including Behcet disease, ankylosing spondylitis, rheumatoid arthritis, and Kawasaki disease, may be associated with aneurysm formation. No randomized trials exist to guide recommendations for detection or surveillance of aneurysms for these inflammatory diseases. Therefore, indications for aneurysm repair should be the same as in noninflammatory disorders.

ANEURYSMS OF OTHER ARTERIES

Coronary artery aneurysm (CAA) is an abnormal dilatation of a localized or diffuse segment of the coronary vasculature, with a diameter of ≥1.5 times the adjacent normal coronary artery. The incidence of this disease is 0.3% to 4.9%, with right coronary artery involvement in 68% of cases.[101] The most common etiology of CAA is atherosclerotic coronary artery disease, with a reported frequency of 50% to 90% depending on the series. Approximately 10% to 20% of cases are attributed to Kawasaki disease, infectious septic emboli, Takayasu arteritis, collagen vascular/connective tissue disorders, or as a complication of coronary stenting.[101–103] In rare instances, genetic predisposition to CAA has been reported.[104] The pathogenesis of this disease is poorly understood; an inflammatory process involving all layers of the coronary artery leading to medial elastin degradation may be a common pathway in the pathology of CAA.[101,103] This is supported by observations that patients with isolated CAA have higher levels of serum C-reactive protein and interleukin-6 than do patients with and without atherosclerotic coronary artery disease. In addition, elevated levels of plasma-soluble adhesion molecules, such as vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin, have also been detected in patients with CAA. Finally, activation of MMPs, particularly MMP-3, has been associated with CAA in humans.[101]

Multiple imaging modalities, including echocardiography, cardiac CT (FIGURE 72.4C), and MRI, can be used to confirm the diagnosis of CAA. However, the gold standard for diagnosis continues to be coronary angiography, which provides detailed anatomic information regarding the size, location, shape, and number of aneurysms.

The low frequency of the disease, and the lack of prospective randomized trials in patients with CAA, prohibits an evidence-based management protocol. However, CAA is clearly not a benign condition; the 5-year mortality rate in untreated CAA was reported to be 29%.[105] At present, options include medical therapy, stent insertion, or surgical excision. If a coronary aneurysm is three or more times greater than the reference vessel diameter, surgery is usually recommended due to the high risk for compression, rupture, or thrombosis.[101] Aneurysmal coronary arteries are susceptible to thrombus formation as a result of the loss of laminar flow in the dilated segment. This risk factor, along with the high concurrence of atherosclerosis in patients with CAA, makes antiplatelet therapy important in the overall management. Although anticoagulation therapy with warfarin would appear to be a reasonable approach for the management of CAA, there is no evidence to support this treatment option over aspirin; its use is based on the patients' clinical context and physician preference.[103]

Popliteal artery aneurysms (PAAs) are localized dilatations of the popliteal artery >2 cm in diameter or more than 1.5 times the reference artery caliber. PAAs are the most common extraaortic arterial aneurysms, accounting for up to 70% of peripheral aneurysms.[106] They have been documented in 55% of patients with AAA, with a male predominance of more than

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95%.[107] Unlike AAA, complications of PAAs are more related to thromboembolism than rupture. Patients usually present with acute or chronic ischemia of the lower extremity due to thrombus formation or distal embolization. Microembolic symptoms are manifested as "blue toes," and mortality and amputation rates with this condition may be as high as 20%.[108] The exact pathophysiology of PAAs is unknown; however, associated factors include atherosclerosis and hypertension, especially in the aging population. The etiology in a small minority may be attributed to trauma, infection or mycotic disruption of the arterial wall, fibromuscular dysplasia, or inflammatory arteritides such as Behcet disease or Takayasu arteritis. A congenital form has also been proposed in small series of young patients.[109] Diagnosis is suggested by popliteal pulse contour and confirmed by duplex ultrasound, CT angiography (FIGURE 72.4D), MRA, or invasive angiography. Repair of PAAs has been recommended for all symptomatic patients, and for asymptomatic aneurysms >2.0 cm in diameter.[110] Open surgical treatment with arterial bypass grafting has been the traditional method to treat PAAs. More recently, endovascular repair with stent grafting has become a reasonable treatment option in anatomically suitable patients, with patency rates comparable to surgical treatment in some studies.[110]

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CHAPTER

73 Intracranial Hemorrhage and Subarachnoid Hemorrhage

TIMOLAOS RIZOS AND THORSTEN STEINER

INTRACEREBRAL HEMORRHAGE

Epidemiology, Risk Factors and Prognosis

Intracerebral hemorrhage (ICH) is an extravasation of blood into the brain parenchyma. Hemorrhagic stroke accounts for approximately 15% of all strokes.[1,2] Advances in treatment (in the context of specialized neurologic and neurosurgic intensive care units) have resulted in improved survival over the last decade.[3] However, the mortality rate in patients with ICHs remains high; approximately 50% of patients die in the first 3 months,[4–6] with half of these patients dying within the first 2 days after symptom onset.[4] Moreover, ICH is associated with significant morbidity. For example, after 6 months only 20% of patients are functionally independent.[4]

ICH is most common in elderly persons.[7] The crude risk ratio for age (every 10-year increase) was calculated to be 1.97.[7] The incidence of ICH is higher in African American and Asian populations compared with whites. The Northern Manhattan Study revealed that Hispanic adults from the Caribbean and Black adults have a higher risk for ICH than whites.[8] Especially in elderly patients, cerebral amyloid angiopathy (CAA) is an important risk factor for primary ICH.[31,32] Many modifiable risk factors for ICH have been identified and prevention of these factors may reduce the incidence of ICH. Arterial hypertension is one of the most important risk factors for ICH. In elderly patients and in patients with untreated or uncontrolled hypertension, the risk of ICH is doubled.[7,17,18] The frequency of arterial hypertension in ICH has been estimated at 70% to 80%[4] and the crude odds ratio (OR) for hypertension is 3.68.[7] Although hypertension is the most important modifiable risk factor for prevention of ICH recurrence, data on optimal blood pressure control are lacking.[19,20]

The use of oral anticoagulation with warfarin is associated with an 8- to 19-fold increase in the risk of ICH.[21,11] The risk of ICH in patients taking oral anticoagulants is estimated to be 0.3% to 3.7% per year (when the international normalized ratio [INR] is between 2.0 and 4.5).[15] Each increase in INR of 0.5 elevates the bleeding risk by a factor of 1.4. The risk is particularly high in patients whose INR exceeds 3.9.[22] Because of the rising prevalence of atrial fibrillation—which is the most frequent indication for long-term therapy with vitamin K antagonists—the burden of oral anticoagulant-associated ICH is expected to rise considerably over the next several years.[14,15,16] Although more studies are needed, newer oral anticoagulants, including factor Xa inhibitors may reduce the risk for ICH when used for prevention of stroke in patients with atrial fibrillation.[23,24] High-dose aspirin has also been implicated as a risk factor for ICH risk, particularly in elderly patients with untreated hypertension.[25] Aspirin use (at doses that reduce the risk of myocardial infarction and ischemic stroke) is associated with an absolute risk increase in ICH of 12 events per 10,000 persons.[25] In a pooled analysis from 21 cohorts, aspirin use at the time of ICH onset was independently associated with increased mortality, but not with poor functional outcome.[25] The combined use of aspirin and clopidogrel further increases the risk of ICH.[25] Other risk factors for ICH have been reported, including alcohol consumption,[26,7] cigarette smoking, cocaine use,[7] high cholesterol level,[27] Vitamin E intake, and racial variations.[28,29]

Age over 80 years represents a risk factor for unfavorable outcome. In addition, hematoma volume and hematoma location contribute to prognosis and outcome.[9,8] Specifically, large infratentorial and deeply located hematomas have unfavorable prognoses.[4] ICH associated with the use of oral anticoagulants accounts for 10% to 18% of all ICH and results in particularly high rates of secondary hematoma enlargement (up to 54% of patients) and mortality (up to 67%).[10–13]

Pathophysiology

The majority of ICH (80% to 85% of all ICH) occur following the spontaneous rupture of small arteries and arterioles that are damaged by chronic arterial hypertension (up to 70% cases) or amyloid angiopathy (up to 30% cases) (FIGURES 73.1 and 73.2).[17,33] These are termed "typical" or "primary" ICH. The location of the hemorrhages is usually deep, but they may also occur in the posterior fossa (brainstem and cerebellum).[4] In the case of CAA, hemorrhage is frequently located in the occipital or parietal region.[34] Miliary aneurysms (Charcot Buchard aneurysms) and lipohyalinosis, both associated with chronic arterial hypertension, have also been proposed as etiologic factors.

The remaining cases (15% to 20% of all ICH) are categorized as "atypical" or "secondary" ICH. These are related to multiple causes, including arteriovenous malformation (AVM), cavernoma, neoplasms, aneurysms, vasculitis, Moya-Moya disease, sinus venous thrombosis, eclampsia, and coagulopathies (e.g., liver malfunction and hemophilia). They also occur in the context of trauma or ischemic stroke, and in association with oral anticoagulation and/or antiplatelet treatment (particularly when given in combination), and drug consumption (i.e., amphetamines, cocaine).

Clinical Presentation

A frequent characteristic feature of ICH is the sudden onset of focal neurologic deficits during physical activity.[1] Presentations of neurologic deficits depend on the bleeding location and hematoma volume. For example, a small hematoma in

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FIGURE 73.1 "Typically" located primary ICH in the left basal ganglia.

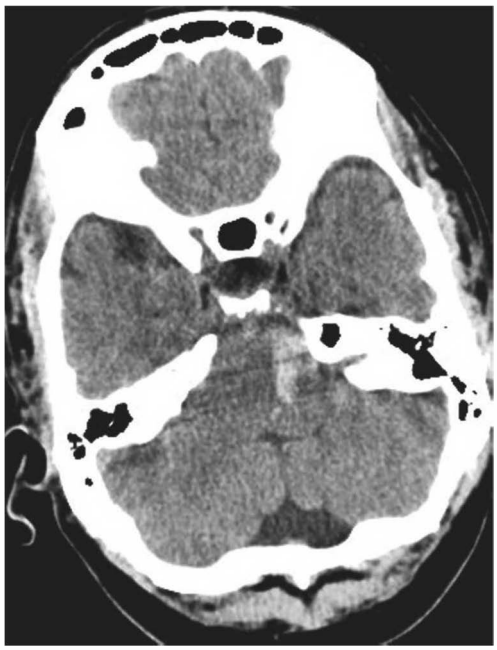

FIGURE 73.3 In this comatose patient, a brainstem hemorrhage was present. Though small in size, the bleeding caused severe symptoms including a high-grade right-sided arm paresis, ataxia, a complex oculomotoric dysfunction and dysphagia.

the brainstem (FIGURE 73.3) may produce profound neurologic deficits, whereas a similarly sized hematoma within supratentorial structures may cause only mild deficits. Bleeding in the basal ganglia may result in sensorimotor hemiparesis on the contralateral side. Bleeding in thalamic structures typically presents with paraesthesia or sensory loss. Symptoms of patients with lobar hematomas (~20% of ICH) include aphasia, neglect, hemiparesis, and hemianopia. Hematomas in frontal regions may lead to neuropsychological disorders. Brainstem symptoms, including diplopia, dysarthria, dysphagia, facial weakness, and cerebellar signs such as vertigo and ataxia can be observed in infratentorially located hematomas (see FIGURE 73.3). ICH in the brainstem or supratentorial ICH with impending or apparent herniation may lead to decreased consciousness and coma. In supratentorial ICH, unequal pupils with a dilated pupil on the side of the supratentorial hematoma (resulting from pinching the third cranial nerve to the clivus bone) indicates herniation of the temporal lobe (FIGURE 73.4). Clinical deterioration is frequent in ICH, often within the first 24 hours after onset of symptoms. Underlying mechanisms include hematoma enlargement, development of perihematomal edema, hydrocephalus, and seizures. As a result, continuous monitoring of vital parameters is indicated.

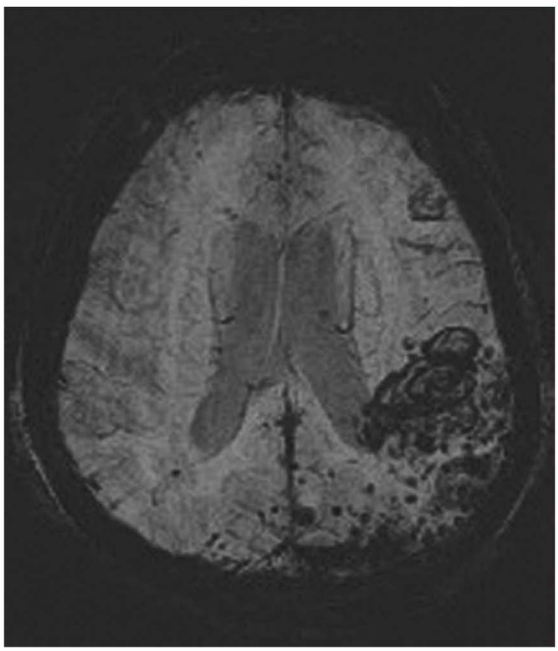

FIGURE 73.2 Cerebral amyloid angiopathy. In this patient, a left occipital ICH was seen on CT, subsequent MRI identified multiple CAA-related microhemorrhages (Susceptibility weighted images). In the CAA, depositions of b-amyloid in the media and adventitia of small- and mid-sized arteries can be found.

Diagnosis

It is important to distinguish between ICH, ischemic stroke, and subarachnoid hemorrhage (SAH). Despite efforts to establish clinical "rules" for differentiating ICH from ischemic stroke,[35] definitive diagnosis requires cranial imaging (cranial computed tomography [CCT] or magnetic resonance imaging [MRI]). The sensitivity for detecting acute ICH using noncontrast-enhanced CCT approaches 100%. Acute ICH presents as hyperdensity on computed tomography images (in patients with anemia, the hematomas may be isodense). During the course

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FIGURE 73.4 Large supratentorial ICH in a comatose patient presenting with a dilated pupil on the right side due to pushing of the Nervus oculomotorius to the clivus bone. Because of subsequent herniation of the temporal lobe, the patient died shortly after hospital admission.

of absorption, the hematoma becomes isodense, and finally hypodense. With the implementation of susceptibility-weighted T2* sequences in standard examination protocols, the sensitivity of MRI in detecting hyperacute and acute ICH is comparable to that of CCT.[36] In hyperacute hemorrhage, these sequences (for a 1.5-Tesla investigation) reveal characteristic hyperintense signals. In the chronic stage, hemosiderin causes a hypointense signal on T2- and T2*-weighted sequences. Whether further imaging studies (with the exception of a 24-hour scan to detect subclinical hematoma enlargement) are needed in patients with "typical" ICH (a previous diagnosis of arterial hypertension and localization of the hematoma in the putamen, pallidum, internal capsule, pons, or cerebellum) or with suspected CAA (periventricular white matter lesions) is controversial.[37] However, in younger patients without known arterial hypertension and in patients with suspected secondary bleeding (e.g., lobar bleedings or so-called "atypical" ICH) further diagnostic work-up is required to clarify the etiology of the hemorrhage. Computed tomography angiography (CTA), magnetic resonance angiography (MRA), and digital subtraction angiography (DSA) provide additional information in these patients. CTA provides a rapid and sensitive method for identifying vascular pathology, including aneurysms (>3 mm), and AVM. However, in patients with large ICH, hemodynamic changes may obscure detection of AVM by MRA or CTA. In these cases, DSA should be performed. MRA is the optimal technique for detecting underlying vascular malformations of low-pressure/venous systems (i.e., cavernoma and hemorrhagic tumors). Diagnosis of sinus venous thrombosis (which can cause secondary hemorrhage) requires venous MRA (contrast-enhanced) or DSA.

Complications

Hematoma Enlargement

The most important complication associated with ICH is enlargement of the hematoma (FIGURE 73.5). Hematoma expansion is frequently associated with neurologic deterioration and has an unfavorable outcome. The mechanisms of hematoma expansion are not fully understood. They may involve local tissue distortion, disruption of small vessels, blood-brain barrier disruption, and local coagulopathy due to the release of tissue thromboplastin.[30] ICH expansion may reflect continued bleeding or rebleeding. In approximately one-third of patients, the hematoma increases approximately 33% within the first 4 hours of onset. An additional 12% of patients suffer hematoma enlargement within the next 20 hours.[38] Different predictors of hematoma expansion have been identified: large initial hematoma volume, irregular hematoma contours, heterogeneity of density on CT images, spot signs on CTA within the hematoma, liver dysfunction, hypertension, hyperglycemia, and high alcohol consumption.[39–41,43] However, reliable radiographic predictors of ICH enlargement do not currently exist.[42]

Intraventricular Hemorrhage and Hydrocephalus

Concomitant bleeding into the ventricles (intraventricular hemorrhage [IVH]) is present in up to 40% of patients with ICH and portends a worse outcome.[30] Furthermore, IVH is frequently associated with obstructive hydrocephalus, which often requires temporary or permanent intraventricular drainage (FIGURE 73.6). Risk factors for further enlargement of IVH include baseline mean arterial blood pressure above 120 mmHg, larger baseline ICH volume, and existence of IVH on the baseline CT scan. Predictors of death or severe disability include older age, lower baseline Glasgow coma score (GCS), larger baseline ICH volume, IVH growth >2 mL, and existent IVH at baseline or at 24 hours.[44]

Perihematomal Brain Edema

Perihematomal brain edema occurs is some patients with ICH. At one time, it was considered to represent ischemic penumbra (the term generally used to define ischemic but still viable cerebral tissue). However, the use of MRI has failed to confirm the existence of salvageable tissue. Indeed, perihematomal hypoperfusion has been shown to follow metabolic changes rather than true tissue ischemia.[47] A more likely mechanism for perihematomal brain edema is an underlying inflammatory response to the release of thrombin and other end products of coagulation from the hematoma, and secondary disruption of the blood–brain barrier.[45,46,48–50] Although studies are lacking, it is interesting to speculate that the use of anti-inflammatory drugs would mitigate this complication.[51]

Conservative Treatment

General Measures

ICH is an emergency and has to be treated as such. Although treatment options are limited, advances in supportive care have reduced mortality of these patients during the last several years. Indeed, previous studies have shown that treatment at specialized neurologic units can reduce mortality and improve functional outcome.[1] In these settings, patients may be continuously monitored for vital parameters and neurologic status using

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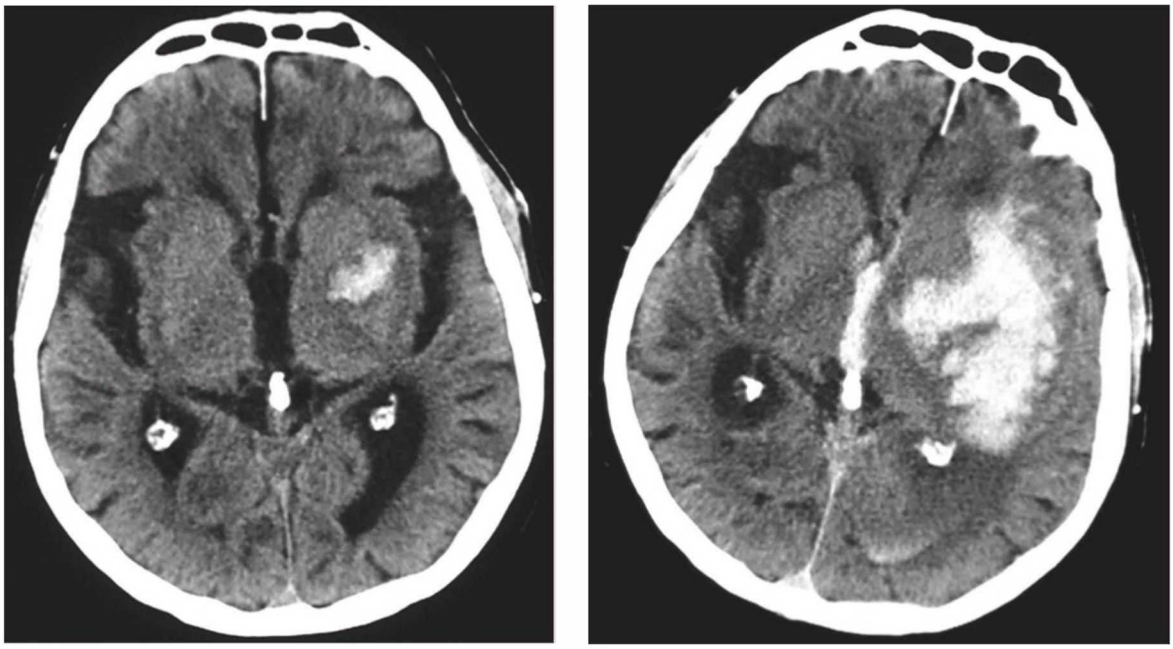

A B

FIGURE 73.5 Left basal ganglia hematoma in a patient presenting with a mild hemiparesis on the right side **(A)**. Three hours after admission, the patient was found comatose with slight unequal pupils (**right < left**). The follow-up CT scan revealed massive expansion of the formerly relatively small hemorrhage **(B)**. The clinical condition further deteriorated and the patient died 26 hours after admission.

standardized scores (e.g., National Institutes of Health Stroke Scale, Scandinavian Stroke Scale, Hemphill-ICH-Score). Airway management including tracheal intubation and ventilation is frequently necessary in unconscious patients.[52] Elevated body temperature (>37.5°C) and hyperglycemia should be avoided and rapidly treated. Further aspects of general treatment are the prevention of decubital ulcerations and infections. Early mobilization and rehabilitation are recommended for clinically stable patients.[1]

Blood Pressure Management

Arterial hypertension is one of the most important risk factors for ICH and blood pressure monitoring and management is the most important aspect of ICH treatment. In ischemic stroke, aggressive lowering of the blood pressure is associated with increased neurologic deterioration.[53] However, small-volume ICH is not associated with detectable ischemic penumbra,[47] and thus the risk-benefits of treating high blood pressure in this patient population may favor such intervention. Indeed, The INTEnsive blood Pressure Reduction Trial[54] revealed that early intensive blood pressure-lowering treatment is clinically feasible, well tolerated, and seems to reduce hematoma growth in ICH. However, patients with severe injury (GCS 3-5) were excluded from this study, and it is still not known whether aggressive blood pressure reduction confers an improved clinical outcome. It is generally recommended that cerebral perfusion pressure (CPP) be monitored particularly in patients with elevated intracranial pressures, and CPP (mean arterial pressure minus intracranial pressure) should be kept above 60 to 80 mm Hg.

Hemostatic Therapy

Previous efforts have focused on the role of hemostatic agents in controlling bleeding and preventing hematoma expansion in ICH. Most of these strategies, including tranexamic acid, epsilon-aminocaproic acid, and aprotinin, have failed to demonstrate efficacy. However, the use of recombinant factor VIIa (rFVIIa) may hold more promise. A prospective randomized controlled phase IIB trial demonstrated the potential of rFVIIa to reduce hematoma expansion and to improve clinical outcome.[55] A subsequent phase III trial (FAST-Trial) confirmed that 80 μg/kg of rFVIIa IV reduced hematoma expansion significantly when given within 4 hours. However, in contrast to the Phase IIB study, rFVIIa did not improve clinical outcome,[56] and was associated with a dose-dependent increase of thromboembolic events.[57] Additional studies are needed to determine the risk-benefit profile of rFVIIa treatment according to patient age, time to treatment, volume of ICH, and presence of IVH.[58]

Treatment of ICH Related to Oral Anticoagulants

In patients with ICH associated with the use of oral anticoagulants, normalizing coagulation is of utmost urgency. Although fresh frozen plasma may be used, it requires large volumes (which increase the risk of congestive heart failure and pulmonary edema) and is associated with substantial time delays before normalization of the INR.[59,60] Thus, prothrombin complex concentrate (PCC) is preferred by many emergency physicians. PCC, a pooled plasma product containing factors II, IX, X, and (in some products) VII, can be stored in the emergency room, enabling prompt availability. However, plasma concentrations of the clotting factors can vary considerably between

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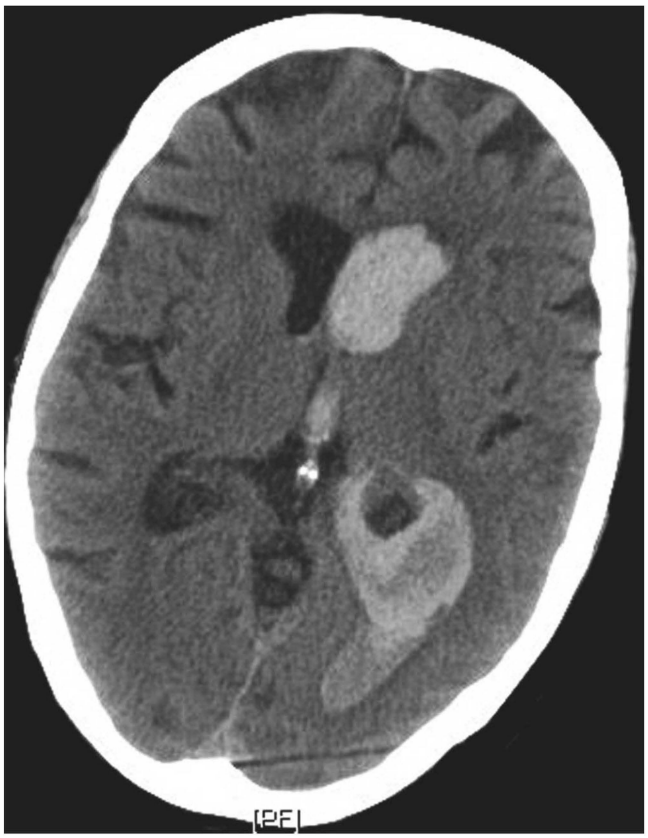

A

B

C

FIGURE 73.6 ICH patient with isolated IVH. **A**: initial CT scan at presentation revealing a large left-sided IVH, the left ventricle is occluded by a blood clot. In this patient bilateral external ventricular drains (EVDs) were placed to anticipate obstructive hydrocephalus. **B**: Furthermore, the EVD can be used to perform intraventricular treatment with rt-PA to hasten clot lysis. Treatment with rt-PA was performed in this patient for 6 days, the CT scan on day 10 after the initial scan reveals only small blood clots in the posterior parts of both lateral ventricles **(C)**.

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different manufacturers and lots, and thus it is difficult to predict the dose needed to reverse the INR.[61] Though rFVIIa can rapidly normalize INR in subjects receiving anticoagulation treatment with warfarin, its effect on bleeding duration is unclear and thus routine use of rFVIIa is currently not recommended.[62,1] Treatment with coagulation factors should be combined with Vitamin K because the half-life times of coagulation factors are shorter than the half-life of warfarin. The exact time point at which the physician can safely continue oral anticoagulation after ICH in the individual patient remains unknown. It may depend on hematoma volume and location, but prospective studies with a high number of patients are needed. Given that the annual embolic risk in patients with mechanical heart valves is about 5% to 10%, discontinuation of anticoagulation for a period of 10 to 14 days seems to be an acceptable trade-off (translated 2-week risk of embolism: 0.2% to 0.4%).[63]

Treatment of ICH Related to Heparin

When ICH develops in patients being treated with unfractionated heparin, rapid normalization of coagulation (i.e., the activated partial thromboplastin time) with protamine sulfate (PS) is the treatment of choice. A relevant side effect of PS is hypotension; maximum infusion rate should not exceed 5 mg PS/min and the total dose should not exceed 50 mg of PS.[34]

Treatment of ICH Related to Fibrinolysis

Patients with ischemic stroke who have been received thrombolytic therapy with recombinant tissue-type plasminogen activator (rt-PA) may develop ICHs. While "any" hemorrhage after stroke treatment with rt-PA is found in approximately 30% of patients, symptomatic hemorrhage (as defined by the presence of clinical deterioration attributable to hemorrhage) occurs in 3% to 9% of cases.[64,65] Symptomatic hemorrhages in these patients are often serious and multifocal. Mortality at 30 days is 60% and above. To rapidly correct the systemic fibrinolytic state, platelet and factor VIII substitution is recommended.[1]

Management of Increased Intracranial Pressure

Increased intracranial pressure caused by cerebral edema and mass effects is frequently seen in patients with high-volume ICH and is associated with high morbidity and mortality. Because elevated intracranial pressure compromises CPP, it must be monitored for and treated in order to maintain a target CPP >60 to 70 mm Hg. Preventative measures include avoidance of situations that might increase the intracranial pressure (e.g., pain, fever, constipation, hyponatremia, and psychological/physical stress), treatment with sedation and analgesia, and maintenance of sufficient venous flow in the jugular veins (i.e., by positioning the head 30 degrees upright). Osmotic diuretics such as glycerol and mannitol (20%) can be used to lower elevated intracranial pressure. A risk of this approach, especially with mannitol, is excessive depletion of the intravascular volume, secondary renal failure, and rebound elevations in ICP. Therefore, serum levels of electrolytes, renal function, and osmolality must be carefully monitored (target osmolality: 300 to 320 mOsmol/L). Another approach is to administer hypertonic saline (Hyper-HAES: NaCl 7,5%; hydroxyethyl starch [HES] 6%). However, this treatment carries the risk of hypernatremia. In some patients, hyperventilation can rapidly reduce elevated ICP but the effect is transient and therefore this strategy is not useful for long-time therapy.

Management of Intraventricular Hemorrhage

Clots or compression from IVH may lead to obstruction of cerebrospinal fluid (CSF) outflow at the level of the 3rd and/or 4th ventricle and secondary hydrocephalus. In such cases, ventricular drainage may be required to prevent herniation. Ventricular drainage can also be used for administering rt-PA into the ventricles to hasten clot lysis. Indeed, preliminary data show that the use of low-dose rt-PA can be safely administered to stable IVH clots and may increase lysis rates[69] (FIGURES 73.6A-C). Although an increasing number of centers are using this approach, further studies are needed to demonstrate positive effects on clinical outcome.

Surgical Treatment

Supratentorial Primary ICH

There is little evidence that surgical intervention (beyond the placement of ventricular drains) improves patient outcome in primary ICH. The Surgical Trial in Intracerebral Hemorrhage, the largest multicenter randomized trial comparing hematoma evacuation with conservative treatment to date, failed to demonstrate an outcome benefit in the surgical group. However, *post hoc* analysis suggested that patients with superficial hematoma (<1 cm from the surface) might benefit from evacuation.[70] Based on these data, evacuation of the hematoma within 96 hours may be considered in patients with a superficial primary hemorrhage. However, very early craniotomy may be associated with an increased risk of recurrent bleeding. Although hemicraniectomy is feasible in certain patients with primary ICH,[71] its efficacy remains to be determined. Studies are currently in progress to determine the benefit of multimodality therapy, including minimally invasive surgery, clot aspiration, and local application of rt-PA.

Infratentorial Primary ICH

Little evidence exists for surgical evacuation and craniotomy in patients with infratentorial hemorrhage. Cerebellar (or brainstem) hematomas can result in serious and devastating compression of brainstem structures. At this location, hematomas may lead to compression of the 4th ventricle or the aqueduct with subsequent hydrocephalus and increased intracranial pressure. In these cases, clot evacuation or posterior craniotomy should be considered even in older patients.[1,2,72]

Other Treatment Options

Treatment options in patients with AVM and in patients with intracranial dural arteriovenous fistulae (FIGURE 73.7) usually include a combination of neurosurgery, radiotherapy, and endovascular approaches.[73] The choice of therapy depends on size, proximity to critical areas, venous drainage, and accessibility.[74] One approach that is frequently used is to reduce AVM size by endovascular embolization and radiotherapy before surgical excision. The risk of bleeding recurrence in AVM is about 18% within the 1st year.[75] The estimated annual bleeding rate for cavernoma bleedings of the low-pressure system is lower than for AVM and these bleeds are usually not life threatening. In patients with cavernomas, treatment varies from observation or radiotherapy (in patients with asymptomatic or inaccessible lesions) to surgical excision (in those with symptomatic and accessible lesions).

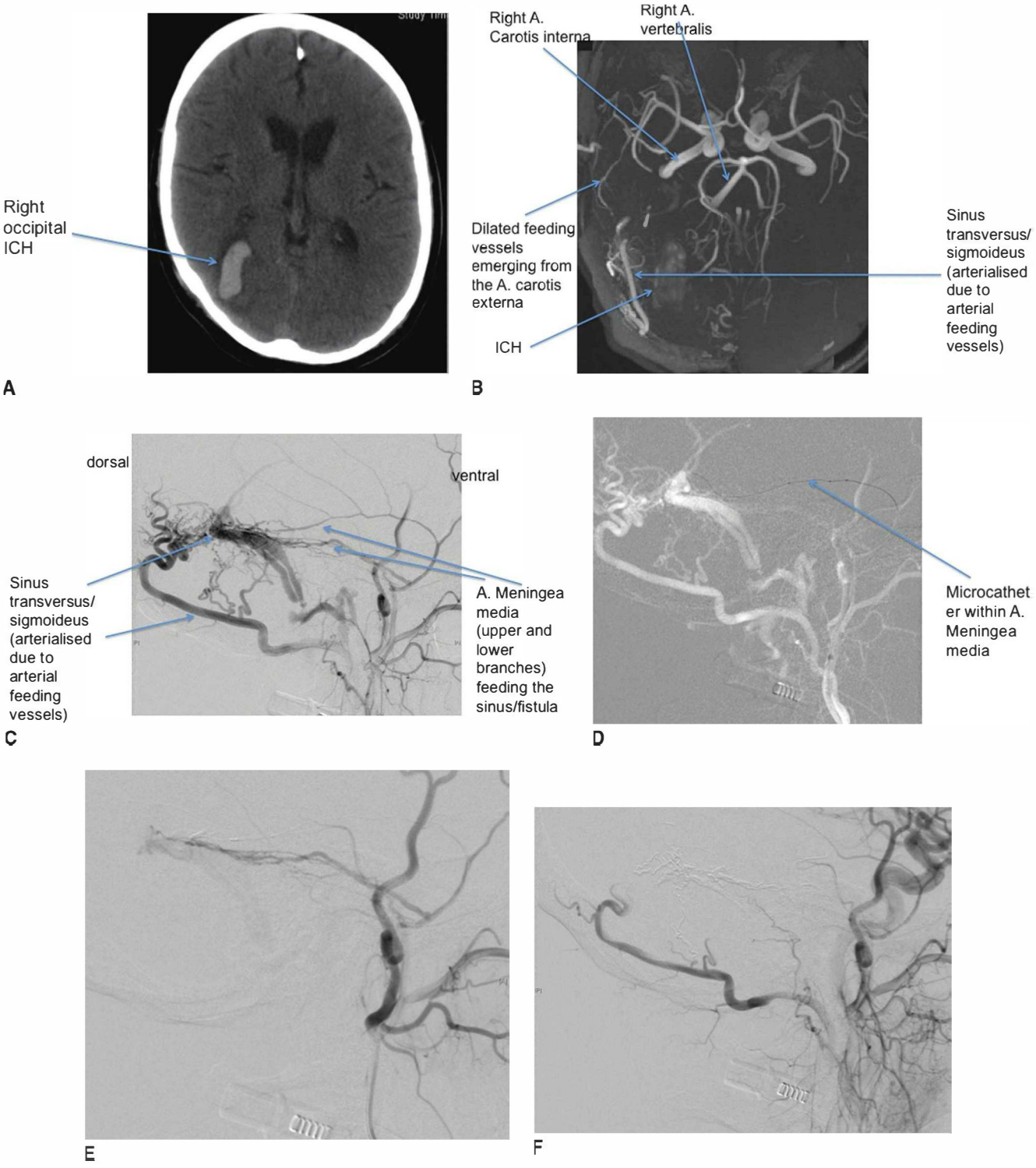


FIGURE 73.7 Series of a patient with dural arteriovenous fistula. **A**: Initial CT scan revealing an occipital ("atypical") ICH; **B**: Subsequent MRI-Angiography showing a intracranial dural arteriovenous fistula with feeding vessels from branches of the external carotid artery (A. meningea media and A. occipitalis). **C**: Initial vessel situation on angiographic images. **D**: A microcatheter placed within the A. meningea media to occlude the fistula using a liquid embolic agent. **E**: Situation after occluding the upper feeding part using a liquid embolic agent. **F**: Situation after complete occlusion.

SUBARACHNOID HEMORRHAGE

Epidemiology, Risk Factors, and Prognosis

In the majority of cases, SAH is caused by the rupture of an intracranial aneurysm. Cranial aneurysms are found in up to 5% of all autopsies. In asymptomatic individuals, MRI imaging detected an overall incidence rate of aneurysms in the general population of 6.8%, rising to 10.5% in persons with a family history of SAH.[76] However, rupture of an intracranial aneurysm causing SAH occurs in 6 to 7 per 100,000 person-years. Interestingly, the age-adjusted annual incidence of SAH considerably varies between countries: A multinational WHO study reported 2 cases per 100,000 persons in China and 22.5 per 100,000 in Finland.[77] SAH is often fatal; population-based mortality rates are as highs as 45% and the majority of deaths occur within the 1st few days after SAH.[78,79,80,81–83] Approximately 10% to 15% of patients die before reaching the hospital,[79,84] and one-third of surviving patients require lifelong care because of neurologic deficits.[84,79]

The incidence of SAH increases with age and occurs most commonly between 40 and 60 years of age (mean age 50 years).[85] Women over 40 years old are more likely to develop SAH, but under the age of 40 men are more often affected.[86,87] The risk for Africans Americans is 2.1 times that of whites[84] and the incidence of SAH increases with age, with a mean age at presentation being 55 years.[84] Modifiable risk factors include arterial hypertension, smoking,[95–98] heavy alcohol consumption,[99,33] and drug consumption.[79] Although hypertension control appears to reduce SAH severity,[100] the effect of treating modifiable risk factors on SAH occurrence has yet to be tested in randomized controlled trials.

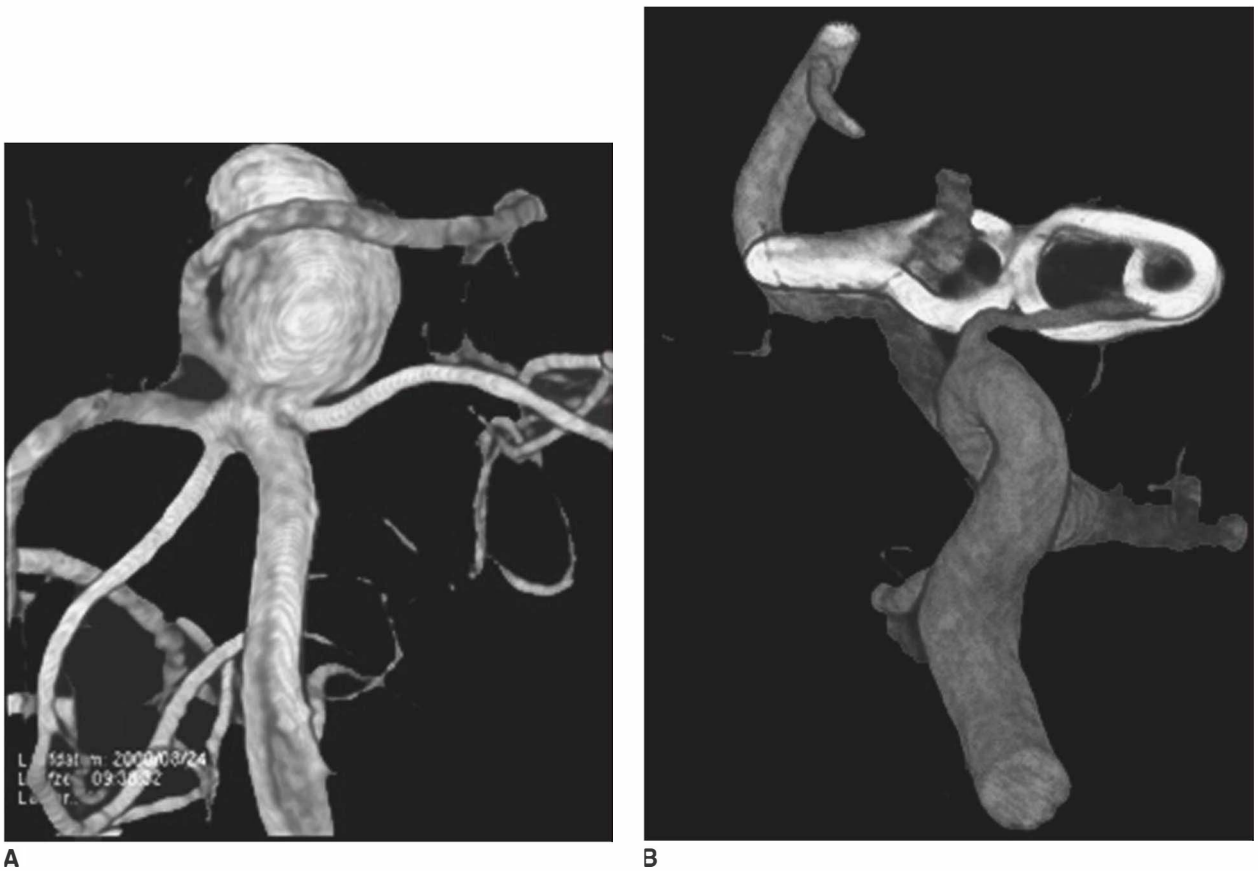
SAH is also associated with heritable disorders of connective tissue (Ehlers-Danlos disease IV, neurofibromatosis type I, Marfan syndrome) and with autosomal-dominant polycystic kidney disease.[79] Furthermore, aneurysms are frequently found on feeding vessels of AVMs.[103]

In patients with a history of a ruptured aneurysm, the annual rate of new aneurysm formation in about 1% to 2% per year[104–111] and patients with multiple intracranial aneurysms are perhaps more vulnerable to develop new aneurysms.[109,112,113] However, it remains unclear whether this is a consequence of genetic or acquired factors.

Unfavorable prognostic factors include older age, decreased level of consciousness at presentation, posterior circulation aneurysms, high quantity of blood in the subarachnoid space, and the presence of intraventricular blood.[84]

Pathophysiology and Location

Eighty five percent of all SAH are attributable to saccular aneurysms, the majority of which are located in the circle of Willis (FIGURE 73.8).[79] Approximately 10% of SAH are caused by nonaneurysmal perimesencephalic SAH (FIGURE 73.9).[79] The remaining



A B

FIGURE 73.8 **A**: Giant aneurysm of the basilar artery. **B**: Anterior communicans aneurysm after neurosurgical clipping

FIGURE 73.9 Typical prepontin SAH localization. No aneurysm was found on CTA, MRA, and conventional DSA.

5% are caused by AVM, dural arteriovenous fistula, dissection of intracranial arteries, septic aneurysms, or traumatic brain injuries (without contusion).[79] The most common sites for ruptured aneurysms are the posterior communicating artery (where it arises from the carotid artery; 41%), anterior communicating artery and anterior cerebral artery (34%), and middle cerebral artery (20%). Rupture in the posterior circulation (including the vertebrobasilar artery) is present in 15% of cases.[114] Importantly, in up to 20% of patients, multiple aneurysms can be detected.[115]

SAH can cause profound reduction in cerebral blood flow, reduced cerebral autoregulation, and acute cerebral ischemia.[124–128] Mechanisms of SAH-induced brain injury include increased intracerebral pressure with subsequent decreased CPP,[124,129,130] reduced availability of nitric oxide,[128,131] acute vasoconstriction,[125,132] microvascular platelet aggregation,[133] activation of collagenases and loss of microvascular collagen,[134] and decreased microvascular perfusion and increased permeability due to endothelial barrier antigen.[133,134] Unfortunately, advances in the understanding of the mechanisms of brain injury induced by SAH have yet to translate into therapeutic breakthroughs.

Clinical Presentation

Although the clinical presentation of aneurysmal SAH is one of the most striking and distinctive in medicine, many persons with SAH do not receive instant medical attention and many SAH cases are misdiagnosed.[88,89,90–94] Typically, patients with ruptured aneurysms report instantaneous onset of acute and severe headache ("thunderclap headache"),[116] often located in the neck and accompanied by acute nausea, vomiting, and neck stiffness. Reduced level of consciousness, hemiparesis, or other neurologic focal symptoms is common in severe SAH. In some patients, seizures or delirium are the first symptoms. Preretinal hemorrhages (Terson syndrome) may indicate an abrupt increase in intracranial pressure and is associated with increased mortality.[84] However, SAH might be missed when classic symptoms are absent. Sentinel thunderclap headaches may occur as the only symptom and reflect a "warning leak." Depending on the size and localization of the aneurysm, patients may present with single or combined cranial nerve palsy. For example, painful third nerve palsies are highly suspicious for aneurysm of the posterior communicating artery.[84] Several grading scores are available to evaluate the severity of SAH[117–121] (Table 73.1).

Diagnosis

Imaging

In almost all cases, diagnosis of SAH can be confirmed by CCT. Sensitivity of CCT decreases from 98% within the first 12 hours after onset of symptoms[79] to 50% on the 7th day.[84] Sensitivity of cranial MRI is comparable to that of CCT if performed on the 1st day.[79] CTA is the preferred tool for detecting aneurysms in SAH patients,[122,123] but additional DSA is frequently needed. Because of the possibility of three-dimensional visualization, CTA and MRA are often helpful for planning treatment especially in large or complex aneurysms.[84] However, conventional catheter angiography remains the gold standard for detecting aneurysms.[84] Because in many patients multiple aneurysms can be found, all cerebral vessels have to be carefully evaluated.[101] In patients with negative results in DSA, examination should be repeated 7 to 14 days after initial presentation if suspicion for the presence of aneurysms remains strong.[84]

Cerebrospinal Fluid

In patients with a typical clinical history of SAH and negative CCT, lumbar puncture (LP) is mandatory (presuming that no contraindications for LP are present). The CSF may be xanthochromic. Normal CSF excludes SAH within the prior 2 to 3 weeks. The value of the traditional "three tube test" whereby CSF is collected in three consecutive tubes for red cell count remains unclear.[79,84]

Complications

Vasospasm

Vasospasm of large cerebral basal arteries occurs in up to 70% of patients with aneurismal SAH. This complication typically begins 3 to 5 days after hemorrhage, peaks at 5 to 14 days, and gradually resolves over 2 to 4 weeks.[138,139] Transcranial sonography is commonly used for detecting and monitoring vasospasm.[84] Modalities such as MRI or CT with diffusion/perfusion sequences have also been shown to be advantageous in guiding the management of vasospasm.[142–144] Vasospasm is often associated with radiographic evidence of diminished brain perfusion and up to 20% of patients with vasospasm develop ischemic stroke or die despite maximal therapy.[140,141] Thus, these patients require aggressive treatment at experienced centers.

Hydrocephalus

Hydrocephalus is often observed in SAH patients. In up to 30% of cases, hydrocephalus occurs early in the course of the disease, and is more common in patients with poor clinical grade and large amounts of subarachnoid blood.[135,136] Occlusion develops when the 4th ventricle, the aqueduct, and/or the 3rd ventricle is obstructed

Table 73.1

Clinical classifications of subarachnoid hemorrhage

	WFNS		Hunt and Hess	Fisher	Modified Fisher
Grade	GCS	Focal deficit[a]		Appearance of hemorrhage	
0	N/A	N/A	N/A	N/A	No SAH or IVH
I	15	None	No symptoms, minor headache, minor neck stiffness	None evident	Minimal/thin SAH, no IVH in both lateral ventricles
II	13-14	None	Moderate to severe headache, neck stiffness, no neurologic deficit other than cranial nerve palsy	Diffuse deposition of subarachnoid blood, no clots, and no layers of blood >1 mm	Minimal/thin SAH with IVH in both lateral ventricles
III	13-14	Present	Somnolence, confusion, minor neurologic deficit	Localized clots and/or vertical layers of blood 1 mm or greater in thickness	Thick SAH[b], no IVH in both lateral ventricles
IV	7-12	Present/none	Sopor, moderate or severe neurologic deficit (hemiparesis)	Any thickness with IVH or parenchymal extension	Thick SAH[b], with IVH in both lateral ventricles
V	6-3	Present/none	Coma, signs of herniation	N/A	N/A

WFNS, World Federation of Neurological Surgeons; GCS, Glasgow coma score; IVH, intraventricular hemorrhage; SAH, subarachnoid hemorrhage.
[a]Cranial nerve palsies are not considered as focal deficit.
[b]Completely filling ≥1 cistern or fissure.

by intraventricular blood. On the other hand, hydrocephalus aresorptivus is a result of malfunctioning Pacchioni granulations, which normally reabsorb the CSF. Delayed hydrocephalus (up to weeks after onset of symptoms) is present in up to 25% of patients. Risk factors include older age, ventricular hemorrhage, poor clinical condition at presentation, and female gender.[137]

Other Complications

Additional complications in patients with SAH include seizures, pulmonary edema, cardiac malfunction, hyponatremia, inappropriate antidiuretic hormone secretion, and cerebral salt wasting.[84] The incidence of bleeding recurrence is particularly high within the first 2 weeks[84] and case fatality rates in these patients are up to 70%.[101] Thus, a critical therapeutic goal in all patients is to prevent rebleeding by early aneurysmal occlusion (by surgical or endovascular means).[101]

Conservative Treatment

General Measures

SAH is a serious medical emergency and patients should be treated in neurologic or neurosurgic critical care units at centers with neurovascular expertise. The main goal of treatment is to prevent medical and neurologic complications. Hypertension may increase the risk of rebleeding. Although controlled studies are lacking, monitoring and treating blood pressure to maintain CPP is recommended.[101] Antifibrinolytic therapy to prevent rebleeding in SAH has been investigated. Although older studies demonstrated overall negative effects of antifibrinolytic agents, recent evidence supports the use of an early, short course of antifibrinolytic agents in combination with other therapeutic modalities.[101] While oral treatment with the calcium antagonist nimodipine is indicated to reduce poor outcome in aneurysmal SAH patients,[145] treatment is recommended in all patients with SAH. The value of other calcium antagonists remains uncertain.[101]

Management of Vasospasm

Treatment of vasospasm starts with early management of the ruptured aneurysm.[101] If vasospasm is present, a reasonable approach is to employ the so-called triple-H therapy (hypervolemia, hypertension, hemodilution) to enable sufficient cerebral perfusion and oxygenation.[101] Catecholamines (e.g., dobutamine and norepinephrine) may be needed to achieve higher systolic pressures (up to 240 mm Hg). Because of the risks associated with aggressive therapy (e.g., pulmonary edema, cardiac failure, cerebral edema, rupture of aneurysm, and electrolyte imbalances), these patients should undergo occlusion of the aneurysm and be carefully monitored and followed in critical care units.[84] Another approach for controlling vasospasm is cerebral angioplasty and/or selective intra-arterial vasodilator therapy in combination with or alternatively to triple-H therapy.[101]

Management of Hydrocephalus

For acute chronic hydrocephalus, intermittent external ventricular catheter drainage is required to prevent increased intracranial pressure with subsequent brain tissue damage and herniation. In cases of chronic hydrocephalus, ventriculo–peritoneal or ventriculo–cardiac shunts are frequently necessary to prevent further brain damage.[84]

Surgical and Neuroradiologic Treatment

Because of the high risk of early rebleeding in aneurysmal SAH, which is often associated with fatal outcome, aneurysm occlusion should be performed early and is indicated as secondary prophylaxis in most cases.[101] Occlusion should be performed prior to the development of vasospasm (i.e., within the first 72 hours). Two types of treatment are available for patients with ruptured intracranial aneurysms: endovascular detachable-coil treatment and neurosurgical intervention with craniotomy and clipping. The multicenter international subarachnoid aneurysm trial reported that in patients who are suitable for both treatments, endovascular coiling is more likely to result in independent survival at 1 year than neurosurgical clipping.[146] However, the risk of late rebleeding was higher after endovascular coiling than after neurosurgical clipping.[146] While randomization of patients was based on the "uncertainty principle," older patients (>70 years) and patients with posterior aneurysms and aneurysms of the media cerebellar artery were underrepresented in the study. Therefore, the decision to use an endovascular or neurosurgical approach should be made on an individual basis.[101] Rebleeding is increased in incompletely wrapped, coated, clipped, or coiled aneurysms; aneurysms should be completely obliterated whenever possible.[101]

Primary Prophylaxis

While the prognosis of SAH is poor and the frequency of asymptomatic aneurysms is relatively high, elective screening has been discussed but is currently only recommended on an individual basis.[101] The management of patients with proven unruptured aneurysm remains controversial.[102]

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CHAPTER

74 Retinopathy

JING CHEN, ANDREAS STAHL, AND LOIS E. H. SMITH

The eye is one of the most important sensory organs that enables us to perceive the environment to ensure species survival. The light-sensitive part of the eye, the retina, is composed of several transparent neuronal layers and functions similarly to a film in a camera. Light focused through the cornea and lens passes across the transparent retinal layers to interact with light-sensitive pigments in the photoreceptor outer segments, causing a signal transduction cascade. The signal generated in the photoreceptor ultimately propagates a series of action potentials across the retina, through the optic nerve to the neurons in the visual cortex of the brain, and results in the perception of an image (FIGURE 74.1). The essential function of the retina in light perception and processing is dependent upon a well-organized nonleaky vascular network that supplies vital oxygen and nutrients and clears waste. In retinal vascular diseases, the normal function of the ocular vasculature is disrupted, often leading to loss of barrier function with resulting plasma exudation and hemorrhage and, in most severe cases, disturbance of retinal neuronal function and loss of vision.

Epidemiologic studies have shown that proliferative diabetic retinopathy (PDR) accounts for the highest incidence of acquired blindness in the working-age population[1,2]; choroidal (subretinal) neovascularization in age-related macular degeneration (AMD) represents the leading cause of blindness in people over the age of 65[3,4]; and retinopathy of prematurity (ROP) is a major cause of acquired blindness in children[5] (FIGURE 74.2). In addition, there are many other ocular diseases that have a vascular component, such as retinal vein occlusion (RVO), Norrie disease, familial exudative vitreoretinopathy (FEVR), Coats disease, sickle cell retinopathy, retinitis pigmentosa, and others. This chapter summarizes our current knowledge of the physiology of the ocular vasculature and pathophysiology of several common vascular eye diseases, as well as cellular and molecular mechanisms that regulate the growth of ocular vessels.

OCULAR VASCULATURE IS COMPOSED OF THREE SEPARATE VASCULAR BEDS: HYALOIDAL, CHOROIDAL/UVEAL, AND RETINAL VESSELS

Hyaloid Vasculature

The hyaloid vasculature is a transient embryonic vascular bed that develops during embryonic and fetal stages to provide blood supply to the developing eye. The hyaloid vascular plexus originates from the optic nerve, extends through the primitive vitreous, and surrounds the developing lens. The hyaloid vessels eventually regress with completion of eye development, usually before 34 weeks of gestation in humans. In rare diseases such as Norrie disease and FEVR, the hyaloid vasculature fails to regress, resulting in persistent fetal vasculature, which is associated with poorly developed neural retina and impairment of vision.

Choroidal Vasculature

The choroidal vasculature is part of the uveal system of vessels that perfuse the iris, ciliary body, and choroid (FIGURE 74.1). During optic cup development, the primitive choroidal vascular system is formed from the surrounding mesoderm. Later, following the development of retinal pigment epithelium (RPE), this primitive vascular layer expands further,[6] until the capillaries completely encircle the optic cup. A definitive choriocapillaris layer appears at around 8 to 12 weeks of gestational age.

The choroidal vasculature consists of larger posterior vessels and a small-caliber anterior choriocapillaris (FIGURE 74.2), adjacent to the RPE. The choriocapillaris is a sinusoidal vascular plexus and is the site of the greatest blood flow (65% to 85%) in the eye.[7] The choriocapillaris provides oxygen and nutrients to the outer retina, particularly the RPE and photoreceptors that have the highest metabolic rate in the body and therefore have a very high oxygen demand. The choriocapillaris is highly fenestrated and allows blood flow beneath the basal surface of the RPE that digests shed photoreceptor outer segments and clears other photoreceptor waste. The RPE with tight junctions on the apical surface adjacent to the photoreceptors constitutes the outer blood retinal barrier (BRB). Similar to the blood brain barrier, the BRB is vital to preserve tissue integrity and function of the retina. The inner BRB is formed by vascular endothelial cells of the inner retina with tight junctions. The endothelial cells abut Müller cell endfeet and pericytes. This inner BRB is compromised in diabetic retinopathy, leading to retinal edema and often severe visual impairment. In exudative AMD in contrast, it is the outer BRB that is compromised when choroidal blood vessels (without tight junctions) grow through Bruch membrane and into the subretinal space, breaking through the RPE's BRB. This leads to edema of the macula, the part of the retina that is responsible for central vision and higher visual functions like reading or recognizing faces.

Retinal Vasculature

In the eye, 20% to 30% of blood flow is supplied by the retinal circulation, serving mainly the inner retina. In humans, retinal vascularization starts *in utero* at about 16 weeks of gestational age and is completed at approximately 40 weeks of gestation, right before birth. The retinal vessels develop as the hyaloid vessels regress. During development, retinal vessels grow first in the most superficial (i.e., innermost) layers of the retina and

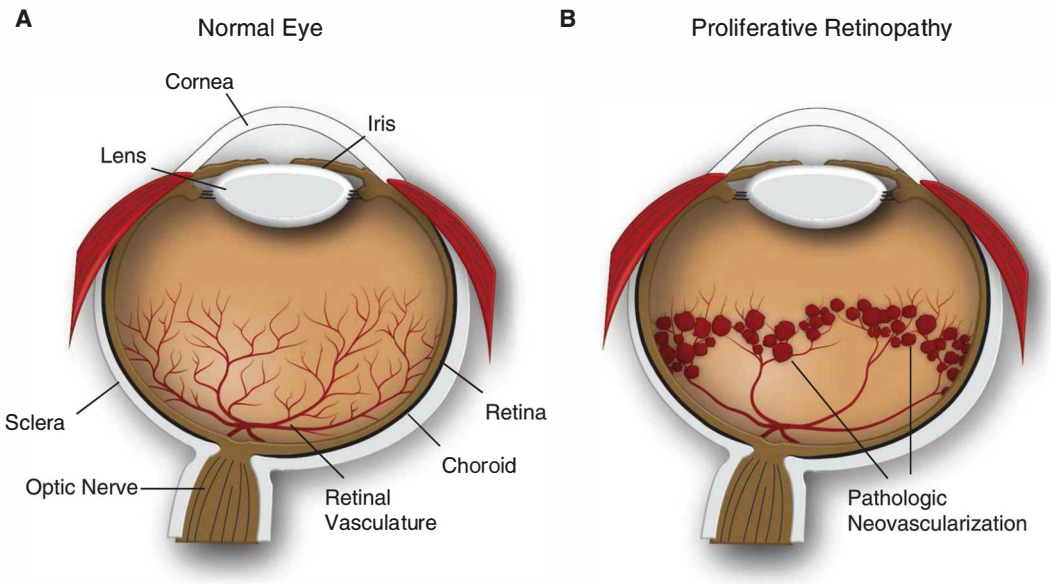

FIGURE 74.1 Schematic illustration of **(A)** normal eye and **(B)** proliferative retinopathy with pathologic neovascularization.

sprout radially from the optic nerve toward the ora serrata, the peripheral edge of the retina. They reach the nasal side of the ora serrata at about 36 weeks of gestational age, and the temporal retina at approximately 40 weeks of gestational age. As the superficial layer is almost completed, retinal vessels dive into the retina to form first a deep and then an intermediate layer along with a well-organized network of interconnecting vessels to complete three vascular layers: a superficial vascular layer that lies in the inner part of the nerve fiber layer, an intermediate layer in the inner plexiform layer, and a deep layer in the outer plexiform layer.[8] Similar to the RPE, the retinal vessels maintain BRB integrity.[9]

Under pathologic conditions when the BRB is disrupted, the retinal vessels become more permeable resulting in retinal edema and vision loss. This is seen with ROP and diabetic retinopathy, when retinal vessels proliferate to form fragile leaky neovessels. RVO is also a common retinal vascular disease. In the early stages of RVO, plasma leakage and hemorrhage is due to increased intraluminal pressure in the obstructed vein; in later stages it is due to vascular endothelial growth factor (VEGF)-induced proliferation of leaky neovessels and VEGF-induced increased permeability of normal vessels.

Among these three blood vessel systems in the eye, the inner retinal vasculature is the most extensively studied. The retina is an excellent model of vascular development, vessel remodeling, and vascular maturation. Studies of retinal vasculature have greatly expanded our basic understanding of the factors that regulate normal and pathologic vascularization including the relationship between oxygen and retinal vascular growth.

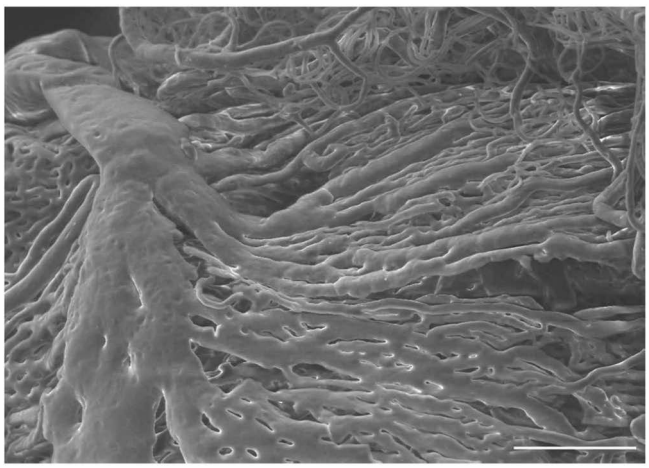

FIGURE 74.2 Vascular cast of choroidal vasculature taken with a scanning electron microscope. Scale bar: 200 μm. Image courtesy of Dr. Przemyslaw Sapieha, University of Montreal, Montreal, Canada.

OXYGEN REGULATES BLOOD VESSEL GROWTH IN THE EYE

The hypothesis that oxygen-sensitive molecules control the development of retinal vasculature originates from early observations that capillaries grow more profusely near venules carrying oxygen-depleted blood than around arteries with fully oxygenated blood.[10–12] The vascular pattern of some eye diseases also supports the idea that oxygen (or lack of it) regulates blood vessel growth in the retina. Pathologic retinal angiogenesis in ROP and diabetic retinopathy is preceded by an initial loss of retinal vessels with resulting retinal ischemia, suggesting that hypoxia is an important stimulator of new blood vessel growth.[13] The timing and spatial pattern of normal retinal vascularization is coordinated with the differentiation, maturation and increasing metabolic demand of neural and glial cells. The wave of maturation of neural retina creates a moving wave of "physiologic hypoxia" that guides the development of new vasculature from the center toward the periphery of the retina.[14] VEGF, an essential growth factor for vascular growth and

survival is induced in a pattern that coincides with retinal blood vessel development both temporally and spatially.[15,16,17] As new vessels form, bringing oxygen and suppressing VEGF, the wave moves forward toward the developing retina.

Retinal Tissue Senses Oxygen Tension through Hypoxia-Inducible Factor

The increased expression of VEGF and some other blood vessel growth factors in response to low tissue oxygen levels is mediated in large part through the transcription factor hypoxia-inducible factor 1 (HIF1), which functions as a global regulator of O_2 homeostasis.[18–20] HIF1 is a heterodimeric protein composed of an oxygen-sensing α unit and a constitutively expressed transcription activating β subunit. Under normoxic conditions, a proline residue in HIF1α is hydroxylated by Egl9 enzyme, facilitating the interaction between HIF1α and von Hippel-Lindau protein, resulting in the ubiquitylation and proteasomal degradation of HIF1α.[21–23] Under hypoxic conditions, HIF1α escapes prolyl hydroxylation and dimerizes with HIF1β. The HIF1 heterodimer then translocates to the nucleus and activates target genes by binding to a hypoxia responsive element in the promoter region.[18] In addition to VEGF, erythropoietin (Epo) is an important target gene of HIF1 that plays an important role during retinal blood vessel homeostasis.[24,25,26] Epo is a glycoprotein hormone that is best known for its stimulation of erythropoiesis. However, Epo has also potent neuroprotective and proangiogenic effects. In the vitreous of patients with diabetic retinopathy,[26,27] ROP[28] or RVO,[29] Epo is upregulated along with VEGF, which is one of the master regulators of both normal retinal vessel development and proliferative retinopathies.[15,30,31]

VEGF Guides Retinal Vascularization Following an Astrocytic and Neuronal Template

Although tissue oxygen tension is an essential stimulus for retinal vessel growth, hypoxia alone does not generate the organized pattern of retinal vascularization and the structural alignment among retinal vessels, astrocytes and neurons that is seen during normal retinal development. It has been suggested that several other cellular guides contribute to this highly organized pattern of retinal vessel growth. Astrocytes grow along the inner surface of the retina preceding the vessels. It is assumed that this astrocytic template secretes VEGF and other factors in a tightly controlled manner to create a highly specialized micro milieu to guide the filopodia of sprouting endothelial cells in the right direction.[8,32–34] It is also postulated that the relative hypoxia of the deeper retinal layers during development results in a VEGF gradient that favors sprouting from the superficial layer downward, resulting in the formation of first the deep and then the intermediate layers of capillary networks.[15,16] If the astrocytic template guides vascular growth in the retina, what is it that guides the formation of the astrocyte template during retinal development? One theory postulates that astrocyte growth follows the radially oriented ganglion cell axons,[8,33] which synthesize platelet-derived growth factor (PDGF) to stimulate astrocyte growth and alignment.[35] The radial cellular pattern in the retina is likely to result from complex and highly regulated reciprocal cellular interactions: retinal neurons serve as the primary template and secrete PDGF to stimulate astrocyte growth and patterning; astrocytes in turn respond to increasing hypoxia during retinal maturation by increasing the synthesis and secretion of VEGF to induce and sculpt the formation of a retinal vascular plexus. When the vascular plexus becomes functional, it alleviates tissue hypoxia, decreasing VEGF expression and limits further excessive vascular growth.[36,37]

Remodeling of Retinal Vasculature Is Also Dependent on Oxygen

Once formed, the superficial layer of retinal vessels is remodeled before sprouting to the deeper layer,[38] which is regulated by the specific needs of the local tissue for oxygen and nutrients. Maturation of the superficial plexus leads to a less dense network with finer vessels compared to the structures formed during early development. Relative hyperoxia around arteries is considered an important factor for pruning of existing vasculature. Local hyperoxia would lead to local suppression of VEGF, which is an endothelial survival factor for immature capillaries. Reduction of VEGF leads to regression of these immature capillaries in areas with excess vasculature.[39,40] Leukocytes from the circulation also participate in vessel remodeling through induction of endothelial cell apoptosis, a process considered most relevant in the context of early vessel loss in diabetic retinopathy.[38]

VEGF during Development of the Choroidal Vasculature

Choroidal vascular development is less well studied than the development of the retinal vasculature. Clinically the retinal vasculature can be readily observed funduscopically in humans, while the choroidal plexus is blocked from view by a dense layer of pigmented RPE cells. Furthermore, in animal models, retinal vasculature development can readily be studied due to the postnatal development of retinal vessels in rodents. Retinal flat mounts expose retinal vessels well. The choroidal vasculature, in contrast, is less readily quantifiable during development. Nevertheless we know that formation and maturation of the choroidal vasculature is tightly associated with signals produced by RPE cells.[41] In particular, VEGF is highly expressed by RPE cells at the time of choriocapillaris formation,[42,43] suggesting involvement of RPE-derived VEGF in the development of choroidal vessels. Furthermore, in colobomas, failure of RPE differentiation results in defective development of the choroid and sclera,[44] and in patients with exudative AMD, degeneration of RPE cells and associated overproduction of VEGF leads to pathologic activation and sprouting of the normally quiescent choriocapillaries.

VEGF and Wnt Signaling during Formation and Regression of the Hyaloid Vasculature

The hyaloid artery penetrates into the optic cup in early development and extends through the vitreous to form branches that envelop the forming lens. Retinal vasculature development coincides with hyaloid vessel regression.[6,45] The mechanism governing both formation and regression of hyaloid vessels is still incompletely defined. VEGF was suggested to trigger the growth of hyaloid vessels because VEGF is expressed in the portion of the lens closest to the forming vessels.[42,46,47] In addition, patients

with VEGF-induced endothelial cell hyperplasia also have disorganized and persistent hyaloid vessels, further supporting a role of VEGF in hyaloid vessel formation.[48]

Interestingly, an entirely different process has been uncovered for hyaloid vessel regression (and retinal vascular formation) from studies investigating a group of rare hereditary eye diseases, Norrie disease, and FEVR. These diseases have in common persistent hyaloid vessels and incomplete formation of the retinal vasculature with a lack of a deep retinal plexus formation.[49] They arise from a number of mutations in the canonical Wnt-β–catenin signaling pathway: Wnt ligands Norrin or Wnt receptors Frizzled4 and Lrp5 are involved and mediated in part through macrophages.[50,51]

OCULAR DISEASES WITH VASCULAR PATHOLOGY

Many retinal eye diseases result from disruption of the normal blood supply. The subsequent hypoxia often triggers pathologic neovascularization. In diabetic retinopathy and ROP, abnormal proliferating retinal vessels extend into the vitreous. In AMD, choroidal vessels can proliferate pathologically into the normally avascular subretinal space. RVO is another common eye vascular disease, which in its late stages can lead to pathologic retinal vessel proliferation. The following sections discuss the clinical features of these common ocular vascular diseases and our current understanding of the pathogenesis and treatment options.

Retinopathy of Prematurity

ROP is a major cause of blindness in children in the developing and developed world despite current surgical and laser treatment in the late stage of the disease.[52] Formerly known as retrolental fibroplasia, ROP was originally described in the 1940s when the condition was first connected with oxygen supplementation after premature birth.[53] Initially, no treatment was available. Major advances came in the 1980s, when cryoablation of avascular retinal areas was shown to be partially effective in preventing blindness in ROP infants.[54] Laser photocoagulation soon supplanted cryotherapy.[55] However, although ablation treatment can reduce the incidence of blindness by approximately 25% in infants with late-stage disease, it does not improve the chances of good visual acuity. Preventive and less destructive therapies for ROP are thus highly desirable.

Risk Factors of ROP

ROP was associated with excessive oxygen use shortly after the initial description of the disease.[56,57] Controlled supplemental oxygen is now delivered to premature infants to maintain adequate blood levels of oxygen but at the same time limit the development of ROP.[58] However, even with monitored oxygen use, the incidence of ROP has increased over the last decades, probably due to the increased survival of infants with very low birth weight.[59] Oxygen use, early gestational age and low birth weight are major risk factors for ROP.[60–62] However, the postnatal factors of low postnatal weight gain, as well as low insulin-like growth factor (IGF)-1 blood levels are as important as gestational age and birth weight in determining ROP progression, as well as increased blood glucose level, which is also recognized as a risk factor.[63–69]

Pathogenesis of ROP: Two Phases

ROP is a biphasic disease consisting of an initial phase of impaired retinal vessel growth followed by a second phase of vascular repair that is—in severe cases—accompanied by pathologic vessel proliferation in the retina and often into the vitreous. Infants born prematurely have an incompletely vascularized retina at birth. During the first phase of ROP, the peripheral avascular zone of premature infants becomes vascularized too slowly to keep up with the rising metabolic demand of the maturing neurosensory tissue. The severity of ROP is to a large extent determined by the size of avascular retina (FIGURE 74.3), which in turn is dependent on gestational age at birth. The earlier an infant is born, the less vascularization of the retina has occurred. In addition to prematurity, a hyperoxic environment relative to that found *in utero* and additional oxygen treatment, which is often needed for survival of prematurely born infants, contributes to the slowing (or even cessation) of normal vascular growth. *In utero*, the normal PaO_2 is 30 mm Hg, compared to a PaO_2 of 60 to 100 mm Hg of an infant breathing room air.[70] As a consequence, *in utero* the blood is only approximately 70% saturated with oxygen compared to 100% in infants in room air. This first phase of impaired retinal vascularization signifies the first phase of ROP, which spans from birth to about 30 to 32 weeks of gestational age.

During the second phase of ROP, the retina changes from hyperoxic to hypoxic. It is believed that this change is induced by increased metabolic demand of the maturing neurosensory retina. During this phase neuronal and glial cells in the avascular areas of the peripheral retina are oxygen and nutrient deficient. This local tissue hypoxia leads to upregulation of proangiogenic growth factors that induce the ingrowth of new blood vessels. This second phase of ROP, characterized by hypoxia-triggered increased blood vessel growth, begins around 32 to 34 weeks of gestational age. However, in severe cases of ROP, with large avascular areas, the upregulation of angiogenic factors is excessive and induces not only organized growth of functional vessels but also uncontrolled sprouting of pathologic vessels in the retina and from the retina proper into the normally avascular vitreous. These preretinal neovessel proliferations are very similar to the pathologic neovascularization seen in other proliferative

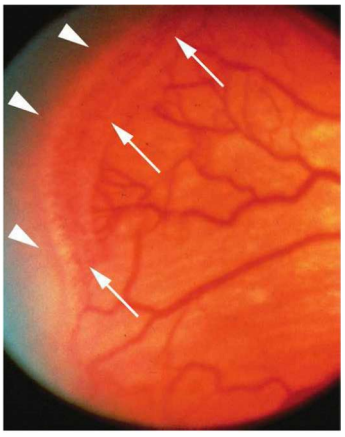

FIGURE 74.3 Fundus image from an infant with retinopathy of prematurity (ROP). *Arrows* indicate the prominent ridge demarking the vascularized retina from the avascular peripheral areas (*arrowheads*).

retinopathies such as diabetic retinopathy or the late stages of RVO. Several severe complications can arise from these pathologic neovessels: first, the preretinal nature of some of these neovessels exert tractional forces leading to detachment of the retina from the underlying RPE; second, the unorganized structure of these neovessels leads to plasma leakage and bleeding into retina and/or vitreous. These complications can lead to blindness or severe visual impairment. Even with treatment, patients who progress to these severe stages of ROP have usually extremely limited chances of developing normal or near-normal visual function.

VEGF in ROP

VEGF is an oxygen-regulated factor essential for normal retinal vascular development and important in ROP. As one of the most potent proangiogenic molecules, [71,72,73] VEGF can be secreted by a wide variety of cell types in hypoxic conditions.[74,75,76] Elevated VEGF levels are found in the vitreous of patients with retinal neovascularization,[75,77] including those with ROP.[78] In the neovascular second phase, antiangiogenic treatment using an anti-VEGF aptamer or anti-VEGF antibodies or antibody fragments might be beneficial. However, although anti-VEGF therapies have produced beneficial results in patients with neovascular AMD, the efficacy and safety of anti-VEGF treatments in ROP have still to be determined. As a consequence, anti-VEGF therapy in the second stage of ROP cannot be recommended at the time of writing as too little data exists on potentially severe side effects of anti-VEGF treatment in premature infants.

Growth Hormone and Insulin-like Growth Factor in ROP

The fact that prematurity is one of the most significant risk factor for ROP suggests that factors involved in growth and development are critical for ROP development. GH and IGF-1, which mediates many of the mitogenic aspects of GH, are both oxygen-independent growth factors. A pituitary hormone was associated with the development of retinal neovascularization after pituitary ablation was found to have an ameliorative effect on PDR.[79–81] Studies in animal models of ROP then found that GH mediated through IGF-1 is important in the development of retinal neovascularization. These studies identified a molecular mechanism by which IGF-1 modulates VEGF effects during retinopathy.[31,82]

IGFs (IGF-1 and IGF-2) are important in fetal growth and development during all stages of pregnancy.[83] Serum concentration of IGF-1, but not IGF-2, increases with gestational age and correlates with fetal size.[84,85] IGF-1 levels rise significantly in the third trimester of pregnancy.[83] After preterm birth of infants, IGF-1 levels fall from the *in utero* levels due to the loss of IGF-1 provided by the placenta and the amniotic fluid. In age-matched premature babies, mean serum levels of IGF-1 are directly correlated with the severity of clinical ROP[66–68,86] and low postnatal serum IGF-1 levels are at least as strong a determinant of risk for ROP as gestational age at birth and birth weight.[66,67] Low postnatal serum IGF-1 levels in preterm infants also correlate with slower brain growth and might account for abnormal neural retinal function in ROP.[68] In patients with genetic defects of the GH/IGH-1 axis, very low levels of IGF-1 directly cause decreased vascular density in the retina as evidenced by fewer vascular branching points.[87] This accumulated evidence suggests that early low serum IGF-1 levels are associated with vessel loss and may be contributing to early vessel degeneration in phase I of ROP that sets the stage for hypoxia leading later to proliferative retinopathy. Early restoration of IGF-1 to uterine levels might prevent ROP development by allowing normal retinal vascular development.

Based on the understanding of the roles of VEGF and IGF-1 in both phases of ROP, a rationale for the evolution of the disease has emerged (FIGURE 74.4). Retinal vessel growth requires both IGF-1 and VEGF. In premature infants, IGF-1, which is normally supplied by placenta and the amniotic fluid, is at very low levels after birth since the premature liver cannot replace the loss. Blood vessel growth therefore slows or stops since IGF-1 is needed to allow maximum VEGF signaling for vascular endothelial growth and survival. With oxygen levels higher than *in utero*, particularly with oxygen supplementation, VEGF is suppressed. Thus, both prematurity and oxygen contribute to the suppression of normal vessel growth and vessel loss in phase I of ROP. As the infant grows and the retina matures without an adequate vasculature to supply oxygen, it becomes oxygen starved, which increases expression of VEGF. In the meantime, as the infant's organs and systems continue to mature, IGF-1 levels rise slowly, eventually reaching a level that allows VEGF signaling to stimulate blood vessel growth. The result is the destructive neovascular proliferation in the phase II of ROP, with blinding consequences.

Current and Future Therapeutic Targets for Treating ROP

Although ROP in most infants regresses spontaneously, in severe cases ablation of avascular retina (which supplies angiogenic factors) is required to prevent loss of vision. This was achieved

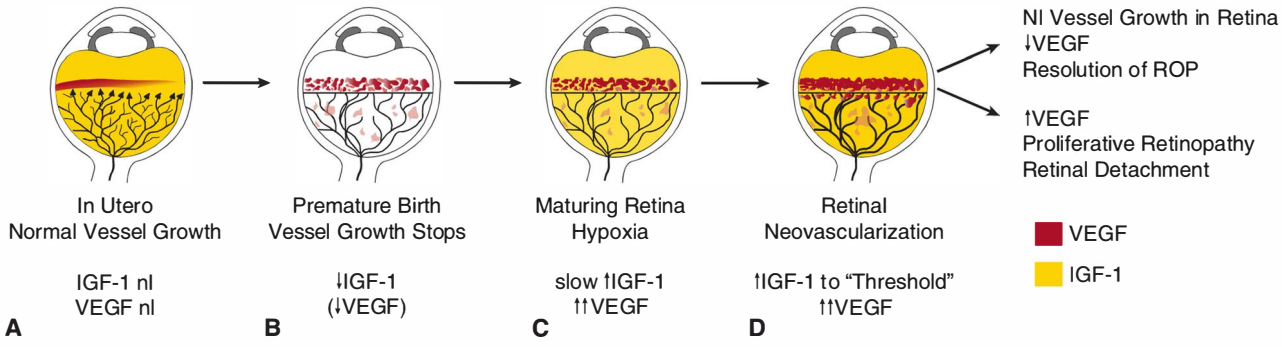

FIGURE 74.4 Schematic representation of IGF-I/VEGF control of blood vessel development in retinopathy of prematurity (ROP): **(A)** *in utero*, **(B)** premature birth, **(C)** retinal maturation, **(D)** retinal neovascularization. Nl: normal.

with cryotherapy in earlier years and has been replaced by laser photocoagulation, which is less invasive and can be more precisely controlled. Recent advances in ROP research suggest a number of new ways to medically intervene in ROP progression. The extent of the second destructive phase of ROP is determined by the amount of avascular retina in phase I. Therefore addressing the problem of persistent avascularity in peripheral retina by promoting increased functional vascularization of the postnatal retina is a very appealing approach to treat ROP. Systemic replacement of IGF-1 to those levels found *in utero* might prevent the disease by allowing normal vascular development after premature birth. This physiologic approach has been taken in a clinical trial to evaluate supplementation of IGF-1 (and IGFBP-3) to *in utero* levels with neurologic development and ROP severity in premature infants evaluated as primary outcomes.

The current understanding of ROP pathogenesis also makes clear that timing is critical in any medical intervention, since the two phases of ROP require very different approaches. Inhibition of either VEGF or IGF-1 in the second phase might prevent pathologic neovascularization while inhibition of either growth factor early after birth can—at least theoretically—severely impair normal blood vessel growth. On the other hand, providing VEGF or IGF-1 early might promote vessel growth, whereas late supplementation in the neovascular phase might exacerbate destructive neovessel formation. In the fragile neonate, any intervention must be made very carefully. Any approach to medically intervene in ROP must therefore be closely monitored and ideally should be guided by measurement of individual growth factor levels with a return to normal physiologic levels at each phase as the goal.

Diabetic Retinopathy

Diabetic retinopathy is one of the major complications of diabetes and in its late stages often progresses to proliferative disease.[88] It is the most frequent cause of legal blindness in the population of 30 to 70 year olds. Before the advent of intensive insulin therapy with the goal of near normalization of blood glucose levels, diabetic retinopathy afflicted over 90% of diabetic patients after 20 years of disease.[89,90] Despite the importance of tight glycemic control in the prevention of retinopathy shown in these clinical studies, it is not possible to achieve this goal in all patients. Diabetic retinopathy remains a persistent complication, especially with the increasing epidemic of obesity and type II diabetes.

Risk Factor of Diabetic Retinopathy

Both type I and type II diabetic patients are at risk of developing diabetic retinopathy. After 20 years of disease, nearly all type I diabetic patients and about 60% of type II diabetic patients develop some stage of retinopathy. Poor glycemic control, high cholesterol, and smoking are all associated with higher risk. In addition, pregnancy may also cause a rapid increase in progression of diabetic retinopathy. Genetic factors also play a role as diabetic patients with Down syndrome almost never develop diabetic retinopathy, possibly due to protection from elevated level of antiangiogenic endostatin.[91]

Pathogenesis of Diabetic Retinopathy

The development of diabetic retinopathy is a slow process that can occur over decades in two phases, nonproliferative retinopathy and proliferative retinopathy. Nonproliferative retinopathy or background retinopathy can be seen as early as 1 year after diabetes onset and begins with slow vessel destruction with vascular basement membrane thickening, pericyte loss, increased leukocyte adherence, and the development of microaneurysms and acellular capillaries. Visual loss is not usually associated with these early changes in retinal vessels. However, increased vascular permeability can lead to exudation of plasma into the retinal tissue and vision-distorting central macular edema. As the disease progresses, vascular occlusion is seen not only in retinal capillaries but also in larger venules and arterioles. The midstage of this disease is characterized by capillary nonperfusion, increased vessel tortuosity, leakage resulting in intraretinal exudates, retinal infarcts (cotton wool spots), as well as areas of hemorrhage (FIGURE 74.5). All these changes lead to vascular dysfunction and poor perfusion, resulting in retinal ischemia and hypoxia, which sets the stage for the second phase of the disease, abnormal vasoproliferation.

In patients with PDR, the nonperfused retina becomes hypoxic, which in turn leads to an increased expression of hypoxia-induced angiogenic growth factors including VEGF to trigger retinal neovascularization. The new vessels growing on the surface of the retina or into the vitreous are fragile and have increased permeability, increasing the risk of vitreous hemorrhage and retinal edema (FIGURE 74.5). Formation of fibrous membranes, associated with this preretinal pathologic neovascularization, can cause traction on the underlying retina and lead to retinal detachment and potential blindness. The proliferative stage of diabetic retinopathy is in many aspects similar to the proliferative stage of ROP. However, the underlying pathologies that initiate vessel loss in the first phase of each disease differ significantly. In ROP, premature birth with all its associated changes in oxygen and loss of factors normally provided *in utero* dictates progression of retinopathy; in diabetic retinopathy, progression in phase I is significantly determined by the quality of glycemic control, by dyslipidemia and by endocrine dysfunction.

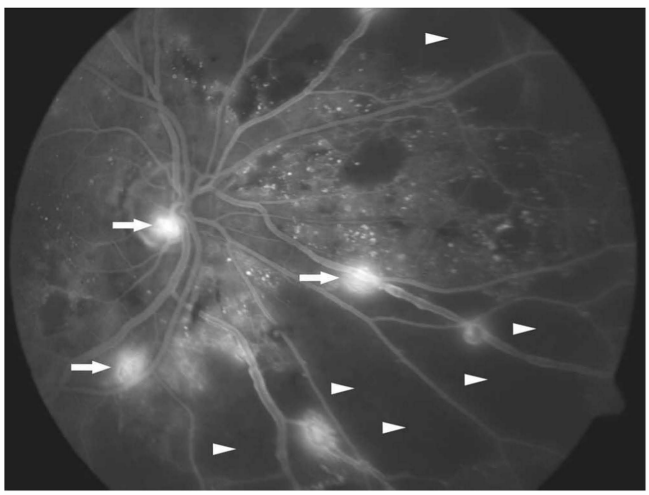

FIGURE 74.5 Fluorescence angiogram from a patient with PDR. *Arrows* indicate neovessel proliferation and *arrowheads* indicate avascular retinal areas. Image courtesy of Dr. Christoph Ehlken, University Eye Hospital Freiburg, Germany.

High blood glucose levels (or rapid changes in glucose levels) are associated with many alterations in cellular metabolism, including vascular endothelial dysfunction.[92] Polyol accumulation in experimental hyperglycemia is associated with a thickened basement membrane, pericyte loss, and formation of microaneurysms.[93] This pathway converts excess intracellular glucose into sugar alcohols via aldose reductase, resulting in increased sorbitol level that in turn is hypothesized to damage vascular cells. Formation of advanced glycation end products, nonenzymatic binding of glucose to protein sides chains, is another well-characterized pathway linked with pericyte loss and microaneurysm formation in diabetic retinopathy.[94] Hyperglycemia can also lead to oxidative stress and increased formation of reactive oxygen species,[92] which may cause vascular damage. In addition, chronic activation of protein kinase C (PKC) in hyperglycemic conditions is associated with perturbed vascular cell homeostasis including increased vascular permeability, contractility, and apoptosis.[95] PKC is activated by the overflow of glycolytic intermediates into synthesis of diacylglycerol, which is a potent stimulus for PKC. Together these processes are thought to modulate diabetic retinopathy progression through their effects on cellular metabolism, as well as production and signaling of growth factors such as VEGF, GH, IGF-1, transforming factor β (TGF-β), and pigment epithelium-derived factor (PEDF) that can all modulate the angiogenic process in the retina.

Growth Factors in Diabetic Proliferative Retinopathy

Increased levels of angiogenic growth factors VEGF and Epo are found in the vitreous of patients with diabetic retinopathy.[75,77,96] Suppression of both factors in animal models of retinopathy suppresses retinal neovascularization illustrating their potential roles in retinal neovascular formation. GH and IGF-1 are also associated with diabetic retinopathy[80] and in animal models inhibiting GH or IGF-1 suppresses retinal neovascularization.[31,82] There is accelerated diabetic retinopathy progression in states of elevated IGF-1 during puberty, pregnancy, and upon rapid improvement of metabolic control.[97,98] Growth factors that may inhibit retinopathy are TGF-β and PEDF. TGF-β is produced by pericytes in the retina. Levels of TGF-β are usually high in normal eyes and may inhibit abnormal endothelial proliferation.[99] Patients with active PDR have lower vitreal levels of TGF-β, which may promote pathologic angiogenesis.[100] PEDF is expressed by RPE and is also an inhibitor of angiogenesis.[101] Systemic injection of PEDF reduces retinal neovascularization in the mouse model of retinopathy.[102]

Treatment of Diabetic Retinopathy

At present, tight glycemic control remains the most effective proven treatment to slow the progression of diabetic retinopathy.[103,104] However, with intensive insulin therapy also comes an increased risk of hypoglycemia, particularly for type I diabetic patients, affecting overall morbidity and mortality. Other therapeutic approaches are necessary. Tight control of dyslipidemia, often present in type II diabetic patients, also protects against progression of retinopathy.[105] In addition, intravitreal triamcinolone, a long acting steroid, decreases diabetic macular edema and increases visual acuity. Similarly, anti-VEGF compounds are currently under investigation for the same purpose. When the disease progresses to the proliferative stage, current standard treatment is ablation of avascular peripheral retina by photocoagulation. Through destruction of hypoxic tissue, the level of hypoxia-induced angiogenic growth factors is reduced and pathologic vessel proliferation is attenuated. However, this approach also irreversibly damages neuronal retina.

Age-Related Macular Degeneration

AMD is the primary cause of blindness in the elderly in developed countries. Because the pathology primarily affects the macula, the central retina associated with highest retinal sensitivity and visual function, AMD can cause severe visual loss (FIGURE 74.6A). In the late stages, AMD can progress into two forms: the nonexudative (dry) type and the exudative (wet) type.

Pathogenesis of AMD

The normal function of retinal photoreceptors is dependent upon a well-functioning adjoining RPE, which phagocytoses the spent photoreceptor outer segments, shed daily, to recycle photopigments (such as rhodopsin) and membrane lipids (such as docosahexaenoic acid). The RPE also transports nutrients and breakdown products between the photoreceptors and the adjoining choroidal vascular plexus, separated from the RPE by Bruch membrane. Therefore the photoreceptors, choroidal vessels, and RPE have an interdependent relationship, and loss of any one component causes dysfunction of the others. This dysfunction is often seen first in the macula because the densely packed photoreceptors here have the highest oxygen consumption and metabolic rate of any tissue in the body.[106]

Clinically, early dry AMD begins with the usually asymptomatic appearance of drusen, seen as white or yellow spots beneath the retina and the RPE on Bruch membrane (FIGURE 74.6B). These deposits increasingly separate the RPE from the underlying choroidal vasculature, interrupting normal function of the RPE and leading to photoreceptor dysfunction and degeneration, a hallmark of late-stage dry AMD. During progression from early AMD to later stages, patients with "wet" AMD develop pathologic blood vessels that sprout from the choroidal plexus, through Bruch membrane and sometimes even through the RPE layer into the subretinal space beneath the photoreceptors (FIGURE 74.6C). Increased leakiness from these neovessels can lead to accumulation of fluid or blood, resulting in detachment of RPE and/or neurosensory retina from the underlying Bruch membrane. Wet AMD is associated with rapid deterioration of visual function. In some cases the choroidal neovessels hemorrhage into the subretinal space, acutely blocking vision in the macula.

Several growth factors, including VEGF, basic fibroblast growth factor, and TGF-β, have been identified in human choroidal neovascularization at increased levels.[107,108] Although VEGF plays a central role in the development of choroidal neovascularization, development of invasive choroidal neovascularization (CNV) may require both elevated VEGF levels and defects in Bruch membrane,[109–114] as defects in Bruch membrane are visible in many ocular conditions with CNV, accompanied by varying degree of inflammation.[115] Increased oxidative stress, which is proinflammatory and can cause invasion of macrophages resulting in the local destruction of Bruch membrane, is another important mechanism in the pathogenesis of AMD.[116] The presence of macrophages has been demonstrated in the outer side of Bruch membrane in eyes with drusen, possibly attracted by RPE secreted monocyte chemoattractant proteins.[117] Increased oxidative stress can also lead to overexpression of many proangiogeneic growth factors by RPE that further exacerbates CNV.

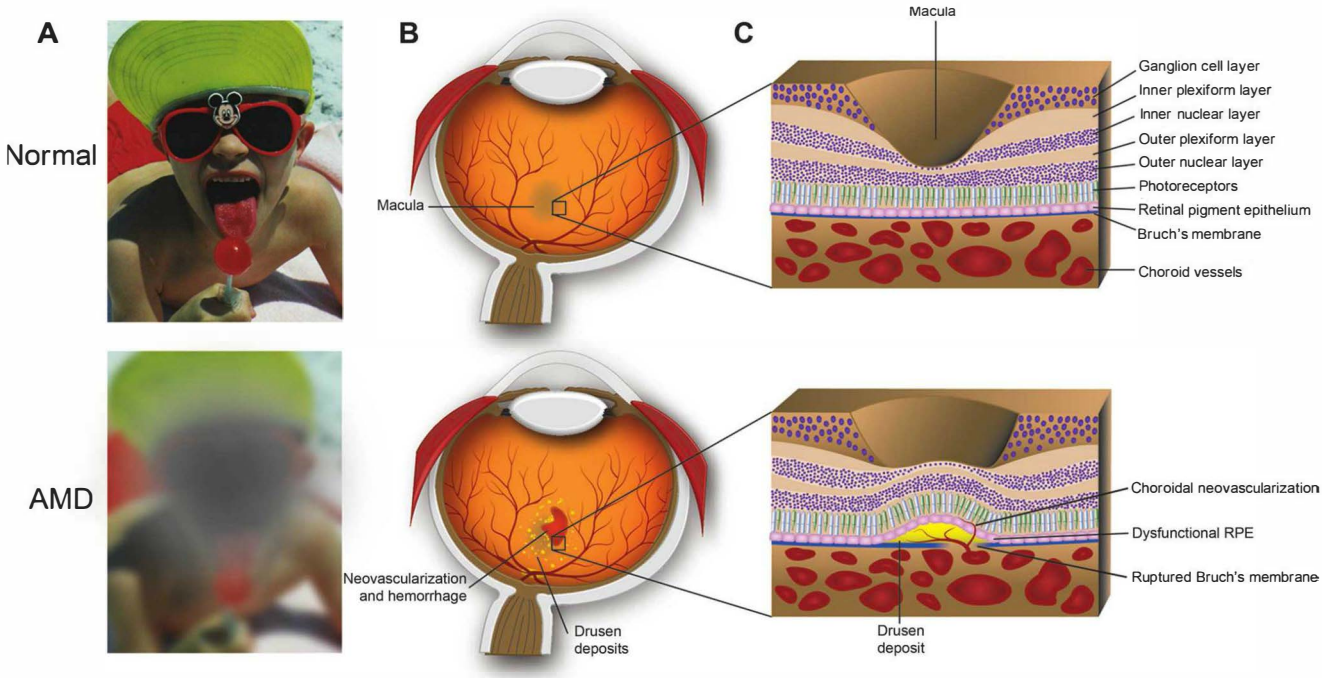

FIGURE 74.6 Loss of central vision in AMD patients. **A:** A representation of the visual field seen by a person with normal vision (**upper**) or seen by an AMD patient with a loss of central color vision (**lower**). **B:** A schematic drawing of a normal human eye (**upper**) compared with an eye with AMD (**lower**) depicting deposits of drusen (*yellow spots*) and choroidal neovascularization with subretinal hemorrhage in the macula (*red*). **C:** A schematic cross section of a normal eye through the macula, showing retinal neuronal layers, RPE, Bruch membrane, and choroid vessels (**upper**). In AMD, the intimate relation between photoreceptors, RPE, and choroid is disrupted by drusen (lipid and cellular debris-containing deposits) (**lower**). The formation of drusen separates RPE from Bruch membrane and the underlying choroidal vessels in association with RPE atrophy and photoreceptor degeneration (hallmarks of dry AMD). In wet AMD with choroidal neovascularization, abnormal leaky choroidal vessels proliferate and penetrate the altered Bruch membrane protruding into the subretinal space, causing hemorrhage and rapid loss of vision.

Hypoxia is also hypothesized to play a role in the development of CNV. Hypoxia-induced VEGF is secreted by RPE basally (toward the choriocapillaris). VEGF receptors are expressed on the choroidal endothelium facing the RPE, suggesting that VEGF is a survival factor for choriocapillaris in a hypoxia-driven feedback mechanism.[117] With age, especially in AMD, Bruch membrane is thickened and there is accumulation of lipids likely resulting in decreased permeability.[118,119] These changes may result in less efficient diffusion of VEGF across Bruch membrane to reach the choriocapillaris, leading to vascular atrophy and consequently hypoxia in the outer retina. In response, RPE would then increase the expression of VEGF, which would, in the presence of a rupture in Bruch membrane, attract invasive choroidal neovessels into the subretinal space. In addition to the upregulation of angiogenic factors such as VEGF, hypoxia also suppresses a potent antiangiogenic factor, PEDF. Therefore hypoxia leads to an inversion of the VEGF/PEDF ratio, which may promote pathologic choroidal angiogenesis.[120] In line with this pathomechanistic scenario, PEDF levels are decreased in the vitreous of patients with AMD and in animal models of CNV.[121,122]

Risk Factors of AMD

A twin study by Seddon et al.[123] found that genetic factors explain 46% to 71% of the variation in AMD disease severity, while environment explains 19% to 42%. Many of these risk factors for AMD are related to inflammation. These include environmental factors, such as smoking and low omega-3 fatty acid intake,[124] and genetic factors, such as polymorphisms in the genes for complement factor H,[125] other complement related genes such as C3, and the *ARMS2/HTRA1* gene.

Current Treatment of AMD

Currently there is treatment available only for the exudative form of late-stage AMD, consisting of anti-VEGF therapies to slow the activity of choroidal neovascularization.[126,127] In addition, progression of mid-stage AMD to late AMD can be slowed by dietary supplementation with vitamins and antioxidants,[128] which is consistentwith the role of oxidation stress in this pathologic process.[116] Higher intake of dietary lipids thought to be anti-inflammatory (omega-3 long-chain polyunsaturated fatty acids and fish) is associated with decreased likelihood of developing late stage AMD.[124]

Rare Inherited Vascular Eye Diseases

There are several rare hereditary eye diseases with similar retinal vascular defects as ROP. FEVR, Norrie disease, and Coats disease (also known as exudative retinitis) have in common incomplete and abnormal retinal vascular growth. Poor vascular growth results in retinal hypoxia, followed by hypoxia-stimulated abnormal proliferative vascular growth that can cause retinal

detachment and blindness. Persistent fetal hyaloid vessels are also a common clinical feature in FEVR and Norrie disease.

In FEVR as well as in Coats disease and Norrie disease, the pathologic process starts with incomplete vessel growth in the peripheral retina followed by abnormal vessel proliferation usually seen by age 10. The progression can continue into adolescence. The closer the child is watched and treated, the better the chances of preserving vision. FEVR progression usually stops by age 20. With a similar pattern but shifted timelines compared to FEVR, Norrie disease and Coats disease tend to present in early childhood often with more severe vision loss than in FEVR patients.

The prevalence of these complex diseases is unknown. FEVR can occur with all three forms of genetic inheritance: recessive, dominant, and X-linked. X-linked FEVR is clinically similar to Norrie disease, which almost always leads to blindness and is sometimes accompanied by hearing loss and mental retardation. Norrie disease in contrast is caused by the X-linked gene Norrin, a Wnt signaling ligand. Coats disease thought to be a result of somatic mutations is also linked to mutations in the Norrin gene. Some of the dominant and recessive FEVRs are caused by different genetic defects in the Wnt signaling receptors Frizzled4 and LRP5. These genetic links suggested that canonical Wnt-β–catenin pathway, a pathway fundamentally important for embryonic development and disease, is also critical for retinal vascular growth and hyaloid regression. Currently there is no treatment for these inherited vascular diseases. Further investigation of the Wnt/Frizzled pathway might lead to novel therapeutic approaches to treat or prevent these diseases as well as other ocular vascular diseases characterized by persistent hyaloid vessels and incomplete retinal vascularization.

Retinal Vessel Occlusion

Retinal vessel occlusions can affect both the arterial as well as the venous retinal vasculature. Retinal artery occlusions (RAOs) usually present with sudden, painless loss of vision, usually unilaterally. In most patients, the cause is embolism of a retinal artery by a dislodged thrombus from for example an atherosclerotic plaque. In that respect, RAOs resemble ischemic strokes where small brain arteries become blocked by embolic particles. Similar to ischemic strokes, revascularization attempts with recombinant tissue-type plasminogen activator have been tried in patients with RAO.[129] In addition, selective intra-arterial thrombolysis in the affected retinal artery has been proposed but has failed to yield superior results over standard care in the randomized, prospective multicenter EAGLE study.[130] One of the main limitations of lysis therapy in RAO patients is that therapy can usually only be initiated several hours after the occlusive event and can be associated with severe side effects like hemorrhage. For the highly metabolically active retinal tissue mere minutes of arterial nonperfusion might be enough time to undergo irreparable damage, irrespective of successful lysis or reperfusion in the later stages.

Retinal vein occlusion (RVO) represents a much more common entity where therapeutic intervention can in many cases successfully improve disease outcome. Pathophysiologically, RVOs are thought to differ from other venous occlusive diseases like deep vein thrombosis in that in most RVO patients the crucial changes appear to be in the vessel wall and not primarily in rheologic abnormalities. While in younger patients, RVO can sometimes occur as a result of genetically determined hypercoaguable states, for example, Factor V Leiden disease, in the large majority of patients above the age of 45, vessel wall alterations are thought to be the main risk factors for RVO. In that respect, RVOs share many of the risk factors of cardiac ischemic disease where small coronary arteries become occluded, also often due to primary vessel wall alterations[131] often but not only in veins. Due to the close proximity of arterial and venous vessels in the eye, alterations in the walls of retinal arteries can impact blood flow in the neighboring vein. This becomes especially apparent in branch retinal vein occlusions that almost exclusively occur at arteriovenous junction sites where the artery often appears to impinge the crossing vein (FIGURE 74.7).

Visual impairment in patients with both branch and central retinal vein occlusion is usually less acute than in arterial occlusion, and is to a large extent determined by secondary changes like retinal edema and/or macular ischemia. In the early stages, retinal edema is mainly the result of increased intraluminal pressure in the occluded vein. The occluded vein still receives unperturbed arterial and capillary inflow but is unable to drain blood through the occluded section, increasing pressure. Thus blood and plasma leak into the surrounding retinal tissue. In the later stages, hypoxia develops around the occluded area due to impaired blood circulation. Hypoxia-induced upregulation of VEGF then further increases leakage of plasma from the occluded vein into the retinal tissue through its permeability-enhancing properties. In severe cases of impaired circulation, macular ischemia can develop leading to severe and irreversible vision loss independent of macular edema.

It is important to distinguish between the causes of visual impairment in RVO patients. To date, no therapy exists that has proven reliably efficient in removing the venous obstruction.[132] Similarly, no recommendation can be provided on the use of systemic or locally applied fibrinolytic or anticoagulant therapies given the limited available evidence.[133] Anti-VEGF therapies, on the other hand, have had remarkable effects in patients with macular edema.[131,134–137] Different from wet AMD,

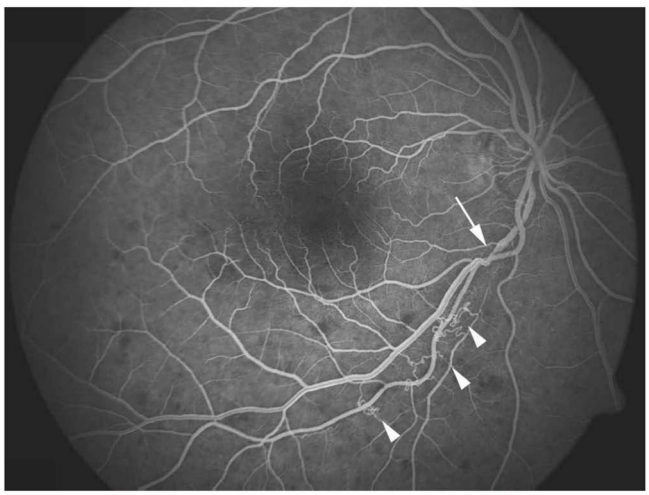

FIGURE 74.7 Fluorescence angiogram from a patient with RVO. The initial occlusion has resolved but the impingement of the retinal vein at the junction with a retinal artery is still visible (*arrow*). Collateral vessels (*arrowheads*) have formed to establish a new perfusion equilibrium. Image courtesy of Dr. Christoph Ehlken, University Eye Hospital Freiburg, Germany.

anti-VEGF therapy in RVO is not used to block the angiogenic properties of VEGF but the vasopermeability effects of VEGF leading to macular edema. It has been found that macular edema, even over relatively short time, leads to irreversible loss of photoreceptor cells.[138] Reduction of edema thus might have important implications in the long-term visual prognosis of RVO patients. As most RVOs eventually in later stages develop collateral circulation through newly-formed functional shunt vessels, limiting macular edema with anti-VEGF therapy in the earlier stages appears to be a sensible and promising strategy to increase final photoreceptor function. However, even (or especially) with anti-VEGF therapy it remains of utmost importance to continuously monitor RVO patients for the development of avascular and severely hypoxic retinal regions. If avascular regions develop, they might initiate—similar to diabetic retinopathy and ROP—a late stage of proliferative retinopathy with pathologic formation of preretinal neovascularizations and consequent complications like neovascular glaucoma and/or retinal detachments.

CURRENT AND FUTURE THERAPY FOR PATHOLOGIC ANGIOGENESIS

Pathologic retinal angiogenesis, which is morphologically distinct from normal retinal vessels often leads to blindness. When the vascular lesions have reached an advanced stage, aggressive measures are often required. Until recently the mainstay of treatment has been laser photocoagulation of the nonvascularized retina in diabetic retinopathy and ROP and photodynamic therapy in AMD.

The aim of laser photocoagulation is to induce the regression of new vessels, by obliterating ischemic areas to reduce expression of hypoxia-induced angiogenic growth factors.[139] Despite photocoagulation, however, retinopathy progresses in a significant proportion of patients. In AMD patients, the introduction of photodynamic therapy offered the first selective treatment for CNV, allowing for closure of choroidal neovessels with relative sparing of the overlying retina. However, the visual results still left room for improvement. With the advent of anti-VEGF therapies, visual function was for the first time improved in patients with late-stage AMD.[126,127] Several anti-VEGF therapies are currently in use or in late-stage clinical trials. Avastin (bevacizumab), a recombinant humanized monoclonal antibody against VEGF, was the first antiangiogenesis drug approved by FDA in 2004 for treatment of colon cancer. It was also the first anti-VEGF drug that was used to treat exudative AMD in off-label clinical application. Macugen (Pegaptanib), a 28-base oligonucleotide (aptamer) that binds VEGF,[140] was approved for the treatment of the wet AMD marking the official advent of anti-VEGF therapy for ocular disease. More recently, Lucentis (ranibizumab), a humanized monoclonal antibody fragment against VEGF, was also approved for the treatment of wet AMD.

Both Macugen and Lucentis are currently being investigated also for treatment of diabetic macular edema in large clinical trials. However prevention of vessel loss will be even more important in the treatment of retinopathy, as the extent of pathologic neovascularization depends in large part on the initial vascular loss and resultant tissue hypoxia. There are currently many active areas of research aiming at treating proliferative retinopathies in these early disease stages that are characterized by vascular loss and/or vascular dysfunction. Among these are dietary interventions with omega-3 polyunsaturated fatty acids that may help to alleviate ischemia by improving regrowth or sustainability of compromised retinal vasculature.[141] Neuroprotective agents may also play an important role by preventing degeneration of the neural retina to sustain functional vascular/neuronal cross-talk. Finally, anti-inflammatory agents may be important in curbing the inflammatory and often destructive activation of host defense cells that accompanies retinal (or subretinal) neovascularization.

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CHAPTER 75

Overview of Therapy of Bleeding Disorders

GILBERT C. WHITE, II, SAM SCHULMAN, AND VICTOR J. MARDER

Historically, once it was recognized that blood clotting factors were components of plasma, the notion of treatment with plasma to correct deficiency states followed quickly. The decay of clotting factor activity in plasma that was stored led to the development of freshly frozen plasma. The problems encountered with fresh frozen plasma—fluid overload and the inability to achieve high clotting factor levels because of the fluid overload—led to efforts to concentrate the clotting factors. The terrible transmission of blood-borne viral illnesses by the concentrates, especially AIDS and hepatitis C, led to efforts to purify factors and develop recombinant products (FIGURE 75.1). Knowledge of the genes for clotting factors, generated by the efforts to develop recombinant products, now drives efforts aimed at gene therapy and proteins with improved function (Chapter 79). Along the way, additional treatments evolved. The discovery and characterization of fibrinolytic enzymes led to development of inhibitors of fibrinolysis, which are used as adjuncts for treatment of bleeding disorders, and profibrinolytics, which are used in the treatment of thrombosis. An analog of vasopressin, DDAVP (1-desamino-8-D-arginine vasopressin), used in diabetes insipidus, was discovered to induce secretion of von Willebrand factor and thereby increase von Willebrand factor and factor VIII levels in patients with milder forms of von Willebrand disease and hemophilia. As a result, the treatment of bleeding disorders has come a very long way from these initial treatments, and today's clinician caring for patients with inherited bleeding disorders now has a highly sophisticated panel of products that are biologically stable, effective, and safe. This section reviews the various products used in the treatment of hemorrhagic disorders and the approaches in specific situations. The treatment of specific inherited coagulation factor deficiencies, such as hemophilia and von Willebrand disease, is discussed in the chapters dealing with those inherited disorders.

Acute hemorrhage is often a medical emergency and the clinician must have an approach and treatment strategy in mind when called. Knowledge of the agents available, their action, and their toxicities are important in this decision. The clinician must be able to quickly assess the source and the cause of the bleeding. It is important to determine if there is a structural lesion, an acquired bleeding defect, and/or an underlying inherited disorder. Often the input of multiple specialists—surgeons, radiologists, gastroenterologists, and hematologists—is required. The formulation of a treatment plan may require both blood replacement and hemostatic agents. These issues are discussed along with specific approaches for particular situations in Chapter 76. For acute hemorrhage in hemophilia and in acquired inhibitors, the reader is also referred to Chapters 51 and 57. Additional approaches in bleeding disorders associated with disseminated intravascular coagulation and liver disease are referred to Chapters 98 and 126. Treatment of acquired and inherited platelet and vascular disorders is described in Sections III-B and III-C.

Chapter 77 describes the use of antifibrinolytic agents. These have become important adjunct therapies for either the treatment of acute or chronic hemorrhage or for the prevention of hemorrhage. Antifibrinolytics are especially useful in the treatment of chronic hemorrhage from tissues that contain high levels of fibrinolysis activators such as the gastrointestinal tract.[1–3] Antifibrinolytics have been used in combination with clotting factor concentrates to reduce factor administration in hemophilia and in combination with bypassing agents for dental work and other treatments in hemophilic individuals with inhibitors.[4–6]

An important question for the first quarter of the 21st century is, can we make even better products for hemorrhagic disorders? Given the discoveries over the past decade or more regarding the structure and function of clotting factors, the feasibility of making products that have altered properties is real. Products for hemophilia A and B are currently leading the way in this regard. Nevertheless, there remain significant knowledge gaps. One important gap is a lack of understanding of the mechanisms of clearance of the various clotting factors. In the case of factor VIII, for example, a number of candidate mechanisms have been proposed, including the low-density lipoprotein receptor–related protein on hepatocytes,[7,8] the high mannose receptor (CD206) on macrophages and other cells,[9] and the Gilbert-Ashwell asialoglycoprotein receptor on hepatocytes,[10] but none appear robust enough, at least in animal models. Most investigators in the field believe that von Willebrand factor is a key determinant of clearance based on the significantly shortened survival of factor VIII in the absence of von Willebrand factor. This would suggest that the approach to prolonging factor VIII survival may be to prolong that of von Willebrand factor. Another significant gap is the lack of structural information regarding clotting factor interactions and the residues that mediate the interactions. In the past decade, the structures of a number of individual clotting factors have been solved, but technology has not yet permitted determination of the structure of multiprotein complexes, such as the prothrombinase and tenase complexes. Knowledge of these interactions at the atomic level is important to developing ways to regulate the interactions.

Although clearance mechanisms for clotting proteins remain uncertain, efforts are going forward with attempts to develop products with a prolonged half-life, especially those used in the treatment of hemophilia. When one considers that the average hemophiliac treats himself about three times a month, even doubling the half-life would have a beneficial

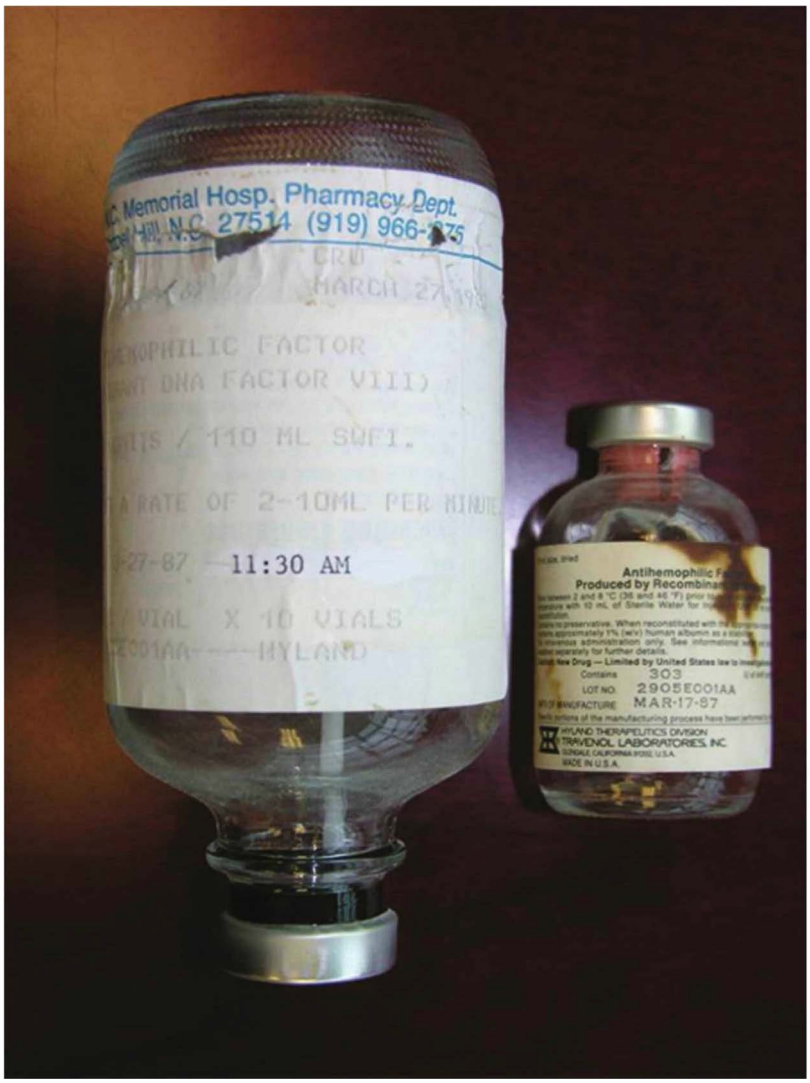

FIGURE 75.1 First vial of recombinant FVIII. Bottle on the right is the initial vial of antihemophilic factor (AHF) (factor VIII) produced by recombinant DNA technology to be infused into a human. The product was produced by Hyland Therapeutics Division, Travenol Laboratories, Inc. (Glendale, CA). The date of manufacture, indicated on the bottle, was March 17, 1987. The lot number was 2905EOO1AA and the vial contained 303 IU of AHF. The inverted bottle on the left is the infusion material reconstituted according to the manufacturer's specifications that was infused in the General Clinical Research Unit at the University of North Carolina on March 27, 1987 at approximately 11:30 AM.

effect. Studies are currently underway with various pegylated forms of factors VIII, IX, and VII and with factors fused to longer-lived proteins such as immunoglobulin fragments and albumin. The real problem with clotting factor therapy, though, is not the frequency of treatment *per se*; it is the intravenous nature of the treatment, the cumulative lifetime expense with insurance caps, and the long-term effect on venous access. For these reasons, the long-range goals of research should be the development of oral agents and more curative approaches such as gene therapy. These and other approaches are discussed in Chapter 79.

In the long term, better knowledge of coagulation biology might be expected to lead to more personalized treatment. Accumulating evidence suggests that individuals have a unique ability to form thrombin, a so-called thrombin potential, that can be assessed by *in vitro* testing.[11–14] Individuals at risk for thrombosis tend to have increased endogenous thrombin potential and individuals at risk for bleeding tend to have decreased endogenous thrombin potential. The actual amount of thrombin that is generated in plasma correlates well with clot structure *in vitro* (see Ref.[15]). For example, excessive generation of thrombin, as might occur with the G20210A mutation in the 3' untranslated region of prothrombin, leads to formation of relatively nonturbid, less permeable clots that are composed of a dense network of relatively thin fibrin strands. Such clots are relatively resistant to fibrinolysis. Conversely, inadequate generation of thrombin, as occurs in hemophilic plasma, leads to the formation of turbid, highly permeable fibrin clots that are composed of thick, loosely woven fibrin strands and are more sensitive to fibrinolysis. It has been suggested that the use of this type of *in vitro* endogenous thrombin generation assay can provide better assessment of thrombotic and bleeding risk and might be used to monitor therapy in bleeding or clotting patients, providing a more personalized approach to treatment.

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CHAPTER

76 Management of Acute Hemorrhage

MICAH J. MOOBERRY, ROMMEL P. LU, AND NIGEL S. KEY

Evaluation and management of the acutely bleeding patient is a common encounter for the hematologist and is often fraught with innumerable challenges. It can additionally involve a wide variety of clinical scenarios, including pediatric, obstetric, surgical, and medical patients. These challenges are often further compounded by a paucity of clinical and laboratory information on which to base decisions. With these factors in mind, this chapter briefly discusses an approach to the initial evaluation of the acutely bleeding patient, including both clinical and laboratory assessments, followed by a discussion of specific therapeutic agents available for the treatment of acute hemorrhage. It concludes with a discussion of several specific bleeding situations and management of complications of antithrombotic therapy.

INITIAL EVALUATION

Clinical Approach

In the ideal setting, the evaluation of acute hemorrhage should be a multistep process beginning with a complete clinical history, followed by a focused physical examination and a directed laboratory evaluation. However, with the advent and now ubiquitous presence of more sophisticated medical diagnostic testing, the interview and examination of the patient often play a secondary role in the medical evaluation. Despite this trend, the clinical evaluation of the acutely bleeding patient is paramount and should be the foundation for further decision making regarding diagnostic testing, as well as possible intervention strategies. Contrary to outpatient bleeding evaluations, adherence to this algorithm may not always be possible in the setting of significant acute hemorrhage, and an abbreviated workup that focuses on pertinent exam and laboratory findings may be all that time allows.

When possible, a detailed history should be obtained, with particular attention paid to prior bleeding episodes, if present. The goal should be to answer several questions with reasonable certainty: (a) Is a bleeding disorder present? (b) If so, is it a disorder of primary or secondary hemostasis? (c) Is it acquired or hereditary in nature? To help make these determinations, the severity and nature (mucocutaneous, intramuscular, intra-articular, etc.) of each prior bleeding episode, as well as the number of bleeding episodes, should be assessed. Specific inquiries should also be made regarding menorrhagia, the presence of bleeding episodes during infancy and childhood, bleeding associated with dental procedures, peripartum hemorrhage, and excessive or unprovoked bruising.

A history of provoked versus spontaneous hemorrhage can offer additional insight. Bleeding events after a hemostatic challenge, such as trauma or surgery, could be indicative of an underlying mild bleeding disorder that had not manifested previously with spontaneous bleeding.[1] Conversely, the absence of bleeding despite the hemostatic challenge of multiple surgeries may suggest the absence of a longstanding bleeding disorder. Timing of bleeding with respect to the hemostatic challenge can also provide clues to whether there is a defect in primary or secondary hemostasis (e.g., immediate bleeding with disorders of primary hemostasis vs. delayed bleeding with disorders of secondary hemostasis) (see Table 76.1).

A thorough family history of bleeding symptoms should also be obtained, querying similar symptoms as those posed to the patient. It should be noted, however, that the absence of a family history of bleeding does not exclude the presence of a congenital bleeding disorder, as there may be no affected family members in the setting of spontaneous mutations or autosomal recessive disorders.[2,3] Obtaining a list of all comorbidities is also worthwhile, as numerous medical conditions are associated with specific coagulation disorders, which may or may not be realized by the patient. Finally, a full review of all current medications can aid in patient management, both from a diagnostic and a therapeutic perspective. This should include an inquiry into nonprescription compounds as well. Obvious culprits in the setting of acute hemorrhage include anticoagulants and antiplatelet agents; however, numerous over-the-counter medications contain nonsteroidal agents that exert antiplatelet effects and are often not considered "medications" by patients unless specifically addressed. Several herbal and nutritional supplements have also been implicated in hemorrhagic events and should be investigated, although they are unlikely to be the sole cause of bleeding complications.[4]

A focused physical examination is also prudent, as this can provide substantial insight into possible pathologic mechanisms and help guide management decisions. Bruises, petechiae, mucocutaneous bleeding, and menorrhagia are often seen in patients with platelet defects, von Willebrand disease (vWD), and other defects in primary hemostasis. In comparison, bleeding into muscles and joints is more characteristic of disorders of secondary hemostasis, such as hemophilia A and B and certain other coagulation factor deficiencies. The exam can also potentially lead to the discovery of previously undiagnosed comorbid conditions that may contribute to a patient's bleeding. Examples include gum hypertrophy in acute myelogenous leukemia (AML) (types M4 and M5); macroglossia, periorbital ecchymoses, and carpal tunnel syndrome in amyloidosis[5,6]; harsh systolic murmur in severe valvular disease; and jaundice,

Table 76.1
Classification of bleeding disorders

Disorders of Primary Hemostasis	Disorders of Secondary Hemostasis
von Willebrand disease	Hemophilia A
Platelet function disorders	Hemophilia B
Inherited	Other factor deficiencies: II, V, VII, X, XI, XIII
Acquired	Factor inhibitors
Drug induced	Fibrinogen abnormalities
Thrombocytopenia	Hyperfibrinolysis
Inherited	Inherited deficiencies
Acquired	Acquired
Drug induced	Connective tissue disorders
Immune mediated	

icterus, spider angiomata, and splenomegaly in severe liver disease, among others (see Table 76.2).

After the clinical assessment of the patient is complete, one should formulate a pretest probability as to whether a bleeding disorder exists or not, and if so, what type. Given the frequency with which "normal" subjects report bleeding symptoms, however, the ability to identify mild bleeding disorders can be challenging.[7–11] To that end, several attempts have been made to develop standardized screening tools that predict the presence of a bleeding disorder.[12–15] To date, however, no prospectively validated clinical tool exists to predict the presence of a bleeding disorder. Notably, the International Society on Thrombosis and Hemostasis recently proposed a Bleeding Assessment Tool consisting of a standardized questionnaire and well-defined interpretation grid to be used in any future studies aimed at description of bleeding symptoms or diagnosing bleeding disorders,[16] but this will need to be validated before it is adopted into clinical use.

Laboratory Evaluation

Evidence has shown that indiscriminate coagulation testing in patients undergoing surgery or other invasive procedures does not help predict risk of bleeding and is therefore not recommended.[17–19] In the setting of acute hemorrhage, however, laboratory testing is vital and should be pursued for several reasons: confirmation of a clinically suspected bleeding disorder, clearly defining the defect if present, and guiding treatment/intervention strategies.

Screening tests of coagulation that evaluate for both primary and secondary hemostatic abnormalities should be performed in all patients with acute hemorrhage. These include a complete blood count (CBC) and platelet count, peripheral blood smear, activated partial thromboplastin time (aPTT), and prothrombin time (PT). These tests are available in most settings, and due to their automated nature, provide reproducible results in a timely manner. In general, these basic tests in conjunction with the clinical assessment should be able to direct further evaluation toward defects of primary or secondary hemostasis. The details of the laboratory evaluation will not be fully covered here (see Chapter 50); however, it should be mentioned that preanalytical variables can have a profound effect on test results and therefore attention should be paid to proper collection and handling of specimens.[20,21]

If all testing for hemostatic defects returns normal, it should be remembered that up to 40% to 50% of suspected bleeding disorders yield a normal laboratory screening evaluation, particularly mild bleeding disorders that probably include heterogeneous platelet function defects.[22,23] Additionally, abnormalities on coagulation tests, particularly in the acutely ill patient, may

Table 76.2
Comorbid conditions and bleeding

Disease	Cause of Bleeding	Other Findings/Manifestations
Acute leukemia	Thrombocytopenia, hyperfibrinolysis in acute promyelocytic leukemia	Pallor, petechiae, gum hypertrophy
Amyloidosis	Acquired FX deficiency, other factor deficiencies (II, V, VII, VIII, IX), hyperfibrinolysis	Macroglossia, periorbital ecchymoses, carpal tunnel syndrome
Ehlers Danlos syndrome	Defect in collagen synthesis	Joint laxity, skin hyperelasticity
Hereditary hemorrhagic telangiectasia	Arteriovenous malformations of multiple sites, GI telangiectasias	Recurrent epistaxis, oral telangiectasias
Hypothyroidism	Acquired vWD	Thin hair, obesity, cold intolerance, dry skin
Liver disease/cirrhosis	Thrombocytopenia, factor deficiencies, hyperfibrinolysis	Hepatosplenomegaly, icterus, jaundice, spider angiomata
Scurvy	Defect in collagen synthesis	Corkscrew hairs, petechiae, perifollicular purpura
Severe valvular disease	Acquired vWD, thrombocytopenia	Harsh murmur
Wilms tumor	Acquired vWD	Flank mass

not be indicative of a primary coagulation defect, but instead be epiphenomena or the result of another acute medical process. Therefore, a suspected primary coagulation disorder should always be confirmed after the acute situation has resolved. In the event that exhaustive laboratory testing does not provide definitive answers as to the cause of the acute bleeding episode, empiric treatment based upon clinical findings and sound medical judgment is a reasonable choice and may be the only course of action available.

THERAPEUTIC APPROACH

Blood Component Therapy

Conservative approach to transfusion therapy has become the standard of care in most situations. In contrast to whole blood, which is utilized primarily in military settings,[24,25] red cell units contain negligible amounts of platelets and coagulation factors and are used to provide increased oxygen-carrying capacity. Plasma-containing blood products, including platelets, are conversely administered to correct hemostatic defects. The primary advantage of blood component therapy is the ability to allocate resources according to individual needs (see Table 76.3). The clinical decision to transfuse should be individualized and not simply based on a given blood count. The underlying mechanism causing the cytopenia or coagulopathy and its clinical risks must be considered as well. However, despite the potential transfusion-associated infectious, metabolic, and immunologic risks, it is important not to withhold blood products to anybody with a life-threatening hemorrhage. The management strategy for transfusion therapy that follows generally refers to adult patients. Guidelines for pediatric transfusion may differ.

Red Cell Transfusion

The decision to transfuse red cells requires a detailed analysis of clinical factors, with the primary goal of restoring tissue perfusion and reversing the effects of shock if already present. Generally, this decision is made on the basis of hemoglobin and hematocrit values in conjunction with the patient's physiologic response to anemia and coexisting medical condition. The administration of red cell transfusions in an actively bleeding patient is often lifesaving, but recommendations about when and how much to transfuse are unclear.[26] For many years, most clinical practices used a hemoglobin/hematocrit threshold of <10 g/dL/30% as transfusion trigger in numerous clinical settings. More recently, however, a pivotal controlled study evaluating transfusion strategies in critically ill patients provided new evidence to guide transfusion practices. This trial demonstrated that a restrictive transfusion approach (i.e., transfusing patients only with a hemoglobin <7 g/dL) was as effective as a liberal strategy (i.e., transfusing patients with a hemoglobin <10 g/dL) and had a lower in-hospital 30-day mortality, cardiac complication rate, and organ dysfunction.[27] However, patients with

Table 76.3

Blood component therapy

Component	Description	Volume	Dosing
Red cells	Prepared by removing plasma from whole blood or apheresis Transfused red cells have a half-life of ~30 d in the absence of processes that would result in red cell loss or premature removal.	200–350 mL	In average-sized adults, one unit of compatible red cells will increase hemoglobin by 1 g/dL and hematocrit by 3%, provided there is no ongoing hemolysis or bleeding. In neonates and older children, a transfusion dose of 10–15 mL/kg is generally given and results in hemoglobin rise of 2–3 g/dL.
Platelets	Collected as RDP or SDP RDPs are derived from whole blood and six units of RDPs constitute one adult dose. SDPs are apheresed platelets and are full adult doses.	One adult dose is 250–300 mL	In adults, expect an increment of approximately 30,000–60,000/μL for each SDP or pooled six units of RDP. In neonates and older children, a dose of 5–10 mL/kg generally results in 50,000/μL increment.
Plasma (FFP, FP24, thawed plasma)	Consists of non-cellular portion of blood. May be prepared from whole blood or collected by apheresis	150–300 mL	10–15 mL/kg (equivalent to 3–4 U in adult)
Cryoprecipitate	Prepared from plasma Each unit contains at least 80 IU of factor VIII:C and 150 mg of fibrinogen in 15 mL plasma.	One adult dose is ~150 mL	One dose (10 U in adult or 1 U per 10 kg body weight in neonates and older children) increases fibrinogen level by 0.5 g/L in the absence of continued consumption or bleeding

Modified from Brecher M, ed. *Technical manual*, 15th ed. Bethesda, MD: AABB press, 2005; Mintz P, ed. *Transfusion therapy: clinical principles and practice*, 2nd ed. Bethesda, MD: AABB Press, 2005.

ongoing hemorrhage were excluded from the study. Thus, it is important to realize the possible limitations of restrictive transfusion strategy in acute settings.

The urgent need for transfusion is uniquely apparent in the operative and trauma settings. Significant hypotension occurs once more than 30% of blood volume is lost and is a late sign of internal hemorrhage. Frequently, red cells are transfused to improve blood pressure, hemoglobin concentration, or both before the cause of bleeding is identified. In the acute trauma setting, the rapidity and amount of blood loss may be inaccurately estimated by the hemoglobin/hematocrit values, thereby making it difficult to exclusively rely on these tests to determine the relationship between blood loss and transfusion requirement. A number of factors must be considered besides hemoglobin level, such as vital signs, oxygenation, and clinical status, to ensure tissue demands for oxygen are met. This is especially applicable in patients with limited cardiac reserve or significant atherosclerotic vascular disease, in whom the heart may be unable to increase total body oxygen delivery in the face of anemia, without risk of myocardial ischemia. A transfusion study in elderly patients with acute myocardial infarction showed a lower short-term mortality when patients were transfused to achieve a hemoglobin as high as 10 g/dL. Evidently, transfusion trigger for red cells must be customized to defined patient groups, and the decision to transfuse must be made on the basis of individual patient characteristics.[28]

Two of the recommendations of the American Society of Anesthesiologists Task Force with practical applications in the management of acute hemorrhage are:

1. Transfusion is rarely indicated when the hemoglobin level is above 10 g/dL and is almost always indicated when the hemoglobin level is below 6 g/dL.
2. The determination of transfusion in patients whose hemoglobin level is 6 to 10 g/dL should be based on ongoing indication of organ ischemia, rate and magnitude of any potential or actual bleeding, patient's intravascular volume status, and risk of complications due to inadequate oxygenation.[29]

Blood Substitutes

Biomedical advances have made the development of hemoglobin-based oxygen carriers (i.e., blood substitutes) possible, but due to safety concerns about increased rate of myocardial infarction, stroke, hypertension, and renal dysfunction in patients who have received them in clinical trials, they are still under development.[30–32]

Platelet Transfusion

Platelet transfusion is used to treat or prevent bleeding due to defect in platelet number or function. In general, most patients do not experience spontaneous bleeding at platelet counts of 40,000 to 50,000/μL unless there is a coexisting platelet dysfunction or other hemostatic defect. Studies indicate that achieving a platelet count of 40,000 to 50,000/μL with platelet transfusion in patients with severe thrombocytopenia might be sufficient to allow an invasive procedure to be safely performed. A threshold of 80,000/μL has been proposed for spinal epidural anesthesia. In those situations in which no excessive bleeding can be tolerated, such as central nervous system or retinal procedures, a platelet count of 100,000/μL is often used. In patients who are severely thrombocytopenic, such as those receiving myeloablative chemotherapy, it is a common practice to maintain a platelet count >10,000/μL in stable nonbleeding patients, and >20,000/μL in febrile nonbleeding patients, based on the observations that serious spontaneous bleeding is unlikely to occur at platelet counts >10,000/μL. When known platelet dysfunction results in microvascular bleeding or before anticipated major surgery, platelet transfusion may be required despite having a seemingly adequate platelet count. However, prophylactic platelet transfusion in patients with immune thrombocytopenic purpura based on platelet count alone in the absence of spontaneous bleeding is discouraged since bleeding is less frequent at equivalent platelet counts, and platelet transfusions are often ineffective. Similarly, prophylactic platelet transfusion practice is discouraged in cases of thrombotic thrombocytopenic purpura (TTP) and heparin-induced thrombocytopenia (HIT), unless there is life-threatening bleeding or in anticipation of major surgery. However, the reluctance to transfuse platelets in TTP and HIT may have been overemphasized, and it remains a controversial topic. In the presence of active bleeding, maintaining a platelet count of 50,000 to 100,000/μL with platelet transfusion support is often practiced. A platelet count threshold of 100,000/μL is often used in patients with severe, noncompressible bleeding such as in the gastrointestinal (GI) tract, intracranial, or intrathoracic areas, while a lower platelet count threshold is used in less severe bleeding. Overall, these guidelines tend to be based on expert recommendations rather than level I evidence.[29,33–38]

Practical issues regarding platelet transfusion include:

1. Response to platelet transfusion is adversely affected by the presence of fever, sepsis, splenomegaly, severe bleeding, consumptive coagulopathy, human leukocyte antigens (HLA) alloimmunization, and treatment with certain drugs (e.g., amphotericin B).[33,39,40]
2. Rh (D)-negative patients, especially women of childbearing age, should receive platelets from Rh (D)-negative instead of Rh (D)-positive donors because of the risk of alloimmunization and development of a clinically significant antibody to Rh (D) antigen, which can cause a hemolytic transfusion reaction from subsequent Rh (D) mismatched transfusions. However, this is not practically feasible at all times because of limited platelet inventory. If platelets from Rh (D)-positive donors are transfused to Rh (D)-negative recipients (especially women of childbearing age), consideration should be given to administering prophylactic Rh (D) immune globulin to prevent sensitization.[41,42]

Plasma Transfusion

Fresh frozen plasma (FFP) contains coagulation proteins involved in the maintenance of vascular integrity, anticoagulation proteins, and proteins involved in fibrinolysis. FFP is frozen at ?18°C or colder within 6 to 8 hours of collection and contains functional quantities of all coagulation factors. Plasma frozen within 24 hours (FP24) and thawed plasma may contain variably reduced levels of factors V and VIII. Despite these processing differences, FP24, thawed plasma, and FFP are generally used for the same indications.[43]

Current published guidelines by multiple organizations consider plasma transfusion appropriate only under specific circumstances. The definitive indications for FFP transfusion are replacement of coagulation factor deficiency in the event of

bleeding with certain coagulopathies (including patients with liver disease, isolated factor V deficiency, disseminated intravascular coagulopathy [DIC], or while on a vitamin K antagonist [VKA]) or TTP. Conditional uses of FFP include prophylactic use in patients with certain coagulopathies prior to invasive procedures, those undergoing cardiopulmonary bypass surgery, or children on extracorporeal membrane oxygenation.

When used to correct multiple coagulation factor deficiencies, the response to plasma transfusion should be closely monitored by follow-up laboratory tests and clinical assessment. A PT and/or aPTT >1.5 times the normal range are typically the recommended triggers for plasma transfusion, although this cutoff is being challenged by several groups.[29,44–46] Despite these guidelines, requests for FFP are the most frequent inappropriate orders received by the blood bank. The most frequent reason for these inappropriate orders is for correction of a prolonged international normalized ratio (INR) in the absence of bleeding. In clinical practice, prophylactic plasma transfusion prior to invasive procedures including neurosurgical interventions has become routine. It is suggested that inappropriate FFP orders occur because of three assumptions: (a) Elevation of the PT/INR will predict bleeding in the setting of a procedure. (b) Preprocedure administration of FFP will correct the prolonged clotting time results. (c) Prophylactic transfusion results in fewer bleeding events. However, these three assumptions about the relationship of FFP transfusion and decrease in INR, especially in patients with mildly prolonged INRs, are unproven. Studies in patients undergoing liver biopsies, bronchoscopic biopsies, renal biopsies, central line vein cannulation, thoracentesis, and angiography have repeatedly demonstrated that the PT and aPTT are not predictive of hemorrhage. Furthermore, plasma transfusion prior to an invasive procedure to correct a mild to moderately abnormal INR neither corrects the INR nor reduces the perceived bleeding risk.[47–50,51,52] Therefore, transfusion for patients not meeting current FFP guidelines does not reliably reduce the INR and exposes patients to unnecessary transfusion risk.

Practical issues regarding plasma transfusion include:

1. A significant degree of change in the INR following FFP transfusion may not be seen until the INR is >1.7, largely because the INR of donor plasma itself ranges between 1.0 and 1.3. Thus, the difference in coagulation activity between donor plasma and patient plasma is so small that plasma transfusions produce minimal demonstrable effect on the patient's INR.[51] Administering vitamin K may be a therapeutic option to consider in patients who have a presumed coexisting vitamin K deficiency.
2. If plasma is to be transfused, its timing should be carefully considered. If correction of a markedly abnormal PT or aPTT is required before surgery, FFP is better given just before the patient is called to the operating room, and not the night before. Several coagulation factors have very short half-lives, and if FFP is given 8 hours preoperatively, those factors will be cleared from the circulation by the time surgery begins.[53]
3. Many transfusion protocols suggest administering plasma after the patient has received 4 to 6 U of red cells, but recent studies in trauma are challenging this practice and are suggesting earlier rather than later plasma administration[54,55] (see section "Massive Transfusion Therapy").

Cryoprecipitate Transfusion

Cryoprecipitate is the precipitable protein fraction derived from frozen plasma thawed at 1°C to 6°C. It contains concentrated levels of fibrinogen, von Willebrand factor (vWF), factor VIII, factor XIII, and fibronectin in a small volume, but it is generally administered as a source of fibrinogen. Patients with hemophilia A or vWD disease should only be treated with cryoprecipitate when appropriate factor VIII concentrates or factor VIII concentrates containing FVIII: vWF are not available.[56]

Since FFP contains all coagulation factors, FFP transfusion should supply the depleted hemostatic proteins, although a large volume of plasma is usually required to fully replete significant deficiencies. This becomes a challenge in patients who are unable to tolerate larger intravascular volumes, such as those with significant cardiac, pulmonary, renal, and/or hepatic disease. In patients with ongoing bleeding with a fibrinogen level <0.8 to 1.0 g/L, cryoprecipitate transfusion is recommended.[29]

Massive Transfusion Therapy

Massive transfusion has been defined differently across studies, but in general it refers to transfusion of at least 10 U of red cells in the first 24 hours after trauma.[30] In lethal hemorrhagic shock, transfusion of blood products is performed empirically and is not guided by laboratory tests but is based on clinical parameters.[57] Hemorrhagic shock is a severe and life-threatening condition. Over 30% of military casualties receive blood transfusion and about 6% require massive transfusion.[58] In patients with severe traumatic injury, there is an association between rising transfusion requirements and increased risk of multiorgan failure and worsened clinical outcomes.[59–61] The increase in mortality is correlated with the number of red cell units transfused (22%, 30%, 50%, and 59% mortality in the groups receiving 1 to 10 U, 11 to 20 U, 21 to 40 U, and >40 U, respectively).[60] In the absence of timely and robust tests of coagulation during acute trauma, massive transfusion protocols were designed to provide rapid delivery of blood products to patients with life-threatening hemorrhage.

In the setting of exsanguinating hemorrhage, transfusion therapy is usually based on the number of red cell units administered and expressed as plasma to red cells (P:RBC) and platelets to red cells (PLT:RBC) ratios. Retrospective studies of trauma resuscitation protocols, especially from military experiences, indicate that traditional transfusion protocols do not achieve normal hemostasis and are designed to treat dilution, while earlier and aggressive treatment of coagulopathy with a P:RBC ratio of 1:1 to 1:2 and a PLT:RBC ratio of 1:1 (using random donor platelets [RDPs]) or 1:6 (using single donor platelets [SDPs]) have indicated a possible survival advantage compared to recommendations in existing transfusion guidelines. Currently, there is no standardized massive transfusion protocol in the United States but many trauma centers have their own protocols, with early use of blood type "O" Rh (D)-negative red cells and "AB" plasma in anticipation of further transfusion needs. These observations of RBC:P:PLT ratios of 1:1:1 (1 U red cell, 1 U plasma and 1 U RDPs), practically expressed as 6:6:1 (6 U red cells, 6 U plasma and 1 U single donor apheresed platelets), albeit retrospective, are paving the way to a changing paradigm of transfusion therapy in the military environment and are beginning to receive attention in the

civilian trauma setting.[62–65,66] Whether this high ratio of plasma to red cells would decrease mortality in nonmassively transfused patients is unknown.

Trauma-Induced Coagulopathy

The presence of a coagulopathy occurring early after traumatic injury is a well-recognized independent predictor of mortality.[67] The factors underlying the development of coagulopathy are multiple and traditionally were thought to include dilution of coagulation factors and platelets, compromise of the coagulation system by infusion of crystalloids and colloids, increased fibrinolysis and hypocalcemia.[68,69] The interplay between hypothermia, acidosis, and coagulopathy has been referred to as the "lethal triad." In patients who are already acidotic, massive transfusion of red cells increases acid load.[68,70] Trauma patients receiving large volumes of plasma may develop hypocalcemia due to the chelating citrate anticoagulant present in plasma. Liberal red cell transfusion, although improving oxygen transport, does not replace coagulation factors and platelets. All these factors, which may occur simultaneously, can potentially contribute to coagulopathic bleeding.[71]

Recent observational studies have described a distinct coagulopathy in trauma referred to as trauma-induced coagulopathy (TIC), Acute Traumatic Coagulopathy, or Acute Coagulopathy of Trauma Shock. TIC is a syndrome of "nonsurgical bleeding." The classic model of trauma coagulopathy occurring during resuscitation has been challenged by recent clinical observations, specifically studies showing that TIC may be present in 20% to 30% of patients upon arrival at the emergency room. This coagulopathy is present even prior to massive transfusion, fluid resuscitation therapy, or the development of hypothermia, and is independently associated with a four- to fivefold increase in mortality compared to patients who do not have coagulopathy upon initial presentation.[67,72,73] A combination of massive tissue injury and shock is believed to underlie the pathogenesis of TIC.[74] This situation is in contrast to the traditional understanding of posttraumatic coagulopathy in resuscitated patients that develops as a consequence of medical intervention. TIC is characterized by systemic anticoagulation and hyperfibrinolysis that manifests as a bleeding phenotype with mild prolongation of the INR (>1.3) and aPTT (>1.5 times normal) with preservation of fibrinogen levels and platelet counts.[75] Its mechanism is unclear but it does not appear to be a consumptive coagulopathy; instead, widespread activation of the protein C pathway has been implicated.[76] Recognition of this coagulopathy has stimulated revision of massive transfusion protocols. Early use of plasma, platelets, and cryoprecipitate together with red cells is advocated to reduce overall blood product use and potentially avoidable deaths from hemorrhage after trauma. This development in modern trauma care emphasizes the concept that not all bleeding can be controlled surgically.[74,77,78]

Pharmacologic Therapy

Prothrombin Complex Concentrates

Prothrombin complex concentrates (PCCs), otherwise known as intermediate purity FIX concentrates, were initially used primarily for the treatment of hemophilia B; however, they are rarely used for that indication now due to the availability of newer FIX preparations. Derived from pooled human plasma, these virally inactivated agents contain the vitamin K–dependent factors II, VII, IX, and X in varying amounts. Earlier preparations had considerable variability in their compositions;[79] however, this limitation has improved over the past 15 years with newer forms, and current composition is in line with levels listed by the manufacturer.[80] Due to increased thrombogenicity and risk of DIC seen with earlier forms, most agents now also contain varying levels of anticoagulants, including protein C, protein S, antithrombin, and heparin.[81] Earlier forms also contained variable amounts of activated factors,[82] particularly FVIIa and FIXa, which have been greatly reduced or eliminated in current preparations.[80] Although most current concentrates contain sufficient levels of all four vitamin K–dependent factors, several so-called three factor PCCs, which contain relatively low FVII levels, are also marketed, and are the only agents currently available in the United States.[83]

Although not licensed as such in the United States, the principal indication for PCCs currently is reversal of the effects of oral anticoagulation with VKAs (see section "Complications of Antithrombotic Therapy"). PCCs are also occasionally used as replacement therapy in patients with congenital or acquired deficiencies of FII or FX.[84,85] Although not licensed for this indication, PCCs have additionally been used in treatment of severe hemorrhage requiring massive transfusion,[86] but this practice is only widespread in certain European countries.[87,88] Other reported uses include treatment of bleeding due to vitamin K deficiency, severe liver disease[89,90] or after liver transplantation. In the latter two conditions, these agents are commonly used when volume status is of concern and the use of FFP risks volume overload. It should be noted, though, that PCCs do not contain all coagulation factors that are deficient in liver dysfunction, most notably FV and fibrinogen, and supplementation with FFP and/or cryoprecipitate may be needed to adequately treat active hemorrhage.

Potency labeling and dosing recommendations for PCCs are based on International Units (IUs) of FIX.[91] One IU of PCC per kg body weight generally increases the plasma activity of FIX by approximately 1 IU/dL. Doses are subsequently calculated based upon the goal FIX level, or in the case of VKA overdose, by INR. Typical doses are 25 to 50 IU/kg and can be repeated every 24 hours as needed. The most feared complication of PCC administration is thromboembolism, which has been reported in numerous case series.[92,93] With recent advances in preparation of these agents, including addition of anticoagulants and minimization of activated factors, it appears that thromboembolic events are relatively uncommon.[89,94] Despite this trend, however, PCCs should still be used with caution when the risk for thromboembolism is deemed to be high, such as in the setting of severe liver disease. PCC use is relatively contraindicated in the context of DIC due to thrombotic risk; however, this is less of a problem with the newer preparations that may be used in hemorrhagic forms of DIC when volume limitations preclude the use of FFP. Other adverse events reported include allergic, and even anaphylactic, reactions, although both are rare.[87]

Recombinant Factor VIIa

Recombinant factor VIIa (rFVIIa) was first introduced in the 1980s as a hemostatic agent for patients with hemophilia A and B with known inhibitors, and was approved for this indication in most countries by the late 1990s.[95] Since that time, its off-label use has become increasingly widespread, particularly in the setting of acute massive or life-threatening hemorrhage.

Its mechanism of action is thought to be related to enhanced platelet-surface thrombin generation.[96,97]

Recombinant FVIIa is currently approved for the prevention and treatment of bleeding in congenital hemophilia A and B with inhibitors, acquired hemophilia, and congenital FVII deficiency. Additional approved uses in Europe include bleeding in Glanzmann's thrombasthenia in the presence of antibodies to glycoprotein (GP) IIb–IIIa and/or HLA, with past or present refractoriness to platelet transfusions.[97] As mentioned above, off-label use of rFVIIa is widespread despite lack of definitive evidence regarding efficacy in most settings. Reported uses include bleeding due to anticoagulation, intracerebral hemorrhage, surgical bleeding, postpartum or obstetric hemorrhage, FXI deficiency, and liver disease, among others. Large-scale trials in these areas are limited, and additionally, results of existing trials have often resulted in conflicting data. In general, however, rFVIIa use is deemed appropriate by most experts when uncontrollable hemorrhage is encountered. Consensus panels in numerous countries have additionally made recommendations on appropriate use of rFVIIa, which are congruous with this approach.[98–100]

Another area of interest regarding the use of rFVIIa has been in the setting of civilian and military trauma, where uncontrolled massive bleeding contributes to up to 40% of early in-hospital deaths.[101,102] Several early phase trials appeared to demonstrate efficacy of rFVIIa in reducing bleeding and transfusion requirements.[103–107] This effect appeared to be more significant with blunt trauma, as opposed to penetrating trauma.[108] Data on mortality benefit were mixed, with several trials showing benefit in both early and overall mortality[103,109–111]; however, a recent phase III clinical trial was terminated prematurely due to low likelihood of showing improvement in primary endpoints of morbidity and mortality.[112] Overall, aggressive transfusion therapy remains the standard of care in trauma (see section "Blood Component Therapy"); however, rFVIIa continues to be used in cases where hemorrhage continues despite these measures. At this time, more trials are needed to further assess its efficacy in the setting of trauma.

Dosing regimens reported for rFVIIa in off-label uses vary tremendously by study, making interpretation of efficacy data difficult. Reported doses range from 5 to 200 μg/kg. In the setting of hemophilia, dosing regimens are more standard. Typical doses for bleeding in hemophilia are 90 μg/kg every 2 to 3 hours × three doses, however recent studies in hemophilia have shown that a single dose of 270 μg/kg is equally effective.[113,114] With no clear dosing guidelines in off-label uses, a reasonable starting point is 90 μg/kg, repeating doses every 2 to 3 hours if bleeding persists. Lower doses may be considered where there is heightened concern for thrombotic events. Of note, newer bioengineered forms of rFVIIa with greater potency and/or more rapid onset of action are in clinical development.[115–118]

Adverse events related to use of rFVIIa are mostly restricted to thrombotic events, including stroke and myocardial infarction. A recent review of safety data reported an incidence of <1% of serious thromboembolism in hemophilia patients treated with rFVIIa.[119] Thrombotic events have been reported with off-label use of rFVIIa as well; however, most placebo-controlled trials have not been powered to demonstrate a significant increase in thrombosis. To address this issue, a recent review evaluated 35 randomized, placebo-controlled trials where rFVIIa was used off-label to treat life-threatening hemorrhage. Results showed no difference in rates of venous thromboembolism; however, there was an increased rate of arterial events associated with rFVIIa use (5.5% vs. 3.2%). This effect was more pronounced in patients 65 years or older (9.0% vs. 3.8%), with particularly high rates seen in individuals 75 years or older (10.8% vs. 4.1%).[120] Thus, given the risk of thrombosis, caution should be taken when using rFVIIa in older patients or persons with known or high risk of atherosclerotic disease.

Antifibrinolytics

Antifibrinolytic agents include the synthetic lysine analogues ε-aminocaproic acid (EACA) and tranexamic acid (TXA), as well as the bovine-derived serine protease inhibitor aprotinin. The former two agents exert their pharmacologic effect by blocking the interaction between fibrin and plasminogen through reversibly binding to the lysine-binding site of plasminogen, subsequently preventing its activation and transformation to plasmin.[121–124] Aprotinin, on the other hand, is a nonspecific serine protease inhibitor of multiple enzymes, including trypsin, chymotrypsin, kallikrein, activated protein C, thrombin, and plasmin, and has effects on both coagulation and inflammation.[125,126]

Aprotinin has been primarily used in the setting of cardiac surgery, where it was initially shown to decrease blood loss and transfusion requirements with coronary artery bypass grafting.[127–133] Other perioperative uses have included prevention of blood loss in liver transplantation[134–137] and in orthopedic surgeries,[138] although both have been less well studied. Concerns about the use of aprotinin in cardiac surgery due to associated adverse events, including renal failure, myocardial infarction, stroke, and death, were raised after publication of results from several observational and cohort studies.[139–142] Subsequently, a large scale, prospective, blinded, randomized, controlled trial confirmed an increased risk of death with aprotinin as compared to EACA and TXA, and the study was prematurely terminated.[143] Based upon these results, aprotinin has now been removed from the market and is no longer available for clinical use.

The lysine analogues, EACA and TXA, are also used for prevention of blood loss in cardiovascular surgeries; however, they have been much less extensively studied.[144] Most studies have shown a reduction in perioperative blood loss with both agents, with mixed results regarding reduction in transfusion requirements and need for reoperation. Use of these agents has not been clearly associated with an increased risk of adverse events, including thrombotic events[145,146]; however, most of the studies have not been adequately powered to address this question conclusively. At this point, larger studies are needed to thoroughly assess their safety profiles in this setting. Both agents have also been evaluated in orthopedic and liver transplant surgeries and have been shown to reduce bleeding and transfusion requirements, albeit again with somewhat variable results.

In the setting of acute hemorrhage, lysine analogues have been used in a variety of situations to control bleeding. Both agents have long been used as adjuncts for the treatment of bleeding in patients with hemophilia and vWD undergoing dental extractions, in which setting they reduce the amount of coagulation factor replacement required.[147–151] In conditions associated with hyperfibrinolysis, such as liver disease, acute promyelocytic leukemia, and α_2-antiplasmin deficiency, they have also proven effective.[152–154] Other situations in which these agents are used include bleeding associated with

thrombocytopenia,[155–157] prevention of bleeding with oral surgery while on anticoagulation,[158–161] traumatic hyphema,[162–165] menorrhagia,[166–170] and postpartum hemorrhage (PPH).[171–173] Regarding the latter two indications, TXA has recently been Food and Drug Administration (FDA) approved for use in menorrhagia.[174] While data on the use of lysine analogues for PPH are relatively sparse at this time, efforts are currently underway to perform a large-scale, international, randomized, controlled trial assessing TXA in obstetric hemorrhage, particularly in low and middle income countries.[175]

Due to their ability to decrease blood loss and transfusion requirements in surgery, antifibrinolytics have also been evaluated in the trauma setting. A previous systematic review concluded that there were not enough data to support or refute the use of antifibrinolytics in the setting of trauma, based upon a small number of trials.[176] More recently, a large scale randomized, placebo-controlled trial evaluated TXA in trauma and found a significant reduction in bleeding as well as all-cause mortality when compared to placebo.[177] Based upon these results, the authors of the study concluded that TXA should be considered in all bleeding trauma patients as an adjunct to transfusion. Notably, despite the benefit seen in trauma in this large study, no benefit, and perhaps harm, has been shown with use of antifibrinolytic medications in the setting of isolated intracranial hemorrhage, where they are not routinely recommended.[178]

EACA and TXA are available in both intravenous (IV) and oral formulations. TXA is 7 to 10 times more potent than EACA,[179,180] which accounts for their different dosing regimens. Both agents have a half-life of approximately 1.5 to 2 hours, and dose reductions should be made for renal impairment, as both drugs are eliminated unchanged by the kidneys.[181] Typical EACA doses are up to 5 g orally or intravenously every 6 hours, with a maximum dose of 30 g/d. A continuous infusion of 1 g/h may also be used after a loading dose.[182] An oral solution is also available for swish/spit versus swish/swallow dosing with oral surgery. Typical TXA doses are 1 to 1.5 g orally three to four times daily or 500 to 1,000 mg IV three times daily.[183] Dosing regimens used in the setting of cardiothoracic surgery are highly variable for both medications and typically include a loading dose, maintenance infusion, and occasionally a pump priming dose.[184]

The side effects of EACA and TXA are typically dose-dependent and usually involve the GI tract. Symptoms include headache, nausea, vomiting, dyspepsia, and diarrhea, which are relatively more common with EACA and often subside with dose reductions. Hypotension and bradycardia may occur with excessively rapid IV administration of either agent. Hypersensitivity reactions, such as rash, and rarely anaphylaxis occur with TXA.[185–187] An increased incidence of seizures has also been documented with TXA, especially higher dosing regimens.[188,189] Rare reports of myopathies, including rhabdomyolysis, have been reported with EACA, particularly with extended use.[190–192] Despite the theoretical risk of thrombosis with both agents, there have only been isolated reports of venous or arterial thrombosis,[193–199] and no clear causal relationship has been established in meta-analyses and other reviews of the literature.[146,200,201] Few prospective safety data, however, exist on the use of these agents. Therefore, it is recommended to avoid their use in DIC, concurrently with PCCs (particularly activated forms), with thrombotic disorders, or with estrogen use due to the increased potential for thrombotic events. These agents are also relatively contraindicated in the setting of upper urinary tract bleeding due to the risk of ureteric obstruction.[150,202–204]

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a V2 synthetic analogue of vasopressin that works, at least in part, by promoting release of ultralarge vWF multimers from endothelial cell Weibel-Palade bodies via a cyclic-adenosine monophosphate mediated pathway, resulting in a three- to five-fold increase in circulating levels of vWF and FVIII.[205–209] Other potential mechanisms for its hemostatic effects are likely and have been explored, but the full extent of its *in vivo* effects are yet to be established.[210–213] Its primary use is in the management and prevention of mild to moderate acute hemorrhage in type 1 vWD and mild hemophilia A[214,215] (see Chapters 51 and 52). It has also proven to be effective in managing acute bleeding in acquired platelet function defects, as well as certain inherited platelet disorders, although with variable outcomes depending on the type of defect.[216,217] Other reported uses include Ehlers-Danlos syndrome,[218–220] factor XI deficiency,[221,222] uremia,[223–225] liver disease,[226] and bleeding associated with antiplatelet agents.[227,228]

Dosing is available in intravenous, subcutaneous, and intranasal forms. The typical IV dose is 0.3 μg/kg, infused over 15 to 30 minutes, and it can also be given subcutaneously. Peak vWF and FVIII levels are reached 30 to 60 minutes after IV administration and 60 to 90 minutes after subcutaneous administration, and remain elevated for 6 to 10 hours after infusion.[229,230] Doses can be repeated as clinically indicated every 12 to 24 hours; however, tachyphylaxis typically occurs after three to four doses, reducing its efficacy with repeated use.[231,232] An intranasal form (Stimate) is also available, with recommended dosing of 150 μg (patient weight <50 kg) or 300 μg (patient weight > 50 kg) and similar pharmacokinetics to that seen with subcutaneous dosing.

Common side effects are typically mild and include tachycardia, flushing, and headache. Given its antidiuretic effect, hyponatremia and resultant seizures have been documented, but more commonly occur in children <2 years of age.[233,234] Care should be taken to avoid aggressive hydration with hypotonic solutions in patients receiving DDAVP, and serum sodium levels should be monitored if repeated doses are given. A rare but serious side effect includes arterial thromboses, particularly acute stroke and myocardial infarction,[235–238] and for this reason caution should be used when treating the elderly or persons with known atherosclerotic disease.

MANAGEMENT OF BLEEDING IN SPECIFIC SITUATIONS

Medical Bleeding

Acute hemorrhage outside of the surgical setting is often encountered and may be due to numerous factors, including mechanical, pharmacologic, or hematologic perturbations. Specific management decisions are, in large part, based upon the underlying cause of the bleeding. For example, hemorrhage due to esophageal varices is most definitively treated by local measures such as banding or sclerotherapy, although adjunctive treatment with transfusions or pharmacologic agents may also be indicated if an underlying coagulopathy exists.

Conversely, other medical conditions are commonly associated with a bleeding tendency due to specific underlying hemostatic abnormalities, several of which warrant further discussion.

Renal Disease

Bleeding is a common and potentially serious complication of acute and chronic renal failure. Although multifactorial in etiology, the pathogenesis of hemorrhage in uremia is largely due to numerous platelet abnormalities,[239] which are at least in part caused by dialyzable uremic toxins.[240,241] Other contributing factors include abnormal nitric oxide production, as well as anemia and the consequences thereof. As this population is also at heightened risk of atherosclerosis and thrombosis, medications such as aspirin and others with anticoagulant properties often further contribute to bleeding susceptibility.

Historically, DDAVP has been the primary pharmacologic agent for treatment of acute bleeding with uremia, where it has been proven to shorten bleeding times and prevent bleeding complications with renal biopsy.[223] Its utility, however, has declined recently due to the widespread use of recombinant erythropoietin, which has made the hemostatic abnormalities associated with uremia much less prevalent due to increased red cell mass, thereby leading to improved blood rheology and increased interaction of platelets with the vessel wall.[242] Due to the delayed hematologic response to erythropoietin, however, it is also reasonable to transfuse red cells to the acutely bleeding uremic patient to improve these rheologic factors quickly and thus improve hemostasis, with a goal hematocrit >30%. Additionally, consideration should be given to urgent dialysis to facilitate improvement in hemostatic parameters, although this should not be the only intervention pursued.[243] Finally, attention should be given to the potential contribution of medications affecting hemostasis, such as aspirin or heparin, and appropriate interventions taken to counteract these effects (see section "Complications of Antithrombotic Therapy").

Liver Disease

Chronic liver disease is characterized by numerous abnormalities in hemostatic parameters, and has classically been considered a substantial risk for bleeding complications. More recently, a view of a fragile balance in coagulation between deficiencies in both procoagulant and anticoagulant pathways, leading to both increased bleeding and thrombotic risk, has emerged.[244] Contributors to the bleeding risk in liver disease may include decreased procoagulant clotting factors,[245] thrombocytopenia secondary to both hypersplenism and decreased thrombopoietin synthesis,[246] platelet dysfunction,[247] dysfibrinogenemia secondary to abnormally glycosylated fibrinogen,[248] hyperfibrinolysis secondary to impaired hepatic clearance of plasminogen activators and/or reduced hepatic synthesis of inhibitors of fibrinolysis,[249] and vitamin K deficiency secondary to decreased enterohepatic recirculation of bile salts.[250]

Due to the complexity of the coagulopathy observed in liver disease, as well as the limited prospective, evidence-based data regarding therapeutic interventions, specific universal recommendations for acute bleeding management are not possible. In general, however, hemostatic abnormalities should be identified and corrected, if possible, typically through the initial use of transfusions, though this practice has undefined efficacy. Platelets should be given for profound thrombocytopenia, with goal platelet level of >50,000[251]; however, platelet recovery to this level may not be possible if there is coexistent splenomegaly. Although indiscriminate attempts at correcting the PT and aPTT in nonbleeding patients is not recommended due to their poor correlation with bleeding risk,[252] FFP infusions are usually given in the event of acute hemorrhage. The utility of FFP is impaired due to the substantial volumes required to improve and correct hemostatic defects, the concomitant risk of volume overload in this susceptible patient population, and lack of clearly defined thresholds in coagulation parameters for which administration is indicated.[253] Finally, cryoprecipitate is commonly given for fibrinogen levels <100 mg/dL[254] and should be considered as well.

Alternative interventions for persistent bleeding or as adjuncts to transfusion therapy may be useful but should be tailored to the specific clinical situation and coagulation defect present when possible. Antifibrinolytic agents such as TXA and EACA[255] should be considered when hyperfibrinolysis is present, although this distinction may be difficult to make due to the current lack of validated assays of global hemostasis/fibrinolysis. PCCs may be useful when tenuous volume status precludes the use of aggressive FFP administration, although it should be remembered that these agents only contain the vitamin K–dependent factors and may not be sufficient alone to achieve hemostasis. Furthermore, there have been no controlled trials using PCCs in this patient population, and therefore they should be used with caution.[256] Finally, rFVIIa has been used to manage acute hemorrhage in liver disease, albeit with mixed results,[257,258] and data from prospective, well-designed trials are generally lacking. Despite this lack of clear evidence, it is generally thought to be a reasonable "rescue" treatment when there is ongoing hemorrhage despite aggressive therapy based upon anecdotal reports of successful administration.[259,260] In addition to the scant data regarding efficacy of these adjunctive therapies, there are even fewer data regarding their safety in the setting of liver disease. Therefore, the use of antifibrinolytics, PCCs, and rFVIIa should take into account their potential thrombotic risk and be avoided in circumstances where risk of thrombosis is deemed to be high.

Obstetric Bleeding

PPH is generally defined as blood loss >500 mL following vaginal birth, or >1,000 mL following cesarean section,[261] though this definition has limitations due to inherent inaccuracies with blood loss measurement. There are numerous potential causes for PPH, with uterine atony being the most common.[262–264] Other local causes may include tissue injury, ruptured uterus or vessels, and abnormal placentation.[265] Initial management options include emptying the uterus, stimulating the uterus to contract with massage or uterotonics, uterine tamponade or compression, and potentially uterine devascularization (via surgical ligation or embolization of uterine arteries) or hysterectomy if warranted.[266]

In addition to local causes of bleeding, a systemic coagulopathy may also contribute to PPH. Causes of coagulopathy in the peripartum period include DIC secondary to placental abruption, retained products of conception, hypertensive complications of pregnancy, HELLP syndrome (hemolysis, elevated liver enzymes, low platelets), septic complications, or amniotic

fluid embolus, as well as coagulopathy secondary to massive hemorrhage itself and/or hemodilution.[267] As with other forms of DIC, any underlying pathology should be identified and treated promptly, if present (i.e., delivery of retained products). In addition to local measures performed by the obstetrician, volume resuscitation and transfusion support are the initial mainstays of therapy. Although not yet largely accepted, some centers are moving toward transfusion protocols similar to those being utilized in trauma settings, with early and aggressive use of plasma components and platelets in addition to red cells[268] (see section "Massive Transfusion Therapy"). Data from clinical trials to guide management of transfusion in obstetrics, however, are lacking. Some advocate assessing the role of early fibrinogen replacement as a first step[269] based upon existing evidence that fibrinogen levels are the only coagulation parameter independently associated with worsening PPH.[270] Despite the lack of clear transfusion guidelines, however, most would advocate transfusing platelets for a platelet count > 50,000; FFP for a PT and/or aPTT >1.5 times the normal range; and cryoprecipitate or fibrinogen concentrate for fibrinogen levels <100 mg/dL, though 150 to 200 mg/dL may be a more useful threshold.[271] As with trauma, attention should also be paid to correction of hypothermia, acidosis, and hypocalcemia.

If hemorrhage is unresponsive to maximal blood component therapy and local measures, off-label use of rFVIIa has been shown to be efficacious in retrospective, cohort, and case–control studies,[272–274] and should be considered. No prospective, randomized studies have been published to date, however, assessing either its efficacy or safety in obstetric hemorrhage. With regards to timing, most recommend using rFVIIa only after failure of other conventional therapies, including invasive therapies, but prior to hysterectomy if possible. Furthermore, its use should not delay or be a substitute for a potentially life-saving procedure.[265,271,275,276]

Even fewer data are available regarding the use of antifibrinolytics for PPH, although one prospective, randomized trial did show a modest reduction in acute blood loss with TXA compared to placebo.[277] More prospective studies are needed to validate the efficacy and safety of antifibrinolytics and rFVIIa before they should be considered for routine use in PPH, though both agents appear promising. Finally, both rFVIIa and antifibrinolytics may increase risk for thrombosis; therefore, caution should be used when using these agents where thrombotic risk is deemed high, such as in the setting of overt DIC.

Surgical Bleeding

Hemorrhage in the surgical setting can be challenging both from both a diagnostic and therapeutic standpoint. When confronted with the bleeding surgical patient, the first step should be to determine whether the bleeding is "surgical" in nature or due to a defect(s) in hemostasis. "Surgical" bleeding is the most common cause of bleeding, and is best controlled with local measures by the surgeon. In addition to operative techniques, numerous topical agents are available to help control bleeding and promote hemostasis in the surgical setting. Mechanical hemostats that form barriers to bleeding include gelatin-based, collagen-based, cellulose-based, polysaccharide-based, albumin-based, and inorganic agents. Potential adverse effects include compression of adjacent structures due to swelling, infection, adhesion formation, and foreign body reactions.[278] Two active hemostatic agents are currently in use. Topical thrombin from bovine, human-pooled plasma, and recombinant forms are marketed. All three have been compared and are functionally equivalent in terms of efficacy, with 95% of patients achieving hemostasis within 10 minutes of application.[279] Fibrin sealant, or fibrin glue, is a two-component hemostat and sealant composed of fibrinogen and thrombin. Certain preparations also contain FXIII and/or antifibrinolytic agents such as aprotinin or TXA.[280] Hematologists should be aware of potential adverse events related to the use of agents containing bovine thrombin, as coagulopathy due to development of cross-reactive antibodies to bovine FII and FV has been reported.[281–284] Although the development of antibodies is relatively common, they do not result in clinical coagulopathy in most patients.[285]

Nonsurgical causes of bleeding should be suspected when there is slow, diffuse bleeding at the surgical site or when there is evidence of bleeding outside of the surgical site. Common causes of coagulopathy in the perioperative period include consumption of platelets and coagulation factors, as well as dilutional coagulopathy secondary to crystalloid and blood product administration during the surgery. Routine laboratory assays such as CBC, PT, and aPTT can help guide decisions to transfuse platelets, FFP, and/or cryoprecipitate; however, it should be noted that routine coagulation studies may not be entirely reliable in this setting.[286] Other secondary causes of coagulopathy should also be entertained, such as DIC, and the underlying conditions be treated, if present. Further interventions beyond transfusion support should be based upon the clinical context, with therapies directed at the underlying hemostatic defect (e.g., antifibrinolytics for mucosal hemorrhage posttonsillectomy). Finally, rFVIIa may be given off-label if there is continued life-threatening hemorrhage despite maximum surgical and medical therapy.[287]

Complications of Antithrombotic Therapy

Antiplatelet Agents

Antiplatelet agents are commonly used in both primary and secondary prevention of atheroembolic disease. Aspirin, one of the most widely used agents in the Western World, works by irreversibly binding to cyclooxygenase-1, thereby inhibiting thromboxane A2 production in all exposed platelets and subsequently causing mild to moderate platelet dysfunction.[288,289] Aspirin use increases the risk of major hemorrhage, typically in the form of GI and intracranial hemorrhage, although the absolute annual increased risk is quite modest (0.13%).[290] The mainstay of reversing aspirin effect in the setting of acute hemorrhage is platelet transfusion. Given the rapidity with which aspirin is metabolized to an inactive form, this strategy is appropriate as long as aspirin has not been taken in the last hour.[291] DDAVP is another therapeutic option shown to reverse the effect of aspirin, although large clinical studies in this area are limited.[292,293]

Thienopyridine derivatives, such as clopidogrel and ticlodipine, are antiplatelet agents that exert their effects through blockade of the platelet adenosine diphosphate (ADP) receptor ($P2Y_{12}$). These agents are frequently used for secondary prevention in coronary artery disease and cerebrovascular disease, and are often used in conjunction with aspirin. Not surprisingly, combined use of aspirin and thienopyridines is associated with higher risk of bleeding.[294] Transfusion of platelets has been shown to reverse the antiplatelet effect seen with clopidogrel

use,[295] and is probably the best management option in acute hemorrhage. DDAVP has also been shown to improve platelet reactivity and hemostatic parameters *in vitro* when administered to subjects receiving clopidogrel,[296] as has rFVIIa[297]; however, little clinical evidence exists for their use in this setting. Newer thienopyridine derivatives have recently come on the market as well, such as prasugrel and ticagrelor, but little evidence regarding bleeding management is available at this time.

GP IIb/IIIa inhibitors are commonly used, and have shown considerable efficacy in improving outcomes in patients with acute coronary syndromes and in percutaneous coronary intervention.[298,299] Management of bleeding complications with these agents varies based upon which agent is used due to major differences in their respective pharmacokinetics. Eptifibatide and tirofiban are similar in that they rapidly bind and dissociate from GP IIb/IIIa receptors, thus requiring increased serum levels.[300] Transfusion of platelets is therefore ineffective due to the fact that the number of drug molecules present in plasma overwhelms the number of GP IIb/IIIa receptors by several orders of magnitude.[301] Both agents are renally excreted, with half-lives of 2.5 hours and return to baseline platelet function in 4 to 8 hours of discontinuation.[302,303] Therefore, discontinuation of the drug is the most important factor in acute hemorrhage. Both agents can be removed with hemodialysis[304] as well, making this a viable option in the setting of renal insufficiency. *In vitro* studies have additionally shown that fibrinogen and platelet supplementation in combination,[305] or desmopressin,[293] may help to restore platelet function in patients treated with these agents, although clinical data are lacking.

Contrary to the above agents, abciximab binds with high affinity to platelet GP IIb/IIIa receptors with little free drug in plasma.[306] Despite its short half-life of 15 to 30 minutes, abciximab has a prolonged biologic half-life with 50% inhibition of platelet function still present 48 hours after drug discontinuation.[307] Because little free drug circulates in the plasma compartment, platelet transfusion can reverse the hemostatic defect by replenishing the number of viable GP IIb/IIIa receptors,[308] and should be used whenever emergent reversal is required due to hemorrhage. With regard to adverse effects related to GP IIb/IIIa inhibitors in general, thrombocytopenia has been reported with all agents and should additionally be evaluated if a patient receiving these medications has bleeding complications.

Vitamin K Antagonists

VKAs have been the mainstay in oral anticoagulants for >60 years and are commonly used in the setting of venous thromboembolism, mechanical heart valves, and atrial fibrillation. VKAs exert their anticoagulant effect by inhibiting vitamin K epoxide reductase, an enzyme that regenerates vitamin K back to its active, reduced form. This reduced form of vitamin K serves as a cofactor in the γ-carboxylation of the vitamin K–dependent factors II, VII, IX, X, protein C, and protein S, a step necessary to promote their coagulant activities.[309] Several preparations exist, including acenocoumarol, warfarin, and phenprocoumon, which differ mainly with regards to their half-lives (9 hours, 36 to 42 hours, and 90 hours, respectively),[310] directly impacting management of treatment-associated bleeding.

Bleeding is not an uncommon clinical scenario in patients receiving VKAs, with risk of major bleeding reported to be between 0.9% and 1.4% annually with warfarin.[311] Factors associated with increased risk of bleeding include intensity and duration of anticoagulation,[312,313] recently initiated anticoagulation,[314,315] older age,[314,316] unstable INRs,[317] comorbid conditions such as hypertension[318] or chronic renal disease,[315] concomitant aspirin use,[319] among others. Per American College of Chest Physicians (ACCP) guidelines, asymptomatic elevation in INR and mild warfarin-associated bleeding can typically be managed with lowering and/or omitting a dose(s). In this setting, vitamin K is also typically recommended, however, only if the INR is >5. For INR 5.0 to 9.0, vitamin K at a dose of 1 to 2.5 mg orally may be used if there is mild bleeding or in the asymptomatic patient with a presumed increased risk of bleeding. For INR > 9.0, vitamin K 2.5 to 5 mg orally is recommended in both scenarios.[320]

With serious or life-threatening hemorrhage, the goal is rapid reversal of anticoagulation with vitamin K and FFP or PCCs. Intravenous vitamin K has been shown to correct the INR more rapidly at 6 and 12 hours, with similar correction at 24 hours, when compared to oral vitamin K.[321] Therefore, vitamin K 5 to 10 mg IV by slow infusion is recommended for severe hemorrhage to promote more rapid improvement in coagulation parameters. Although the risk of anaphylaxis with IV vitamin K exists, it is quite low and has been described in only 3 per 10,000 doses with the more modern micelle preparations.[322] Intramuscular vitamin K should be avoided due to the risk of intramuscular bleeding, and subcutaneous administration has been shown to be inferior to IV administration[323] and should not be used.

Although FFP contains all the coagulation factors needed to correct the INR, its use in rapid reversal of anticoagulation due to warfarin is hindered due to the large volumes required, time needed to crossmatch and prepare the plasma, and inability to infuse rapidly.[324] Subsequently, PCCs are typically recommended over FFP, when available, in the setting of severe hemorrhage, although prospective trials comparing them with FFP are currently lacking. The optimal dose of PCCs has not been clearly established; however doses of 25 to 50 U/kg have been proven effective.[325,326] Tailored dosing based upon INR at presentation has been shown to have similar efficacy as well.[327] It should be noted that "three factor" PCC use may result in inadequate correction of the INR, which can be improved with the addition of several units of FFP, based upon a retrospective case series.[83] This has, however, recently been challenged in a prospective cohort study.[328] Therefore, supplemental FFP administration should be considered when using "three factor" PCCs, particularly if the INR does not adequately correct with PCC alone. Finally, rFVIIa has been used to treat warfarin-associated bleeding; however, data are mainly limited to *in vitro* changes in coagulation assays or clinical outcomes in retrospective, nonrandomized studies in which most subjects also received FFP.[329] A recent prospective, placebo-controlled, exploratory phase I trial additionally showed no improvement in a skin punch biopsy bleeding model with rFVIIa, although there was improvement in coagulation parameters.[330] Further studies are needed in this area; however, at this time rFVIIa cannot be recommended as frontline therapy for warfarin-associated bleeding. It may be considered in the setting of refractory bleeding despite standard therapy, or in low dose as an adjunct to vitamin K and PCCs, particularly "three factor" PCCs.

Heparins

Unfractionated heparin exerts its anticoagulant effect by binding to antithrombin and augmenting its inhibition of certain coagulation factors by 1,000-fold. It inhibits FIIa to a much

greater extent than FXa and has a half-life of 1 to 2 hours.[331] Given its short half-life, minor bleeding may be managed by simply discontinuing heparin. In cases of severe hemorrhage, protamine can be used as an antidote to rapidly reverse its anticoagulant effect. Protamine doses of 1 mg for every 100 U of heparin administered over the last several hours should be given to attempt complete reversal.[331] The maximum dose of protamine is 50 mg, and infusion should be done slowly (5 mg/min) to decrease the risk of histamine-associated hypotension and bronchoconstriction.[332] Due to its short half-life of 10 minutes, repeat dosing may be needed to reverse therapeutic doses of subcutaneous heparin.[310] It should be noted, however, that protamine has an anticoagulant effect of its own and excess administration can lead to increased bleeding.

Low molecular weight heparins (LMWHs) are derived from heparin, generally have longer half-lives, and exert their anticoagulant effects primarily through antithrombin-mediated inhibition of FXa. Protamine neutralizes the anti-IIa activity of LMWHs, and only partially affects the predominant anti-Xa activity of these drugs.[333,334,335] Recommended doses of protamine for LMWH-associated bleeding are 1 mg for every 100 anti-Xa U of anticoagulant given within the last 8 hours. A second dose of 0.5 mg protamine for every 100 anti-Xa U can be administered if bleeding persists.[331] Data regarding the effectiveness of this intervention are mixed and mainly limited to case reports.[336–338] No proven antidote is available for newer agents, such as fondaparinux, idraparinux, and danaparoid; however, *ex vivo* data suggest that rFVIIa may reverse the anticoagulant effects of some of these agents, including LMWHs.[339] Clinical results are again limited to case reports that showed mixed results, but this approach is reasonable in the setting of life-threatening hemorrhage or persistent bleeding despite conservative measures.

Other Anticoagulants

Direct thrombin inhibitors (DTIs), including lepirudin, bivalirudin, and argatroban, are typically administered via continuous infusion and have relatively short half-lives.[340] Thus, prompt discontinuation of the drug with acute hemorrhage is an important factor and may be all that is necessary with less serious bleeding. No clearly proven reversal agent exists for this class of medication, which presents a serious challenge in the setting of acute bleeding. Activated PCC and rFVIIa can improve coagulation *ex vivo*[339] as well as bleeding in animal models,[341] but their clinical utility remains to be seen. Recombinant FVIIa has been evaluated in several case reports, albeit with mixed results.[342] DDAVP has also been shown to improve bleeding time in animal models[343]; however, it has not been evaluated fully in humans. Finally, hemodialysis can remove all three DTIs and is another management option,[331] particularly with renal impairment and the associated toxicity with the renally eliminated preparations lepirudin and bivalirudin.

New oral anticoagulants have either recently become available or are expected to be available shortly, including the IIa inhibitor dabigatran, and the Xa inhibitors rivaroxaban and apixaban. No antidote exists for these agents, which is a cause for concern in the setting of acute hemorrhage, particularly as they become more commonly used. As with the DTIs, discontinuation of the medication is an important factor with associated bleeding. *Ex vivo* data suggest that rFVIIa may antagonize the anticoagulant effect of some of these agents; however, no clinical data exist at this time.[344] Further studies are needed to better guide management of bleeding with these newer agents.

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CHAPTER

77 Therapy with Antifibrinolytic Agents

DAPHNE B. STEWART AND VICTOR J. MARDER

After hemostasis is established in an injured vessel, a highly orchestrated process follows to heal the vessel and to reestablish blood flow. To a large extent, this process is mediated through locally controlled fibrinolysis by activation of the plasminogen–plasmin enzyme system (see Chapters 20 and 21). Briefly, plasminogen binds to lysine residues on fibrin and is converted to plasmin by plasminogen activators (PAs) released by endothelial cells, through cleavage of the heavy chain at Arg560-Val561.[1] Trace amounts of plasmin liberate an amino-terminal 76-residue portion of plasminogen,[2] converting glu-plasminogen to lys-plasminogen, which binds to fibrin more efficiently[3] and is more sensitive to PA activation,[4–6] thereby promoting further conversion of plasminogen to plasmin.[7,8] Just as coagulation inhibitors such as antithrombin and tissue factor pathway inhibitor limit the initial hemostatic process to the site of vessel injury, fibrinolytic inhibitors such as plasminogen activator inhibitors, α_2-antiplasmin, and α_2-macroglobulin contain the fibrinolytic reaction to the site of injury.

Antifibrinolytic agents (AFs) are best viewed as prohemostatic drugs that act in two situations. The first is to prevent bleeding caused by an excess of plasmin activity (see FIGURE 77.1), such as in the setting of primary menorrhagia.[9,10] The second situation is to retard physiologic fibrinolysis following slow or ineffective hemostasis (see FIGURE 77.1), such as may occur in a hemophiliac.[11,12] AFs may improve hemostasis in a wide variety of bleeding states, reducing blood loss and transfusion. Useful properties of AFs are ready whole body distribution following oral or intravenous administration, potent inhibition of plasmin, and no associated tachyphylaxis. When used appropriately to diminish an exuberant fibrinolytic response, these agents do not induce or potentiate coagulation. Thus, lower levels of D-dimer were present in patients treated with ε-aminocaproic acid (EACA) (vs. placebo) before, during, and after cardiac surgery, demonstrating inhibition of fibrinolysis, but levels of thrombin–antithrombin complexes and soluble fibrin were the same as in placebo-treated patients, indicating no activation of coagulation.[13] However, AF agents have the potential to unmask subclinical thrombosis, such as in a patient with unrecognized, compensated, disseminated intravascular coagulation (DIC) (see Chapter 98).

There are three distinct clinical situations in which antifibrinolytic therapy has been proven to be useful: (a) systemic hyperfibrinolysis, (b) hypocoagulability, and (c) localized lesions in the absence of systemic hyperfibrinolysis or hypocoagulability.[14–17] The field has undergone a sea change due to two significant circumstances:

1. Withdrawal of the preeminent AF agent, aprotinin, from the market due to evidence of increased mortality following its use
2. The expansion of the indications for AF agents to the treatment of trauma-induced hemorrhage, an indication that will likely dramatically increase the number of patients treated

MODE OF ACTION OF ANTIFIBRINOLYTIC AGENTS

The AF agents fall into three categories:

1. Natural plasma inhibitors of plasmin and of PAs,[18,19] which are not the subject of this chapter (see Chapter 21)
2. Synthetic lysine analogs, such as *trans-p*-aminomethylcyclohexane carboxylic acid (tranexamic acid, TXA) and 6-aminohexanoic acid (EACA)[21–24]
3. Aprotinin (Trasylol, bovine pancreatic trypsin inhibitor), a 58-residue polypeptide (M_r = 6,500) isolated from bovine lung, parotid gland, or pancreas,[20] is a dose-dependent inhibitor of plasmin[17] and at higher concentrations of other serine proteases (trypsin, kallikrein, chymotrypsin).[14,16,17] Recent studies show an increased risk for death and renal failure after treatment with aprotinin, so this drug is no longer marketed for use in the United States or Europe, although it remains available for research.

Biochemistry

The lysine analogs (TXA and EACA) prevent excessive plasmin formation by occupying plasminogen's lysine-binding site (LBS) for fibrin, thereby precluding plasminogen localization to fibrin for activation by PA (see FIGURE 77.2). Lysine analogs also bind to tissue plasminogen activator (tPA) kringles, interfering with plasminogen activation.[18,21] *In vitro*, these agents bind to the LBS of plasminogen, resulting in a conformational change that may actually accelerate activation,[19,21,22,25] and binding to plasmin kringles reduces the binding by plasmin inhibitors, which could theoretically increase fibrinolytic activity.[26,27] Yet, the major action of these compounds *in vivo* is antifibrinolytic, primarily by blocking the association of plasminogen and tPA on the fibrin surface.[28,29] Although plasmin may still be generated, fibrinolysis is prevented.

The seemingly disparate effects may be explained by the two-step process required for binding of plasminogen to fibrin (see FIGURE 77.2). Plasminogen is activated by proteolytic cleavage that exposes the serine–histidine catalytic site, a cleavage that also promotes binding to fibrin[30] between LBSs on plasminogen and lysine residues on fibrin. Without proper binding, proteolysis cannot proceed. This two-step requirement explains the seeming discrepancy of *in vivo* inhibition[29,31–36] and *in vitro* activation.[22,25–38] *In vitro*, EACA and TXA accelerate plasminogen activation by altering plasminogen's conformation and making it more susceptible to proteolytic action by activators. However, lysis of a fibrin substrate is inhibited because the same binding phenomenon also blocks the LBS. Therefore, plasmin may be

	Normal Response to Injury	Bleeding Tendency	
		Abnormal Hemostasis, Normal Fibrinolysis	Normal Hemostasis, Excessive Fibrinolysis
Injury Incurred	Platelet Adhesion, Aggregation, Formation of Primary Plug	Delayed Plug Formation	Normal Primary Plug Formed
	↓	OR	↓
Hemostasis	Thrombin Burst, Fibrin Formed and Cross-Linked	Inadequate Plug Reinforcement	Fibrin Formed and Cross-Linked
	↓	↓	↓
Fibrinolysis	Delayed Clot Resolution	Normal Rate of Clot Resolution	Accelerated Fibrinolysis
	↓	↓	↓
Net Effect on Bleeding	Bleeding Resolves, Vessel Patency Re-Established	Prolonged Primary Bleeding or Late Rebleeding	Delayed Rebleeding

FIGURE 77.1 Schematic of body's hemostatic response to vascular injury and effect of imbalances between hemostasis and fibrinolysis. Under physiologic conditions, when an injury is incurred, hemostasis proceeds culminating in fibrin generation and reinforcement of the platelet plug, followed by delayed clot resolution through fibrinolysis. A bleeding tendency arises when either arm is disrupted. If hemostasis is inadequate, normal fibrinolysis will occur and cause premature clot resolution with bleeding. As well, if hemostasis is normal, yet balanced by enhanced fibrinolysis, a clot will dissolve prematurely and result in bleeding.

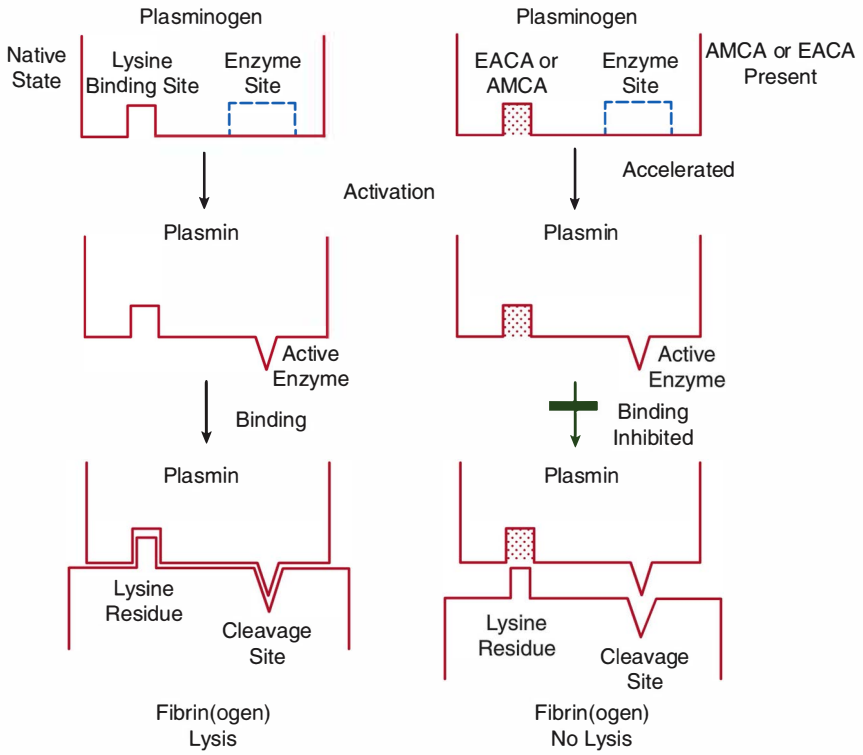

FIGURE 77.2 Schematic diagram of the *in vitro* mode of action of synthetic lysine analogs on plasminogen activation and plasmin proteolysis of fibrin(ogen). ε-aminocaproic acid (EACA) and *trans-p*-aminomethyl-cyclohexane carbolyxic acid (TXA) accelerate plasminogen activation by inducing a conformational change in the molecule; however, they inhibit fibrinolysis by blocking the lysine binding site necessary for the binding of plasmin(ogen) to its substrate. Therefore, even though plasmin may be rapidly formed, it cannot reach the fibrin substrate because its binding site is occupied by the synthetic inhibitor. The accelerated activation as shown in the figure is from the *in vitro* experiments; it remains to be demonstrated *in vivo*.

produced, even rapidly, but without binding to fibrin; there is no proteolytic fibrinolysis by the serine enzyme site.

EACA and TXA are structurally similar to lysine and inhibit the interaction between fibrinogen lysine residues and plasminogen LBSs, especially those located on kringle 1.[39–42] Subtle differences in the synthetic analogs markedly affect their inhibitory potential, as exemplified by the approximately 6- to 10-fold higher molar potency of TXA in comparison with EACA[43] and the ineffectiveness of the *cis* form of TXA in comparison with the *trans* form.[43] Other active analogs of lysine include *p*-amino methylbenzoic acid and 4-amino ethylbicyclo-[2.2.2]-octane-1-carboxylic acid, which are 5- to 10-fold and 100-fold, respectively, more potent than EACA.[44]

Aprotinin is a nonspecific serine protease inhibitor of the Kunitz family. Its active residue, 15-lysine, binds reversibly to its target's catalytic site and inhibits numerous serine proteases, including trypsin, plasmin, chymotrypsin, kallikrein, elastase, urokinase, and thrombin.[17] *In vivo*, aprotinin binds directly to plasmin at 137 kallikrein inhibitor units (KIU) per mL, whereas it binds to kallikrein at >250 KIU per mL. Side chain Lys15 of aprotinin binds to the aspartate side chain in the specificity pocket of plasmin. Aprotinin blocks activation of protease-activated receptor-1 on platelets, preventing thrombin-induced platelet aggregation, a mechanism thought to be particularly important in the platelet dysfunction acquired during exposure to cardiopulmonary bypass circuits.[45] Aprotinin inhibits the inflammatory cascade at multiple levels, protects against kallikrein-induced generation of bradykinin, decreases neutrophil degranulation and extravasation at inflammatory sites, decreases expression of proinflammatory glycoproteins (CD11b) on neutrophils, and attenuates complement generation.[46,47]

Pharmacology

TXA and EACA are rapidly absorbed from the gastrointestinal (GI) tract, allowing oral or intravenous administration,[48–50] with peak plasma levels at 1 to 2 hours after oral dosing. Elimination is primarily via renal excretion and metabolism, approximately 65% excreted in the urine unchanged and 10% as an inactive metabolite, adipic acid. Approximately 85% of an intravenous dose is cleared within 3 hours, but because EACA penetrates the entire extravascular space, urinary excretion may be detected for 12 to 36 hours.[49] The drugs cross the blood–brain barrier,[51] enter joint fluid and synovial membranes,[52] are excreted at low levels into breast milk, and pass through the placenta.[53]

The recommended dose for EACA has been derived from *in vitro* studies and shown to be effective for the treatment of hyperfibrinolytic states; however, the minimal dose required to inhibit normal or enhanced local fibrinolysis is not known. EACA is usually administered as an intravenous priming dose of 0.1 g/kg over 20 to 30 minutes, followed by a continuous infusion of 0.5 to 1 g/h or an equivalent intermittent dose every 1, 2, or 4 hours, generally until acute bleeding is controlled.[35] Continuous intravenous infusion of 0.5 g/h may be a useful regimen for serious acute bleeding (personal observation). Plasma levels of at least 0.001 M (13 mg/100 mL) are adequate to inhibit *in vivo* fibrinolysis.[29,33,43,54] Urinary concentration is generally 75- to 100-fold higher than plasma[49]; therefore, much lower doses may be used to control urinary tract bleeding (3 g EACA per day). As renal clearance approximates endogenous creatinine clearance,[48] patients with renal insufficiency should have their doses reduced according to creatinine clearance.

The serum half-life of TXA is similar to EACA, 1 to 2 hours, and it is also rapidly excreted unchanged in the urine, more than 90% in 24 hours.[50,55] Compared with EACA, TXA is much more potent, inhibiting fibrinolysis at much lower plasma concentrations, with inhibitory effect lasting for 7 to 8 hours. The recommended oral dose is 25 mg/kg, three times daily. Intravenous infusion is delivered at a dose of 10 mg/kg, three times per day, or if used for surgical prophylaxis of bleeding, 10 mg/kg loading dose followed by continuous infusion of 1 mg/kg/h. Although studies with aprotinin have demonstrated a clear dose–response curve with regard to reduction in fibrinolytic bleeding, assessment of a dose response among lysine analogs is limited by a lack of randomized, placebo-controlled trials.[56]

Unlike the orally bioavailable lysine analogs, aprotinin must be administered parenterally. Following its distribution phase, the plasma half-life of aprotinin is 150 minutes, and its terminal half-life is 5 to 10 hours. Aprotinin is primarily reabsorbed in the proximal tubules, with only approximately 10% excreted unchanged in the urine. Ultimately, the drug is taken up by tubular endothelium, stored in phagolysosomes, and slowly degraded by lysosomal enzymes.[20] Aprotinin dosing is measured as KIU, or the amount of aprotinin to decrease two biologic units of kallikrein activity by 50%. Aprotinin is associated with a risk for anaphylaxis, so an intravenous test dose of 10,000 KIU must be given to exclude hypersensitivity at least 10 minutes prior to the loading dose.[20] Clinically significant decreases in surgical blood loss and transfusion requirements correlate with higher doses of aprotinin.[57–60]

Side Effects and Complications

EACA and TXA are associated with nasal stuffiness, conjunctival injection, skin rash, and dose-related GI discomfort, including nausea, vomiting, and diarrhea.[35,50] A literature review of EACA use between 1972 and 1995[61] found 33 cases of clinically significant myopathy, notably with prolonged administration of high doses (16 to 36 g/d for >28 days) and that patients recovered after discontinuing the medication.

Aprotinin, a bovine-derived protein, is associated with hypersensitivity reactions, including skin eruption, pruritus, dyspnea, and fatal anaphylactic shock in 0.3% to 0.6%.[56] Although exceedingly rare with first exposure, the incidence of anaphylaxis may be as high as 2.7% upon second exposure.[62] A pretreatment test dose must be given to exclude those at risk for hypersensitivity, although anaphylaxis has occurred following a nonreactive test dose.[62] Efforts to decrease incidence of anaphylactic shock have not proven effective. Aprotinin is associated with dose-related renal dysfunction and increased need for dialysis,[63,64] with higher risks in patients with baseline renal impairment or those taking renal toxic medications. When combined with off-pump bypass surgery and angiotensin converting enzyme inhibitor (ACEI) use, aprotinin nearly tripled the risk for postoperative renal dysfunction.[65] Additional side effects of aprotinin include vasodilation, generally transient and preventable with slow drug administration, and prolongation of the partial thromboplastin time and activated clotting time due to inhibition of the intrinsic clotting cascade.

Serious systemic thrombotic complications have been reported following the use of antifibrinolytic therapy, although these complications are most clearly associated with a concurrent thrombogenic stimulus, and case reports of devastating, systemic thrombotic episodes following antifibrinolytic therapy are reported,[61–73] generally in association with conditions predisposing to coagulopathy. Central venous catheter thrombosis may occur more commonly following antifibrinolytic therapy,[74–77] and prophylactic antifibrinolytic therapy used during joint replacement or prostate surgery is associated with an increased risk of venous thrombosis.[60,78,79] Lysine analogs have been used successfully to treat postpartum hemorrhage and hemorrhage associated with *abruptio placentae*, and a retrospective analysis of 2,102 pregnant women showed only 2 of 256 TXA-treated patients versus 4 of 1,846 matched controls developed thrombotic complications,[80] and the authors concluded that there was no thrombogenic effect of TXA in this high-risk group. Clots in the urinary tract have been noted following AF therapy in hemophilic and hemoglobinopathy patients with active genitourinary bleeding, explained as inhibition of urokinase allowing the generation of lysis-resistant clots.[81,82,83,84–93] AF therapy is best reserved for patients with excessive, protracted genitourinary bleeding for whom surgical intervention is contemplated.

Recent retrospective analyses of over 60,000 patients treated with AF agents during cardiac surgery suggest an increased risk of all-cause mortality associated with aprotinin use,[81,82,83] (relative risk 1.32 to 1.64 versus lysine analog or placebo), although thrombotic disease has not been clearly implicated to explain the mortality excess. Five studies evaluating cardiac graft patency after cardiopulmonary bypass grafting surgery, comparing aprotinin-treated patients with controls, included more than 1,300 patients and demonstrated no increased rate of graft occlusion among those treated with aprotinin.[84–88] Secondary thrombotic outcomes following treatment with AF agents during cardiac surgery show variable results. In the Cochrane Systematic Review of 2007, aprotinin use did not affect the incidence of myocardial infarction, stroke, or renal dysfunction following cardiac surgery,[89] and there was no association between treatment with TXA or EACA and increased thromboembolic events. An observational study of more than 4,000 patients comparing adverse outcomes following exposure to aprotinin or the lysine analogs suggests a significantly increased incidence of myocardial infarction (55% increase), stroke (181% increase), and renal failure requiring dialysis (more than 200% increase).[83] In contrast, the meta-analysis by Brown et al. reported no difference between aprotinin, TXA or EACA, or placebo in the risk of myocardial infarction or stroke.[94] More recent trials utilizing aprotinin have reserved the drug for patients with higher risk of bleeding, limiting the retrospective comparison to patients treated without the drug.[95] These observational studies led to the randomized blood conservation using antifibrinolytics in a randomized trial (BART) comparing aprotinin to TXA and EACA, which found a 50% increase in death at 30 days among aprotinin-treated patients.[96] The data suggest that among patients undergoing cardiac surgery without concurrent thrombogenic stimulus, aprotinin confers a greater risk for adverse outcomes compared with the lysine analogs, and although no definitive evidence exists, suggests a greater tendency to thrombosis.

CLINICAL APPLICATION

Indications for AF therapy include interventions for ongoing hemorrhage or prophylactic administration to prevent bleeding, for which systemic or local fibrinolysis plays a contributory role (Table 77.1).

Fibrinolytic Hemorrhage

Systemic Hyperfibrinolysis

Many pathologic states may generate hyperplasminemia through the release of endothelial stores of tPA in sufficient quantities to overcome fibrinolytic inhibitors and convert plasminogen to plasmin (see Chapters 77 and 98). Plasmin, in turn, degrades fibrinogen and fibrin, and previously stable or new hemostatic sites are at risk of bleeding. Acute conditions that may induce systemic hyperfibrinolysis include heat stroke, hypoxia, hypotension, cardiothoracic surgery, and treatment with thrombolytic agents. Chronic diseases (neoplasm, cirrhosis) may be associated with a systemic hyperfibrinolytic and hemorrhagic state characterized by a shortened euglobulin lysis time, elevated levels of plasma tPA, decreased plasminogen and fibrinogen, and elevated D-dimer. In most cases, tPA release is of limited duration, and an essential therapeutic consideration is directed to the underlying condition.

Acute, systemic hyperfibrin(ogen)olytic states responding dramatically to EACA have been reported in portal hypertension following bowel surgery, a plasminogen-activator–secreting tumor, and with amyloidosis.[97–99] Chronic hyperfibrinolysis may exist with some neoplasms and with the rare congenital α_2-antiplasmin deficiency, and is manifest as a bleeding tendency effectively controlled with chronic antifibrinolytic therapy.[100] Acquired α_2-antiplasmin deficiency may be seen in acute promyelocytic leukemia, systemic amyloidosis, and metastatic adenocarcinoma, and hemorrhagic complications may be successfully controlled by treatment with AF agents[101–104] Bleeding may accompany treatment with systemic thrombolytic therapy. Because the lysine analogs inhibit plasminogen-activator–mediated fibrinolysis, these agents are reasonable antidotes for acute bleeding attendant PA treatment, although efficacy has not been documented by clinical trial.

Cardiac Surgery

Cardiopulmonary bypass is associated with significant perioperative bleeding, on average ranging from 750 to 1,250 mL and requiring median transfusion of 1.5 to 2.2 U of packed red blood cells.[105] The extracorporeal bypass circuit and the surgical wound both induce thrombin and plasmin generation, continuing for up to 24 hours postoperatively.[106] Laboratory findings of thrombosis and fibrinolysis, namely, high prothrombin fragment 1 + 2, high plasma D-dimer, shortened clot lysis time, and increased tPA levels,[107,108] are demonstrated in blood collected from the chest wound[109–125] helping to explain the significant blood loss. Perioperative bleeding leads to a high rate of cardiac reoperations[126] and patients requiring blood transfusion during cardiac surgery have a significantly decreased life expectancy.[127,128] Concern for the morbidity of significant bleeding and laboratory evidence supportive of a state of hyperfibrinolysis, have led to the extensive prophylactic use of AFs during cardiac operations to block the physiologic manifestations of hyperfibrinolysis and thereby minimize perioperative blood product transfusions.[109]

Table 77.1

Clinical application of antifibrinolytic therapy

Bleeding State	Clinical Association	Use of Antifibrinolytic Therapy	Comment
Systemic hyperfibrinolysis	Spontaneous plasminemia or following therapeutic thrombolysis	Rare, since hyperfibrinolytic state usually self-limited	If DIC present, AF therapy is contraindicated
	Cardiac surgery, with or without cardiopulmonary bypass	Prophylactic administration indicated to lessen blood loss, particularly in high risk circumstance, for example, repeat sternotomy, prolonged pump time, preoperative aspirin use	TXA, EACA decrease blood loss and transfusions vs. placebo. Aprotinin is modestly more effective than TXA/EACA in decreasing transfusions, but increases all-cause mortality
	Liver transplant	Prophylaxis useful prior to graft reperfusion to reduce perioperative bleeding and transfusion requirements	Adults with hepatocellular dysfunction more prone to hyperfibrinolytic bleeding; case reports of thrombotic events suggest use may be best guided by coagulation testing
	α_2-antiplasmin deficiency	Lysine analogs indicated for acute bleeding and prophylaxis	Chronic administration controls lifelong bleeding state in congenital deficiency
Defective hemostasis, followed by normal fibrinolysis	Hemophilia, von Willebrand disease	Indicated to control mucosal bleeding and as adjunctive prohemostatic treatment postoperatively or following acute bleed	Reduces blood loss and use of factor concentrates after dental extraction, synovectomy, joint replacement; likely useful in all postoperative settings
	Chronic anticoagulation	Indicated to prevent interruption of therapeutic anticoagulation during dental procedures	May be useful in treatment of minor bleeding episodes in chronically anticoagulated patients
	Thrombocytopenia or qualitative platelet dysfunction	Indicated for acute bleeding episodes to reduce platelet transfusions	Less effective if platelet count $<10{,}000/\mu L$
Normal hemostasis, followed by enhanced local fibrinolysis	Upper urinary tract	Indicated to control bleeding from essential hematuria, renal disease postrenal biopsy, and prolonged, spontaneous hematuria in sickle cell disease or hemophilias	Higher risk for obstructive clot formation in hemophiliacs treated simultaneously with factor replacement
	Lower urinary tract	May be useful in patients undergoing prostatectomy who have high risk of bleeding	Studies with lysine analogs demonstrate reduced blood loss, without effect on transfusion rate
	Dysfunctional uterine bleeding	Indicated for treatment of essential menorrhagia, menorrhagia due to IUD use or cervical conization	Underlying uterine pathology must be excluded Role in treatment of postpartum hemorrhage undergoing study
	Subarachnoid hemorrhage	Reduces incidence of rebleeding, but currently, AF treatment not indicated	Treatment associated with higher rate of vasospasm and ischemic events
	GI bleeding	Indicated to treat acute bleeding episodes from gastric erosions, ulcers, varices	Proven to decrease rate of rebleeding, need for surgical intervention, and mortality
	Traumatic hyphema	Indicated in patients with high risk for rebleeding (e.g., aspirin use)	Lysine analogs decrease rate of rebleeding without vision improvement
	Mucous membranes	Indicated for recurrent epistaxis; may be useful for excessive bleeding following tonsillectomy or sinus surgery	May be given orally or topically as packing component

(Continued)

Table 77.1

Clinical application of antifibrinolytic therapy *(Continued)*

Bleeding State	Clinical Association	Use of Antifibrinolytic Therapy	Comment
	Cavernous hemangioma	Used to reverse consumptive coagulopathy prevalent in large lesions	Lysine analog therapy leads to tumor shrinkage and improved symptoms
	Chronic urticaria	Indicated for cases resistant to standard therapy	Effective in subset with enhanced local fibrinolysis; elevated D-dimer may predict response to AF treatment
	Hereditary angioedema	Long-term prophylaxis against attacks	No effect on symptoms during acute episode
Blood conservation	Orthopedic surgery	Indicated to reduce blood loss in total knee replacement and total hip arthroplasty	Reduces total blood loss and blood transfusion requirements
	Neurosurgery	Indicated for multilevel spinal surgery and meningeal procedures with systemic bleeding	AF therapy reduces blood loss and transfusion
	Trauma	Indicated in patients presenting after traumatic events with significant bleeding	Associated with decreased mortality and decreased death due to bleeding

DIC, disseminated intravascular coagulation; TXA, *trans-p*-aminomethyl-cyclohexane carboxylic acid; EACA, *ε*-aminocaproic acid; IUD, intrauterine device.

Prior to 2006, aprotinin was the most widely used AF agent for cardiac surgery. Its use was first described in 1987 in a trial of 22 patients undergoing repeat sternotomy.[110] A Cochrane Database Systematic Review in 2001 evaluated 61 trials of aprotinin in cardiac surgery involving over 7,000 patients and concluded that aprotinin use decreased the rate of red blood cell transfusion by 30% and decreased the need for reoperation due to bleeding by 60%.[89] However, the risk of hypersensitivity reactions led the American College of Cardiology and the American Heart Association to restrict the use of aprotinin in primary cardiac surgery for patients at high risk of blood loss,[111] but as aprotinin showed greater benefit in patients undergoing repeat sternotomy procedures,[112,113] its use was routinely recommended for these patients.[114] Although less well studied, two randomized trials of aprotinin in patients undergoing lung resections, who were deemed at high risk for bleeding, demonstrated significantly reduced blood loss, need for transfusion, and need for re-exploration due to bleeding in the treated group versus placebo.[115,116] A randomized, double-blind, placebo-controlled trial of 70 patients undergoing myocardial revascularization by total arterial grafting showed that aprotinin reduced the proportion of patients requiring transfusion (39% vs. 77%) and significantly decreased the number of units transfused (0.8 vs. 2.6).[117]

A study of 56 patients undergoing cardiac surgery found lower blood loss over the first 24 hours postoperatively among patients treated with EACA, an effect that was more marked in patients with cyanotic heart disease or on cardiopulmonary bypass for longer than 1 hour.[118] The largest trial of EACA prophylaxis randomized 350 patients undergoing elective cardiac surgery to EACA or placebo and found that patients treated with EACA had significantly less postoperative blood loss, fewer red blood cell transfusions (2.8 U vs. 4.2 U), and fewer reoperations for bleeding,[119] without an increase in thrombotic complications. Early reports exploring the use of EACA reported rare adverse events, including DIC unmasked by therapy,[67] and significant intracavitary bleeding with the formation of lysis-resistant clots.[11,107]

Toward finding the optimal dosing schedule, one trial of 90 patients undergoing primary cardiopulmonary bypass surgery randomized three groups: TXA administered prior to bypass, TXA after bypass, or saline placebo. The number of units of transfused blood was significantly less in the TXA pre-bypass group versus control (0 vs. 4.5).[120] A larger trial of 210 randomized patients undergoing primary or repeat sternotomy procedures or valve operations to prophylactic treatment with high-dose TXA prior to initiation of bypass or saline placebo, and found a smaller proportion of patients treated with TXA required blood product transfusion support (13% vs. 31%),[121] without an increase in thrombotic events. In two similar trials of prophylactic TXA in pediatric cardiac surgery, one trial of 41 patients undergoing repeat sternotomy treated with high-dose TXA had significantly decreased transfusion support,[122] whereas another study of 85 patients showed decreased blood loss without a difference in transfusion versus placebo.[123] A trial that compared patients with on-pump (n = 51) versus off-pump (n = 51) procedures, randomized to prophylactic treatment with high-dose TXA or placebo found reduced perioperative blood loss in both on- and off-pump groups, greater with on-pump procedures, with TXA use.[124] Prior to elective cardiac surgery, aspirin use is typically discontinued to prevent excessive bleeding, but continuing aspirin may be beneficial by improving survival.[129] A meta-analysis by McIlroy et al.[130] of trials of prophylactic AF agents in patients receiving aspirin within 7 days of cardiac surgery found no effect of aspirin coadministration on the benefit of AF agents to reduce bleeding.

Topically applied AF therapy has been utilized as a prophylactic measure to reduce postoperative bleeding following cardiac surgery, the hypothesis being that the pericardium is an important site of fibrinolytic activity[131] and that the pericardium is a barrier to absorption of medications.[132,133] In a meta-analysis of topical AFs[134] administered directly into the sternal wound and into the pericardial cavity, the data suggest that topical AF agents reduce postoperative chest tube drainage (by a mean of 220 mL) and postoperative red blood cell transfusions (by 1 unit per patient), compared with placebo. These data are similar to results after systemic antifibrinolytic therapy.[94]

The trials cited above all utilized blood loss and transfusion requirements as the primary study outcomes. Head to head trials comparing aprotinin with the synthetic lysine analogs in cardiac surgery show a modest hemostatic advantage for aprotinin,[96] and a meta-analysis of 211 trials (21,000 patients) showed a small reduction in red blood cell transfusion (relative risk 0.83).[135] Although aprotinin was widely adopted for its slight superiority over the synthetic lysine analogs in terms of blood loss and blood transfusions, recent trials have found aprotinin use to come at a substantial cost in increased mortality and postoperative renal insufficiency[136] (see Table 77.2). Three observational cohort studies published between 2007 and 2008 suggested a higher risk of mortality associated with aprotinin exposure during cardiac surgery.[81,82,83] From prospectively collected observational data on more than 4,300 patients, Mangano et al.[83] found a 48% increased risk of mortality at 5 years in aprotinin- versus placebo-treated patients, with no such effect on mortality associated with TXA or EACA (vs. placebo). Schneeweiss et al.[81] analyzed a European database including patients undergoing cardiac surgery and receiving prophylactic treatment with aprotinin (n = 33,517) or EACA (n = 44,682), and found a 64% increase in the risk for death following treatment with aprotinin. The BART trial of 2,331 high-risk patients undergoing cardiac surgery found aprotinin was associated with a lower rate of massive hemorrhage (9.5% vs. 12.1% for EACA or TXA), but there was a 6% risk of death at 30 days versus 3.9% and 4% amongst patients treated with EACA or TXA (relative risk = 1.53).[96] The conclusion of the study was that the modest benefit of aprotinin in terms of bleeding was offset by increased mortality and that aprotinin should not be used in high-risk cardiac surgery.

Given the discordance between hemostatic effectiveness and survival outcomes, future trials of AFs must address clinical endpoints, not just blood loss and transfusion requirements. Although specific patients may derive benefit from aprotinin, at present, this agent is not used in the United States, Canada, or Europe. Concerns remain regarding the safety of the synthetic lysine analogs, for example, TXA is associated with more seizures compared with aprotinin.[137]

Table 77.2

Antifibrinolytic therapy in cardiac surgery and effect of mortality

	Study Type	Study Population	Outcome	Conclusion
Fergusson et al.[96]	Double-blind, randomized, controlled	N = 2,331 High risk cardiac surgery	Massive bleeding: Aprotinin 9.5% TXA 12.1% EACA 12.1%	RR massive bleeding: 0.79 (0.59–1.05)
	Aprotinin vs. TXA vs. EACA		Mortality at 30 days: Aprotinin 6% TXA 3.9% EACA 4%	RR mortality: 1.55 (1.06–2.22) Aprotinin use not justified in cardiac surgery
Mangano et al.[362]	Multicenter, observational, prospective trial	N = 3876 undergoing CABG and CPB	Mortality at 5 y: Aprotinin: 20.8% TXA: 14.7% EACA: 15.8%	HR for death after Aprotinin: 1.48 (1.19–1.85)
	AF or no antibleeding agent		Control: 12.7%	No increased risk of death with TXA or EACA.
Schneeweiss et al.[81]	Prospective, multicenter cohort Aprotinin vs. EACA	N = 78,199 undergoing CABG	Mortality prior to hospital discharge: Aprotinin 4.5% EACA 2.5%	RR of death after Aprotinin: 1.64 (1.50–1.78)
Shaw et al.[82]	Prospective, single center cohort Aprotinin vs. EACA vs. control	N = 10,275 undergoing CABG	Mortality at 30 days: Aprotinin: 6.4% EACA: 2.4% Control: 2.2%	HR for death after Aprotinin: 1.32 (1.12–1.55)

TXA, tranexamic acid; EACA, aminocaproic acid; RR, relative risk; AF, antifibrinolytic agent; CABG, coronary artery bypass grafting; CPB, cardiopulmonary bypass; HR, hazard ratio.

Liver Transplantation

Many coagulation defects are present in the setting of end-stage liver disease, including increased fibrinolytic activity due to impaired hepatic clearance as well as enhanced endothelial release of tPA. The central role played by the liver in clearing circulating tPA was demonstrated by Illig et al. in a study of patients undergoing aortic clamping during repair of abdominal aortic aneurysms.[138] Patients who underwent supraceliac clamping of the aorta compared to those requiring infrarenal clamping experienced a reversible primary fibrinolytic state manifest by a shortened euglobulin lysis time, increased circulating tPA, and decreased α_2-antiplasmin concentration, likely caused by hepatic hypoperfusion. Orthotopic liver transplantation is associated with still increased fibrinolytic activity related to ischemic insult to the mesenteric vascular bed and release of activators from the transplanted graft during the anhepatic and reperfusion phases of transplantation.[139] To reduce hemorrhage associated with hyperfibrinolysis, an AF agent is commonly administered just prior to graft reperfusion.[140] In the study by Segal et al.,[141] patients treated with aprotinin during liver transplantation had a blunted increase in circulating tPA, prolonged euglobulin lysis time, decreased peak D-dimer levels, and increased circulating α_2-antiplasmin concentration during the reperfusion phase compared with control subjects, consistent with reduced systemic fibrinolytic activity.

Prophylactic treatment with AF agents during orthotopic liver transplantation shows variable results. The European Multicenter Study on the Use of Aprotinin in Liver Transplantation trial, a randomized, double-blind, placebo-controlled study of 137 patients undergoing liver transplantation, demonstrated lower intraoperative blood loss as well as considerably reduced transfusion requirements in patients treated with aprotinin versus placebo, although no differences occurred in 30-day mortality or incidence of thrombotic events.[142] Low-dose (2 mg/kg/h), continuous TXA during transplantation demonstrated decreased laboratory parameters of fibrinolysis, but no effect on blood loss or transfusion requirement versus placebo.[143] A prospective, placebo-controlled study of 132 patients comparing prophylactic treatment with high-dose TXA (10 mg/kg/h) and EACA (16 mg/kg/h) to placebo demonstrated significantly reduced transfusion requirements for patients treated with TXA, but not for EACA, versus placebo.[144] Blinded comparison of TXA and aprotinin in 127 patients showed no difference in blood loss, transfusion requirements, mortality, or thrombotic events between groups.[145] Prophylactic AF agents may not be useful in all clinical situations, and may even lead to adverse events.[114,140] Coagulation changes and excessive intraoperative bleeding due to hyperfibrinolysis are less common in children undergoing liver transplant, possibly because most are being treated for cholestatic rather than hepatocellular liver failure,[146] and adults with primary biliary cirrhosis have less laboratory evidence of hyperfibrinolysis than patients with cirrhosis due to hepatocellular disease.[147] A meta-analysis of 23 studies including 1,400 patients undergoing liver transplantation and treated with aprotinin or TXA found that both drugs significantly decreased the transfusion rate with no difference in rate of hepatic artery thrombosis, venous thrombotic events, or 30-day mortality.[148] Retrospective analysis of nearly 1,500 patients transplanted for liver disease who were exposed to aprotinin or no AF agent showed an increased risk for reversible renal dysfunction with no difference in the rates of hepatic artery thrombosis, venous thrombosis, or long-term mortality.[149,150] A review of prophylactic administration of AFs during orthotopic liver transplantation reviewed by the Cochrane Database in 2009 concluded that there is no effect on perioperative morbidity or long-term mortality, although the review cautioned that its findings are not robust.[151]

α2-Antiplasmin Deficiency

α2-antiplasmin deficiency may be a congenital condition associated with a severe, lifelong bleeding tendency in homozygotes,[152,153] as well as an acquired deficiency seen in settings of severe liver disease, DIC, and myeloproliferative disorders.[154] The bleeding clinically seen in deficiency is due to premature dissolution of hemostatic plugs by robust, unimpeded plasmin activity.[155] Both lysine analogs, EACA and TXA, are used to treat acute bleeding episodes. They may also be employed in prophylactic doses prior to invasive procedures.[156]

Localized Fibrinolysis with Defective Hemostasis

The formation of a stabilized and solid hemostatic plug is dependent on a maximal thrombin "burst,"[157] allowing activation of factor XIII to cross-link fibrin polymers and attenuate local fibrinolysis by activation of the thrombin-activatable fibrinolytic inhibitor.[158,159] Therefore, any state in which full thrombin generation is impaired, as in hemophilia and other hereditary coagulation disorders, is prone to hemorrhage due to fibrinolytic dissolution of inadequate hemostatic plugs.[160,161] Although systematic trials of AF agents have been tested predominantly in hemophiliacs, results are reasonably generalized to patients with other congenital or acquired hemostatic defects.

Hemophilia

Thrombin generation is considerably decreased in hemophilia because feedback amplification of the clotting cascade requires factor VIII and factor IX.[162,163] Accordingly, fibrin clots formed in patients with hemophilia are sensitive to accelerated fibrinolytic degradation. *In vitro* study of factor VIII-deficient plasma shows that the presence of TXA increases clot stability fourfold.[164] AF therapy has been used in hemophilia (a) as prophylaxis to prevent hemarthrosis; (b) for treatment of hematuria; (c) for treatment of gum bleeding and epistaxis; (d) as adjunctive, prohemostatic treatment during and following surgery and (e) in patients with factor VIII inhibitors. Although some studies have concluded that a combination of factor concentrate and AF agent produces an enhanced therapeutic effect,[165,166] the weight of evidence indicates that EACA and TXA may be useful for treatment of individual bleeding episodes but are not effective for prophylaxis against hemarthrosis.[167–169]

Treatment of intractable *hematuria* due to bleeding from the upper urinary tract may lead to renal functional impairment or extrarenal obstruction in hemophiliacs treated with factor replacement.[170] However, additional risk probably occurs with the use of AF agents because they prevent or retard dissolution of clots, which may lead to frank renal obstruction[92,93,171,172,173,174–196,197,198–208] that may contribute to mortality.[209–224] An early review of 66 patients with hemophilia treated with EACA for severe hematuria reported improvement in symptoms in 52 patients, but with renal obstruction in 10.[93] A recent report of four cases of children with protracted hematuria due to hemophilia or sickle cell trait described complete cessation of bleeding with oral EACA every 6 hours without complications.[174] Therefore, upper urinary tract bleeding should be managed conservatively

unless bleeding is protracted, requires transfusion, or if surgical intervention is considered.

The addition of AF agents to cryoprecipitate in 10 hemophilic patients with *gum bleeding*[175] improved hemostasis and recovery. A case report describes a patient with an acquired factor VIII inhibitor and severe *epistaxis* who was successfully treated with oral EACA.[176] Combined prophylaxis with EACA or TXA and factor IX in eight patients with hemophilia B permitted dental extractions without bleeding.[177] A single center report of 534 dental procedures, including 288 oral surgeries performed using EACA adjunctively, reported only a 1.9% incidence of severe bleeding complications..[178]

Although factor replacement therapy is effective in providing normal hemostasis after all types of major surgical procedures in hemophiliacs,[179] the addition of AFs reduces blood loss and the amount of factor VIII or factor IX concentrate required by an average of more than 80%.[177,180–184] It seems reasonable to suggest that patients with hemophilia undergoing other surgical procedures can be similarly supported by jointly administered AF and factor replacement therapy, as seen, for example, in synovectomies.[185,186] Among patients with acquired hemophilia, TXA and EACA are useful adjuncts to factor bypassing agents and recombinant factor VIIa for nonelective surgery[187] and for acute bleeding episodes.[188]

Chronic Anticoagulation

AF use has proven effective as a hemostatic agent in patients on chronic warfarin anticoagulation. A randomized trial of aTXA mouthwash after oral surgery in patients on chronic anticoagulation for a prosthetic cardiac valve was effective in reducing the incidence of bleeding episodes, as compared to the saline control group.[189] A meta-analysis of eight trials found that patients receiving continuous anticoagulant therapy and using AF mouthwashes had no greater bleeding than patients treated with interrupted anticoagulation or reduced anticoagulation.[190]

Thrombocytopenic and Thrombocytopathic States

Because qualitative and quantitative platelet deficiencies lead to fragile hemostatic plugs, AF therapy should be useful in these settings. A hemostatic effect of EACA has been demonstrated in patients with bleeding due to immune thrombocytopenia or thrombocytopenia due to induction chemotherapy for leukemia[191–194] and in Bernard-Soulier (giant platelet) syndrome[195,196] in the control of obstetric bleeding. In a single-center series of 77 patients with chemotherapy-induced severe thrombocytopenia and hemorrhage, treatment with EACA resulted in complete resolution of hemorrhage in 66% of patients, and a 17% rate of partial response or 50% decrease in platelet transfusion requirement.[197]

Normal Hemostasis with Localized Fibrinolysis

Bleeding can result from a localized fibrinolytic effect caused by an imbalance between heightened local lysis and normal hemostasis.[10,97] Tissues that are rich in PA might be more likely to be affected by such imbalances, for example, in the uterus, which increases PA concentration during menstrual bleeding.[198] Important sites of heightened fibrinolytic activity include the vascular endothelium; cells that line glands, ducts, tubules, or body spaces (kidney, prostate, endometrium, meninges, pleura, pericardium, and peritoneum); invasive tumors and cells; and fluids exposed to or draining such sites. In fact, the earliest clinical applications of AF therapies were for bleeding at sites of known, enhanced fibrinolytic activity: prostate, bladder, uterus, and uterine cervix.

Upper Urinary Tract

The renal pelvis is rich in urokinase, which may be the underlying defect in essential hematuria. Andersson et al.[199] reported the successful treatment of 13 patients with "*essential hematuria*" with EACA and TXA, and others have demonstrated that AF therapy can be dramatically successful for prolonged upper urinary bleeding in sickle cell disease.[200–206] However, there is an increased risk of clot formation in the renal collecting system when AF agents are used.[207] This complication is usually of minor importance, but in some patients, the clot is large enough to impair renal function,[91,92,173] appearing as an abrupt clearing of blood from the urine and flank pain, with radiologic evidence of hydroureter or hydronephrosis. Such clots may be treated with intraureteral urokinase,[208] but their size may be minimized by a high urine output before and during AF therapy. Posttransplantation or postrenal biopsy hematuria has been treated with EACA.[209–212]

Lower Urinary Tract

Bleeding after *prostatectomy* is enhanced by urokinase, which diffuses into the operative site to dissolve newly formed hemostatic plugs.[10,11,15,16,97,199,213–215] Eight randomized trials in the 1960s and 1970s evaluating EACA therapy after prostatectomy demonstrated a significant decrease in blood loss when compared to placebo[214,216–223]; a ninth study found similar results with TXA treatment.[224] In these trials, there was a twofold to fivefold reduction in urinary tract blood loss during the postoperative period, but no effect on the volume of intraoperative blood loss. Although the difference was statistically significant, the findings were not as relevant clinically, because the bleeding in control patients rarely required transfusion. Thromboembolic side effects of treatment were no greater than in control groups and were not reduced by the concomitant use of prophylactic heparin.[225] A prospective, double-blind study of EACA versus placebo administered immediately following prostatectomy in 61 patients failed to demonstrate any effect of EACA on blood loss,[223] leading the authors to conclude its use was not warranted, and a trial of prophylactic TXA in 136 men undergoing elective transurethral resection of the prostate demonstrated decreased operative blood loss in the treatment group, but without an effect on transfusion requirements.[226] AF agents have been used to control lower urinary tract bleeding associated with benign prostatic hyperplasia or prostatic carcinoma,[35,215] but Smart suggested that EACA- and TXA-associated clots in the lower urinary tract pose a problem after prostatectomy.[227] Interestingly, although aprotinin is a potent *in vitro* AF agent, it was not beneficial in a single study of lower urinary tract bleeding.[228] Given the lack of efficacy in preventing transfusion, it has been suggested that AF therapy should be reserved for those patients who demonstrate excessive bleeding at the time of prostatectomy.[97]

Dysfunctional Uterine Bleeding

Higher uterine PA activity is present during the secretory phase when compared with the proliferation phase of the menstrual cycle,[229] and higher mean PA levels on the first day of menses are demonstrated in patients with primary menorrhagia than in those with normal menstrual blood loss.[230] Treatment of

essential menorrhagia has been studied by several groups,[35,231,232] and the efficacy of AFs was clearly demonstrated by Nilsson and Rybo[233] in a double-blind clinical trial. In the latter study, 37 women received EACA or placebo during two successive menstrual cycles, with the menstrual blood loss significantly different, 120 mL for placebo-treated patients and 52 mL for EACA-treated patients. Later studies of EACA and TXA[234–237] confirmed these data, especially when AFs are taken during the first 3 days of menstrual bleeding.[10,97] No benefit was seen in women with bleeding secondary to uterine fibroids who were treated with TXA.[238] Patients with von Willebrand disease–associated menorrhagia responded to chronic TXA with reduced blood loss.[239] A systematic review published in the Cochrane database in 2000 concluded that AF therapy leads to a reduction in blood loss compared with placebo or other medical therapies such as oral progestogens or nonsteroidal anti-inflammatory drugs.[240] Concern for thrombotic complications with chronic use has not been borne out.[241]

Whether increased bleeding secondary to the placement of an intrauterine device (IUD)[242] is caused by the higher fibrinolytic activity in the endometrium[243] or by edema, inflammation and necrosis, AF therapy decreases the menstrual blood loss. In an uncontrolled study before and after IUD placement, Kasonde and Bonnar[244] noted that menstrual blood loss increased from 43 to 82 mL in 28 untreated women, whereas there was a diminished increase (from 43 to only 54 mL) in patients treated with EACA. A double-blind study in 65 women showed a similar increase in blood loss from 36 to 65 mL in control patients but no increase (mean <4 mL) in patients treated with TXA.[245–277]

The cervix contains a high concentration of PA, which contributes to excessive bleeding in some patients after *cervical conization*. In a double-blind study by Rybo and Westerberg[245] treatment with TXA beginning after surgery and continuing for 12 days, led to significantly reduced blood loss (from 79 to 23 mL). 7 of the 23 women treated with placebo had unexpected severe bleeding after the fifth postoperative day, requiring resuturing of the operative site, a complication that was not seen in the TXA-treated group. Similar findings were reported by Grundsell et al.[246]

Given the high concentration of PA in the female genital tract, recent interest in the use of AFs in the prevention and treatment of maternal hemorrhage has launched the World Maternal Antifibrinolytic Trial, a randomized, placebo-controlled trial to evaluate the role of TXA for the treatment of postpartum hemorrhage.[247]

Although providers must rule out underlying pathology in patients with menorrhagia or postoperative bleeding,[248] it would not be unreasonable to attempt to control excessive uterine bleeding from any cause in the absence of DIC with AF therapy while the diagnostic workup is proceeding, and it may be useful as an adjunctive therapy once treatment of the underlying disorder has been initiated.

Subarachnoid Hemorrhage and Intracranial Surgery

Although cerebral tissue is not rich in tPA activity,[198] there are localized regions of high concentration in the meninges and the choroid plexus,[249] and measurable amounts of PA present in the cerebrospinal fluid after cerebral injury[250,251] may contribute to lysis of fragile intra-aneurysmal thrombi and contribute to "rebleeding" following the initial event in 6% to 20% of the at-risk population.[252–255] The data from clinical trials employing AF therapy in the setting of *ruptured intracranial aneurysm* is conflicting. Although the initial, uncontrolled studies suggested that therapy after subarachnoid hemorrhage was useful in preventing recurrent hemorrhage,[256,257–266] controlled trials of EACA or TXA are discordant.[267–273] Three small controlled studies failed to show benefit with AF therapy.[270–272] Concern regarding increased thromboembolic complications[274] inspired two large multi-institutional studies.[275,276] The Cooperative Aneurysm Study[254,255,275] was based on 672 patients and showed rebleeding in 11.7% of 467 patients receiving AF treatment, compared with 19.4% of 205 patients without AF therapy. However, the rate of focal ischemic events was higher in the AF group (32.4% vs. 22.7%), as was the incidence of hydrocephalus (14% vs. 7%). The incidence of clinical venous thromboembolic complications and the overall mortality rates were not different. A second large trial comprising 479 patients was organized as a randomized, double-blind, placebo-controlled trial[276] and showed essentially the same mortality rates at 3 months for TXA and placebo (35% vs. 37%). Although the rate of rebleeding was significantly lower in the TXA group, the incidence of cerebral infarction was higher. Thus, although AFs probably decrease rebleeding, they do so at the cost of an increase in cerebral ischemia and infarction, leading to an unchanged mortality rate with treatment.[278] In an effort to exploit improved methods to control cerebral vasospasm, Roos et al. undertook a randomized, placebo-controlled trial of TXA in 462 patients with aneurysmal hemorrhage in which all patients were treated with calcium antagonists to minimize vasospasm. Although TXA considerably reduced the incidence of rebleeding, there was no effect on primary functional outcome at 3 months.[279] A recent case–control study by Starke et al. addressed the question of early use of EACA to prevent rebleeding, with required discontinuation of the drug by 72 hours from symptom onset to avoid exposure during the period most associated with ischemic injury. The results showed a significant decrease in rebleeding (2.7% vs. 11.4% in untreated patients), no difference in ischemic complications, and a trend toward improved mortality.[280] Interestingly, all patients were evaluated with lower extremity Doppler ultrasound, and the EACA-treated patients had an eightfold increased risk of developing deep venous thrombosis (DVT).[280]

Gastrointestinal Tract

Certain pathologic lesions that cause upper and lower GI bleeding have been associated with increased fibrinolytic activity, either within the tissues themselves or in veins draining the affected organ.[281] Six prospective, controlled clinical trials[281–286] used TXA in patients with upper GI bleeding and demonstrated significant benefit. The study by Cormack et al.[282] conducted in the early 1970s demonstrated that treatment with TXA was superior to placebo in patients with undiagnosed bleeding distal to the esophagogastric junction in terms of a fatal outcome, the need for surgery, or continued bleeding that required blood transfusion. There was, however, no difference in the failure rate in patients with bleeding secondary to esophageal varices. A prospective, double-blind study comparing TXA and placebo in 154 patients with upper GI bleeding from verified, benign lesions, demonstrated a significantly decreased need for blood

transfusion or surgical intervention after TXA treatment.[286] In the study by Biggs et al.[283] of 200 randomized patients with acute upper GI hemorrhage, there was a significant difference in the requirement for surgical intervention to control bleeding, occurring in 23 of 97 control patients but in only 7 of 103 TXA-treated patients. Unlike the study by Cormack et al.,[282] this study showed benefit in patients with esophageal varices as well as in those with gastric ulcers and gastric erosions. Barer et al.[285] randomly assigned 775 patients with acute upper GI bleeding to TXA, cimetidine, or placebo, and showed a reduced mortality from 13.5% in the control group to approximately 7% in either of the treatment groups. Meta-analysis of the effect of fibrinolytic inhibitors on mortality from upper GI hemorrhage included six randomized, controlled trials involving 1,267 patients and concluded a short-term course of TXA (2 to 7 days) resulted in a 20% to 30% reduction in the rate of rebleeding and a 30% to 40% reduction in mortality.[287] Other upper GI lesions improved by antifibrinolytic therapy are giant hypertrophic gastritis (Ménétrier disease) and gastric antral vascular ectasia, through a reduction in incidence of rebleeding.[288,289]

Patients with cirrhosis often have laboratory evidence of a chronic, systemic lytic state, with elevated D-dimer and tPA concentrations in the blood. Although the incidence of bleeding is higher in those patients with more advanced cirrhosis and with higher circulating D-dimer and tPA levels,[290] clinical efficacy of AFs may be attributable more to inhibition of local fibrinolysis at lesions prone to bleed than to correction of the systemic fibrinolytic state.[291] Evidence for this hypothesis is provided by the work of Cox et al.,[281] who found a high level of plasmin activity in gastric veins of patients with peptic ulcers when compared to a control population, and by Oka and Tanaka,[292] who demonstrated increased fibrinolytic activity in the mucosa of the esophagus and stomach in patients with cirrhosis and upper GI bleeding. The use of AF therapy in bleeding complicating portal hypertension and cirrhosis deserves further clinical study.

Increased amounts of PA have been demonstrated in the rectal mucosa of some patients with active ulcerative colitis.[288,293] Although an uncontrolled study[294] suggested that the bleeding in such patients was controlled with AF therapy, two controlled trials[295,296] give conflicting results. Alternative approaches such as direct administration of agent by enema may prove more useful,[297,298] but trials have not been performed.

Traumatic Hyphema

Increased local fibrinolytic activity is present in circumscribed areas of the eye, specifically in the canal of Schlemm, ciliary body, iris, and proximal scleral vascular plexus.[299] This fibrinolytic activity presumably prevents obstruction of intraocular fluid drainage and glaucoma, and may be responsible for clot lysis following ocular trauma, but on the other hand, such fibrinolytic activity may also contribute to rebleeding after blunt eye trauma. Therapy with AFs has been attempted as a prophylactic measure against such rebleeding,[300,301] which occurs in as many as 30% to 40% of patients, approximately 2 to 6 days after the injury. A favorable effect of AF therapy in preventing secondary bleeding after traumatic hyphema was reported in studies based on historical controls[300,302–305] and in three controlled trials.[301,306,307] In a controlled study of 238 patients, oral TXA decreased the incidence of secondary hemorrhage from 26% in those receiving placebo to only 10% ($P = 0.008$).[308] A similar benefit has been noted in a study of 118 patients in which topical TXA was as effective as oral TXA in reducing secondary hemorrhage (2 of 64 vs. 12 of 54 patients receiving placebo).[309] A 5-day course of topical EACA versus placebo decreased the rate of rebleeding in 51 patients presenting with traumatic hyphema, and there was a nonsignificant trend toward more rapid improvement in vision in the treatment group.[310] Yet the study by Taboul et al.[311] suggests that the incidence of secondary hemorrhage after traumatic hyphema is quite low in patients without added risk factors such as aspirin ingestion and, therefore, that routine use of EACA (or any antifibrinolytic) is not warranted. Taken together, the data seems to indicate that on balance, AF therapy is a reasonable approach in preventing rebleeding following traumatic hyphema, at least in those who do have added risk factors for hemorrhage.

Mucous Membranes

AF therapy has been studied in patients undergoing tonsillectomy and adenoidectomy,[312–314] demonstrating a significant decrease in blood loss when compared with other treatment modalities, although the amount of blood loss may not suffice to recommend such agents for routine use. AF therapy has been a useful adjunct to control bleeding after minor oral surgery for tonsillectomy in von Willebrand disease.[315,316] A randomized blinded trial of 30 patients treated with topical TXA or EACA versus placebo administered by spray at the conclusion of endoscopic sinus surgery demonstrated topical TXA to be superior to EACA or placebo in promoting hemostasis at the surgical site.[317] A trial of TXA for patients with epistaxis showed a significant decrease in the number and severity of bleeding episodes.[318] AF treatment might reasonably be attempted in patients with unexplained serious postoperative mucosal bleeding that requires unsuturing or packing or in those with recurrent, unexplained epistaxis.

Cavernous Hemangioma

Patients with multiple giant hemangiomata (Kasabach-Merritt syndrome) present a clinical dilemma particularly resistant to medical therapies, with progressive enlargement of vascular tumors that often require extensive surgery. Ongoing consumption of platelets and fibrinogen suggests that thrombi are continually formed and dissolved within the hemangioma. This led Neidhart and Roach[319] and, later, Warrell and Kempin[320] and Ortel et al.,[321] to apply the novel approach of inducing *in situ* thrombosis by the administration of EACA, either alone[319] or with concomitant cryoprecipitate infusion.[320] Tumor shrinkage, symptomatic improvement, or improvement of laboratory evidence of consumption were obtained in all cases. A similar approach has been applied successfully in a reversal of a consumption coagulopathy associated with Klippel-Trenaunay-Weber syndrome.[322,323] Variable success has been achieved using AF agents in hereditary hemorrhagic telangiectasia,[324] but, as noted in an editorial,[325] systematic studies are needed to establish this therapy as definitely effective.

Chronic Urticaria

Chronic urticaria is a rare condition, generally managed with antihistamine and steroid therapy, which, when severe, can activate the coagulation and fibrinolytic systems. A pilot study demonstrated that patients with chronic, therapy-resistant

urticaria respond to combination therapy with low molecular weight heparin and TXA, in particular those patients with D-dimer elevation.[326]

Hereditary Angioedema

The syndrome of hereditary angioedema, with recurrent attacks of swelling of arms, legs, abdomen, or oropharynx, is due to mutations in the C1 inhibitor gene leading to an abnormal protein, incapable of inhibiting complement proteases, kallikrein, and plasmin.[327] Although ineffective for the management of acute symptoms, the lysine analogs are effective in long-term prophylaxis. One crossover trial of TXA and placebo in 18 patients with hereditary angioedema demonstrated complete cessation of attacks in 7 of 18 patients during the TXA treatment period, with recurrence during the placebo crossover.[328] Another report cited long-term benefit of EACA prophylaxis, with 90% decreased frequency of attacks in two patients.[329]

Applications to Reduce Blood Loss

Given their role in promoting hemostasis, AF agents have been employed in surgical settings to control bleeding and reduce patient exposure to allogeneic blood products. In conjunction with desmopressin and technical approaches such as hypotensive anesthesia or hypothermia, they may reduce hemorrhage.[330]

Orthopedic Surgery

Three placebo-controlled trials have demonstrated that TXA significantly decreases perioperative blood loss and transfusion requirements associated with primary total knee replacement surgery.[331–333] Hiipala et al.[331] randomized 75 patients undergoing total knee replacement to TXA or placebo and demonstrated that fewer patients required perioperative transfusion (17/39 vs. 34/38, $P < 0.00003$) as well as a lower mean number of transfused units (1 vs. 3.4, $P < 0.0001$). A randomized, double-blind trial in 127 patients undergoing bilateral total knee replacement treated with TXA, EACA, or placebo showed that 7.5% of treated patients required blood transfusion compared with 38% of placebo-treated patients ($P < 0.001$).[334]

Similar benefit has been shown for patients undergoing primary total hip arthroplasty. Husted et al.[335] randomized 40 patients to TXA or placebo, and demonstrated a significant decrease in postoperative blood loss, decreased proportion of patients receiving transfusions ($P = 0.04$), and no difference in thrombotic events. Rajesparan et al.[336] randomized 73 patients to bolus TXA given at anesthesia induction versus placebo and showed that TXA significantly reduced total blood loss and transfusion rate without affecting the incidence of venographically documented DVT. Claeys et al.[337] demonstrated the efficacy of preoperative bolus injection of TXA over placebo in reducing total blood loss and transfusion volume, although there was a higher rate of DVT in the treatment group (3 vs. 0 events). Bolus TXA (20 mg/kg) prior to orthognathic surgery significantly improved total blood loss versus placebo use (878 mL vs. 1,287 mL, $P < 0.001$).[337,338] Meta-analysis of trials of prophylactic TXA in orthopedic surgery evaluated 12 trials and concluded that treatment with TXA significantly reduces total perioperative blood loss and the proportion of patients requiring transfusion (odds ratio 0.26).[60]

Placebo-controlled trials evaluated aprotinin prophylaxis during hip replacement surgery and generally have demonstrated a reduced rate of allogeneic blood transfusion (vs. placebo), without an increased incidence of thrombotic events. Janssens et al.[339] evaluated 40 patients and demonstrated a reduction from 3.4 transfused units in the placebo group to 1.8 in the treatment group ($P < 0.001$). Murkin et al.[340] randomized 225 patients undergoing hip replacement surgery to treatment with high-dose versus low-dose aprotinin or saline placebo, and demonstrated that a significantly lower proportion of aprotinin-treated patients required allogeneic blood transfusion (6% vs. 12%). Samama et al.[341] found no dose-related effect on transfusion requirements in high versus low-dose aprotinin treatment. Jeserschek et al.[342] demonstrated that high-dose aprotinin versus placebo significantly reduced the requirement for intraoperative blood transfusion (1.4 U vs. 3.1 U). Confirming the importance of delivering aprotinin continuously, a randomized study of 40 patients undergoing hip replacement surgery who received a single prophylactic injection of aprotinin did not demonstrate any benefit in reducing blood loss.[343]

Neurosurgery

Spinal surgery is associated with significant blood loss and transfusion support.[344] A randomized trial comparing TXA and placebo in 64 patients undergoing spinal surgery showed TXA had a 49% decrease in total blood loss and 80% decrease in the rate of transfusion ($P < 0.007$ and $P < 0.008$).[345] Among 182 patients treated with EACA or placebo, the rate of RBC transfusion was lower in patients receiving EACA (2 U vs. 2.8 U), although the amount of allogeneic blood transfused was the same.[346] A trial comparing intermediate-dose aprotinin with EACA or placebo demonstrated significantly decreased blood loss after aprotinin, less so with EACA, compared with placebo (3,628, 4,056, and 5,181 mL, respectively).[59] The results of a meta-analysis of 18 clinical trials utilizing aprotinin, TXA, or EACA in spinal surgery, confirmed all three agents decreased total blood loss and transfusion volume. However, aprotinin and TXA were associated with the greatest effects, while the effect of EACA became significant as the complexity of the spinal surgery increased.[344]

AF therapy may reduce blood loss when administered into the surgical field. In a pilot trial to evaluate topical AF therapy, 30 patients were randomly assigned to TXA or placebo administered directly into the spinal wound; those treated with topical TXA had a 50% decrease in postoperative blood loss.[347]

The endothelium lining the meninges is a rich source of tPA and fatal episodes of systemic hyperfibrinolysis have been reported following surgical resection of benign lesions.[348,349] One patient with life-threatening perioperative bleeding associated with elevated levels of circulating tPA was successfully resuscitated with high-dose aprotinin.[350] In a study of 100 patients undergoing craniotomy for an intracranial meningioma or vestibular schwannoma, randomized to low-dose aprotinin versus placebo, intraoperative blood loss was reduced by 50% in the group treated with aprotinin (1,014 to 508 mL, $P = 0.028$).[351]

Trauma

Given the efficacy of AF therapy to reduce blood loss and transfusion volume in patients undergoing surgery, its utility to moderate the bleeding and coagulopathy that occurs with trauma has been explored. Over 20,000 adult trauma patients with clinical evidence of hemorrhage or deemed to be at risk for significant bleeding were randomized to treatment with TXA versus saline placebo. [352] Treatment with TXA was associated with a modest reduction in all-cause mortality at 4 weeks (relative risk 0.91, $P = 0.0035$) and a decreased risk of death due to bleeding

(RR 0.85, $P = 0.0077$). The deceased mortality was independent of blood loss and the study's design did not permit conclusions to clarify the mechanism by which TXA reduced mortality.

Pediatric Surgery

Results in pediatric patients are comparable to those in adults. One randomized trial of 41 pediatric patients undergoing repeat cardiac surgery received either high-dose TXA or placebo and showed a 24% decrease in blood loss in the TXA-treated group (26 mL/kg vs. 34 mL/kg, $P = 0.03$).[122] In pediatric cardiac surgery, a randomized, double-blind trial of high-dose aprotinin in 60 patients demonstrated decreased perioperative blood loss as well as a significantly reduced proportion of patients requiring blood transfusion support (13% vs. 47%).[353]

In a study of pediatric posterior spinal fusion surgery for scoliosis, TXA was compared to placebo in a randomized, double blind trial, and showed a significantly decreased rate of RBC transfusion.[354] One randomized trial of 59 anterior or posterior spinal fusion procedures demonstrated decreased blood loss and RBC transfusion (1 vs. 2 U, EACA vs. placebo).[355] In 44 pediatric patients undergoing spinal fusion surgery, aprotinin decreased blood loss and transfusion requirements compared with placebo (1.1 U vs. 2.2 U).[58] As a hemostatic adjunct for patients requiring surgical intervention while on extracorporeal membrane oxygenation, a retrospective analysis at a single center demonstrated a decreased incidence of surgical site bleeding with no adverse thrombotic events in 298 treated patients.[356] A meta-analysis of AF therapy in pediatric patients at high risk for bleeding while undergoing major surgery included five studies of patients undergoing scoliosis surgery and 23 trials of cardiac surgery, treated with either TXA or aprotinin, and found both agents significantly reduced red blood cell transfusion compared to placebo (4 and 7 mL/kg respectively).[357]

Fibrin Sealants

Fibrin sealants containing fibrinogen, thrombin, and factor XIII have been used to enhance local hemostasis and adhesion during surgical procedures, and some commercially available fibrin sealants contain an AF agent (bovine aprotinin, TXA) as well.[358] Although these agents have been used effectively in uncontrolled trials in hemophiliacs undergoing dental extractions, hypospadias repair, endonasal surgery, and tracheoesophageal fistula repair,[359–361,171] there are no randomized trials comparing the efficacy of fibrin sealant products with and without AF additives.[358] Among children treated with aprotinin containing fibrin sealant during cardiac surgery, 40% exhibited circulating, aprotinin-specific antibody 6 weeks postoperatively, suggesting that a test dose remains imperative to avoid anaphylaxis even with topical application.[172,359–362]

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CHAPTER

78

Platelets, Plasma, and Specialized Coagulation Concentrate Therapies

THOMAS S. KICKLER

The biggest driving force in improving the safety of transfusion therapy in bleeding disorders has been the concern by patients of disease transmission. Safety has been accomplished not only by improved donor screening and testing but also through technologic improvements of blood product production and recombinant production of clotting proteins. The purpose of this chapter is to review the properties and clinical indications of platelets, plasma, and coagulation factor concentrates.

PLATELET TRANSFUSION THERAPY

Platelet Concentrates and Pheresis Platelets: Platelet transfusions are available as platelet concentrates or as apheresis units,[1] the former prepared from whole blood by centrifugation and the latter collected by pheresis devices. Platelet concentrates are separated from whole blood by first preparing platelet-rich plasma, from which the platelets are centrifuged. The contents of the platelet concentrates are highly variable, but 50 mL of platelet concentrate has at least 5.5×10^{10} platelets. A buffy coat method uses centrifugation to prepare a buffy coat, from which platelets are separated by an additional centrifugation. The white cell contamination is approximately 10^8 per bag using the platelet-rich plasma method of concentration, but considerably lower (10^6 per bag) for platelets prepared by the buffy coat method. Lower white blood cell (WBC) content in buffy coat platelet preparations may be advantageous in reducing alloimmunization and febrile transfusion reactions.[1,2,3] Platelet concentrates obtained from platelet-rich plasma method versus the buffy coat method do not show any functional difference *in vitro*, and comparative clinical trials have not been done.

Pheresis platelets are collected from donors by continuous processing of large volumes of blood and removal of platelets using an automated system. Most devices have WBC contamination of $<10^6$ per bag.[1] An advantage of pheresis platelets is that enough platelets can be derived from a single donor to provide a therapeutic dose, in contrast to platelets prepared from whole blood collection that requires pooling of platelets from four to six donors, which increases donor exposure risk.

Platelet Storage Lesion: In the United States, platelet blood components are licensed for a storage time of 5 days at room temperature, a limitation that constrains inventory control and can lead to wastage of this resource and periods of platelet shortages. Two factors lead to the short shelf life, first the risk of bacterial contamination and second the deterioration of platelet function. Platelets are stored at room temperature, as storage at 6 to 10°C leads to loss of function and viability, but room temperature (22°C) storage promotes bacterial growth of that may have been introduced at the time of blood collection. The risk of bacterial contamination is decreased by shortening storage time and by bacterial testing and pathogen inactivation procedures.[4]

The platelet storage lesion is characterized by (a) irreversible change in platelet shape from discoid to spherical, (b) generation of lactic acid from glycolysis, with an associated decrease in pH, (c) release of cytoplasmic and granule content causing a decrease in *in vitro* measures of platelet function and ADP-induced shape change, and (d) reduction in *in vivo* recovery and survival. Many of the biochemical changes are related not just to the active metabolism of platelets but to preparation technique and storage conditions. Consequently improvements in bag composition and platelet additive solutions that maintain platelet metabolism during storage are under evaluation.[2,4,5]

Clinical Applications of Platelet Transfusions

Thrombocytopenia: Platelet transfusions in thrombocytopenic patients enhance endothelial support functions by plugging fenestrations between endothelial cells. Table 78.1 outlines platelet transfusion guidelines for clinical situations, including issues of long-term support.[6,7,8] There has been considerable interest in defining the lowest safe platelet concentration at which bleeding is unlikely, so that fewer transfusions are consumed. Investigators have now shown that a prophylactic platelet transfusion threshold of 10,000/μL in adult patients with acute leukemia is safe,[9,10,11] thereby decreasing platelet utilization, with only a small adverse effect on bleeding and with no effect on mortality. Thus, prophylactic transfusions should be given to patients with amegakaryocytic thrombocytopenia if the count falls below 10,000/μL, and at values between 5 and 10,000/μL, transfusion can be held if the patient is stable and if no other conditions make spontaneous bleeding likely. These conditions include blast crisis, anticoagulation with heparin for disseminated intravascular coagulation (DIC), drugs that affect platelet function, uremia, and recent invasive procedures, including lumbar punctures or placement of central venous catheters. A study in patients after autologous peripheral blood stem cell transplantation demonstrated the safety and feasibility of therapeutic platelet transfusions, with one-third of transplants performed without platelet transfusions, reducing platelet transfusion by 50% compared with historical controls when the practice of prophylactic transfusions was in place.[11]

Platelet Dosing: While there have been a few small studies addressing platelet dosing,[12,13,14] a well-designed dose-ranging study was recently completed.[15] In this large study by Slichter et al., 1,351 adult and pediatric hematology–oncology patients were randomized to receive low (1×10^{11}), medium (2.2×10^{11}), and high dose (4.4×10^{11}) platelets per m^2 body surface area. Among the 1,272 evaluable patients, WHO grade 2 or higher bleeding was not significantly different between the three groups. This study suggests that fewer transfused platelets can

Table 78.1

Platelet transfusion guidelines

Prophylactic Platelet Transfusion Thresholds:

- Acute leukemia: For adult patients, a threshold of 10,000/μL is recommended; transfusion at higher levels may be necessary in the newborn or in patients with hemorrhage, high fever, hyperleukocytosis, rapid fall in platelet count, or coagulation abnormalities
- Hematopoietic cell transplantation: Same as for acute leukemia, with similar exceptions
- Chronic stable severe thrombocytopenia: Many patients can be observed without prophylactic transfusion, reserving transfusion for episodes of hemorrhage or during times of active treatment
- Solid tumors: Evidence supports the benefit of prophylactic transfusion at a threshold of 10,000/μL or less; a threshold of 20,000/μL should be considered for patients receiving aggressive therapy for bladder cancer, as well as for those with demonstrated necrotic tumors
- Surgical or invasive procedures: A platelet count of 40 to 50,000/μL is sufficient to perform major invasive procedures safely, in the absence of associated coagulation abnormalities; certain procedures, such as bone marrow aspiration/biopsy, can be performed safely with counts <20,000/μL; lumbar puncture in children is safe at platelet counts >10,000/μL
- Prevention of alloimmunization using leukoreduced blood products: It is appropriate to provide leukoreduced blood products to patients with acute myelogenous leukemia from the time of diagnosis to ameliorate the problem of alloantibody-mediated refractoriness to platelet transfusion; currently, this is not indicated in patients with cancer RBCs or treatments that do not produce significant and sustained thrombocytopenia
- Diagnosis of refractoriness to platelet transfusion: A CCI of 7,500/m²/μL is recommended as a definition of a satisfactory response; a diagnosis of refractoriness should be entertained only when at least two consecutive ABO-compatible transfusions, stored <72 h, result in poor increments
- Management of refractoriness to platelet transfusion: Patients with alloimmune refractory thrombocytopenia (i.e., poor increments in association with alloantibody detected using lymphocytotoxicity or antiplatelet antibody assays) are best managed with HLA-A and HLA-B antigen–selected donors or from compatible donors identified using platelet crossmatching techniques. Alloimmunized patients do not benefit from unmatched prophylactic platelet transfusions that fail to raise the platelet count, Such patients should be transfused only for hemorrhagic events

RBC, red blood cells; CCI, corrected count increment; ABO, blood group system; HLA, human leukocyte antigen.

prevent clinically relevant bleeding. It should however be noted that the total number of platelets transfused ended up being higher in the low-dose platelet group who received a median of five transfusion events versus three in the other two groups. Thus while the dose may be decreased it is not apparent that the total number of platelets needed to sustain a patient through a period of aplasia will be decreased.[15]

Platelet Transfusion in Specific Disorders

Consumptive Thrombocytopenias: Thrombocytopenia may develop on the basis of increased consumption, for example, autoimmune or drug-induced thrombocytopenia, DIC, and thrombotic thrombocytopenic purpura (TTP). Thrombocytopenia in these conditions can be quite severe, and profound bleeding is not uncommon, so platelet transfusions may be considered necessary in such patients. In the immune-mediated thrombocytopenias, the destruction of allogeneic platelets is as likely as are autologous platelets, and platelet transfusion is not the only therapeutic means to treat this form of thrombocytopenia.[16] There has been no documentation of increased morbidity from using platelets in DIC despite some concern that their use may increase disease severity by fueling the ongoing activation of coagulation. In TTP, the use of platelet transfusions has been said to provoke acute clinical deterioration[17] but there are also cases in which patients received platelet transfusions without difficulty, although they were already undergoing plasma exchange therapy.[18]

Qualitative Platelet Disorders: The most common acquired qualitative platelet abnormalities are either drug-induced or caused by uremia, others occurring in patients with myeloproliferative disorders, dysgammaglobulinemias, or secondary to mechanical cardiac assist devices. No controlled trials have been conducted to determine what an appropriate platelet dose should be, but one recommendation is for at least 10 platelet equivalent units of platelets should be given for severe bleeding or for extensive surgery.[19] Platelet transfusions can improve the bleeding time in patients taking aspirin if the transfused platelets constitute 10% of the platelet population,[20] an observation of importance in cardiac patients who require emergency intervention. Whether one can extend this observation to other antiplatelet drugs such as clopidogrel is unclear. In those patients where clopidogrel cannot be stopped preoperatively within 72 hours of surgery, blood loss is higher and platelet transfusions are frequently given.[21]

Inherited qualitative platelet disorders such as Glanzmann thrombasthenia and Bernard-Soulier syndrome can be manifest by severe bleeding, particularly following trauma. Platelet transfusions are effective in these disorders until the patient becomes alloimmunized to human leukocyte antigens (HLAs)

or to a platelet glycoprotein receptor such as the IIb/IIIa or Ia/IX complex, after which platelet transfusions are ineffective. In this situation, recombinant factor VIIa has been used successfully.[22]

Platelet Transfusion Refractoriness: Failure to achieve an expected increment to a platelet transfusion (refractoriness) may be caused by immune or nonimmune processes, most commonly due to HLA alloantibodies.[23,24] Before labeling a patient as being refractory to platelet transfusions, one should document low platelet recovery after using ABO identical platelets on two sequential transfusions.[25] The platelet count increment is calculated using the following formula:

$$\text{Corrected platelet count increment} = \frac{(\text{Posttransfusion} - \text{pretransfusion platelet count}) \times 10^{11} \text{Body surface area } (m^2)}{\text{Number of platelets transfused}}$$

This formula takes into account the dose of platelets given in total number of platelets and is corrected for the body surface area of the patient. Facilitating this calculation is the labeling of the platelet bag with the number of platelets in the bag by the facility producing the platelet transfusion. An acceptable corrected platelet count increment should be >7,500 platelets per $m^2/\mu L$ within 1 to 60 minutes after the transfusion, and >4,500 platelets per $m^2/\mu L$ if measured 18 to 24 hours later.

Immunologic Basis of Platelet Transfusion Refractoriness: Serial platelet transfusions result in decreasing platelet count increments. These transfusion failures result from the induction of alloantibodies to HLA-A and -B locus antigens.[6] Antibodies to human platelet alloantigens such as HPA-1a (PLA1) are only rare causes of platelet transfusion refractoriness. ABO compatible transfusions are associated with about a 20% greater increment than those that are not compatible. Some patients with blood group O who have high isoagglutinin titers to group A or group B antigens present on platelets may also fail to achieve adequate platelet increments.[25]

HLA antibodies are measured using lymphocytotoxicity or enzyme-linked immunosorbent assay (ELISA) testing. Some patients may have decreases in or a loss of HLA antibody, either permanently or transiently, yet still be successfully transfused with platelet concentrates. HLA antibodies may be transient, and after their disappearance, patients are not refractory to platelets, even though they are not HLA matched. Some patients may have antibodies to HLA class I and yet not have platelet transfusion failures, because these HLA antigens may not be well expressed on platelets.[26–28]

There are two broad types of HLA antibodies produced in response to platelet transfusions. The first type recognizes epitopes unique to a particular HLA allele, referred to as antibodies to private specificities and including A2 or B12. The second type of HLA antibodies recognize more than one gene product. These antibodies recognize structural similarities between gene products (cross-reactive epitopes) or identical epitopes present on different gene products of different alleles, and are referred to as antibodies to public epitopes. Traditionally, HLA serology has placed the greatest emphasis upon classifying the private antigens, although more attention has recently been given to the clinical importance of public HLA specificities, the best known examples of which are Bw4 and Bw6. These antigens are encoded by a diallelic system and are associated with two different groups of HLA-B class I antigens. Other public antigens carried by HLA-B class I antigens have been divided into four cross-reactive groups, B5, B7, B8, and B12. The observation that the specificity of HLA antibodies in multiply transfused individuals is generally against private epitopes suggests that matching for these public antigens is not necessary.[29–31]

Table 78.2 outlines alternative approaches to selecting platelets for an alloimmunized patient. In patients with an unusual HLA type, such as in some ethnic groups, HLA matching may be difficult. Relying only on HLA matching may have shortcomings and in some cases it is overly restrictive, in that some HLA-B loci antigens are not located on platelets. Therefore, in addition to antigen matching there has been interest in adopting an approach to red cell compatibility. In this paradigm, one first selects a red cell phenotype that matches the patient's phenotype, and then performs a major crossmatch. This same approach can be used for platelets by also performing a platelet crossmatch. Unlike red cell pretransfusion testing, there are no accepted standards for selecting platelet transfusions in patients with alloantibodies to platelets.[23,24]

Table 78.2

Two approaches to selection of platelets for alloimmunized patients

Approach A
Determine HLA phenotype and ABO type of the recipient
Screen patient's serum for (a) HLA antibodies typically done by ELISA testing, or (b) antibodies to human platelet antigens (if there is a history of poor response to HLA-identical platelets)
Select from the donor pool those units with the most compatible HLA antigens and, if possible, ABO systems
Or
Approach B
Crossmatch available platelet units without regard to patient or donor HLA type (circumvents the need for HLA typing and provides a means of finding compatible platelets when antibodies to human platelet antigens are present); then obtain 10-min and 18–24-h posttransfusion platelet counts, not only to assess transfusion outcome but also to guide the selection of donors for future transfusions

Nonimmune Refractoriness: Platelet transfusions may not result in an increment if the stored platelets are defective. Splenomegaly is a major cause of platelet transfusion failure. Normally, approximately 30% of a patient's platelet mass is contained within the spleen, but with increases in splenic size, up to 90% of circulating platelets can be sequestered. Characteristically, splenic sequestration is associated with a reduced 1-hour platelet recovery but a normal survival. Fever and infection also cause decreased platelet survival. One study noted that platelet transfusion requirements were increased by 50% in febrile patients, even more so in patients with major infections and DIC. Medications may cause platelet refractoriness, including amphotericin, vancomycin, antithymocyte globulin, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor, and interferons.[32–34] Gamma radiation of platelet transfusions either prior to transfusion or prior to storage period does not affect platelet transfusion survival.

Management of Platelet Transfusion Refractoriness and Bleeding: When no compatible platelets can be found for an alloimmunized patient who is bleeding, or may be undergoing invasive procedures, therapeutic modalities have included splenectomy, corticosteroids, plasmapheresis, administration of intravenous gamma globulin, and repeated platelet transfusions. Except for intravenous immunoglobulin, there is little evidence that any of these work.[23,24,35] Kickler et al.[35] performed a randomized, placebo-controlled clinical trial, investigating the use of intravenous immunoglobulin (400 mg/kg for 5 days) in patients with alloimmunized thrombocytopenia. An incompatible platelet transfusion from the same donor was used before and after the patient received study drug or placebo. Although platelet recovery at 1 to 6 hours was satisfactory in five patients after intravenous immunoglobulin treatment, 24-hour survival was not improved in most of these patients. This small study suggests that the kinetics of platelet destruction may be improved by intravenous immunoglobulin to an extent to permit the performance of invasive procedures. When all conventional methods fail to increase the platelet count to hemostatic levels, an alternative is continuous platelet transfusion. Although this approach does not result in an increased platelet count, transfused platelets may permit platelet plug formation or help to maintain endothelial integrity, but clinical trial has not tested this assumption.

Prevention of Alloimmunization: Animal studies show that depletion of contaminating leukocytes from donor blood components is effective in prevention of alloantibody response to major histocompatibility complex.[36] In the Trial for the Reduction of Alloimmunization to Platelets, 534 leukemic patients receiving induction chemotherapy were studied during an 8-week period for the development of platelet alloantibodies. HLA antibodies developed in 45% of patients in the control group, compared to 18% in the filtered platelet concentrate group and 17% in the filtered apheresis group.[37] Not all patient groups may benefit from leukoreduction, for example, cardiac surgery patients who are not immunosuppressed may still develop HLA antibodies even with leukoreduced platelet transfusions.

PLASMA THERAPY

Table 78.3 lists plasma products and their properties. Clinicians are often aware of only fresh frozen plasma (FFP), whereas in reality, many transfusion services rely on plasma frozen within 24 hours, rather than 8 hours; plasma frozen within 24 hours has approximately 15% lower levels of factors V and VIII. Coagulation factor activity is well maintained in thawed plasma stored at 1 to 6°C for up to 5 days. An average unit of plasma has a volume of about 300 mL. Today, clinicians use plasma primarily for replacement therapy when a specific clotting concentrate is not available and for patients undergoing therapeutic plasma exchange for TTP.[38,39] Because plasma can be frozen for up to 1 year, some blood centers release the plasma for use when the donor returns at least 112 days later, and the infectious disease tests are found negative for hepatitis B, C, and human immunodeficiency virus (HIV). The 112-day release date for retested plasma was chosen because it exceeds the period during which virally transmitted diseases can occur despite negative infectious disease testing.

Methods for pathogen inactivation include solvent detergent treatment and photoinactivation using either psoralen or methylene blue. Solvent detergent plasma has lower levels of protein S and there is concern about increasing thrombosis in patients with TTP.[38]

Indications for Plasma

When plasma was first introduced, it was used as a plasma expander, promoter of improved wound healing, and nutritional support for correcting hypoalbuminemia, but safer and more effective means are available using nonblood products such as crystalloid with dextran. Plasma therapy is a poor method for nutritional support,[39] and there is no evidence that plasma improves wound healing. Frozen plasma carries the risk of viral disease transmission, similar to that associated with the use of red cell transfusions. Allergic reactions to plasma are common, as high as 3%, the most serious complication being transfusion-related acute lung injury (TRALI). In some locales, plasma from multiparous women is not used to make plasma, so as to circumvent the passive transfusion of white cell antibodies that provoke the potentially fatal TRALI syndrome. There is no single test or intervention that cannot be done to prevent TRALI. Most important strategy is preventive, where donors implicated in TRALI can no longer donate plasma or platelets by pheresis.

Congenital or Acquired Coagulation Factor Deficiencies: Patients with hemophilia with factor VIII levels of 5% to 10% rarely have spontaneous bleeding, and with factor VIII levels >50%, effective surgical hemostasis is anticipated. Therefore, the volume of transfused plasma should be sufficient to achieve approximately 30% coagulation factor levels, approximately 10 to 15 mL/kg of body weight. One of the limiting factors in dosing plasma is how well the patient can accommodate the increased plasma volume. In surgery where there is active bleeding, volume overload may not be an issue, whereas in a patient with severe liver disease, volume overload is a limiting factor.[38,39,40] FFP is used for correcting congenital or acquired coagulation deficiencies of factors II, V, VII, X, XI, and XIII, and adequate hemostasis occurs at factor concentrations of 0.2 to 0.3 U/mL (20% to 30%). Depending upon the half-life and extent of injury or surgery, additional treatments with FFP or factor concentrates may be needed.

Reversal of Warfarin: In the setting of active bleeding, emergency surgery, cardioversion, or invasive procedure where there is inadequate time to wait for vitamin K reversal, plasma is used to correct a prolonged international normalized ratio >1.6. Reversal may require 5 to 8 mL of plasma per kilogram body weight,[38,39] but prothrombin complex concentrates (PCCs) can be utilized for this purpose. PCCs achieve more rapid and

Table 78.3

Plasma products and their properties

Plasma Type	Preparation Characteristics	Composition	Comment
FFP	Separated from whole blood and frozen at –18°C or colder within 8 h of collection	Volume varies between 180 and 300 mL and plasma proteins and all coagulation factors	A 200-mL bag of FFP would contain approximately 200 U of each coagulation factor
Plasma frozen within 24 h of collection	Frozen within 24 h of whole blood collection	Levels of factors V and VIII are reduced approximately 15% compared to FFP	
Liquid plasma	Liquid plasma is separated no more than 5 d after the expiration date of whole blood	Stable coagulation factors are equivalent to FFP, but the labile factors of V and VIII are significantly reduced	Rarely should this product be used for replacement therapy in massive transfusion or severe liver disease and associated bleeding
	The supernatant plasma after the removal of cryoprecipitate material	Deficient in fibrinogen, factor VIII, and von Willebrand multimers	Has been used in therapeutic pheresis, and in TTP
Cryoprecipitate	Cryoprecipitate is prepared by thawing FFP at 1–6°C and recovering the precipitable material and then stored at 18°C or colder for up to 1 y	Contains more than 150 mg of fibrinogen, more than 80 U of factor VIII, von Willebrand multimers, and factor XIII in 15 mL of volume	Used in replacement therapy of hypofibrinogen, in preparation of fibrin sealants used in surgery, and in uremic platelet dysfunction

complete warfarin reversal than FFPs, but they carry the risk of thromboembolic complications. FFP when given in sufficient amount to correct hypoprothrombinemia may result in volume overload that may end up putting the patient in the intensive care unit. It should be noted that FFP contains citrate that when given rapidly in large volume may induce tetany by reducing ionized calcium, but not to an extent that affects coagulation. A systematic review of the use of recombinant factor VIIa (rFVIIa) to reverse warfarin provided no quality evidence for its use in this situation, so FFP still has a role.[38,41,42]

DIC: In many patients with DIC, severe hypofibrinogenemia may be present, but FFP alone may be insufficient to raise the fibrinogen to hemostatic levels of 100 mg/dL. Fibrinogen is concentrated 15-fold or greater in cryoprecipitate, which is the blood component of choice for correcting acquired hypofibrinogenemia. Transfusion of cryoprecipitate in doses of 1 bag per 5 kg of body weight will increase the fibrinogen levels by 80 mg/dL. The half-life of fibrinogen is 3 to 5 days,[43] and additional cryoprecipitate should be given on the basis of fibrinogen concentration.[43–45,46]

Massive Transfusion: Several factors contribute to bleeding in the setting of massive transfusion, including transfusion of packed red cells without clotting factors, large volumes of crystalloid leading to clotting factor dilution, and development of concurrent bleeding diathesis from DIC. Monitoring of coagulation should guide transfusion therapy, noting that thawing of plasma may take up to 1 hour, so empiric therapy based on clinical judgment may be the best approach in the face of serious bleeding.[43–45] Chapter 129 contains further description of transfusion therapy in surgical situations.

SPECIALIZED COAGULATION CONCENTRATES

Plasma Clotting Factor Concentrates

Tables 78.4A to 78.4K lists all the Food and Drug Administration (FDA) licensed blood product derivatives or recombinant therapies used for treating hemostatic disorders. Lyophilization of factor concentrates was introduced in the 1970s, leading to the widespread availability of a stable transfusion product that could be kept without freezing and permitted the introduction of home therapy by many patients with hemophilia. The introduction of viral inactivation and enhanced donor screening, along with the avoidance of paid plasma donors have resulted in highly safe plasma derivatives. The development of recombinant concentrates has provided for the safest possible form of factor replacement.[46,47,48] Since the introduction of recombinant clotting factors, clinical experience has shown that they are similar in efficacy as agents purified from plasma.[47]

Gel and ion exchange chromatography were used to increase the specific activity of factor VIII preparations to approximately 100 U of factor VIII per mg of protein. Polyethylene glycol precipitation followed by affinity chromatography of factor VIII/von Willebrand complex using heparin agarose columns is one method used to obtain relatively high purity concentrate of factor VIII and von Willebrand protein; other manufacturers employ monoclonal antibodies directed to factor VIII, producing a product of factor VIII that has >3,000 U of factor VIII per mg of protein. These affinity chromatography products

Table 78.4A

Products licensed in the United States for treatment of hemophilia A

Recombinant Factor VIII Concentrates

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Human or Animal Plasma-Derived Protein used in Culture Medium	Generation	Specific Activity of Final Product (IU Factor VIII/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?
Advate	Baxter	1. Immunoaffinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80)	None	Third	4,000–10,000	Yes
Helixate FS	Bayer (distributed by CSL Behring)	1. Immunoaffinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80)	Human plasma protein solution	Second	4,000	Yes
Kogenate FS	Bayer	1. Immunoaffinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80)	Human plasma protein solution	Second	4,000	Yes
Recombinate	Baxter	Immunoaffinity chromatography	Bovine serum albumin	First	1.65–19	Yes
ReFacto	Wyeth	1. Immunoaffinity chromatography 2. Solvent/detergent (TNBP/Triton X-100)	Human serum albumin (manufactured by Instituto Grifols, Spain, OctaPharma, Sweden)	Second (B-domain deleted)	9,110–13,700	Yes
Xyntha	Wyeth	1. Solvent/detergent (TNBP/polysorbate 80) 2. Nanofiltration	None	Third (B-domain deleted)	5,500–9,900	Yes

Table 78.4B

Products licensed in the United States for treatment of hemophilia A

Immunoaffinity Purified Factor VIII Concentrates Derived from Human Plasma

Product Name	Manufacturer	Method of Viral Inactivation	Heparin in Final Vial	Specific Activity of Final Product (IU Factor V78-4I/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?
Hemofil M	Baxter	1. Immunoaffinity chromatography 2. Solvent/detergent (TNBP/octoxynol 9)	None	2–20	Yes
Monoclate-P	CSL Behring	1. Immunoaffinity chromatography 2. Pasteurization (60°C, 10 h)	None	5–10	Yes

Table 78.4C

Products licensed in the United States to treat hemophilia B

Recombinant Factor IX Concentrate

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Stabilizer in Final Vial	Heparin in Final Vial	Human or Animal Plasma-Derived Protein Used in Culture Medium	Generation	Specific Activity (IU Factor IX/mg Total Protein)	Viral Safety Studies in Humans with This Product?
BeneFIX	Wyeth	1. Affinity chromatography 2. Viral filtration	Sucrose	None	None	Third	200–360	Yes

are referred to as very high purity, in contrast to gel and ion exchange products, which are referred to as high purity.[48,49,50,51]

Viral inactivation steps were developed in the 1980s to prevent hepatitis and HIV. Careful longitudinal studies were performed to document the safety of this approach and the pitfalls, namely, the inability to inactivate nonenvelope viruses by detergent.[50,51] Dry heat activation concentrates heated at 68°C for 32 to 72 hours are partially effective for HIV, but not for hepatitis. Prolonged dry heating at 68°C for 144 hours is used for some products, moist heating under pressurized vapor at 60°C for 10 hours for others, the latter appearing to prevent transmission of hepatitis C or HIV, but not parvovirus. A newly available human fibrinogen concentrate is heated and undergoes glycine precipitation to inactivate or remove viruses. Solvent detergent treatment of concentrates to disrupt viral lipid membranes is used in conjunction with chromatographic purification, and no cases of hepatitis C or B, or HIV have occurred with this approach. Most of the currently available factor concentrates are treated with solvent detergent.[50,51,52]

Recombinant-Derived Factor VIII and IX Concentrates

Several recombinant coagulation formulations are available for the treatment of hemophilia A.[46,47] A larger treatment of inherited and acquired hemophilia is in Chapter 51. B-region deleted recombinant factor VIII product is more stable than the natural full-length rFVIII, making production in mammalian cell culture less difficult, but it may be less bioactive than the other recombinant factor VIII products. One product was stabilized through coexpression of von Willebrand factor, and another through supplementation of the culture medium with small synthetic compounds. The first generation of recombinant FVIII were full length copies of VIII and had either human- or animal-derived protein exposure. The second generation of recombinant VIII was either B-domain deleted or full length and also had human- or animal-derived protein exposure. The current third generation recombinant FVIII have no human or animal protein exposure and are full length. Whether

Table 78.4D

Products licensed in the United States to treat hemophilia B

Coagulation Factor IX Concentrates Derived from Human Plasma

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Heparin in Final Vial (U/IU Factor IX)	Specific Activity of Final product (IU Factor IX/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?
AlphaNine SD	Grifols	1. Dual affinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80) 3. Nanofiltration (viral retention filter, <70 kDa)	0.04	>150	Yes
Mononine	CSL Behring	1. Immunoaffinity chromatography 2. Sodium thiocyanate 3. Ultrafiltration	None	>190	Yes

Table 78.4E

Products licensed in the Unites States to treat hemophilia A and von Willebrand disease

Concentrates Derived from Human Plasma that Contain Factor V78-4I and von Willebrand Factor

Product Name	Manufacturer	Method of Viral Inactivation	Heparin in Final Vial (U/mL)	Specific Activity of Final Product (IU vWF/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?	FDA Approved for Von Willebrand Disease?	Ratio of vWF: FV78-4I
Alphanate	Grifols	1. Affinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80) 3. Dry heat (80°C, 72 h)	1.0	>5	Yes	Yes	0.4–1:1
Humate-P	CSL Behring (GmbH)	1. Pasteurization (60°C, 10 h)	None	1–2	Yes	Yes	1.8–2.8:1
Koate-DV1	Talecris	1. Solvent/detergent (TNBP/polysorbate 80) 2. Dry heat (80°C, 72 h)	None	9–22	Yes	No	Unknown
Wilate	Octapharma	1. Solvent/detergent (TNBP/octoxynol-9) 2. Dry heat (100°C, 2 h)	None	>60	Yes	Yes	1:1

Table 78.4F

Products licensed in the United States to treat inhibitors

Anti-inhibitor Coagulation Complex (Activated Prothrombin Complex) Concentrate Derived from Human Plasma (For use in Patients with Inhibitors to Factor VIII or IX)

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Heparin in Final Vial (U/mL)	Specific Activity of Final Product (IU factor/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?
FEIBA	Baxter	1. Vapor heat (10 h, 60°C, 190 mbar plus 1 h, 80°C, 375 mbar) 2. Nanofiltration, 35 mm	None	0.8	Yes

Table 78.4G

Products licensed in the United States to treat inhibitor patients

Recombinant Factor VIIa Concentrate for use in Patients with Inhibitors to Factor VIII or IX

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Stabilizer in Final Vial	Heparin in Final Vial (U/mL)	Human or Animal Plasma-Derived Protein Used in Culture Medium	Generation	Viral Safety Studies in Humans with This Product?
NovoSeven RT	Novo Nordisk	1. Affinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80)	1. Mannitol, 25 mg/mL 2. Sucrose, 10 mg/mL	None	Newborn calf serum	Second	Yes

Table 78.4H

Products licensed in the United States to treat rare bleeding disorders

Human Plasma-derived Concentrate to Treat Fibrinogen (Factor I) Deficiency

Product Name	Manufacturer	Method of Viral Inactivation	Stabilizer in Final Vial	Heparin in Final Vial (U/Unit Factor IX)	Specific Activity of Final Product (mg Factor I/mg Total Protein After Addition of Stabilizer)	Viral Safety Studies in Humans with This Product?
RiaSTAP	CSL Behring	1. Pasteurization (60°C, 20 h)	Human albumin	None	20	Yes

Table 78.4I

Products licensed in the United States to treat rare bleeding disorders

Recombinant Factor VIIa Concentrate for Treatment of Factor VII Deficiency

Product Name	Manufacturer	Method of Viral Depletion or Inactivation	Stabilizers in Final Vial	Heparin in Final Vial (U/mL)	Human or Animal Plasma-Derived Protein Used in Culture Medium	Generation	Viral Safety Studies in Humans with This Product?
NovoSeven RT	Novo Nordisk	1. Affinity chromatography 2. Solvent/detergent (TNBP/polysorbate 80)	1. Mannitol, 25 mg/mL 2. Sucrose, 10 mg/mL	None	Newborn calf serum	Second	Yes

Table 78.4J

Products licensed in the United States to treat rare bleeding disorders

PCCS Derived from Human Plasma for use in Patients with Deficiencies of Factors II and X

Product Name	Manufacturer	Method of Viral Inactivation	Heparin in Final Vial (U/Unit Factor IX)	Specific Activity of Final Product (Iu Factor IX/mg Total Protein After Addition of Stabilizer)	Relative Content of Factors (FIX = 100)	Viral Safety Studies in Humans with This Product?
Bebulin VH	Baxter	1. Vapor heat (10 h, 60°C, 190 mbar pressure plus 1 h, 80°C, 375 mbar)	<0.15	2.0	X > II > IX > VII	Yes
Profilnine SD	Grifols	1. Solvent/detergent (TNBP/polysorbate 80)	None	4.5	II > IX = X > VII	Yes

Table 78.4K

Products licensed in the United States to treat rare bleeding disorders

Factor XIII Concentrate

Product Name	Manufacturer	Method of Viral Inactivation	Heparin in Final Vial (U/unit Factor XIII)	Specific Activity of Final Product (IU Factor XIII/mg Total Protein After Addition of Stabilizer)	Relative Content of Factors (FXIII = 100)	Viral Safety Studies in Humans with This Product?
Corifact, factor XIII concentrate	CLS Behring	$Al(OH)_3$ adsorption/ Vitacel defibrination, ion exchange chromatography and heating to 60°C	None	Stated on vial	Not available	No

recombinant VIII versus plasma-derived VIII promotes inhibitor formation with greater frequency is still an active question of investigation.[50,51]

Structurally and therapeutically, recombinant factor IX is comparable to monoclonal plasma-derived factor IX.[53,54] The only observed difference is the recovery recombinant factor IX at approximately 72% that of a plasma factor IX product, due to minor differences in the posttranslational modifications.

Prothrombin Complex Concentrates: Anti-inhibitor coagulant complex is a freeze-dried sterile human plasma fraction with Factor Eight Inhibitor Bypass Activity, hence the trade name FEIBA. FEIBA contains factors II, IX, and X, mainly nonactivated, and factor VII, mainly in the activated form, as well as 1 to 6 U of factor VIII coagulant antigen per mL, and traces of factors of the kinin-generating system.[46] FEIBA is indicated for the control of spontaneous bleeding episodes or to cover surgical procedures in hemophilia patients with inhibitors.[55] Other prothrombin complexes contain variable amounts of vitamin K dependent clotting factors and one has been tested for reversing the anticoagulant effect of warfarin and for severe surgical bleeding where platelet and plasma infusions were ineffective.[56]

Recombinant Factor VIIa: Production of recombinant human factor VIIa requires a mammalian expression system. The gene for human FVII, isolated from chromosome 13, is transfected into a baby hamster kidney cell line that secretes factor VII into the culture medium in its single chain form. The product is purified with murine monoclonal antifactor VII antibodies and is subjected to treatment with 0.1% Triton X-100 to inactivate enveloped viruses. Subsequent ion exchange chromatography further purifies the product and causes autoactivation, producing the activated form.[57]

Administration of rFVIIa enhances thrombin generation through direct activation of FX independent of FVIII or FIX, on thrombin-activated platelet surfaces, ensuring a full thrombin burst needed for formation of a fully stabilized fibrin plug.[57,58] rFVIIa is approved by the U.S. Food and Drug Administration for treating bleeding episodes in patients with hemophilia A or B when inhibitors to these factors are present. In Europe, rFVIIa is approved for use in alloimmunized patients with Glanzmann thrombasthenia, who are bleeding. Experimental evidence of rFVIIa for warfarin reversal in normal volunteers has not demonstrated that its use reduces blood loss.[41] In one randomized placebo controlled trial after cardiac surgery, rFVIIa showed benefit for treating bleeding, but this was at the expense of serious adverse events, including stroke.[52]

Activated Protein C (Drotrecogin-Activated): Drotrecogin α (activated) is a recombinant form of human-activated protein C (APC) that is manufactured as an inactive zymogen, enzymatically activated by thrombin cleavage and purified using monoclonal antibodies.[59] APC exerts an anticoagulant effect, along with its cofactor protein S, by inactivating factors Va and VIIIa, two critical cofactors that promote thrombin formation. APC is approved for use in severe sepsis, on the basis of decreased mortality in high-risk patients with sepsis. APC therapy increases the risk of bleeding and is contraindicated in patients with active internal bleeding, recent spinal or cranial surgery or trauma, presence of an epidural catheter, or an intracranial neoplasm or mass lesion.[59] There are no recommendations for individual dose adjustments on the basis of laboratory studies.

Antithrombin: There are two forms of antithrombin concentrate: (a) a lyophilized preparation isolated from pooled plasma by a modification of the Cohn fractionation technique with viral attenuation achieved by heat treatment at 60°C for at least 10 hours, with some products also nanofiltered, and (b) a recombinant harvested from transgenic goat milk, further purified by flow filtration, heparin affinity column, and nanofiltration, and with specific measures taken to ensure the absence of prions.[60] Antithrombin serves as a control mechanism for coagulation activation in the circulation, inhibiting thrombin, factor Xa, and other serine proteases much more efficiently than when those factors are bound to platelets or fibrin. Antithrombin is approved for use in the treatment of patients with hereditary antithrombin deficiency, as prophylaxis against thrombosis following surgery or obstetric procedures, and for therapeutic use when such patients have thrombosis. Debate continues about the use of antithrombin concentrates in patients with "acquired" antithrombin deficiency, such as in cases of sepsis and DIC, or in those with relative heparin resistance during surgery and extracorporeal circulation.[60] The product is not approved for pediatric use. Replacement therapy is designed to achieve levels of 120%, with subsequent doses administered empirically to keep levels

at 70% to 120%. In stable antithrombin-deficient patients, the half-life of infused antithrombin is 60 to 90 hours after an initial half-disappearance time of 22 hours. In acute DIC, the half-life is usually 50% that of stable patients, and may be as short as 4 hours.

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CHAPTER

79

New Approaches for the Therapy of Bleeding Disorders, Including Gene Therapy

GILBERT C. WHITE, II AND HAROLD R. ROBERTS

Better understanding of the molecular mechanisms of blood coagulation and the structure and function of blood clotting factors, along with advances in recombinant DNA technology have placed us on the threshold of remarkable new approaches to the treatment of bleeding disorders. Gene therapy is the most visible of these new approaches, but mechanisms now exist to actually make coagulation factors that are better than the native proteins. Some of these new approaches are the subject of this chapter.

HISTORICAL PERSPECTIVE

The treatment of bleeding disorders has traditionally been through the use of fresh frozen plasma or plasma-derived coagulation factor preparations. The first of these coagulation factor preparations was cryoprecipitate, which was developed by Judith Graham Pool in 1964. Cryoprecipitate was enriched in factor VIII, as well as fibrinogen, von Willebrand factor, and fibronectin, and for the first time provided a method of increasing factor VIII levels with less volume overload. Later came other coagulation factor preparations, including glycine-precipitated purified factor VIII and barium sulfate–purified prothrombin complex concentrates containing factors II, VII, IX, and X. These were produced commercially and revolutionized the treatment of hemophilia A and B, leading to home treatment. The prothrombin complex concentrates were also effective in the treatment of patients with coagulation factor inhibitors, allowing treatment of these individuals for the first time. The mechanism of action was uncertain, but activated clotting factors in the concentrates were thought to "bypass" the inhibitor block in the coagulation cascade. Although these crude plasma-derived concentrates changed the treatment of hemophilia, they contained blood-borne viruses that were transmitted to hemophiliacs. By the early and middle 1970s, hepatitis B and a new non-A, non-B form of hepatitis, later renamed hepatitis C, were becoming epidemic in the hemophilic population. Even worse, human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) appeared in the hemophilic population in the early 1980s and eventually led to the untimely death of thousands of patients with hemophilia. In response to these blood-borne infections, the academic–industry complex developed physical and chemical methods for inactivating hepatitis, HIV, and other blood-borne viruses, used monoclonal antibodies to factors VIII and IX to generate antibody affinity-purified ultrapure forms of both factors, and developed recombinant DNA techniques for producing factors VIII and IX, resulting in synthetic forms of both factors that were essentially free of blood-borne contaminants. As a result of these and other advances, current products used to treat hemophilia are the safest ever developed.

Despite their safety, the current products have some drawbacks. They are expensive, require intravenous administration, and must be administered frequently because of the short biologic half-life of the normal coagulation factor proteins. For the average hemophiliac, who might bleed as many as 50 times a year or more, the cost of replacement therapy with current products can be as much as $50,000 per year. Prophylaxis, the treatment to prevent bleeding and the damage to joint and tissues, requires treatment two to three times a week or 100 to 150 times a year, with costs in excess of $50,000 and considerable damage to veins from frequent venipuncture. Developing technologies to make clotting factors that are cheaper, last longer after infusion into patients, or those that could be administered by alternative routes (including oral) would be advantageous. Gene therapy is another approach that has received considerable attention. This chapter reviews these new approaches to the treatment of bleeding disorders. The focus of the chapter is primarily on hemophilia A, but the concepts can be applied broadly to all bleeding disorders.

GENE THERAPY APPROACHES

Gene therapy is literally the use of genes, DNA or RNA, as treatment of a particular condition or disorder. In most cases, it is the proteins produced from the genes that have the intended effect in gene therapy, although DNA itself can have effects. For monogenic disorders like hemophilia or cystic fibrosis, the intent of gene therapy is curative. The aim is to use normal genes to express enough normal protein to correct the abnormality. One of the great promises of gene therapy is the potential ability to cure monogenic disorders such as hemophilia. For complex multigenic disorders, such as atherosclerosis, diabetes, or cancer, the aim of gene therapy is modification of the disease process. The goal might be to express a protein or gene that is toxic to a cancer cell or inhibits smooth muscle proliferation in atherosclerosis.

The origins of gene therapy lie in the remarkable advances in recombinant DNA technology that occurred in the middle and latter half of the last century. The ability to isolate and manipulate DNA, and the development and use of viral and nonviral plasmid vectors to introduce DNA into cells where the DNA products could be expressed were critical developments. In one

of the early reports using direct injection of DNA into cell nuclei, Jaenisch and Mintz reported injecting SV40 DNA into mouse blastocysts and finding SV40 gene sequences developing from the blastocysts in the animals.[1] The notion of using viruses as vectors to introduce new genetic information into cells evolved very quickly. By 1979, several laboratories were employing the SV40 virus, using viral promoter, splicing, and polyadenylation sequences in plasmid vectors to introduce β-globin sequences into cells and drive transgene expression.[2,3,4] Retroviruses, which are adept at gaining entry into cells and inserting their genetic material into the host genome, soon followed. Defective retroviruses in which the malignant transforming gene region was eliminated, were shown to retain the ability to transduce cells.[5–7] The first gene transfer experiments in humans were carried out by Cline in 1980 in two subjects with severe β thalassemia.[8] Nucleated cells, obtained by bone marrow aspiration, were transfected *in vitro* using a pBR vector containing a herpes simplex virus thymidine kinase (*HSVtk*) marker gene, and then returned to the subject intravenously. HSVtk sequences were detected at low levels 1 to 2 weeks after transfection and lasted 3 months in one subject and 9 months in the second.

Hemophilia A is a monogenic disorder which, like sickle cell disease, cystic fibrosis, and muscular dystrophy, has historically been considered as an attractive target for treatment using gene transfer approaches. Primary prophylaxis studies in which clotting factor concentrates have been administered to maintain low levels of factor VIII have shown that near-continuous correction of the clotting factor defect prevents bleeding in unaffected joints and delays progression in diseased joints.[9–11] Logically, more continuous levels of factor VIII, as might be anticipated with gene therapy, provide the rationale for the enthusiasm for this approach in hemophilia A.

Other aspects of hemophilia A add to its attractiveness as a candidate disorder for gene transfer (see Table 79.1). As a monogenic disorder, correction of the factor VIII defect should correct the phenotype. Furthermore, unlike monogenic inborn errors of metabolism, levels of factor VIII are not regulated in any way and do not require feedback or other control as, for example, insulin requires regulation by blood glucose levels. Another important feature is that complete correction of the factor VIII level is not absolutely necessary in hemophilia for gene transfer to be effective. For example, an increase in plasma levels of factor VIII by even as little as 1% to 2% could have a beneficial effect, especially in patients with severe hemophilia. Persistence of expression after gene transfer might also have beneficial effects. Given the biological half-life for current plasma products of approximately 12 hours, expression of factor VIII for even a month would change patients' lives. Factor VIII message is detected in a number of tissues,[12] but as long as the factor VIII that is expressed after gene transfer is processed correctly and has access to blood, it does not matter where the protein is produced. There is no current evidence that synthesis of factor VIII in tissues outside its site of normal synthesis is detrimental to that tissue. Therefore, it is not necessary to target the factor VIII to a particular tissue or to its tissue of natural synthesis. In addition, factor VIII is a secreted protein that would be expected to gain easy access to blood. The gene for factor VIII is well characterized and, based on homology modeling, much is known about the structure of the factor VIII protein and the function of the various structural domains.[13] A low-resolution crystal structure of factor VIII is available.[14] Good clinical and laboratory parameters of efficacy are available. Large and small animal models of hemophilia are available for preclinical trials.[15,16] Finally, safe and effective treatments for hemophilia currently exist, and so subjects can make appropriate decisions about participation in clinical trials without the pressure engendered by the lack of adequate treatment.

Table 79.1

Hemophilia as a target for gene transfer

Monogenic disorders; correction of factors VIII or IX will correct the phenotype
Complete correction of factors VIII or IX is not required; any level of factor VIII will have a clinical effect
Production of factors VIII or IX can occur in any tissue that has access to blood
The genes for factors VIII and IX are well characterized and protein structure is understood
Large and small animal models of hemophilia A and B exist
Safe and effective treatments for hemophilia A and B exist

Gene Transfer Vectors*

Viral Vectors

Viruses have evolved highly efficient mechanisms for entering human cells and using the DNA/RNA replication machinery of the cell to produce viral products.[17] By replacing viral coding sequences with genes of particular interest (e.g., the cDNA for factor VIII) one can use viruses as agents to introduce the gene into cells where they use the cellular machinery to produce therapeutic quantities of the protein. The obvious advantage of viruses as vectors is their native ability to get into cells; the general disadvantage is their toxicity.

Retrovirus Vectors

Retroviruses are enveloped RNA viruses that use reverse transcriptase to convert virion RNA into double-stranded DNA; the DNA is subsequently integrated into the genome of the cell. The surface of the retrovirus consists of a lipid bilayer with protruding membrane proteins, GP41 and GP120, both encoded by the *env* gene. The tropism of the virus, that is the host cell that is targeted by the virus, is determined by these surface proteins. For example, for HIV-1, GP120 mediates the interaction of HIV-1 with CD4 lymphocytes. For gene therapy vectors, it is common to replace the retroviral *env* gene with other sequences that change the tropism of the vector. This can be done to either target a particular tissue or cell or to broaden the tropism of the vector to increase the number of cells that are transduced. Two sequences that are frequently used to broaden the tropism of retrovirus vectors are the amphotropic envelope gene of

*Sections of text on gene transfer vectors and phase I gene transfer trials in hemophilia A and B have also been used by G. C. White in the first edition of the *Textbook of Hemophilia*, eds. Lee, C., Berntorp, E. and Hoots, W. K., Blackwell Publishing, Oxford, UK.

murine leukemia viruses, which is able to interact with a widely expressed phosphate transporter on human cells, and the glycoprotein of vesicular stomatitis virus, which interacts with a ubiquitous receptor on cells. Current retroviral vector production is through a three-part packaging system, consisting of a genome vector that contains the packaging signal and the cDNA of interest, an expression vector that contains the pseudotyped envelope glycoprotein, and an expression vector that expresses the viral *gag* and *pol* genes. These are assembled into vector particles in a packaging cell. The retroviral packaging systems in current use appear safe and are designed to prevent the generation of replication-competent virus. In addition, sufficient modifications are made in the viral sequence to minimize the possibility of homologous recombination with retroviral sequences in target cells. Self-inactivating ("sin") vectors are ones in which the long-terminal repeats (LTRs) have been removed to prevent activation of downstream cellular genes.

Vectors derived from the Moloney murine leukemia virus (MoMLV) were the first retroviral vectors used for gene transfer. MoMLV has a 9-kb cDNA expression cassette and is therefore able to accept the entire 7.1-kb factor VIII cDNA or 1.9-kb factor IX cDNA. Although MoMLV can be produced in large quantities and is able to efficiently gain entry into cells, it only transduces dividing cells,[18] a disadvantage for many tissues such as liver where the rate of cell replication is low. Strategies for inducing cell division in the liver using partial hepatectomy,[19,20] chemical injury,[21] or hepatocyte growth factor[22–25] have been proposed to increase retrovirus-mediated transduction.

More recently, vectors derived from lentiviruses, including HIV-1, have been developed.[26,27] Members of this family of retroviruses include feline immunodeficiency virus, simian immunodeficiency virus, and equine infectious anemia virus.[28–30] Like MoMLV, lentiviral vectors have a 9-kb cDNA expression cassette. These vectors retain some of the attractive features of the Moloney-based retroviral vectors, such as stable integration into the host chromosome and targeted cellular uptake through coat proteins, and they are able to infect nondividing and dividing cells.[26,31] More recent observations provide greater understanding of the mechanisms of nuclear import by lentiviruses and may lead to improvements in cell transduction. During reverse transcription (RT) of HIV-1, a three-stranded DNA flap is formed that is composed of the central polypurine tract from the *pol* gene and central termination sequences. This flap functions as a cis-acting element to enhance nuclear import and improve gene transduction in nondividing cells.[32–35] Other cis-acting DNA elements that enhance transgene expression, perhaps through a mechanism like the DNA flap, have been identified, including the matrix attachment region from immunoglobulin κ[36] or from β interferon.[37,38]

Although retroviral integration is not sequence-specific, there is increasing evidence that it is also not completely random, as was once thought. The ability of retroviruses to successfully replicate themselves depends on integration of the viral DNA into transcriptionally active areas of the host cell chromosome. Integration into silent areas would prevent expression of viral proteins and inhibit replication. Studies to map the site of retrovirus integration demonstrate that different viruses have different patterns of integration.[39–41] With murine leukemia viruses (MLV), for example, transcriptional start sites are favored targets. For HIV-1, active genes are preferential integration targets, especially genes that are activated by infection of the cell by the retrovirus. Regional hotspots also exist.[39] Whether the predilection for active genes results from increased chromatin accessibility, locally bound transcription factors, or some other influence is not clear. In contrast, for avian sarcoma leukosis virus, an avian retrovirus, the virus does not prefer transcription start sites or active genes,[41] a feature that might confer an advantage with regard to gene therapy and the risk of insertional mutagenesis (see below).

Retroviruses insert their DNA into the host chromosome and are therefore termed *integrating vectors*. Because of their integrating properties, insertional mutagenesis is a concern with some retroviruses. Integration of retroviral sequences near protooncogenes has been known to be capable of activating their expression, contributing to tumorigenesis in animal models.[42,43] A report of T-cell leukemia in subjects with X-linked severe combined immunodeficiency syndrome treated by MLV-mediated gene transfer[44] shows that insertional mutagenesis occurs in man. The subjects were among 10 receiving *ex vivo* retrovirus-mediated gene transfer into autologous $CD34^+$ bone marrow cells. Approximately 3 years after treatment, the subjects developed acute T-cell lymphocytic leukemia. The leukemic T-cells showed evidence for retroviral insertion near the LMO2 proto-oncogene promoter. Because of the survival advantage that the cells expressing the retroviral γC-transgene had over the normal T-cells, it has been suggested that the insertional mutation in the LMO2 promoter region resulted in uncontrolled clonal expansion of the corrected T-cells, initially correcting the severe combined immunodeficiency and then resulting in T-cell leukemia.

Adenovirus Vectors

Adenoviruses (Ads) are linear, double-stranded DNA viruses that infect a wide variety of human cells, both dividing and nondividing, including lung, liver, heart, and brain. The 36-kb viral genome consists of a number of early and late genes. Transcription and translation of the early genes modulates cell function to facilitate replication of the viral DNA, whereas the late genes are involved in the structural aspects of viral replication. The icosahedral adenoviral capsid is composed of three major components: the hexon, penton base, and knobbed fiber. Viral entry into cells is accomplished through an initial high-affinity interaction between the knobbed fiber[45,46] and a widely expressed, 46-kDa coxsackie-adenovirus receptor (CAR) on the surface of cells.[47] After the initial interaction with CAR, entry of the virus into the cell proceeds through clathrin-mediated endocytosis, by an interaction between the penton base and $\alpha_V\beta_3$ and $\alpha_V\beta_5$ integrins on the cell surface. Disruption of the viral capsid occurs in the endocytic vesicle and the viral genome enters the nucleus through nuclear pores. In the nucleus, the adenoviral genome does not integrate into the chromosomes of the host cell, functioning instead from an extrachromosomal or episomal template.

Adenoviral vectors characteristically elicit an intense inflammatory response. This is due in part to capsid proteins, which provoke a brisk humoral and cellular immune response.[48] At the same time, Ad is designed to evade the immune response that it elicits.[49,50] Products of the *E1A* gene interfere with nuclear factor κB (NFκB) which is a key regulator of the innate antiviral response by cells. Products of the *E3* gene are also known to subvert the host immune response. Other gene products play a role, as well. One of the goals in the generation of adenoviral vectors

is to retain the high degree of efficiency that these vectors have with respect to cell infection while moderating or eliminating the inflammation that they cause. First-generation adenoviral vectors were deleted in E1 and E3, removing some of the primary mediators of the immune response while increasing the expression cassette to 6.5 kb. The *E2* genes were removed in the second-generation vectors and, in recent vectors, all of the viral coding sequences have been removed, rendering the vector "gutless."[51,52] Although preclinical studies indicate that removing these proteins may reduce the cellular immune response,[53–55] there is also evidence that the virion shell alone elicits substantial cellular responses.[56]

There are at least 47 different adenoviral serotypes, each immunologically distinct. The development of an immune response to one vector serotype generally precludes readministration of that vector. Adenoviral vectors for gene transfer are primarily Ad2 or Ad5.

The only death that was directly attributable to a gene transfer trial occurred with an adenoviral vector in an 18-year-old subject with ornithine transcarbamylase deficiency.[57] The second-generation vector used in this study was based on human Ad5 and was deleted in E1 and E4. Following administration of the vector in the right hepatic artery, altered mental status and jaundice were noted 18 hours later, and the ensuing clinical course was marked by a systemic inflammatory response syndrome with high levels of interleukin-6 (IL-6) and IL-10, biochemically detectable disseminated intravascular coagulation, and multiple organ system failure, leading to death 98 hours following gene transfer. The story of Jesse Gelsinger has been well-chronicled in the news media.[58]

Adenovirus-Associated Virus Vectors

Adenovirus-associated viruses (AAV) are small, nonenveloped, single-stranded DNA viruses, which require helper virus to facilitate efficient replication. In the absence of helper virus-mediated replication, the wild-type virus persists in the host cell in a latent state. The 4.7-kb genome of AAV consists of two inverted terminal repeats (ITRs) and two open reading frames that code for the rep and cap proteins. There are four rep proteins that function in regulating AAV replication and three cap proteins that form the protein coat of the virus. Wild-type AAV stably integrates into the host genome at the AAVS1 region of chromosome 19q13.3-qter.[59] Two of the *rep* gene products, rep68 and rep78, are believed to direct integration to the AAVS1 site.[60] Nine different AAV serotypes have been identified, AAV1 to 9, each with distinct tissue tropism.[61] AAV8 targets primarily liver, whereas AAV1 and AAV7 transduce muscle with high efficiency. The cellular receptor for AAV2, the serotype most commonly used for gene transfer studies, is heparan sulfate, a glycosaminoglycan that is variably present on the surface of most cells.[62] Slow-twitch (slow myosin-expressing) skeletal muscle fibers have higher concentrations of heparan sulfate proteoglycan on their surface and are better transduced by AAV2 than fast-twitch fibers.[63] Interestingly, AAVS1, the site of integration, is closely linked to the slow skeletal troponin *T* gene, TNNT1,[64] raising an interesting correlate with the preference of AAV for slow myofibers. The integrin $\alpha_v\beta_5$ and fibroblast growth factor receptor 1 are coreceptors for AAV2.[65,66]

Typical AAV gene transfer vectors have been derived from AAV2. The ITRs are retained but the rest (96%) of the viral genome has been removed, including the *rep* gene. As a result, AAV vectors currently in general use are unable to integrate into the host chromosome in a site-specific manner, and persistence as integrated sequences appears to be a low-frequency event, if it occurs at all. Efforts to quantify *in vivo* integration in skeletal muscle and liver suggest that at least 99.5% (skeletal muscle) or at least 90% (liver) of persisting AAV vector DNA was episomal, present in large concatemers.[67,68]

The AAV expression cassette is approximately 5 kb, too small to accommodate the whole factor VIII cDNA but large enough to accommodate the factor IX cDNA. Several groups have separately packaged factor VIII light and heavy chain sequences under the control of minimal transcriptional regulatory elements into AAV vectors and demonstrated expression *in vivo* after coinfection.[69–72]

AAV are not associated with any known disease and are not pathogenic.[73] On the basis of this nonpathogenicity, the toxicity associated with AAV has been expected to be low. In preclinical studies, AAV has been well tolerated with no effects on hematopoiesis, liver function, or other organs. Reports of late tumors in mice treated with AAV vectors[74] have not been confirmed and the chromosomal breaks that have been observed in cells following AAV integration[75] are of uncertain significance, especially because integration by AAV vectors lacking the *rep* gene is infrequent. However, in liver-directed human trials for hemophilia B, transient liver enzyme abnormalities have been reported and were associated with loss of transgene expression.[76]

Recent advances in AAV technology include the production of self-complementing (sc) forms of the vector that overcome the rate-limiting complementary-strand DNA synthesis step that is the primary barrier to transduction *in vivo* and lead to faster onset of protein expression and higher transduction efficiency.[77,78] This is accomplished through deletion of the terminal resolution site from one of the AAV terminal repeats (TRs) thereby enabling, after entry into the cell and uncoating, intramolecular base pairing of the viral DNA within the mutant TR that then proceeds to formation of a double-stranded molecule. The disadvantage of scAAV is the reduced insert size. A second advance has been the continued evolution of the identification of naturally occurring AAV serotypes and the idea of cross-packaging vector genomes from one AAV serotype into the capsid proteins of other AAV serotypes, generating "pseudotyped" vectors. This pseudotyping permits novel targeting strategies and can be used to reduce immune neutralization of vector.[79,80,81]

Plasmid Vectors

Nonviral approaches for delivering therapeutic genes as naked plasmid DNA are attractive because plasmid DNA is relatively simple and inexpensive to produce, does not engender cell-mediated immune responses, and does not result in humoral immune responses against the DNA vehicle that would limit the opportunity for repeated delivery. Until recently, most plasmid DNA applications have resulted in short-lived (days to weeks) transgene expression. Recent attempts to use electrical[82] or hydrodynamic[83] approaches to augment target cell uptake of plasmid DNA have resulted in expression of clotting factors lasting months after delivery. By rapidly treating mice through the tail vein with factor VIII expression sequences under the control of transcriptional regulatory elements optimized for liver expression, in large fluid volumes, supraphysiologic factor VIII levels were obtained.[83] Immune responses that are species independent have been reported.[84] Despite the attractive features of

nonviral approaches, current technical limitations reduce the likelihood of human use in the near future.

Spliceosome-Mediated Pre-mRNA Transsplicing

A specific repair mechanism that may be especially applicable to the common inversion mutations that cause approximately 30% of hemophilia A is an approach termed *spliceosome-mediated mRNA transsplicing*.[85] This method is used to repair messenger RNA, rather than DNA. In the final steps of transcription, introns are removed and exons are spliced together to form mature, functional mRNA. The site of splicing is directed by 30 to 40 nucleotides at each end of the intron that form consensus binding sites for the spliceosome, a large ribonucleoprotein complex composed of small nuclear ribonucleoprotein particles that catalyze the splicing reaction. Spliceosome-mediated pre-mRNA transsplicing takes advantage of this normal cellular process to effect RNA repair. The method involves synthesis of a pretranssplicing molecule (PTM) that contains the corrected mRNA fused to a 30 to 40 nucleotide sequence that targets a specific pre-mRNA. The PTM binds to the pre-mRNA, allowing specific transsplicing of the corrected mRNA through normal mechanisms.

Chao et al.[86] used a transsplicing approach to correct the hemophilic defect in factor VIII knockout mice. The repair PTM consisted of a 125-nucleotide binding domain that was complementary to intron 15 of the gene, a spacer sequence, and a strong 3′ splice acceptor site, linked to exons 16 to 26 for the mouse factor VIII cDNA. Correctly transspliced factor VIII was detectable genetically and functionally in the treated mice, with up to 17% factor VIII activity observed. It was estimated that conservatively between 1.6% and 6.3% of mutant transcripts were being repaired.

Enhanced Translational "Read Through"

In prokaryotes and eukaryotes, aminoglycoside antibiotics cause ribosomes to read through stop codons during translation.[87,88] As a result, these antibiotics can suppress nonsense genotypes, increasing expression of the full-length protein and partially restoring the normal phenotype. In tissue culture cells, restoration of gene expression to as much as 20% of wild-type levels has been observed after aminoglycoside treatment.[87] Clinical trials of gentamicin in cystic fibrosis showed that translational "read through" could be obtained in patients who were both homozygous and heterozygous for stop mutations with a reduction in basal potential difference in nasal mucosa and an increase in response to chloride-free isoproterenol solution.[89] Approximately 9% of hemophilia A and hemophilia B mutations are nonsense mutations that might respond to aminoglycoside treatment. Several small preliminary studies have examined the responses to aminoglycoside antibiotics in patients with hemophilia A and B and nonsense mutations with inconsistent results.[90,91,†]

Gene Editing

Cells possess normal DNA repair mechanisms that edit or correct mistakes that can occur during DNA replication. Li et al.[92] took advantage of this gene editing process to correct factor IX expression in a murine hemophilia model. A site-directed zinc finger nuclease that induced double-stranded DNA breaks at a targeted region of the *F9* gene together with a promoterless wild-type cDNA cassette containing *F9* exons 2 to 8 as a corrective donor template were codelivered to liver with an appropriately designed AAV8 vector. With this approach, they were able to achieve partial correction of the gene defect, with expression of up to 6% to 7% functional levels of factor IX for an extended time. Although the levels achieved were only a fraction of what has been achieved in mice with more traditional gene transfer approaches, the study demonstrated the ability to use a targeted gene editing approach to correct gene defects. The potential advantage of this approach is the ability to correct multiple defects, from small deletions to point mutations, over a large segment of the *F8* or *F9* gene using a single system. The key to successful gene editing will be the ability to improve the targeting efficiency, which was only 1% to 3% in the study.

Induced Pluripotent Stem Cell Approach

A number of laboratories have explored the use of induced pluripotent stem (iPS) cells to effect gene transfer. The general idea is to take an accessible tissue, skin being most accessible, extract fibroblasts or keratinocytes from the skin and grow them in tissue culture. The differentiated fibroblasts are reprogrammed to a pluripotent state by the simultaneous expression of four transcription factors: Oct3/4 and Sox2 plus either Nanog and LIN28 or KLF4 and c-Myc.[93–95] The normal *F8* or *F9* gene is then inserted *in vitro* into the iPS cells by homologous recombination, the cells expressing the transgene selected, differentiation initiated under growth factor guidance, and the cells then inserted into the patient. The obvious advantage of this approach is the transplanted cells are immunologically identical with the host, so immune responses other than to the transgene should be nonexistent. Another advantage is that the site of gene insertion in the transduced iPS cells can be determined and cells with insertions in tumor suppressor genes can be eliminated. The highest expressing cells can also be selected for transplantation. Several labs have explored this approach in preclinical models.[96,97] Factor levels of 8% to 12% were observed for out to 3 months.

Codon Optimization

Codon optimization through protein sequence-conserving changes in the DNA codons has evolved as a method to improve the efficiency of gene expression.[98–101] Practically, there are several ways to achieve this, including increasing the guanine-cytosine content (GC content) of the transgene, which increases mRNA stability, removal of cryptic splice sites, removal of RNA instability elements, and utilizing the most frequently used codons for a given amino acid, which typically have high concentrations of tRNAs and therefore have the fastest rates of and most accurate translation. For example, CTC is the most frequently used codon for leucine in man, and human cells generally have developed higher concentrations of tRNA for this codon than for less frequently used codons for leucine. The higher concentrations of tRNA for CTC results in more efficient translation. Doing this for the whole transgene improves gene expression. Current gene therapy trials in hemophilia use codon-optimized *F9* and *F8*.

[†]Sommer, S, unpublished data.

Phase I Gene Transfer Trials in Hemophilia A and B‡

Before gene transfer trials can be carried out in humans, clinical grade vectors—viral, plasmid, or naked DNA—must undergo rigorous testing. Vectors driving expression of factor VIII or factor IX are first tested in tissue culture systems, then in small animal models, usually mice, in large animal models, usually dogs or monkeys, and in factor VIII– or factor IX–deficient models, usually knockout mice and/or hemophilic dogs. These studies test the safety and efficacy of the vectors. Current U.S. National Institutes of Health (NIH), U.S. Food and Drug Administration (FDA), and the European Agency for the Evaluation of Medicinal Products (EMEA) regulations for gene transfer trials are available at http://www4.od.nih.gov/oba/rac/guidelines/guidance.html, at http://www.fda.gov/cber/genetherapy/gtpubs.htm under guidance, and at http://www.emea.eu.int/index/indexh1.htm, respectively. Safety must be rigorously demonstrated before initiating human trials.

Clinical trials have been performed in hemophilia A and B (see Table 79.2).[102,103–107]

Chinese Trial in Hemophilia B

The initial clinical trial in hemophilia was undertaken at Fudan University in China in 1991.[102] This was a phase I trial in hemophilia B using *ex vivo* retrovirus-mediated gene transfer in autologous skin fibroblasts.[108] Two vectors were used: XL-IX, a MoMLV -based vector containing human factor IX cDNA under control of the retroviral LTR, and N2CMV-IX, a Moloney-based vector containing human factor IX cDNA under control of the cytomegalovirus (CMV) promoter.

Two brothers with moderately severe hemophilia B, ages 9 and 13, were studied. Baseline factor IX activity and antigen levels were 2%. Skin fibroblasts obtained by biopsy and maintained in culture were transduced with XL-IX or N2CMV-IX. Following demonstration of *in vitro* synthesis of factor IX, the resulting HBSF-IX cells were injected subcutaneously into multiple sites in the wall of the abdomen and the back at approximately monthly intervals. A total of 6.6 to 11 $\times$ 10^8 cells were injected. No treatment-related side effects were observed. An increase in factor IX was observed in both subjects, as early as 15 to 20 days after the first set of injections. Although the pretreatment variation in factor IX levels was not reported, plasma factor IX antigen and activity following treatment was increased to more than twofold in both subjects and was reported to persist for more than 420 days. There was also a reduced tendency to bleed. One subject received an additional set of injections of HBSF-IX cells at 16 months after factor IX levels had diminished and demonstrated another increase in factor IX levels in response to the injections.

Transkaryotic Therapy (TKT) Inc. Trial in Hemophilia A

In overall design, this trial employed an *ex vivo* approach,[109] similar to that employed in the initial Chinese trial in hemophilia B, except that the TKT trial used a nonviral, plasmid vector rather than a viral vector and the target disorder was hemophilia A rather than hemophilia B. The plasmid used for the TKT trial contained the cDNA for the B-domain-deleted form of human factor VIII under control of the CMV promoter.

A total of 12 subjects, ranging in age from 19 to 71 years, were studied, 9 in a dose-escalation trial, and 3 in a comparison of sites of intraperitoneal injection. Seven of the twelve were HIV-seropositive without AIDS and all were hepatitis C virus (HCV)-seropositive with stable liver function. Dermal fibroblasts obtained from the subjects by excisional skin biopsy were electroporated with the plasmid containing the B-domain-deleted human factor VIII cDNA. Fibroblasts expressing the factor VIII gene were clonally expanded, characterized for the stability of the integrated gene sequences and production of factor VIII, and administered by laparoscopic intraperitoneal injection, either into the fat of the greater omentum, or into the fibrofatty tissue that bound the lesser omentum. Laparoscopy was performed under general anesthesia. A total of 100, 400, or 800 million autologous clonal cells were injected in three cohorts of three subjects per cohort. Follow-up was up to 24 months. There were no serious adverse events related to the study material, the skin biopsy, or the intraperitoneal injection. No inhibitors to factor VIII were reported. Seven of the twelve subjects demonstrated a decreased bleeding frequency and/or factor VIII use following cell transfer, with low levels of factor VIII detected transiently in plasma, up to 1% to 2% of normal, with a maximum of 4%. Injection of cells into the lesser omentum appeared to be less effective than injection into the greater omentum. The first six subjects in this study were reported by Roth et al.[103]

Chiron Trial in Hemophilia A

The Chiron study was designed as a phase I, open-label, multi-institution, single-dose, dose-escalation trial in volunteers with severe hemophilia A using a replication-deficient MoMLV-derived vector to deliver B-domain-deleted human factor VIII cDNA (hFVIII[V]). The vector was complement-resistant and employed a human packaging cell line system with reduced homology between the retroviral vector and the packaging components, thereby allowing production of high-titer virus production without the generation of replication-competent retrovirus (RCR).[110] Expression of the B-domain-deleted factor VIII was driven by the retroviral LTRs.

The study was undertaken in subjects over the age of 18 with severe hemophilia A and no inhibitor.[104] Subjects could be HIV seropositive or seronegative, but were required to have stable CD4 counts over 400 per mm^3 and were excluded if taking RT inhibitors because of their effect of the (hFVIII[V]) on RT. Five doses, 2.2 $\times$ 10^7, 9.2 $\times$ 10^7, 2.2 $\times$ 10^8, 4.4 $\times$ 10^8, and 8.8 $\times$ 10^8 transduction units per kg, were administered in divided daily doses by peripheral vein injection. A total of 13 subjects were enrolled, three at each of the first four doses and one at the highest dose. The infusion of (hFVIII[V]) was well tolerated, with no serious adverse events. There was no acceleration of chronic HIV or HCV disease and all tests for RCR were negative. No factor VIII inhibitor activity was detected in any subject. Although no subject had sustained levels >1%, six patients showed factor VIII levels of more than 1% on at least two occasions, 5 or more days after infusion of exogenous factor VIII. Most elevated levels were in the range of 1.0% to 1.8%, although isolated levels of 2.3%, 3.0%, 4.3%, and 6.2% were reported for three subjects. Elevated levels of factor VIII were detected as early as 8 days following treatment. Peripheral

‡Sections of text on gene transfer vectors and phase I gene transfer trials in hemophilia A and B have also been used by G. C. White in first edition of the *Textbook of Hemophilia*, eds. Lee, C., Berntorp, E. and Hoots, W. K., Blackwell Publishing, Oxford, UK.

Table 79.2

Gene therapy for hemophilia clinical trial summary

	Subject	Age	Dose	Inhibitor	Semen PCR	Peak Factor[a]
Chinese study[102]—hemophilia B *ex vivo* transduction	1	9	11×10^8 cells	No	Unk	5.92%
	2	13	6.6×10^8 cells	No	Unk	4.13%
TKT study[103]—hemophilia A *ex vivo* transfection	1	Unk	1×10^8 cells	No	Neg	<0.8%
	2	Unk	1×10^8 cells	No	Neg	<0.8%
	3	Unk	1×10^8 cells	No	Neg	2.0%
	4	Unk	4×10^8 cells	No	Neg	<0.8%
	5	Unk	4×10^8 cells	No	Neg	1.0%
	6	Unk	4×10^8 cells	No	Neg	4.0%
	7	Unk	4×10^8 cells	No	Neg	<0.8%
	8	Unk	4×10^8 cells	No	Neg	<0.8%
	9	Unk	4×10^8 cells	No	Neg	<0.8%
	10	Unk	8×10^8 cells	No	Neg	<0.8%
	11	Unk	8×10^8 cells	No	Neg	1.1%
	12	Unk	8×10^8 cells	No	Neg	1.0%
Chiron study[104]—hemophilia A retrovirus	2–1	50	2.8×10^7 TU/kg	No	Neg	1.8%
	5–1	33	2.8×10^7 TU/kg	No	Neg	6.6%
	5–2	30	2.8×10^7 TU/kg	No	Neg	3.0%
	3–1	51	9.2×10^7 TU/kg	No	Neg	1.7%
	3–2	55	9.2×10^7 TU/kg	No	Neg	1.3%
	5–3	50	9.2×10^7 TU/kg	No	Neg	6.2%
	2–2	20	2.2×10^8 TU/kg	No	Neg	1.1%
	3–3	43	2.2×10^8 TU/kg	No	Neg	<0.1%
	5–4	18	2.2×10^8 TU/kg	No	Neg	2.6%
	1–1	52	4.4×10^8 TU/kg	No	Pos	<0.1%
	5–5	48	4.4×10^8 TU/kg	No	Neg	1.4%
	5–6	18	4.4×10^8 TU/kg	No	Neg	4.4%
	5–7	20	8.8×10^8 TU/kg	No	Neg	2.1%
Avigen study[105]—hemophilia B muscle-directed AAV	1	38	2×10^{11} vg/kg	No	Neg	3.7%
	2	23	2×10^{11} vg/kg	No	Neg	0.8%
	3	67	2×10^{11} vg/kg	No	Neg	<0.3%
	4	29	6×10^{11} vg/kg	No	Neg	<0.1%
	5	44	6×10^{11} vg/kg	No	Neg	<0.1%
	6	43	6×10^{11} vg/kg	No	Neg	1%
	7	38	1.8×10^{12} vg/kg	No	Neg	<0.1%
	8	30	1.8×10^{12} vg/kg	No	Neg	<0.1%
Genstar study—hemophilia A "gutless" Ad	1	23	4.3×10^{10} vg/kg	No	Unk	N/A
Avigen study[106]—hemophilia B liver-directed AAV	1	63	2×10^{11} vg/kg	No	Pos	<0.1%
	2	49	2×10^{11} vg/kg	No	Pos	<0.1%
	3	20	1×10^{12} vg/kg	No	Unk	<0.1%
	4	22	1×10^{12} vg/kg	No	Unk	<0.1%
	5	31	5×10^{12} vg/kg	No	Unk	11.8%
	6	28	5×10^{12} vg/kg	No	Unk	3%
	7	20	1×10^{12} vg/kg	No	Unk	<0.1%
St Jude's study[107]—hemophilia B liver-directed AAV	1	63	2×10^{11} vg/kg	No	Pos	<0.1%
	2	49	2×10^{11} vg/kg	No	Pos	<0.1%
	3	20	1×10^{12} vg/kg	No	Unk	<0.1%
	4	22	1×10^{12} vg/kg	No	Unk	<0.1%
	5	31	5×10^{12} vg/kg	No	Unk	11.8%
	6	28	5×10^{12} vg/kg	No	Unk	3%

PCR, polymerase chain reaction; AAV, adeno-associated virus; TU, transduction units; vg, vector genomes; Unk, unknown.
[a]Peak factor levels. In the Chiron study, this was defined as the highest level observed in those individuals who demonstrated levels >1% on at least two occasions, 5 or more days after infusion of exogenous factor.

blood mononuclear cells demonstrated the presence of vector gene sequences by polymerase chain reaction (PCR) testing to 6 months in all 10 subjects tested and to 1 year in three of four subjects tested, indicating long-term persistence of vector sequences even at the lowest dose. Pharmacokinetic examination following the infusion of exogenous factor VIII 13 weeks after vector infusion showed a statistically increased half-life (T1/2) and area under the curve compared to prestudy values. Bleeding frequency was decreased in six subjects compared to historical rates. A single, transient, low-level positive signal was detected in a single semen sample from one subject at week 9; no other unusual events or complications were observed.

Avigen Trial in Hemophilia B

The Avigen trial was a prospective, multi-institution, single-dose, dose-escalation, phase I trial to examine the safety of an adeno-associated virus vector to deliver human factor IX to skeletal muscle in subjects with severe hemophilia B.[105,111] Coagulin-B, the recombinant AAV produced by Avigen, which was used in the study, was derived from serotype AAV-2.

A total of seven subjects were enrolled in the trial, three at 2×10^{11}, three at 5×10^{11}, and one at 2×10^{12} vg/kg. Vector was administered under general anesthesia through ultrasound-guided injections in the *vastus lateralis* muscle of the leg. The number of injections ranged from 10 to 72, depending on the dose administered. Muscle biopsies performed 8 to 12 weeks after vector administration showed immunohistochemical evidence for factor IX in the extracellular space in a pattern similar to that observed in preclinical studies. There was no muscle injury or inflammation. Although subjects had detectable pretreatment titers of neutralizing antibodies against AAV that were as high as 1:1,000, gene transfer and expression was demonstrated in each subject by Southern blot on DNA extracted from muscle and by RT-PCR. No antibodies against factor IX were detected. There were no detectable vector sequences in semen at any time. Increased plasma levels of factor IX were observed on multiple occasions beginning approximately 8 weeks after vector administration in one subject with cross-reacting material positive severe hemophilia B receiving 2×10^{11} vg/kg. The highest level was 1.6% at week 10 and increased levels were detected up to at least 22 weeks. A second subject had levels up to 0.8%. Both subjects had reduced requirement for replacement factor.

Genstar Trial in Hemophilia A

The aim of the Genstar trial was to examine the safety of MaxAdFVIII, a "gutless" Ad vector derived from Ad5.[112] The vector was devoid of all viral genes except essential cis-elements and carried a 27-kb expression cassette that contained the full-length factor VIII cDNA under the control of the human 12.5-kb albumin promoter.

The Genstar trial was designed as a phase I, multi-institutional, single-dose, dose-escalation study in subjects with severe hemophilia A with normal liver function. One subject was enrolled in the trial. Infusion of MaxAdFVIII in that subject was associated with high fevers at the time of injection. There was an approximately 50% decrease in platelet count, starting posttreatment day 1 with a return to normal by day 7, and a 10-fold increase in alanine aminotransferase starting on posttreatment day 2 and returning to normal by approximately day 20. The reduction in platelet count and increase in liver enzymes constituted serious adverse events and the trial was placed on hold by the FDA and eventually discontinued.

Avigen Liver-Directed Trial in Hemophilia B

The AAV2 vector used in the second, liver-directed Avigen trial was similar to that used in the initial muscle trial but contained the α_1-antitrypsin promoter and apolipoprotein E-enhancer/hepatic control locus region to drive liver-specific factor IX expression.

The trial was a phase I, single-dose, dose-escalation study to examine the safety of liver-directed AAV-AAT-FIX in severe hemophilia B.[106] A total of seven subjects were enrolled, ranging in age from 20 to 63 years. Three cohorts of two patients each received doses of 2×10^{11}, 1×10^{12}, or 5×10^{12} vg/kg by injection directly into the liver via cannulation of the hepatic artery. The liver-directed injections were well tolerated. Two subjects at the highest dose achieved significant increases in factor IX level, one to 11.8% and the second to 3%. However, expression in both subjects was transient and the loss of expression was accompanied in the subject with the highest expression level by significant liver enzyme abnormalities starting approximately 3 weeks after vector administration.[81,106] A third subject treated with 1×10^{12} vg/kg also developed transient asymptomatic transaminitis. T-cell immune responses to two conserved AAV-2 capsid peptides were identified in this subject. Because of the grade 3 liver toxicity and the lack of persistent effect, the trial was discontinued.

No inhibitors to factor IX were detected. The first two subjects developed PCR-positive signals in sperm. Both subjects cleared vector signal over time and all subsequent subjects were advised to use contraception until the semen was negative.

St. Jude AAV Trial in Hemophilia B

A third AAV trial sponsored by St. Jude's Children Hospital utilized a pseudotyped, scAAV8-based vector containing codon-optimized FIX under control of a liver-specific LP1 promoter (scAAV8-LP1-FIXco).[107] Vector was administered by peripheral vein injection.

A total of six patients with severe hemophilia B have been treated in three dose cohorts. AAV-mediated gene expression was observed in all six subjects, with FIX levels of 2% to 11%. Two subjects in the high dose cohort developed asymptomatic elevation of liver enzymes, one associated with AAV8 capsid protein positive T cells. Treatment with steroids resulted in normalization of liver function and maintenance of FIX expression levels. No inhibitors have been detected.

NEW APPROACHES TO THE SYNTHESIS OF COAGULATION FACTOR CONCENTRATES

Despite intense interest in gene transfer approaches, coagulation factor concentrates continue to be the mainstay of treatment in hemophilia and other bleeding disorders. In the developed world, approximately 85% of patients with hemophilia are treated with recombinant concentrates, either factor VIII, factor IX, or factor VIIa. The commercial synthesis of these factors has not changed substantially since the initial products were introduced in 1990, although the promise of recombinant technology has always been the ability to use it to make better products. With increasing knowledge of the three-dimensional structure of coagulation proteins and better correlates between structure and the function of these molecules, it becomes more feasible to use recombinant technology to make proteins that have improved function. Some of the current directions in developing improved coagulation factor proteins are described here.

Current recombinant clotting factor concentrates are synthesized in well-characterized mammalian cells such as Chinese hamster ovary and baby hamster kidney cells. The proteins synthesized in these cells are fully active and are identical with purified plasma-derived proteins in their ability to participate in blood coagulation. There are six wild-type polymorphic forms of factor VIII (H1-H6), but only two, H1 and H2, are produced recombinantly for clinical use (Recombinate and Advate from Baxter are H1; Kogenate and Kogenate FS from Bayer are H2).[113] There are minor differences between recombinant and plasma proteins that are due in part to differences in the posttranslational modifications that take place in tissue culture cells compared to human cells. All three plasma proteins contain complex *N*- and *O*-linked carbohydrates. In the recombinant proteins, the carbohydrates are attached normally, but the complex structure of the *N*-linked carbohydrates in the recombinant proteins differs from that in the native molecules. Although the carbohydrate changes do not appear to affect the function of the recombinant molecules, it is possible that other unknown functions may be affected. Plasma factor VIII contains six sulfated tyrosine residues at positions 346, 718, 719, 723, 1,664, and 1,680[114] and these appear to be present and fully modified in recombinant factor VIII. Plasma factors IX and VIIa each contain 12 γ-carboxylated glutamic acid (Gla) residues in the amino-terminal portion of the molecule that are important for binding of the proteins to phospholipid surfaces and therefore for the coagulant activity of the proteins. Recombinant factor IX is less than fully γ-carboxylated, with approximately 60% of the recombinant molecules containing 12 Gla residues, 35% containing 11 Gla residues, and 5% containing 10 Gla residues.[115,116] The less than fully modified proteins demonstrated a specific clotting activity that was indistinguishable from the fully modified form. Plasma factor IX contains a single β-hydroxylated aspartic acid residue, which appears to be comparably modified in recombinant and plasma factor IX. In addition, factor IX contains a sulfated tyrosine at amino acid 155 and a phosphorylated serine at amino acid 158 in the activation domain that appear to be important for the distribution of the protein in the circulation. In plasma factor IX, these two residues are fully modified. In recombinant factor IX, Ser158 is not phosphorylated, and Tyr158 is only 15% sulfated.

Synthesis of Properly Modified Proteins

One major area for improvement in the production of recombinant clotting factor proteins is better control of the posttranslational modification of proteins to more closely approximate plasma proteins. For example, the recent identification of the genes for vitamin K carboxylase and vitamin K epoxidase /reductase[117–119] potentially permit better control of the carboxylation process during the tissue culture synthesis of the vitamin K-dependent proteins. More importantly, full sulfation of Tyr155 and phosphorylation of Ser158 in the activation domain of factor IX would be expected to improve the recovery of factor IX and have a beneficial effect in the treatment of hemophilia B. Because changes in the conditions of synthesis or structure of recombinant coagulation factors require extensive preclinical and clinical testing to compare the new product with the old before the changes can be approved by the FDA, the cost of modification can be very high. As a result, the improvement in molecular function must be considerable to make the cost worthwhile.

Active forms of factors VIII and IX have been synthesized in transgenic livestock.[120–123] Velander et al. showed that the factor VIII and IX produced in mammary epithelium is catalytically active and can be easily purified from endogenous milk proteins. Expressed under control of the whey acidic protein or β-lactoglobulin promoter, factor VIII or IX concentration in bovine or porcine milk is over 10-fold to 200-fold higher than in plasma.[121] The porcine mammary gland has been shown to carry out the posttranslational processing necessary for full biologic activity and normal half-life in the human circulation. The advantage of the transgenic approach in milk is the large volume of protein that can be produced and the ease of purification because of the small number of admixed endogenous proteins. In addition, it has been suggested that the large amount of protein that can be produced from transgenic animals makes alternative delivery methods, including oral methods, feasible.

Super-Molecules

One of the promises of recombinant technology is the ability to make proteins that have enhanced functions. In the case of coagulation factors, this might be increased coagulant activity, or an increased half-life in the circulation, or reduced immunogenicity. As the structure and function of clotting factors become better defined, it may be possible to synthesize coagulation factor molecules that are actually better than the native proteins. Early attempts to make such molecules are underway.

Coagulation Factors with Improved Coagulant Function

Proteins with increased coagulation factor activity have been achieved by a number of laboratories. In some cases, this has been accomplished by an actual improvement in the catalytic efficiency of the molecule. For example, the catalytic efficiency of factor IX was increased by the replacement of selected sequences in the catalytic domain of factor IX with the corresponding sequences from factor X.[124] Substitution of selected sequences from the catalytic domain of factor X for the corresponding sequences in the catalytic domain of factor IX resulted in a 56-fold increase in the amidolytic activity of factor IX. There was no significant change in inhibition by *p*-aminobenzoic acid (PABA), an S1 pocket–directed reversible inhibitor of serine proteases, demonstrating that the integrity of the active site was unchanged. Further modifications, including alterations in the 99-loop, a structure unique to factor IX which is believed to shield the active site prior to assembly of the tenase complex, led to more impressive increases in the amidolytic activity of factor IX.[125] Building on these observations, Milanov et al. showed that a triple mutant factor IX (V181I/K265T/I383V—termed ITV) exhibited almost 16% the activity in factor VIII-deficient plasma that wild type factor IX exhibited in normal plasma.[126] In other words, the mutant ITV factor IX was able to by-pass the need for factor VIII. Chang et al.[127] showed that the apparent activity of factor IX could also be enhanced by replacing the epidermal growth factor (EGF)-1 domain of factor IX with the corresponding domain from factor VII. The increased factor IX activity was demonstrated both *in vitro* and in a canine model. The EGF-1 substitution increased the rate but not the extent of factor Xa generation, suggesting that the EGF-1 domain of factor VII was more efficient in promoting assembly of the tenase complex. Although the immunogenicity of the chimeric factor IX-VII molecule was not examined in the canine model, the

substitution of whole domains may preserve the tertiary structure of the domain but the altered protein might be immunogenic. Factor IX with increased activity occurs through a natural mutation, too,[128] an observation that may be of use in gene therapy.

Although factor VIII does not have intrinsic protease activity, the cofactor function of factor VIII in generating tenase activity can be similarly enhanced. Substitution of the factor VIII sequences that direct thrombin cleavage to a site between the A2 and A3 domains with sequences that direct thrombin cleavage of heparin cofactor II, increased factor VIII cofactor activity.[129] The increased factor VIII activity is believed to result from improved cleavage at a critical site in thrombin-induced activation of factor VIII.

Factors with Increased Survival

Another approach to generating improved forms of factor VIII has been to synthesize molecules that have prolonged activity in the circulation. Factor VIII circulates as an inactive procofactor that must be activated by thrombin to participate in the activation of factor X. Factor VIII is then rapidly inactivated by either spontaneous dissociation of the A1 and A2 domains or by further proteolytic cleavage by activated protein C. A critical event in the inactivation of factor VIII is the dissociation of the A2 domain from the light chain and A1 domain (see Chapter 12). The A2 domain exists in a metal-ion complex with the other domains and either falls apart spontaneously or during cleavage by activated protein C. By linking the A2 domain to the light chain, the activity of factor VIII can be considerably prolonged *in vitro*.[130,131] This inactivation-resistant factor VIII shows persistence for up to 40 minutes after activation, a marked increase compared with native factor VIII. The rapid inactivation of factor VIII activity under normal physiologic conditions suggests that there may be biologic advantages to limiting the presence of activated factor VIII in the circulation. Persistence of activated factor VIII might lead to more generalized generation of thrombin and an increased risk of thromboembolism. Modifications of factor VIII that increase its activity—either by decreasing the inactivation of activated factor VIII or by increasing the intrinsic catalytic activity of the protein—must be examined carefully in *in vivo* models before clinical trials proceed in humans.

Interfering with the interaction of factor VIII with receptors involved in the clearance of clotting factors from the circulation constitutes another method to prolong the survival of factor VIII in the circulation. The advantage of this approach is that one does not have to worry about the effect of increased circulation of an activated clotting factor. The low density lipoprotein-related protein (LRP) receptor, a hepatic protein that has been implicated in the clearance of low-density lipoproteins and other proteins from the circulation, has been shown to interact with and bind factor VIII through cysteine-rich low-density lipoprotein receptor class A repeats in the LRP extracellular domain.[132,133] Heparin sulfate proteoglycans may also participate in the clearance of factor VIII by LRP. Conditions that inhibit the interaction of factor VIII with LRP prolong the survival of factor VIII in hemophilic mice.[134] More general approaches to prolong clotting factor half-life include modification with polyethylene glycol (PEG). PEG has been used to prolong the half-life of a number of molecules, including interferon and erythropoietin. Preclinical studies and initial human trials with PEG-factors VIII, VIIa, and IX are currently underway.[135–139]

Another approach to prolong the half-life of clotting proteins is the creation of fusion proteins with long-lived plasma proteins such as immunoglobulin and albumin. Both of these proteins are cleared from the circulation by neonatal Fc receptors (FcRn) on endothelial cells that recirculate the proteins through an endocytic pathway.[140] Clinical trials of factor IX (rFIXFc–B-Long) and factor VIII (rFVIIIFc–A-Long) fused to an Fc molecule are currently underway (see www.clinicaltrials.gov[141,142]) and albumin-fused factor VIII, factor IX, and factor VIIa molecules are in various stages of development.[143–145]

Immunologically Altered Proteins

Inhibitors in hemophilia A are a common and serious complication that renders normal therapy with replacement factor VIII ineffective. Forms of factor VIII that evade or prevent an immune response would be advantageous for patients with inhibitors. One approach to generate antigenically altered factor VIII, based on the observation that porcine factor VIII is able to substitute for human factor VIII in coagulation but is poorly recognized by antibodies to the human protein, has been to engineer chimeras of human and porcine factor VIII.[146,147] The substitution of porcine sequences, either entire domains or selected sequences, into human factor VIII significantly reduced recognition of the molecule by inhibitor antibodies while preserving participation in thrombin generation *in vitro*. Nevertheless, although the porcine/human hybrids were not well recognized by established human inhibitor antibodies, it remains to be determined whether the hybrid molecules are immunogenic themselves. Porcine factor VIII is variably immunogenic in humans and the hybrid sequences might be capable of generating an immune response in some individuals. Despite this, the concept of engineering factor VIII genetically to reduce antigenicity would seem to be a viable approach as we learn more about inhibitor epitopes and the three-dimensional structure of factor VIII. The critical question with this approach is whether we can understand the antigenicity and immunogenicity of proteins well enough to engineer molecules that lack both.

Improved Synthesis of Coagulation Factors

Another area for improvement is in the efficiency of protein synthesis in mammalian cells (see Chapter 5 for general background). The endoplasmic reticulum (ER) is a major site for the folding and assembly of secretory proteins such as factor VIII and factor IX (FIGURE 79.1A).[148] Proteins destined for the cell surface are cotranslationally translocated into the lumen of the ER where the proteins undergo various posttranslational modifications and proper folding.[148,149] These reactions and the orderly movement of proteins through the ER and the Golgi apparatus are facilitated by enzymes and molecular chaperones present in the various compartments of the ER and Golgi. Two homologous protein chaperones that regulate transport of improperly folded glycoproteins through the ER are calnexin, a conserved integral ER membrane protein, and calreticulin, an ER lumenal protein. Calnexin and calreticulin associate transiently and selectively with newly synthesized glycoproteins in the early stages of folding and are thought to promote the interaction of misfolded proteins with disulfide isomerases that enhance proper folding.[150] The oxidizing environment of the ER facilitates the action of these disulfide isomerases to catalyze and monitor disulfide bond formation in an ordered manner.

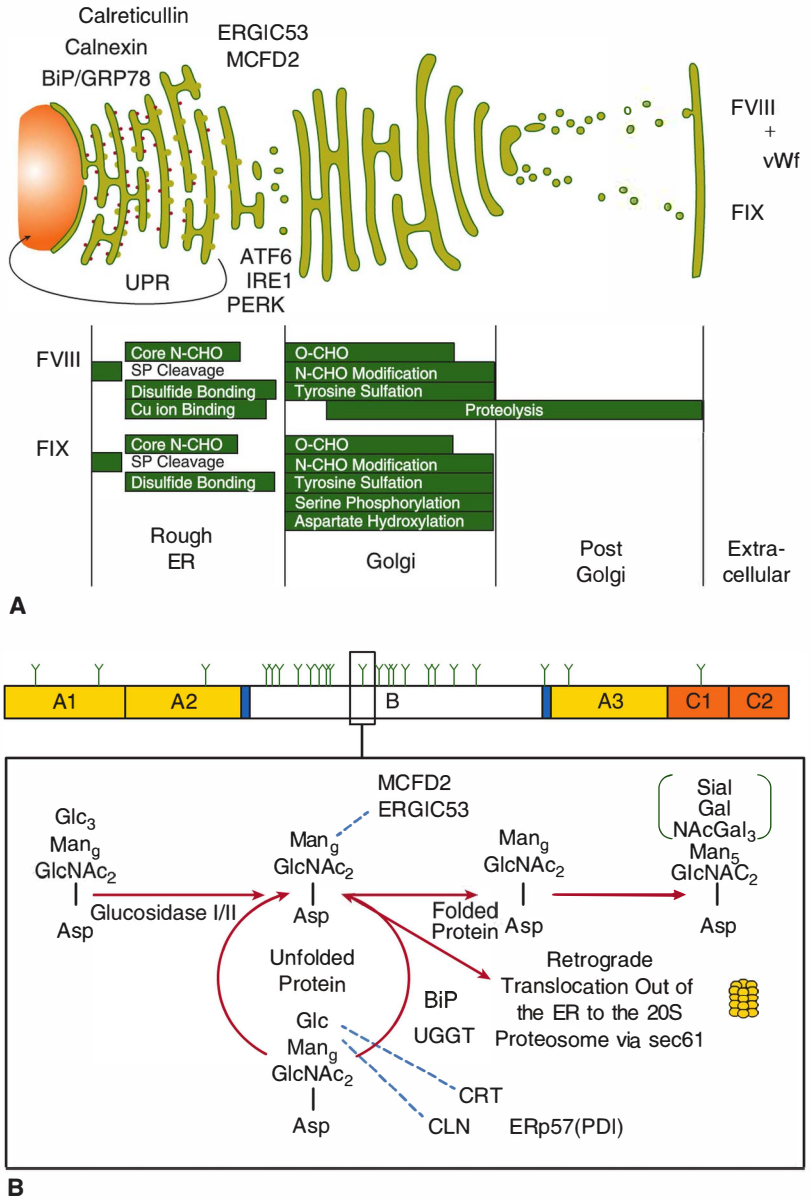

FIGURE 79.1 Cellular biosynthesis and assembly of factor VIII and IX. Secretory proteins like factors VIII and IX undergo complex intracellular processing. **Panel A:** Factors VII and IX are cotranslationally translocated into the lumen of the endoplasmic reticulum (ER) where they undergo various modifications and folding. After cleavage of the signal peptide, factor VIII and factor IX undergo specific posttranslational modifications. Copper atoms are bound to factor VIII in the rough ER. Protein folding begins and disulfide bonds are formed, providing stability to the tertiary structure of the protein. These changes are monitored by chaperone proteins, calnexin, calreticulin, and BiP/GRP78, which provide "quality control" to the folding process. The presence of unfolded protein in the ER leads to an integrated signaling response that transmits information from the ER to the nucleus to affect protein synthesis. ATF6, IRE 1, and PERK are ER transmembrane proteins that initiate transduction of the UPR. Also in the ER, *N*-linked carbohydrates are added to target asparagine residues, a process that is thought to be critical to the monitoring process by chaperone proteins. Once the protein is properly folded, it can bind to the ERGIC53/MCFD2 complex and transit to the Golgi apparatus. In the Golgi, the *N*-linked carbohydrates are modified, *O*-linked carbohydrates are added, and target tyrosine residues are sulfated. Like factor VIII, factor IX undergoes extensive posttranslational processing in the ER and Golgi, but the secretion of factor IX is more efficient than that of factor VIII and there does not appear to be the same critical monitoring of factor IX structure that occurs for factor VIII. In particular, the ERGIC53/MCFD2 complex is not involved in factor IX movement through the ER-Golgi. **Panel B:** The folding cycle for factor VIII. The *N*-linked carbohydrates in factor VIII play an important role in proper folding of the protein in the ER. Following the addition of carbohydrates, the terminal-glucose residues are sequentially removed by glucosidases. A UGGT in the ER senses improperly folded proteins by an unknown process and is activated by the unfolded protein to transfer glucose to the carbohydrate. Calnexin and calreticulin bind to the monoglucosylated protein, leading to an interaction with ERp57, a protein disulfide isomerase that stimulates disulfide bond formation. The protein undergoes another cycle of deglucosidation and, if still improperly folded, interaction with UGGT, monoglucosylation, binding to calnexin and calreticulin, and disulfide bond formation. Once properly folded, the factor VIII binds to the ERGIC53/MCFD2 complex to transit to the Golgi. Conversely, if the protein fails to fold properly, it is eventually translocated out of the ER through an ATP-dependent interaction with BiP. (Adapted from Kaufman RJ. Orchestrating the unfolded protein response in health and disease. *J Clin Invest* 2002;110:1389–1398.)

Prolonged association of calnexin or calreticulin is observed when proteins are misfolded or unable to oligomerize. The calnexin or calreticulin recognition motifs are at least partially determined by the structure of asparagine-linked oligosaccharides. Another resident ER protein, immunoglobulin-binding protein (BiP)/glucose-regulated protein (GRP78), uses energy from adenosine triphosphate (ATP) hydrolysis to promote folding and prevent aggregation of protein in the ER.[151] Feedback mechanisms exist in the ER that also promote proper folding. There is an unfolded protein response (UPR) that senses excess unfolded protein in the ER and generates signals to the nucleus to upregulate chaperone proteins such as calnexin, calreticulin, and BiP to increase proper folding.[148] Amino-terminal fragment (ATF)6, IRE1, and protein kinase-like ER kinase (PERK) are three proteins in the ER that signal to bZip transcription regulators in the nucleus to synthesize new chaperone proteins.

The synthesis of factor VIII has been studied intensely. At a molecular level, glucosylation controls factor VIII transit through the ER–Golgi (FIGURE 79.1B). In the ER, factor VIII undergoes *N*-linked glycosylation. After translocation into the lumen of the ER, a core unit of 14 saccharides (GlcNAc2Man9Glc3) is added to targeted Asp residues. The terminal-glucoses are trimmed, first by glucosidase I, which cleaves the first glucose, and then by glucosidase II, which cleaves the second and third glucoses. If the protein is properly folded, the deglucosylated protein is able to interact with the ER–Golgi intermediate compartment (ERGIC53)/multiple coagulation factor deficiency 2 (MCFD2) complex, probably through the terminal mannose groups on the proteins, and factor VIII is transferred through the intermediate compartment to the Golgi apparatus.[152] If the protein is not folded properly, it is recognized by a UDP-glucose:glycoprotein glucosyl-transferase (UGGT), which transfers a single glucose to the high mannose containing side chains. The activity of the UGGT to add this single glucose is stimulated by the unfolded protein although the sensing mechanism is uncertain. The monoglucosylated protein interacts with calnexin and calreticulin, which direct the interaction of factor VIII with ERp57, a protein disulfide isomerase that stimulates disulfide bond formation, leading to increased protein folding. The protein then undergoes another cycle of deglucosylation by glucosidase I/II, interaction with UGGT, interaction with calnexin and calreticulin, and disulfide bond formation. Once the protein is properly folded, it is no longer recognized by UGGT and is not glucosylated. The high mannose protein is then able to interact with ERGIC53, a mannose-binding cargo protein in the intermediate compartment that transfers the protein to the Golgi apparatus. The interaction with ERGIC53 is facilitated by MCFD2, a calcium BiP that forms a complex with ERGIC53.[153] In the Golgi, the mannose groups are trimmed to a core of GlcNAc2Man5, knocking off ERGIC53, which recycles to the ER to transport more protein to the Golgi. If, despite maximal folding, the protein remains misfolded, it is removed from the ER. This degradative pathway is through a transport protein called sec 61 to the 20S proteasome and is facilitated by BiP.

The synthesis of factor VIII, in particular, is inefficient compared with factor IX, factor VII, or even factor V, which is highly homologous with factor VIII. Studies by Kaufman, Pipe et al.[149] have shown some of the reasons for the inefficient production of factor VIII. First, factor VIII secretion from cells is affected by its interaction with the chaperone protein BiP/GRP78 and subsequent ATP-dependent release. The requirement of ATP hydrolysis by BiP/GRP78 is unique for factor VIII and is not required for release of factor V. Binding of factor VIII to BiP/GRP78 occurs through Phe309 in the A1 domain and mutation of this residue results in improved factor VIII secretion efficiency.[154] Interestingly, Phe309 is adjacent to Cys310, which is a site of copper binding in factor VIII (see Chapter 12). It has been suggested that binding of copper to factor VIII stabilizes the factor VIII structure and BiP/GRP78 may monitor this stability. Second, the B-domain of factor VIII is important for efficient secretion. The B-domain-deleted form of factor VIII is 10 times less efficiently secreted than full-length factor VIII. The important sequences in the B-domain for this are the amino-terminal 226 amino acids of the B-domain, which contain six asparagine-linked glycosylation sites. Mutation of these asparagines residues to glutamine reduces the efficiency of factor VIII secretion, perhaps by reducing the interaction of factor VIII with ERGIC53/MCFD2.[155]

The increased understanding of the transport of secretory proteins like factor VIII through the ER and Golgi will have its greatest application in the synthesis of recombinant proteins for clinical use, not as a method for treatment of hemophiliacs, although methods of increasing protein production in mild hemophilia might be useful. In the manufacture of recombinant clotting factor proteins, any enhancement in the amount of protein synthesized per cell would reduce the cost of production and result in cheaper products for patients, allowing more frequent treatment and prevention of bleeding.

SUMMARY

The treatment of hemophilia and other bleeding disorders is on the threshold of a new era. Clinical trials of gene transfer approaches have been started and although continued advances in the field promise to impact hemophilia in the future. Current understanding of the structure and function of factors VIII, IX, VII, and other clotting factors also promise to lead to the development of new and better proteins. We are at a point in time where we can truly consider the possibility of making proteins that are potentially better than those that exist in nature. However, the pursuit of making such molecules must be done carefully, and with circumspection.

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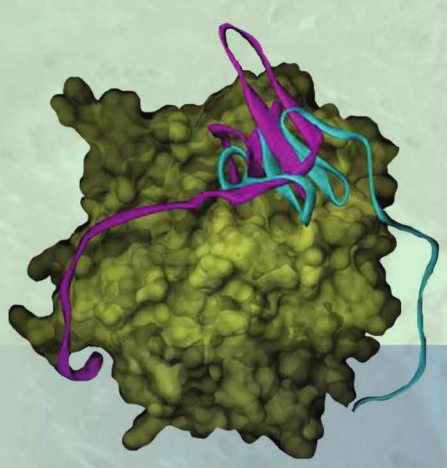
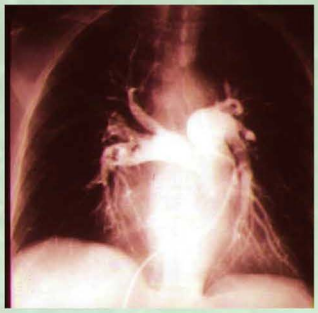
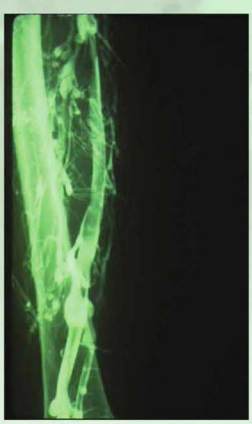


PART IV





THROMBOTIC DISORDERS

CHAPTER 80

Overview of Venous Thromboembolism

SAM SCHULMAN, VICTOR J. MARDER, AND GILBERT C. WHITE, II

HISTORICAL BACKGROUND

Thrombosis as a clinical condition was probably described almost 5,000 years ago, when the Chinese emperor Huang Ti wrote of a likely arterial event, "when it coagulates within the pulse the blood ceases to circulate beneficially; when the blood coagulates within the foot it causes pains and chills."[1] The Greek physician Claudius Galen (131 to 201 AD) coined the term "thrombosis," which means curdling, but the first thorough analysis of the pathogenesis of thrombosis was described by the German pathologist Rudolf Virchow (1821 to 1902).[2] He concluded that "consequences" (rather than causes) "of the obstruction could be grouped into three categories," (a) irritation of the vessel and its surroundings, (b) blood coagulation, and (c) interruption of the blood stream,[2] the combination of which was later named Virchow's triad.

RISK FACTORS FOR VENOUS THROMBOEMBOLISM

Research during the past 3 to 4 decades has identified abnormalities that are etiologic factors for venous thromboembolism (VTE).[3] These abnormalities can be categorized as hereditary or acquired, or, alternatively, as increased coagulation factor activity (factor V Leiden, prothrombin G20210A mutation), decreased coagulation inhibitor levels (antithrombin, protein C, endothelial protein C receptor, protein S), or antiphospholipid antibody disruption of hemostasis. In addition, abnormalities of the fibrinolytic system and protective mutations of coagulation factors (e.g., factor XIII Val34Leu) may have roles in thrombosis. The hereditary thrombophilias (see Chapter 81) mainly contribute to VTE, but there is controversy as to their importance in arterial thrombosis.[4,5] On the contrary, the antiphospholipid syndrome is associated with both venous and arterial thromboembolism (see Chapter 101).

The need for investigation of thrombophilia in patients with VTE is controversial. Screening for thrombophilia in patients without previous VTE, but who are at increased risk (estrogens, pregnancy, major surgery), is hardly ever cost-effective.[6] Acquired risk factors can be temporary, chronic, or permanent. Temporary risks include surgery, immobilization, estrogen exposure, L-asparaginase treatment, long-haul travel, infection, heparin-induced thrombocytopenia, and indwelling catheters. Long-distance travel may or may not present a need for prophylactic therapy; for example, if deep-vein thrombosis (DVT) after an 8-hour car trip 1 month earlier is the only identifiable risk factor, one may be hesitant about the association and whether the thrombosis is provoked or unprovoked. Chronic or permanent risk factors include immobilization such as after spinal cord injury or stroke, inflammatory bowel disease, systemic lupus erythematosis, nephrotic syndrome, cancer, myeloproliferative disorders, and paroxysmal nocturnal hematuria (see FIGURE 80.1 for more information).

The reported incidence of VTE varies from 104 per 100,000 in a study of medical records in Worcester, Massachusetts,[7] to 387 per 100,000 in a cohort study in Sweden.[8] Differences relate to methodology for data capturing, selection of population, and diagnostic techniques. There is a definite need to establish trends accurately in order to show when implementation of preventive measures has had any impact and also to determine how changes of patient demographics (age, body weight) play out in the disease panorama (see Chapter 82).

DIAGNOSIS OF VENOUS THROMBOEMBOLISM

Clinical symptoms and signs of DVT or pulmonary embolism (PE) are not sufficient for diagnosis, but the combination of risk factors and select symptoms and signs is used in clinical prediction, for example, with the Wells score for DVT or PE[9,10] or the Geneva score for PE.[11] The revised Wells scores for DVT and PE allow one to utilize low, moderate, or high probability of VTE for assessing unlikely or likely outcomes.[12,13] Patients with unlikely or low probability of VTE can be assessed with a D-dimer test, which, if negative, allows for a highly negative predictive value for VTE. This strategy allows for a 50% reduction of expensive imaging for VTE diagnosis.

The "gold standard" of imaging of the leg veins using venography has been almost entirely abandoned in favor of noninvasive compression ultrasound. Similarly, computed tomography of the pulmonary arteries (chest CT) for imaging of PE is utilized more often than ventilation–perfusion lung scanning (see Chapter 83). In spite of progress in diagnostic approach, there are unresolved issues, for example, the extent of ultrasound examination, DVT recurrence, diagnosis of PE in pregnancy, and VTE assessment of suspected PE in hospitalized patients with elevated D-dimer levels. The PIOPED III study demonstrated that gadolinium-enhanced magnetic resonance angiography has a sensitivity of 78%, which is suboptimal, and many centers have a high proportion of technically inadequate images.[14] Conversely, multidetector chest CT may be too sensitive, identifying subsegmental filling defects of questionable clinical significance.

The assessment of the emergency room patient with possible VTE requires not only a correct diagnosis but also an assessment of risk. This is of particular importance for patients with PE, for whom there is no high-quality evidence for the safety of outpatient treatment. The Pulmonary Embolism Severity Score, based on identifying risk strata that allow prognostic accuracy,[15,16] describes 30% of patients with low risk who have

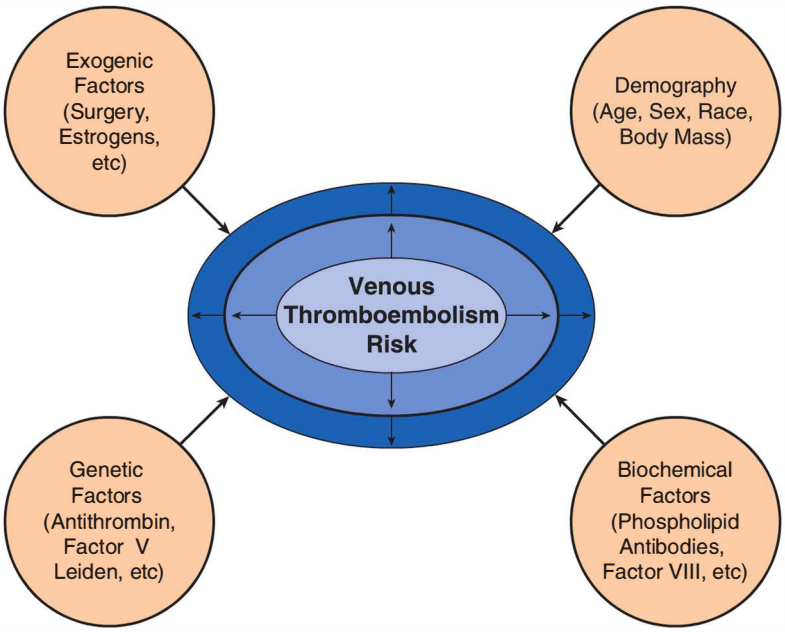


FIGURE 80.1 Risk factors for VTE.

a 30-day mortality of 1% and can be treated as outpatients. For the remaining 70% of patients, cardiac biomarkers (troponin, brain natriuretic peptide [BNP], pro-BNP, and heart-type fatty acid–binding proteins) combined with clinical score may be useful to identify additional patients for home treatment.

LOCATION OF VENOUS THROMBOEMBOLISM

Most DVT occurs in the lower extremity, and consequently almost all randomized controlled trials on management are based on this thrombus location. Upper extremity thrombosis of the brachial, axillary, and subclavian veins constitutes 4% to 10% of DVT,[17] and chronic sequelae with venous insufficiency are not uncommon (FIGURE 80.2). The basilic and cephalic veins are superficial, not deep veins.

Superficial phlebitis is mainly an inflammatory process secondary to chemical or mechanical irritants or in varicose veins with very slow blood flow. It is often complicated by a self-limited clot formation. It is difficult to find any data on the incidence of superficial (thrombo-) phlebitis, but in an Italian study of 15,000 patients seeking advice from their general practitioner for any medical condition, there was a history of this disorder in 10.8% of the women and 4.9% of the men.[18] It is unclear how patients should be selected for 4 to 6 weeks of treatment with low molecular weight heparin (LMWH) or fondaparinux versus short-term anti-inflammatory therapy.[19]

The most common acquired cause for upper extremity DVT is an indwelling catheter; thoracic outlet syndrome accounts for 30% of upper extremity DVT (2 events/100,000/year).

Cerebral vein or sinus thrombosis is diagnosed in two persons per million each year.[20] Patients can present with headache, papilledema with blindness, seizures, hemianopsia, aphasia, hemiparesis, or cognitive disorders, depending upon thrombus location and infarct extension. Nowadays, the diagnosis is usually obtained early with MRI or CT, and appropriate treatments can be started to reduce intracranial hypertension, avoid seizures, and control the clot size (FIGURE 80.3). The prognosis has improved compared to a few decades ago; the ISCVT study of 624 patients showed 80% recovery with fatal outcome in 8%.[21] Residual headache is usually related to chronic intracranial hypertension, and continuing seizure activity occurs in 10% of patients, less so after the first year.

Splanchnic vein thrombosis includes mesenteric, portal, and hepatic (Budd-Chiari syndrome) vein thrombosis. The incidence of Budd-Chiari syndrome or isolated mesenteric vein thrombosis is <1 in a million per year, whereas extrahepatic portal vein thrombosis occurs in at least 4 per million.[22] The majority of mesenteric or portal vein thrombosis occurs in the presence of other disorders, mainly cancer but also chronic myeloproliferative disorders, hepatic cirrhosis, pancreatitis, inflammatory bowel disease, or as a postoperative complication. Hypercoagulability plays an important role with inherited thrombophilia identified in one-third of the patients.

Among patients with thrombosis associated with myeloproliferative disease, the JAK2 V617F mutation is highly prevalent,[23] present in up to 96.5% of patients. There is no controversy regarding the need for anticoagulant therapy, but the duration of secondary prophylaxis must balance the risks of recurrence against that of bleeding.

Renal vein thrombosis is a rare complication in severely ill neonates, often with poor prognosis for the renal function.[24] It also occurs in adults associated with renal transplantation, with nephrotic syndrome with urinary loss of antithrombin and possibly free protein S, or with severe thrombophilia. The condition is best diagnosed with CT angiography.[25]

Retinal vein occlusion (RVO) differs from the other types of unusual-site thrombosis in that it is largely a primary vascular disease, associated with systemic atherosclerotic risk factors such as hypertension, diabetes, and hyperlipidemia. RVO occurs in 5.2 per 1,000 individuals who are 30 or older[26] and 2.1% over 10 years among patients with ocular hypertension.[27] There is controversy regarding the importance of traditional risk factors for DVT, the highest yield provided by tests for antiphospholipid

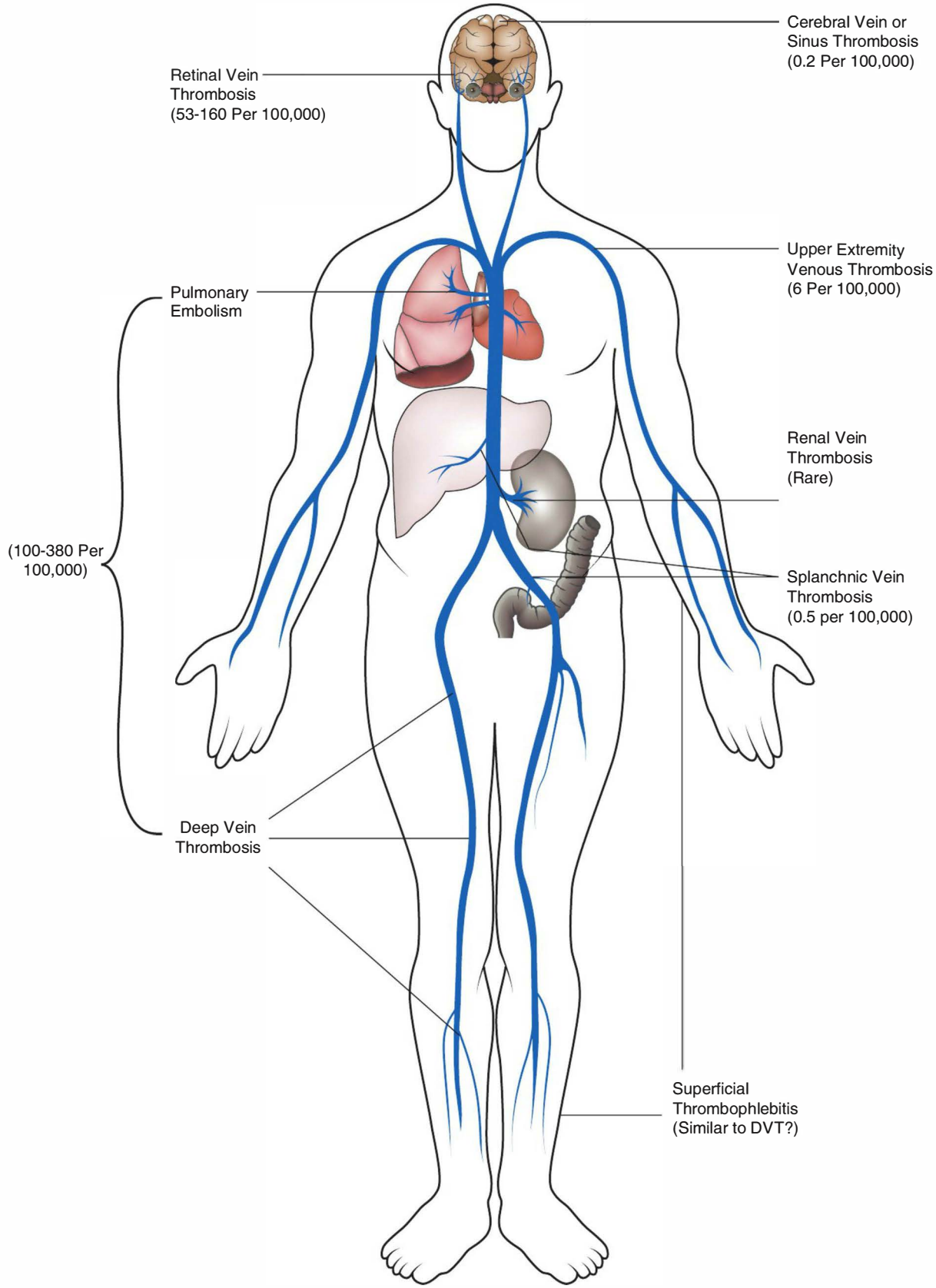


FIGURE 80.2 Locations and annual incidence of VTE.

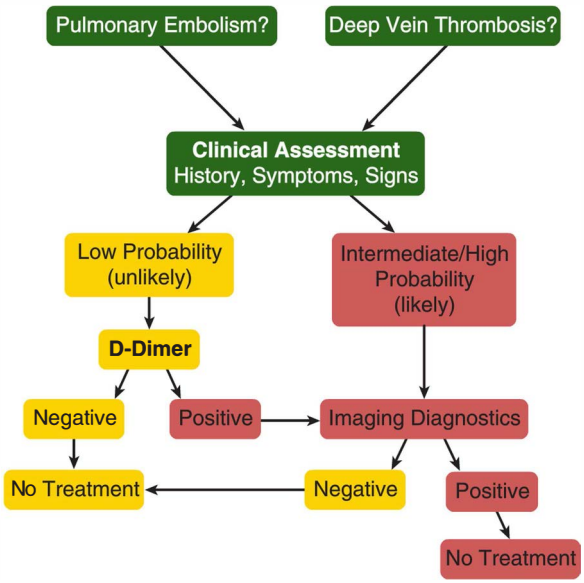


FIGURE 80.3 Simplified diagnostic algorithm for VTE.

syndrome.[28] A randomized study comparing LMWH with aspirin in 58 patients with RVO showed better functional long-term results with anticoagulation, but this result requires confirmation.[29] Unusual sites of DVT are described in Chapter 84.

LONG-TERM CONSEQUENCES OF VENOUS THROMBOEMBOLISM

Patients with DVT have a high risk of recurrence as well as chronic venous insufficiency, the postthrombotic syndrome (PTS) (FIGURE 80.4). The main cause of PTS is damage to the delicate venous valves, with resulting reflux and increased venous pressure in the affected extremity. The clinical findings range from mild swelling in the evening, often combined with symptoms of heaviness or tiredness in the leg, or skin discolorations due to telangiectasia and malleolar flare, to severe edema, iron deposition with hyperpigmentation, lipodermatosclerosis, and, ultimately, venous stasis ulcers[30]; the latter present in 5% of patients after 10 years.[31] The Villalta score has been recommended for quantifying the syndrome.[32] Patients with PE without symptomatic DVT also have an increased risk of developing PTS, 41% at 10 years.[31] Compression stockings should be used to reduce the risk of PTS,[33] but there are some controversies. Once severe PTS has occurred, treatment is challenging. Some relief may be gained with intermittent pneumatic compression,[34] pentoxyphylline,[35] or rutosides.[36]

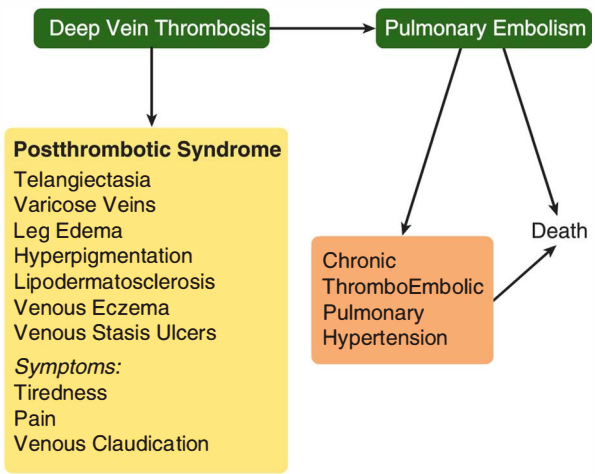


FIGURE 80.4 Sequelae of VTE.

Pulmonary hypertension (mean pulmonary artery pressure ≥25 mm Hg) is an incapacitating long-term sequel that has been reported in 0.8% to 3.8% of patients 2 years after PE.[37,38] It is unclear what transforms the original fragile emboli into fibrotic, endothelialized obstructions that are tightly attached to the arteries. The condition must be suspected when the dyspnea persists or recurs without evidence of new emboli. A screening program with echocardiography several years after an episode of PE was not useful due to a low yield of only 0.57% with chronic pulmonary hypertension.[39] Right heart catheterization is required to assess the pulmonary artery pressures accurately, and central venous saturation and cardiac output are needed to understand prognosis and decide on medical therapy.[40] Pulmonary endarterectomy is the treatment of choice but should only be performed at highly specialized centers. The long-term consequences of VTE are reviewed in Chapter 85.

PROPHYLAXIS OF VENOUS THROMBOEMBOLISM

Prophylactic regimens against VTE have been most extensively evaluated in major orthopedic surgery, notably hip or knee replacements, with primary efficacy being asymptomatic DVT documented by venography and surgical wound bleeding serving as a sensitive safety outcome. Recently, there has been intensive debate around these outcomes. The value of asymptomatic DVT as a surrogate outcome for fatal PE has been questioned.[41] The European Medicines Agency has since 2007 recommended a composite of (a) symptomatic or asymptomatic proximal DVT, (b) symptomatic nonfatal PE, and (c) VTE-related death or any death as the primary outcome;[42] so asymptomatic distal DVT may not be an acceptable surrogate outcome. Variations in the definition of major bleeding have been used during the past two decades,[43] and attempts have been made to harmonize this definition, published as recommendations by the Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.[44,45] The European Medicines Agency refers to this definition in their recommendations.[42,46]

In major orthopedic surgery, prophylaxis against VTE is today given to a high proportion of patients, although the recommended duration and start time are not followed for over 60% of patients.[47] Overall compliance with guidelines in surgical patients is 58.5%, and only 39.5% for medically ill patients, according to the ENDORSE survey.[48] Collins et al.[49] showed in a meta-analysis of more than 70 randomized trials that prophylaxis with low-dose unfractionated heparin (LDUH) reduces fatal PE in the surgical setting from 0.81% to 0.26%. In the studies in medical patients, death was not significantly affected.[50,51] The duration of high VTE risk was recently addressed by the EXCLAIM trial by evaluating extended prophylaxis with LMWH in medically ill patients. Although prolongation of prophylaxis to 28 days reduced the incidence of VTE, this occurred at the cost of a significant increase in major bleeding,[52] a phenomenon

that did not occur in patients receiving extended prophylaxis after hip replacement[53] or hip fracture surgery.[54] Although there are many "excuses" for abstaining from prophylaxis against VTE in medically ill patients, almost as many patients who do not qualify for prophylaxis ended up receiving treatment as did the eligible patients who were not given prophylaxis.[55] Education, local protocols, and national/international guidelines are not sufficient to maintain an acceptable standard,[56] but computer-driven electronic alerts resulted in more frequent use of prophylaxis with a concomitant reduction of VTE (8.2% to 4.9%) in medical patients with a high proportion of cancer (80%).[57]

LDUH, LMWH, and fondaparinux are well-documented prophylactic agents for patients with medical illness or following orthopedic or general surgery.[56] For patients requiring extended prophylaxis, vitamin K antagonists may be utilized, but they are not effective and are challenging for obtaining and maintaining a stable therapeutic level.[58] In medically ill patients or high-risk surgical patients, LDUH (5,000 U) needs to be administered every 8 hours to have a comparable effect with LMWH.[56,59,60] Mechanical prophylactic methods should be combined with pharmacologic prophylaxis for patients at very high risk of VTE or given alone when there is a high risk of bleeding. See Chapter 86 for more details regarding VTE prophylaxis.

During the past 5 years, several new, specific, orally available anticoagulants have been investigated in large programs. The direct thrombin inhibitor dabigatran and the direct factor Xa inhibitors rivaroxaban and apixaban have been approved in some countries for use in patients undergoing hip or knee arthroplasty (see Chapters 86 and 110).

TREATMENT OF ACUTE AND CHRONIC VENOUS THROMBOEMBOLIC DISEASE

The most important advance in VTE treatment during the past two decades has been the change from inpatient to outpatient management of most patients with DVT and some patients with PE. This change was possible with the shift from required monitoring of intravenous unfractionated heparin to the use of subcutaneous LMWH, which does not require routine laboratory monitoring (FIGURE 80.5). Subcutaneous LMWH seems more effective and safer than unfractionated heparin when given intravenously or subcutaneously,[61] and outcomes are similar when LMWH is given in the hospital compared with outpatient administration.[62] The same quality of evidence is not available for outpatient therapy of PE, but with risk stratification, ever more patients at low risk are treated as outpatients.

Promising results of the new anticoagulants from the orthopedic VTE prophylaxis studies are now being replicated in patients with VTE. Oral thrombin inhibitors ximelagatran (withdrawn from the market in 2006) and dabigatran showed similar efficacy as warfarin with seemingly less bleeding.[63–65] The factor Xa inhibitor rivaroxaban demonstrated similar safety and efficacy as a regimen of LMWH followed by vitamin K antagonists,[66] and none of these new agents require laboratory monitoring of coagulation. These agents will simplify treatment and may allow for longer treatment durations to prevent VTE.

There is uncertainty as to whether patients with unprovoked VTE with a 30% risk of recurrence over 10 years[31,67] should be treated for 3 to 6 months or indefinitely. The PROLONG trial showed that 6.2% of patients with a normal D-dimer level at 1 month after stopping warfarin had recurrence, compared with 15% among those with increased D-dimer.[68] However, a 6.2% recurrence rate over 1.4 years may still be higher than acceptable, and a follow-up study demonstrated that with repeated negative D-dimer tests at 1 and 3 months after discontinuing anticoagulation allowed for selection of a population with a 2.9% risk of recurrence.[69] This low rate must be balanced by the risk of bleeding with continued anticoagulation.

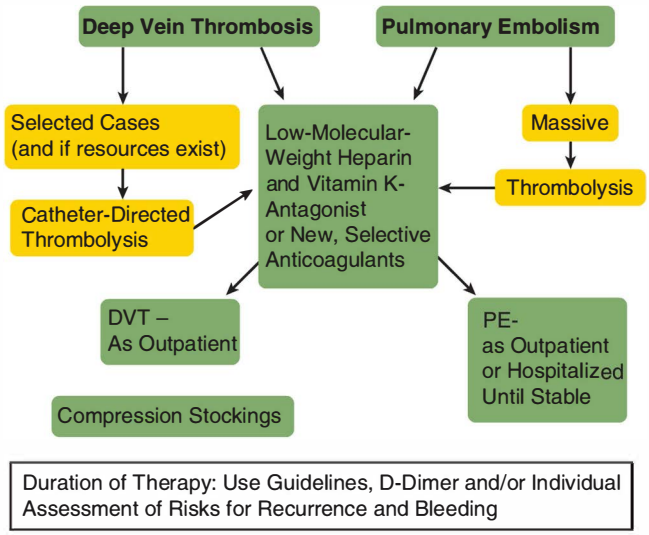


FIGURE 80.5 Treatment of VTE.

Major questions remain regarding the optimization of initial treatment in order to minimize long-term sequelae. Can catheter-directed thrombolysis or pharmacomechanic combinations effectively remove the DVT before the delicate venous valves have been damaged to avoid the PTS[70]? Should other patients with PE than those with hemodynamic instability receive treatment with thrombolysis? Within the next few years, we should have answers to these questions.

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CHAPTER

81 Thrombophilia Genetics

JOSEPH EMMERICH, MARTINE AIACH, AND PIERRE-EMMANUEL MORANGE

Each year, approximately 1 in 1,000 individuals in industrialized countries develops deep-vein thrombosis (DVT) of the lower extremities.[1,2] Between 1% and 2% of these patients die from pulmonary embolism, and as many as 25% suffer the chronic effects of the postthrombotic syndrome. It is therefore important to identify high-risk patients with a genetic predisposition to thrombosis, particularly among those who have recurrent DVT. The terms "hypercoagulability" and "thrombophilia" refer to situations in which constitutional or acquired risk factors for thrombotic events are present.[3]

Hereditary thrombophilia is associated with a higher risk of thrombosis (mainly venous thrombosis) and is the antithesis of hemophilia, a disease associated with a high risk of spontaneous hemorrhage. Female carriers of hemophilia have clotting factor levels approximately 50% of normal and have a reduced risk of thrombosis.[4] In contrast, thrombophilia is mainly an autosomal dominant trait linked to heterozygous loss-of-function mutations affecting inhibitors of coagulation or gain-of-function mutations affecting coagulation factors. Interestingly, half-normal coagulation factor levels are not associated with a bleeding tendency, whereas half-normal levels of coagulation inhibitors are associated with an increased risk of thrombosis.

As far back as 1856, Virchow postulated that thrombosis could be due to "changes in the composition of blood," yet it was only in 1965 that Egeberg[5] published the first case of inherited antithrombin (AT) deficiency, and this was followed by similar reports of protein C (PC) and protein S (PS) deficiency.[6–8] Hereditary thrombophilia was initially considered as a rare monogenic disorder, but this view was challenged in the mid-1980s.[9,10] With the discovery of activated protein C resistance (APCR), a frequent factor V (FV) mutation known as FV_{Leiden} became a change of paradigm of genetic risk factors for thrombophilia and paved the way for studies of gene–gene and gene–environment interactions.[11,12] The main risk factors for venous thromboembolism (VTE) can be divided into acquired factors, genetic factors, and other factors that may vary due to genetic variations yet to be identified[13,14] (see Table 81.1). This chapter focuses on genetic risk factors, the main features of which are summarized in Table 81.2.

COAGULATION INHIBITOR DEFICIENCY

Antithrombin Deficiency

Biochemistry

AT is a 58-kDa plasma protein that regulates coagulation by inhibiting procoagulant serine proteases such as thrombin, activated (a) factor X, and factor IXa (see Chapter 18). AT belongs to the serpin family, members of which share structural features that allow them to form a stable stoichiometric complex with their target serine protease. The reaction between AT and thrombin involves the reactive center loop and the protease active site serine and is increased approximately 1,000-fold by heparin and other glycosaminoglycans (e.g., heparan sulfate) that are present on the endothelial surface. Heparin binding to AT involves amino-terminal amino acids 1 to 47, the A helix, and the D helix.[15] AT is synthesized by the liver and circulates at a concentration of approximately 2.5 μM; levels are decreased by estrogen and heparin therapy.

Clinical Manifestations

AT deficiency is transmitted as an autosomal dominant trait (0.02% prevalence in the general population) and is associated with a high risk of VTE (~1% per year).[16–19] It is prevalent in <2% of unselected patients with a first VTE event, and it confers a higher risk of thrombosis than other thrombophilia. Homozygous AT deficiency may cause death *in utero* or severe, life-threatening thrombotic problems in the perinatal period.[20]

Laboratory Diagnosis

There are two types of hereditary AT deficiency. Type I is the most frequent and is characterized by decreased activity in a heparin cofactor assay and a decreased protein concentration by immunoassay. Type II deficiencies are caused by functional defects, protein concentrations being normal or near normal in immunoassays. The dysfunction may affect the reactive site (II_{RS}) or the heparin-binding site (II_{HBS}) or both (II_{PE}) (pleiotropic effect).[21] Homozygosity is mostly present in patients with type II_{HBS}, with the exception of one patient with type II_{PE} deficiency.[20] Venous and arterial thrombosis may occur during infancy in such patients.[22,23]

The ability of plasma to inhibit bovine thrombin and human FXa in the presence of heparin is the foundation for chromogenic heparin cofactor assays. Concentrations <80% (without heparin or estrogen treatment) call for further investigation, although only patients with severe deficiencies (<60%) are at high risk of thrombosis. Chromogenic assays distinguish type II deficiencies, in that type II_{HBS} has normal activity whereas type II_{RS} has low activity; the risk of VTE is very low in type II_{HBS} deficiency.[24]

Molecular Basis

The human *AT* gene (SERPINC1—GenBank X68793.1) is located on chromosome arm 1q23–25 and it comprises seven exons spanning 1.3 kb and containing 10 Alu repeats.[25] A leader sequence of 32 amino acids is encoded by exon I and by the 5′ end of exon II. The mature secreted polypeptide (432 amino acids) is encoded by exons II to VI. The reactive site, an Arg393–Ser394

Table 81.1

Main risk factors for venous thrombosis

Acquired Risk Factors	Genetic Risk Factors	Other Risk Factors[a]
Age	AT deficiency	Hyperhomocystinemia
History of venous thrombosis	Protein C deficiency	High levels of factor VIII
Surgery	Protein S deficiency	High levels of factor XI
Cancer	Factor V_{Leiden}	High levels of factor IX
Hormonal treatment	Factor II G20210A	High levels of factor VII
Antiphospholipid syndrome	Dysfibrinogenemia	High levels of TAFI
Myeloproliferative disorders	Fibrinogen γ10034T	Low levels of TFPI
Trauma	Non-0 blood group	APCR in the absence of FV_{Leiden}
Plaster cast		
Obesity		

TAFI, thrombin activatable fibrinolysis inhibitor; TFPI, tissue factor pathway inhibitor.
[a]Possible genetic regulation.
From Chandler WL, Rodgers GM, Sprouse JT, et al. Elevated hemostatic factor levels as potential risk factors for thrombosis. *Arch Pathol Lab Med* 2002;126(11):1405–1414; Bertina RM. Genetic approach to thrombophilia. *Thromb Haemost* 2001;86(1):92–103, with permission.

bond, is located in the carboxy terminus of the protein and is encoded by exon VI. The HBS of AT is located in the amino-terminal domain and consists of two regions encoded by exon II and exon IIIa (see Chapter 18).

One of the two alleles is not expressed in type I deficiency, leading to a 50% reduction in the circulating protein. The database lists 80 different point mutations, including microinsertions and deletions; partial or entire deletions have been found in 12 patients.[21]) The high proportion of Alu repeats plays an important role in the generation of large SERPINC1 deletions causing AT type I deficiency.

The type II_{RS} mutations result from amino acid substitutions in the reactive site loop, comprising amino acids 378 (P15) to 398 (P5′), protruding from the surface of the protein. Mutations located in the hinge domain, affecting Ala382 or Ala384 (P10–P12), transform AT into a substrate for thrombin.[24] The Ala384 Ser mutation is particularly frequent in the British population.[25,26] Mutations affecting Gly392, Arg393, and Ser394 (P2, P1, P1′) prevent AT recognition by its target protease.[25]

Most published mutations modifying AT affinity for heparin (type II_{HBS}) are located in the amino-terminal domain encoded by exon II or exon IIIa. Several patients have been found to

Table 81.2

Main features of hereditary thrombophilia

Protein Affected	Antithrombin	Protein C	Protein S	Factor V	Factor II
Gene location	1q23–25	2q13–14	3p11	1q21–22	11p11–q12
Type of mutation(s)		Loss of function Private mutations		Gain of function Arg506Gln	Gain of function G20210A
Frequency in the general population (%)	0.02[a]	0.2–0.4[a]	0.7–2.3[a]	2–10	2–4
Type of assay	Heparin cofactor activity against FXa	Clotting assay or amidolytic assay	Clotting assay or immunoassay for free PS	APCR (second-generation aPTT-based assay) or FV genotyping	FII genotyping
Functional effect	Thrombin and FXa inhibitor	Reduce thrombin generation by inactivation of FVa and FVIIIa		FV variant resistant to APC inactivation	Increases the circulating FII concentration
Risk of VTE	× 10	× 4–5	× 4–5	× 4–5	× 3–4

PS, protein S; APCR, activated protein C resistance; aPTT, activated partial thromboplastin time; APC, activated protein C; VTE, venous thromboembolism.
[a]From Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. *N Engl J Med* 2001;344(16):1222–1231, with permission.

be homozygous for the Arg47Cys mutation or the Leu99Phe mutation.[21,22]

A cluster of mutations located on the carboxy-terminal side of the reactive loop, affecting residues 402, 404, 407, and 429, have a pleiotropic effect (type II_{PE}). The identification of an Asn187Asp mutation in a patient with recurrent thrombosis and apparently normal circulating AT is puzzling.[27] Indeed, this AT variant has a normal function in its native form, but tends to adopt an inactive conformation during storage *in vitro*, leading the authors to suspect that the thrombotic episodes were provoked by fever.

We identified a Phe229Leu mutation in a 13-month-old child with cerebral thrombosis. This mutation appears to generate an unstable variant that spontaneously polymerizes in the circulation.[23] This observation confirms that conformationally altered AT variants with reduced thermostability can be associated with severe thrombosis.[28]

More than 130 different mutations have been reported and are described online at gene database (http://www1.imperial.ac.uk/departmentofmedicine/divisions/experimentalmedicine/haematology/coag/antithrombin/).

Protein C and Protein S Deficiencies

Biochemistry

PC is a vitamin K–dependent zymogen that is activated at the endothelial surface when thrombin binds to thrombomodulin. This reaction transforms thrombin from a procoagulant enzyme into an inhibitor, by activating PC to activated protein C (APC). PC binding to the endothelial protein C receptor (EPCR) augments its PC activation by the thrombin–thrombomodulin complex.[29] In the presence of its cofactor PS, APC degrades activated FV (FVa) and FVIIIa, thereby impeding further thrombin generation.[30] FV inactivation occurs in a biphasic reaction, with rapid cleavage at Arg506, followed by slower cleavage at Arg306. The first cleavage only partially affects FVa activity, whereas full inactivation occurs after the second cleavage at Arg306. PS markedly stimulates the second phase of the inactivation process, by a 20-fold enhancement of Arg306 hydrolysis.[31] The mechanism of FVIIIa inhibition by APC is also biphasic, with cleavage at Arg562 and then at Arg336. FVIIIa inactivation by APC is increased by PS and FV, which act synergistically as cofactors for the reaction.[32] The anticoagulant activity of PS has also been attributed to interaction with the prothrombinase complex, independent of APC.[33,34] Furthermore, PS enhances around 10-fold the inhibition of factor Xa by tissue factor pathway inhibitor-alpha (TFPI-alpha) in the presence of Ca^{2+} and phospholipids.[35] In addition to its anticoagulant function, APC also binds to EPCR in lipid rafts/caveolar compartments to activate protease-activated receptor 1, thereby eliciting anti-inflammatory and cytoprotective signaling responses in endothelial cells. These properties have led to FDA approval of recombinant APC as a therapeutic drug for severe sepsis (see Chapter 123).

PC is synthesized by hepatocytes and circulates at a concentration of approximately 70 nM, with a half-life of approximately 8 hours. APC forms inactive complexes with serine protease inhibitors, mainly protein C inhibitor, but also protease nexin 3, α_1 antitrypsin, and α_2 macroglobulin.[36]

Although PS is mainly produced by hepatocytes, it is also detected in endothelial cells and platelets. In the circulation, PS forms inactive complexes with C4b-binding protein (C4BP). Free PS represents approximately 40% of the total circulating level, and only this fraction has APC cofactor activity. C4BP is a multimeric protein composed of six or seven α chains, plus or minus a β chain. Only isoforms with a β chain (C4BP β+), which normally represent 80% of circulating C4BP, can bind PS. The interaction between PS and C4BP is reversible, but, in the presence of Ca^{2+}, the dissociation constant is $<10^{-9}$ mol/L. All circulating C4BP β+ molecules carry one molecule of PS, so free plasma PS results from a molar excess of PS over that of C4BP β+.[37] PS plasma levels are lower in women younger than 45 years and in those who are pregnant or are using oral contraceptives.[38] During acute-phase reactions, plasma C4BP concentrations increase after stimulation of the C4BP α, C4BP β, and *PROS1* genes by inflammatory cytokines. PS not only suppresses coagulation as an enhancing cofactor for the coagulation inhibitors APC and TFPI but is also a physiologic ligand for the Tyro/axl/Mer-family of receptor tyrosine kinases that mediate an anti-inflammatory regulatory loop of dendritic cell and monocyte inflammatory function.

Clinical Manifestations of Protein C and Protein S Deficiencies

PC and PS deficiencies are transmitted as autosomal dominant traits with incomplete penetrance, and heterozygous subjects belonging to families with the disorder are at risk of recurrent VTE during adulthood. Hereditary PC deficiency was first identified in subjects who had about half the normal PC concentration and a family history of thrombosis. At 45 years of age, 65% of affected members of the family described by Bovill et al.[39] and 50% of affected members of the 24 families described by Allaart et al.[40] were still free of thrombotic events. In prospectively studied asymptomatic members of thrombophilic families, the incidence of VTE was approximately 0.5% per patient-year in patients with PC deficiency and between 0.5% and 1.65% in patients with PS deficiency.[14–18]

The thrombotic risk associated with PC levels <67% was confirmed in a case–control study of unselected patients who developed DVT before 70 years of age, with a relative risk (RR) of approximately 3.[41] Homozygous patients with undetectable PC have a very severe clinical phenotype, including life-threatening thrombotic complications at birth, mainly neonatal purpura fulminans with large bruises that become necrotic and gangrenous. The parents and family members of these homozygous infants have about half the normal PC concentration and are asymptomatic.[42,43] This form of genetically determined PC deficiency was believed to be recessively transmitted.

Heterozygous subjects belonging to families with PS deficiencies are at risk of recurrent thromboembolic disease in adulthood (review in[44]). In heterozygous subjects, the probability of being free of thrombotic events at 45 years of age is approximately 50%.[45] We found that free PS and/or PS anticoagulant activity below the 10th percentile (ELISA < 75%) of control values was indeed associated with a risk of developing VTE.[46] Homozygous PS deficiency, such as homozygous PC deficiency, is a rare disease associated with severe thrombosis, including neonatal skin necrosis and purpura fulminans.[47,48]

Laboratory Diagnosis

Most clinical laboratories now use the snake venom protease Protac-based assay, allowing PC to be specifically and directly activated in plasma.[49] Such one-step assays evaluate the APC

generated after activation by Protac with synthetic substrates (amidolytic assays) or measure the prolongation of the activated partial thromboplastin time (aPTT) (anticoagulant assays). An immunoenzymatic assay measuring the protein concentration in plasma and functional assays measuring enzymatic or anticoagulant activity are used to distinguish several types of PC deficiency. In type I (quantitative) deficiency, which is caused by reduced synthesis of a normal protein, the plasma concentration is low in the three assays; this is the case in most PC deficiencies. Type II (qualitative) deficiency is characterized by normal synthesis of a nonfunctional protein that affects both the amidolytic and coagulation assays when the mutation disturbs the catalytic site but that affects only the coagulation assays when the mutation disturbs the interaction of PC with calcium, phospholipids, or macromolecular substrates (FV and FVIII). Therefore, it is recommended to use coagulation assays to screen patients for PC deficiency. It is difficult to establish normal ranges, as PC levels in subjects with PC gene abnormalities overlap with levels in healthy subjects[49] and vary with age. According to Miletich,[49] the increase in the PC concentration is approximately 4% per decade. Therefore, patients with PC concentrations <70% may have a hereditary deficiency, although values between 55% and 70% must be considered as borderline.

The diagnosis of PS deficiency is complicated by the presence in plasma of two molecular forms, that is, free PS and C4BP/PS complexes. Therefore, to measure the total circulating PS, immunoenzymatic assays have to be performed in conditions in which C4BP/PS complexes are dissociated, such as with highly diluted plasma in anti-PS–coated plates.[50] PS deficiency characterized by a low free PS concentration but a normal total PS concentration was identified by Comp.[50] A monoclonal antibody-based immunoenzymatic assay is available to measure free PS specifically.[51] APC cofactor activity can be evaluated in an aPTT assay after adding diluted plasma to PS-depleted plasma in the presence of purified APC and purified FVa.

According to the International Society on Thrombosis and Hemostasis (ISTH) standardization subcommittee, three types of PS deficiency have been defined on the basis of total PS levels, free PS levels, and APC cofactor activity. Type I deficiency is characterized by low total PS and free PS antigen levels; type II deficiency is characterized by normal free PS and low APC cofactor activity; and type III PS deficiency is characterized by low free PS levels and normal or near-normal total PS levels. Type I and type III deficiencies in fact appear to be two phenotypic expressions of the same genetic disease,[45] and free PS is used to diagnose PS deficiency in these cases. The lower normal limit of total and free PS levels is 65% of the level observed in a pool of normal plasmas. However, the reference range in women younger than 45 years is approximately 55% under the same conditions. It is recommended to use both the clotting assay and the monoclonal based immunoassay specific for free PS to screen patients for PS deficiency.

Molecular Bases of Protein C and Protein S Deficiencies

The human *PC* gene (PROC) maps to chromosome arm 2q13–q14, spans over 11 kb, and comprises a coding region (exons II to IX) and a 5′ untranslated region encompassing exon I.[52] The protein domains encoded by exons II to IX show considerable homology with other vitamin K–dependent coagulation proteins such as factors VII, IX, and X. Exon II codes for a signal peptide, whereas exon III codes for a propeptide and a 38–amino acid sequence containing nine Glu residues. Exons IV, V, and VI encode a short connecting sequence and two epidermal growth factor (EGF)-like domains, respectively. Exon VII encodes both a domain encompassing a 12–amino acid activation peptide that is released after activation of PC by thrombin and dipeptide 156 to 157, which, when cleaved, yields the mature two-chain form of the protein. Exons VIII and IX encode the serine protease domain, with His211, Asp257, and Ser360 forming the catalytic triad.

The database published on behalf of the ISTH coagulation inhibitor subcommittee[53] lists 160 different mutations. The proportion of missense mutations is very high and the spectrum very wide, making the molecular basis of PC deficiencies similar to that of FIX deficiency (hemophilia B). The large spectrum of mutations responsible for PC deficiency is probably caused by a high rate of *de novo* mutations.[54] Among 90 patients with point mutations, selected on the basis of PC levels <65% and with at least one episode of thrombosis, 76 bore a missense mutation. Interestingly, 4.4% of the patients with the missense mutation were homozygous or compound heterozygotes, of whom only one had purpura fulminans at birth.[55] Homozygosity and compound heterozygosity may account for concentrations ranging from <1% to 25%. Only patients with concentrations <5% are at risk of purpura fulminans.[55,56]

The amount of PC produced by the mutant allele (null or plus), as well as genetic status (heterozygous, homozygous, or compound heterozygous), partly accounts for the variable clinical expression. However, these factors do not explain why, in many families carrying a single *PC* gene mutation, more than 50% of the affected members remain asymptomatic. Other putative genetic factors may therefore favor thrombosis or protect against disease expression.

Two kinds of type II deficiency can be distinguished on the basis of plasma assays after PC activation by Protac. The substitution of different residues in the propeptide or the gamma carboxyglutamic acid (GLA) domain always resulted in low PC anticoagulant activity, whereas amidolytic activity was normal. It is not surprising that mutations giving rise to this biologic phenotype affect exon III because both the propeptide and the *N*-terminal region play a major role in the formation of the GLA domain, required for the anticoagulant activity of PC.

A few mutations in exon IX, which encodes the serine protease domain, also affected the coagulation assay but not the amidolytic assay, suggesting that, in addition to the catalytic pocket, this domain encompasses a region or regions required for PC anticoagulant activity. The other mutations in exon IX led to abnormal results in both the coagulation assay and the amidolytic assay.[57,58] The possible structural impact of natural substitutions has been examined by Greengard et al.[59] using three-dimensional molecular modeling.

Two homologous genes for PS map to chromosome 3p11.[52] The active gene, *PROS1*, spans over 80 kb and comprises 15 exons. Because *PROS2* has no open reading frame and shows multiple base changes, stop codons, and frameshifts, it is probably a pseudogene. The 5′ part of the *PROS1* gene shows strong homology with the other vitamin K–dependent proteins, particularly PC. Exon I encodes the signal peptide; exon II encodes the propeptide and the GLA domain; exon III encodes a domain with a high aromatic amino acid content; exon IV encodes a thrombin-sensitive loop; and exons V to VIII encode four EGF-like domains. The 3′ part of the *PROS1* gene

differs from that of all other known coagulation proteins: The last seven exons (IX to XV) encode a sex-hormone-binding-globulin-homologous domain.[56] On screening consecutive patients with unexplained thrombosis and low PS levels, we found mutations in 70% of cases.[60] Simmonds et al.[61] found a mutation in 41% of 34 patients with type I deficiency, selected using criteria similar to ours. The mutations observed in type I deficiency are distributed throughout the coding sequence. More than 200 different mutations are listed in the database.[44,48,62] Other mechanisms may explain why one allele is not expressed in patients with type I deficiency who have no detectable mutation. Because the screening strategy is based on selective amplification of the *PROS1* gene, recombination events between the *PROS2* and *PROS1* genes are not detected by polymerase chain reaction (PCR)-based techniques. However, only three *PS* gene abnormalities involving large deletions have been shown to be responsible for PS deficiencies.[63] No mutations in the promoter domain have been identified (unpublished data).

A single mutation transforming Ser460 to Pro (Heerlen polymorphism), first described by Bertina et al.[64] as a polymorphism, was found in 28 of our 118 patients referred with unexplained thrombosis and PS type III deficiency; however, approximately 50% of the patients also carried the FV Arg506Gln mutation or a *PC* gene defect, suggesting a cooperative effect on clinical expression.[60] The fact that the affected members of seven families carrying the Ser460Pro mutation were all asymptomatic[65] further suggests that this mutation is not itself a major cause of thrombosis. The type I and type III phenotypes were found to be associated with a similar thrombotic risk, and the two phenotypes coexisted in 14 families, leading the investigators to postulate that type I and type III are phenotypic variants of the same genotype.[45] Taken together, these results show that type III phenotypes have a heterogeneous molecular basis and a wide range of clinical consequences. Recently, it has been shown that the cutoff levels of free PS identifying type III individuals at risk for DVT might be far below the normal range in healthy volunteers. Indeed, individuals with free PS level less than the 5th percentile (<41 IU/dL) had 5.6 times increased risk of DVT compared with those in the upper quartile (>91 IU/dL).[66]

PS type II deficiency is fairly infrequent, and only a few mutations have been identified in patients with normal PS concentrations and low APC cofactor activity. Most mutations giving rise to a type II phenotype are located in the amino-terminal part of PS, which is homologous to that of other vitamin K–dependent proteins and encodes the domains interacting with APC.[60]

A database of mutations responsible for defective genes, including PROC and PROS1, is available online at http://www.hgmd.cf.ac.uk.

FACTOR V_{LEIDEN}

Biochemistry of Activated Protein C Resistance

In 1993, Dahlback described three families in whom APC did not yield the expected prolongation of the clotting time in an aPTT assay[67,68]; this defined a new phenotype called APCR. This was found in 15% to 25% of patients with DVT and in 2% to 10% of control subjects.[69,70] The study by Bertina et al.[71] showed that APCR cosegregated with the *FV* gene and with a single mutation (FV_{Leiden}, Arg506 Gln) affecting one of the APC cleavage sites. Most, but not all, cases of APCR are caused by FV_{Leiden}. In the Leiden Thrombophilia Study (LETS), after exclusion of patients with FV_{Leiden}, a relation was observed between APC sensitivity and the risk of thrombosis: the lower the normalized APC sensitivity ratio, the higher the associated risk. The adjusted (i.e., age, sex, and FVIII) odds ratio (OR) for the lowest quartile was 2.5 (95% confidence intervals [CI], 1.5 to 4.2) compared with the highest quartile.[72] In another study, phenotypic resistance to APC was associated with VTE, independently of FV_{Leiden} status; the age- and sex-adjusted OR for VTE was 1.7 (95% CI, 1.0 to 2.7) in participants who had a normalized APC sensitivity ratio of 0.50 to 0.84 compared with those who had a ratio of 0.85 to 1.3.[73] Clinical states with low APC sensitivity that are not caused by FV_{Leiden} may also be acquired, as is the case during pregnancy, with the use of oral contraceptives, or in patients with lupus anticoagulant or high levels of FVIII.

Molecular Basis of Activated Protein C Resistance

FV is a 330-kDa multidomain single-chain glycoprotein, with a plasma concentration of 20 nmol/L (0.007 g/L).[74,75] The FV gene (gene locus on chromosome arm 1q23) spans more than 80 kb and contains 25 exons. The complimentary DNA (cDNA) has a length of 6,672 bp and encodes a preprotein of 224 amino acids. Similar to FVIII, FV is organized into six domains (i.e., A1, A2, B, A3, C1, and C2). FV and FVIII share approximately 40% sequence identity in their A- and C-domains. Thrombin and FXa activate FV by a cleavage at peptide bonds at positions 709, 1,018, and 1,545, thereby releasing the B-domain, which connects the heavy chain (domains A1-A2) to the light chain (domains A3-C1-C2). Upon activation, FVa is formed by the heavy and light chains that are noncovalently associated by a Ca^{2+} ion. FVa is an essential FXa cofactor; its presence in the prothrombinase complex enhances the rate of prothrombin activation 10^3- to 10^5-fold. Downregulation of the procoagulant activity of FVa is accomplished through its inactivation by APC at positions Arg306, Arg506, and Arg679. Cleavage at Arg506 is essential for optimal exposure of cleavage sites Arg306 and Arg679 but results in partial inactivation of FVa (~40% of procoagulant activity remains). The slower Arg306 cleavage is required for complete inactivation of the protein, whereas the third cleavage site (Arg679) is less important. Therefore, any defect on one or more of these three cleavage sites (i.e., Arg506, Arg306, and Arg679) may potentially affect APC inactivation. FVa inactivation is enhanced by PS. The PS effect is highly phospholipid dependent and is mediated by a selective 20-fold stimulation of APC-catalyzed cleavage at Arg306. In addition to its major role in the procoagulant process when activated, FV also has an anticoagulant role as an APC cofactor (such as PS), downregulating FVIIIa activity after cleavage at position 506. Therefore, this dual pathway involving both inactivation of procoagulant FVa by APC and partial proteolysis of intact FV generating an anticoagulant protein is a complex and subtle regulatory system.

Bertina identified the genetic explanation for the APCR phenotype first in 1994, followed closely by three other groups.[71,76–78] A single base mutation, guanine to adenine at position 1,691 of the *FV* gene, is responsible for the Arg506Gln mutation known as FV_{Leiden}. This mutation results in a substantially reduced

anticoagulant response to APC, because FV_{Leiden} is inactivated about 10 times slower than normal FV. This impairment of FVa inactivation increases thrombin generation and explains more than 90% of clinical APCR phenotypes. However, other mechanisms may contribute to the hypercoagulable state, as suggested by similar inactivation of FVa and FVa_{Leiden} by APC in the presence of PS, FXa, and high concentrations of FVa.[68] The fact that FV_{Leiden} is a much less active cofactor of APC than wild-type FV for FVIIIa inactivation may also explain the thrombophilic state. Impaired FVa inactivation and loss of APC cofactor activity contribute equally to the APCR phenotype in subjects with FV_{Leiden}.[79] Interestingly, two other mutations in FV affect the Arg306 cleavage site. Arg306 is replaced by Gly in $FV_{Hong\ Kong}$, and by Thr in $FV_{Cambridge}$.[80,81] $FV_{Hong\ Kong}$ is prevalent (~5%) among the Chinese in Hong Kong, but neither of these latter mutations is associated with an increased risk of developing venous thrombosis.[82] The two FV mutations involving Arg306 yield identical mild APCR patterns. The associated APC resistance is due not only to incomplete FVa inactivation, but also to reduced APC cofactor activity in FVIIIa inactivation.[75] It strongly argues for the importance of the loss of the APC cofactor activity of FV when it is mutated; it is also strengthened by the demonstration that a recombinant FVa, in which the three known APC cleavage sites have been mutated, is still inactivated by APC, although much slower than the wild-type protein.[83] FV Ile359Thr (FVLiverpool) and FV Glu666Asp are two rare mutations also associated with thrombosis and APCR.[75]

Epidemiology

The prevalence of FV_{Leiden} is high in populations of white descent, but low in native populations of Asia, Africa, and Australia.[84] All FV_{Leiden} alleles are carried by the same haplotype, leading to the inference that the mutation occurred only once and spread by a founder effect. The estimated time of the mutation is approximately 30,000 years ago, implying that it took place after the out-of-Africa divergence that occurred approximately 100,000 years ago.[85] Its spread among whites and its high prevalence suggest that FV_{Leiden} is associated with a survival advantage, such as a decrease in severe bleeding after delivery.[86,87]

The frequency of FV_{Leiden} in white populations is between 2% and 15%.[69,70,88–93,94,95] FV_{Leiden} is very rare in China, Korea, Taiwan, and Japan.[96–98] In the United States, FV_{Leiden} is found in 2.21% of Hispanic Americans, in 1.23% of African Americans, in 0.45% of Asian Americans, and in 1.25% of American Indians.[99] The risk of DVT in heterozygous carriers is approximately fivefold higher than in control population (see Table 81.3). The association of FV_{Leiden} with pulmonary embolism is much weaker than with DVT, with a prevalence of FV_{Leiden} <10% versus 15% to 25%,[100] perhaps explained by the formation of a thrombus that is more stable and adherent and less prone to embolism.[101,102] Whatever be the reasons, this imbalance between the risk of DVT and pulmonary embolism, known as FV_{Leiden} paradox, is not caused by a bias in selection and is specific for FV_{Leiden}, as it has not been observed in carriers of the prothrombin G20210A mutation.[102] To date, there is no clear explanation to understand the FV_{Leiden} paradox.[103]

Familial and case–control studies show that FV_{Leiden} is clearly a milder thrombophilic state than heterozygous AT, PC, or PS deficiency.[16–18] The absolute risks of VTE events in subjects with AT, PC, and PS deficiency are between 0.5% and 1.5% per year, compared to only 0.1% to 0.3% per year in heterozygous carriers of FV_{Leiden}. On the basis of these data, it is estimated that the lifetime probability of developing thrombosis in heterozygous FV_{Leiden} carriers is between 5% and 10%.[73,104] A census-based screening study of 15,109 persons between 18 and 65 years of age suggested that the FV mutation could explain approximately 7% of all cases of VTE and could be responsible for at least 50 cases of VTE annually per million community-living adults.[73]

The prevalence of FV_{Leiden} homozygosity in the general population is approximately 1 in 2,500. The thrombotic complications are far less severe than in homozygous PC and PS deficiencies.[105,106] FV_{Leiden} homozygosity was found in 4.1% of 1,200 consecutive patients with juvenile VTE.[107] In a pooled analysis of eight case–control studies on behalf of the ISTH coagulation inhibitor subcommittee, we found FV_{Leiden} homozygosity in 1.3% of 2,310 cases and 0.13% of 3,204 controls.[94] In homozygotes, the risk of venous thrombosis is increased 30- to 140-fold; yet an additional environmental or genetic risk factor is often present at the first thrombotic event.[94,107] The risk of death among FV_{Leiden} carriers is identical to that among noncarriers.[108,109]

Table 81.3

Risk of a first venous thrombosis and of recurrence with FV_{Leiden} and FII G20210A mutation

	Heterozygous FV_{Leiden} Mutation OR (95% CI)	Heterozygous FII G20210A Mutation OR (95% CI)
OR for a first venous thrombosis (94)	4.9 (4.1–5.9)	3.8 (3.0–4.9)
OR for a recurrent event (after 112)		
Ho et al. (2006)	1.41 (1.14–1.75)	1.72 (1.27–2.31)
Marchiori et al. (2007)	1.39 (1.15–1.76)	1.20 (0.89–1.61)
Segal et al. (2009)	1.56 (1.14–2.12)	1.45 (0.96–2.21)

Factor V_{Leiden} and Recurrent Venous Thrombosis

Two studies showed an increased risk of recurrence in heterozygous carriers of FV_{Leiden},[110,111] but nine other studies did not.[93,112–118,119,120] In three systematic reviews (reviewed in[112]), the risk of heterozygous carriers was around 1.4 (Table 81.3). However, because the increased rate of recurrence remains low, long-term anticoagulant therapy is not mandatory after a first thrombotic event in heterozygous carriers of FV_{Leiden}. The risk of recurrence in FV_{Leiden} homozygotes appeared to be increased, mainly from retrospective studies.[22,93,106,107] Nevertheless, a recent and large case–control study in a cohort of families with thrombophilia did not demonstrate any high risk of recurrent venous thrombosis in individuals with homozygous FV_{Leiden} and/or homozygous prothrombin G20210A or double heterozygous carriers of FV_{Leiden} and prothrombin G20210A.[121]

Factor V_{Leiden} and Arterial Thrombosis

The possible link between FV_{Leiden} and arterial thrombosis is elusive.[122–126] Most studies have shown no association between FV_{Leiden} and myocardial infarction. Nevertheless, meta-analysis of 19 studies (17,500 individuals) suggests that FV_{Leiden} may be associated with a 20% increase in the risk of myocardial infarction.[122] In a case–control study of young women (18 to 44 years), the OR for myocardial infarction was 2.4 (95% CI, 1.0 to 5.9), the increased risk being largely confined to current smokers.[127] A meta-analysis of five studies on premature myocardial infarction determined an OR of 1.54 (95% CI, 1.07 to 2.22).[122] Another meta-analysis of six studies determined an OR of 1.34 (95% CI, 0.94 to 1.91) associated with the FV_{Leiden} and myocardial infarction before the age of 55 years.[126] Two studies suggest that the prevalence of FV_{Leiden} is higher in patients with myocardial infarction associated with nonsignificant stenosis or obstruction, as shown by coronary arteriography.[128,129] Any association between myocardial infarction and FV_{Leiden} would be weak and would not justify screening for the mutation in patients with coronary artery disease.

The prevalence of APCR or the FV_{Leiden} mutation in patients who have had stroke has been examined in more than 30 studies, few of which showed a link with ischemic stroke.[130,131] In 100 patients younger than 45 years who survived an ischemic stroke without a cardiac embolic source, FV_{Leiden} was not overrepresented.[131] FV_{Leiden} is associated with cerebral vein thrombosis, with an OR of 3 to 4.[132–136]

Laboratory Diagnosis

Two types of tests can be used for diagnosing APCR: the functional APCR assay and the genetic FV_{Leiden} identification test. The functional test is based on aPTT prolongation after the addition of purified APC and is expressed as an APC sensitivity ratio: aPTT(+APC)/aPTT(–APC). A low APC sensitivity ratio defines APCR. Second-generation APCR tests, which use dilution of test plasma into FV-deficient plasma, have very good specificity and can even be used to test patients taking warfarin.[124,137] It is mandatory to confirm a positive APCR test by direct detection of FV_{Leiden}. The most commonly used genetic test for FV_{Leiden} is based on PCR amplification of the FV gene exon X. The presence or absence of the mutation can be determined with allele-specific probes or restriction enzymes. The advantage of genetic testing is that it avoids ambiguous results and determines heterozygous/homozygous status in patients with a low APC sensitivity ratio.

THE FII (PROTHROMBIN) G20210A MUTATION

Biochemistry

Prothrombin has procoagulant, anticoagulant, and antifibrinolytic activities after its activation into thrombin by the prothrombinase complex. Thrombin acts by activating factors XIII, XI and VIII, V, PC, and the thrombin-activatable fibrinolysis inhibitor (TAFI) and by cleaving fibrinogen to fibrin. Prothrombin is a 72-kDa multidomain single-chain vitamin K–dependent glycoprotein. Prothrombin activation is mediated by FXa, which cleaves prothrombin at Arg271–Thr272 and Arg320–Ile321 to release the catalytic domain from the carboxy-terminal domain. Prothrombin activation is accelerated approximately 300,000-fold in the presence of FVa, phospholipids, and Ca^{2+}. Human thrombin contains an A chain of 36 amino acids and a B chain of 259 amino acids. The prothrombin gene is 21 kb in length and is encoded by chromosome 11 (position 11p11–q12).[138] The gene comprises 14 exons separated by 13 introns, with the 5′ and 3′ untranslated regions that may play regulatory roles in gene expression.

Molecular Characteristics

By extensively screening the prothrombin genes of 28 families with unexplained venous thrombosis,[139] Poort et al. found one heterozygous nucleotide transition (G to A) at position 20210 in the 3′ untranslated region in five probands (18%). In 474 unselected patients with a first episode of venous thrombosis and 474 controls from the LETS, the frequency of the mutation was 6.2% and 2.3%, respectively. In this study, the OR for thrombosis associated with the FII 20210A allele was 2.8 (95% CI, 1.4 to 5.6). Interestingly, the FII 20210A allele was associated with significantly higher prothrombin levels in heterozygotes than in noncarriers (1.32 and 1.05 U/mL, respectively). The plasma prothrombin level was an independent risk factor for thrombosis. Together, these risk factors suggested that FII 20210A acts by increasing prothrombin levels, leading to increased thrombin generation.[140,141] Another possible explanation for the role of the FII level in thrombosis is that elevated prothrombin levels can inhibit APC-mediated inactivation of FVa.[142]

The mechanism by which the G20210A mutation influences prothrombin levels is controversial.[143–147] The increase in protein synthesis could result from more efficient 3′-end formation, increased messenger ribonucleic acid (mRNA) stability, increased translation efficiency, or a combination of these mechanisms. Mutations of the poly(A) signal sequence commonly cause loss of gene function. FII G20210A is the only example of mutation in the cleavage site required for mRNA polyadenylation that increases 3′ end processing. Another rare FII mutation (FII C20221T) was found in a child with arterial thrombosis after allogenic kidney transplantation and in a 28-year-old man with Budd-Chiari syndrome.[147–149] This paradoxical gain-of-function from mutations in the 3′ end of the *FII* gene was explained by unusual 3′ noncanonical sequence elements: The FII cleavage site for polyadenylation and uridine-rich elements in the 3′ flanking sequence seems less efficient than in other genes, thereby favoring gain-of-function mutations.[147] The same authors demonstrated that, compared to the wild-type FII (FII 20210G), the 20210C and 20210T mutations led to a 40% to 50% increase in mRNA expression, and the 20210A mutation led to a 215% increase.

It was reported that an intronic *FII* gene polymorphism, A19911G, influences splicing efficiency and modulates the effects of the FII G20210A mutation on mRNA and protein expression.[150] The 19911G allele is in linkage disequilibrium with the 20210A allele, and heterozygous carriers of the FII G20210A mutation who also have the 19911G mutation on the other allele (19911G homozygotes) have a higher risk of venous thrombosis.

Epidemiology

The prevalence of the FII mutation is high in populations of white descent but low or nil in Asians, American Indians, and African Americans.[151–153] As in the case of FV_{Leiden}, a founder effect explains the high prevalence of the factor II G20210A

mutation in whites.[153] Haplotyping suggests that the mutation arose 20,000 to 30,000 years ago, after the divergence of non-Africans from Africans and whites from Mongoloid subpopulations. A survival advantage based on a protective prenatal effect of the FII G20210A genotype, such as improved embryonic implantation, has been suggested.[154]

The estimated frequency of FII G20210A in white populations is between 1% and 6%.[94,138,155–161] This mutation is more common in southern than in northern Europe, a gradient opposite to that of FV_{Leiden}.[151] The risk of DVT is increased threefold to fourfold in heterozygous carriers of FII G20210A compared to noncarriers (Table 81.3). In two prospective studies, and in a large Italian cross-sectional survey, the risk of thrombosis was lower than in case–control studies.[155,156,160] In the rare FII G20210A homozygotes, the risk of thrombosis is only moderately increased,[141,162–164] often associated with other genetic or acquired risk factors.[164]

FII G20210A and Recurrent Venous Thrombosis

Two studies showed an increased risk of DVT recurrence among heterozygous carriers of FII G20210A,[109,165] but eight other studies did not.[93,114,116,117,119,120,166,167] Although systematic reviews showed an OR of around 1.4 for recurrence, consensus exists that in heterozygous FII G20210A carriers long-term anticoagulant therapy is not mandatory after a first thrombotic event.[112] The risk of recurrence in FII G20210A homozygotes is probably increased, as in double heterozygotes carrying both FV_{Leiden} and FII G20210A, in whom the risk of a primary VTE event is increased 20-fold.[94,115,116,165] As previously discussed for FV_{Leiden}, a recent and large case–control study did not demonstrate any high risk of recurrent venous thrombosis in homozygous FII G20210A carriers.[121]

FII G20210A and Arterial Thrombosis

Similar to FV_{Leiden}, any association of FII G20210A with arterial thrombosis would be very weak.[123–126,168] Most studies have shown no association between FII G20210A and myocardial infarction or stroke. Nevertheless, in populations at low risk of arterial thrombotic events, such as young women, the OR for myocardial infarction is increased fourfold in FII 20210A carriers.[127,169] A meta-analysis of three studies in patients with myocardial infarction before the age of 55 years found an OR associated with the FII G20210A mutation of 1.86 (95% CI, 0.99 to 3.51).[126]

More than a dozen studies have analyzed the association between the FII G20210A mutation and stroke, and none has shown a link with ischemic events.[130] One study, of 72 patients with ischemic stroke before the age of 50 years and no other risk factors, found a fourfold to fivefold increase in the risk of stroke in carriers of the FII G20210A mutation.[170] This increased risk of stroke in young patients was not found in another study.[135] The risk of cerebral venous thrombosis is increased in carriers of the FII G20210A mutation.[136]

Laboratory Diagnosis

Although FII G20210A heterozygotes have 30% higher prothrombin levels than noncarriers on an average, this phenotype cannot be used to identify carriers of the mutation because of a large overlap of prothrombin levels. The only way to reliably detect the mutation is genetic screening after PCR amplification of the 3′ untranslated region of the *FII* gene. Several techniques are based on detection using specific restriction endonucleases, gel electrophoresis, or fluorescent probing.[168] Multiplex PCR-based assay is valuable for concomitant detection of FV_{Leiden} and FII G20210A.[171]

OTHER GENETIC RISK FACTORS

The Protein C Gene Promoter Polymorphism

The 5′ untranslated region of the *PC* gene, which encompasses exon I, has polymorphic sites at positions –1,654 (C/T), –1,641 (A/G), and –1,476 (A/T). Three haplotypes are commonly observed (CGT, TAA, and CAA). In the LETS, individuals homozygous for CGT had a higher risk of venous thrombosis than individuals with the TAA genotype.[172] In the Paris Thrombosis Study (PATHROS), we found that both homozygotes and heterozygotes for the CG haplotype had lower PC concentrations than noncarriers. The CG allelic frequency was considerably higher in patients with VTE than in controls.[173]

The FXIII α Chain Gene Polymorphism

The replacement of Val34 by Leu in the α chain of FXIII modifies the activation process and results in faster activation by thrombin.[174] Unexpectedly, this polymorphism had a protective effect on myocardial infarction.[175,176] In VTE, several case–control studies failed to show any effect of the FXIII polymorphism; however, in a meta-analysis of four of these studies, we found a weak protective effect, with ORs of 0.86 (95% CI, 0.74 to 0.99) and 0.58 (95% CI, 0.41 to 0.82) in heterozygotes and homozygotes, respectively.[177]

Fibrinogen γ10034T

A C to T variant at position 10034 in the fibrinogen gamma chain (rs2066865) is associated with a 2.4 times increased risk of VTE.[178] Around 6% of individuals carry this variant which is associated with reduced $\gamma A/\gamma'$ fibrinogen ratio. Its association with VTE was confirmed in a Dutch population, in an Austrian population, and in a white population in the United States.[179]

Blood Group Non-0

It was known since 1969 that non-0 blood group is associated with an increased risk of VTE.[180,181] Blood group 0 is associated with decreased levels of factor VIII and von Willebrand factor (vWF), due to increased clearance of vWF.[180] A twofold increased risk for all non-00 genotypes, with a synergistic interaction with FV_{Leiden}, was confirmed in the LETS.[181] The attributable risk of VTE to the non-0 blood group is approximately 25% due to the magnitude of the frequency.[182]

X-Linked Thrombophilia (Factor IX Padua)

All genetic causes of thrombophilia reported to date are autosomal dominant, except for a recently described X-linked thrombophilia due to a Leu for Arg mutation in the factor IX gene. This mutation was found as a gain of function of factor IX, as

the male proband had a ratio of activity to antigen of 8 [183]. This mutation was only found in one family and does not play a role in the epidemiology of the disease. On the other hand, it is an excellent paradigm defining thrombophilia as the antithesis of hemophilia (see *Introduction*). Furthermore, it demonstrates that rare mutations in candidate genes could also play a role in unexplained familial thrombophilia.

GENOME-WIDE ASSOCIATION STUDIES

In the last 5 years, the genome-wide association study (GWAS) approach has been the most popular strategy to identify, with huge successes, new susceptibility loci for multifactorial diseases.[184–186] The first large-scale association analysis on VTE was a multistage strategy gathering 3,155 DVT patients and 5,087 controls and focusing on 19,682 single-nucleotide polymorphisms (SNPs) located in known genes.[187] Three new loci were revealed—the *F11*, the *GP6*, and SERPINC1 (encoding AT) genes. Given their role in coagulation and in platelet aggregation, these three genes are obvious candidates for DVT. The associations at the *F11* and *GP6* loci were further confirmed in a meta-analysis of three independent studies,[188] but the functional variant(s) remain to be identified. A second association study investigating 291,872 SNPs spanning the whole genome was carried out in a sample of 419 DVT patients with idiopathic VTE at a young age (<50 years) and 1,228 healthy controls.[188] In this work, the strongest associations were observed at two known loci, the *FV* and *AB0* genes; only one new locus on chromosome 6 (*HIVEP1* gene) was additionally detected, but its effect on VTE risk was modest when compared to those of *FV* and *AB0*.[189] *HIVEP1* belongs to the HIVEP gene family encoding very large sequence-specific DNA-binding proteins containing multiple zinc fingers, but the mechanisms relating *HIVEP1* to VTE risk remain to be elucidated.

To identify genetic factors that may influence the plasma levels of the three C4BP existing isoforms, $\alpha_7\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_0$, a GWAS was conducted by analyzing 283,437 SNPs in the Genetic Analysis of Idiopathic Thrombophilia study.[190] Three SNPs at the *C4BPB/C4BPA* locus were found genome-wide significantly associated with $\alpha_7\beta_0$ levels. One of these SNPs was further found to explain approximately 11% of the variability of mRNA *C4BPA* expression in the Gutenberg Heart Study composed of 1,490 individuals, with no effect on *C4BPB* mRNA expression. The allele associated with increased $\alpha_7\beta_0$ plasma levels and increased *C4BPA* expression was further found associated with increased risk of VTE (OR = 1.24 [1.03 – 1.53]) in MARTHA and FARIVE, two French case–control studies gathering 1,706 cases and 1,379 controls. Interestingly, this SNP was not associated with free PS or total PS. This suggests that it acts on DVT through a PS-independent mechanism that remains to be elucidated.

Through a GWAS, seven SNPs were recently found associated with plasma levels of FVIII and vWF by the CHARGE consortium.[191] These SNPs were located in five genes that have not previously been suspected to be associated with these protein levels. The influence of these loci on thrombosis risk has been investigated in MARTHA and FARIVE. Among the five genes associated with FVIII and/or vWF levels, we observed strong arguments in favor of the *TC2N* gene (also referred to as *MTAC2D1*) as a new candidate gene for VTE, but the functional variants remain to be identified and biologic mechanisms relating it to DVT pathophysiology to be elucidated.[192]

Despite their indisputable successes to generate new candidate genes for VT, GWASs suffer from limitations. Moreover, the new genetic risk factors identified through this approach are not useful in the clinical setting given the very small risk they confer. Thus, other strategies are mandatory to identify the missing heritability of VTE. In a recent epidemiologic study, 30,179 siblings of 19,599 cases of VTE were identified. The siblings had an RR of 3.08 compared with the general population, with a similar increase among siblings of index cases with DVT as among those where the index cases had pulmonary embolism. This emphasized the still unknown genetic component of VTE.[193]

COMBINATIONS OF GENETIC RISK FACTORS AND ASSOCIATION OF GENETIC AND ACQUIRED RISK FACTORS

Combined Defects

There is an increased risk of VTE in patients carrying more than one genetic defect. Indeed, in two studies, 19% and 9.5% of symptomatic probands carrying a *PC* gene mutation also carried the FV_{Leiden} mutation.[194,195] Similar frequencies of combined defects were found in the probands of thrombophilic families carrying a *PS* gene mutation.[60,61] In family members with AT deficiency, coinheritance of FV_{Leiden} increased the risk of DVT.[196] A meta-analysis confirmed that the RR of thrombosis was much higher in carriers of both FV_{Leiden} and FII G20210A.[94] Patients heterozygous for a single genetic risk factor, with the possible exception for AT deficiency, have the lowest risk (RR 3 to 5). These patients may have DVT during adulthood, the risk increasing with age and, often, with acquired risk factors such as prolonged immobilization, surgery, pregnancy, and estrogen therapy. Combined genetic defects are associated with a stronger risk (RR 10 to 20) and may lead to thrombosis in early adulthood; this includes patients carrying two risk alleles, such as homozygotes for FV_{Leiden} and heterozygotes for FV_{Leiden} plus any of the other four genetic risk factors. Finally, homozygosity for PC, PS, or some types of AT deficiency is often associated with very severe early-onset thrombotic disease, and in some cases they could benefit from continuous or discontinuous preventive antithrombotic therapy. Because 10% to 30% of thrombophilic family probands have combined defects, the thrombotic phenotype may not be transmitted as a dominant trait.

Other unknown genes could possibly influence the thrombophilic phenotype. For example, noncarrier members of FV_{Leiden} families have a higher frequency of DVT than the general population does,[76,197] and the Vermont family study showed that other genes control the PC deficiency penetrance.[198,199]

Oral Contraception

The interaction between oral contraceptives and thrombophilia was first established in the LETS, by selecting 155 cases aged 15 to 49 years with a first episode of VTE and 169 matched controls. In this study, the use of oral contraceptives by women carrying FV_{Leiden} led to a 30-fold increase in the risk of VTE, which strongly suggested a synergistic effect.[200] In a meta-analysis of several case–control studies,[94] the risks of using oral contraceptives in FV_{Leiden} carriers were estimated in 517 women 15 to 49 years of age and in 518 controls. The OR (10.28) clearly

indicates a supra-additive effect of the association FV_{Leiden} and oral contraception. The risk of using oral contraceptives associated with FII G20210 was also evaluated in this meta-analysis (OR, 7.14). As already observed,[201] the latter mutation was found to interact synergistically with oral contraception.

The risk of thrombosis is also high in women with AT, PC, and PS deficiencies or homozygous for FV_{Leiden} who use oral contraceptives, particularly in case of AT deficiency, with an annual incidence of 3.4% per person (vs. 0.75% in nonusers).[202,203] Analysis of the LETS dataset led to the conclusion that women with inherited thrombophilia (FV_{Leiden}; FII G20210; and AT, PC, and PS deficiencies) develop VTE not only more often but also earlier than other women.[204] FVIII, FV, and FXI levels above the 90th percentile and FXII levels below the 10th percentile in patients whose first VTE episode occurred when they were taking oral contraceptives were associated with ORs of 10 to 12. However, the joint effect of these risk factors did not exceed the sum of separate effects (additive but not synergistic).[205]

These observations raise the question of whether asymptomatic young women should be tested for thrombophilic factors before the first use of oral contraceptives. Most authors agree that such tests in young women is not desirable because the absolute risk is very low in this population (to prevent one death by pulmonary embolism would require tests on ~500,000), although it might be justified in some cases.[206,207]

Hormone Replacement Therapy

Several observational studies have shown that women taking conjugated or natural estrogen therapy have an increased risk of VTE.[208,209] Few studies have been designed to evaluate the risk of such replacement therapies in thrombophilic postmenopausal women. In the Estrogen in Venous Thromboembolism Trial (EVTET) study,[210] 144 women younger than 70 years were randomly allocated to receive a dose of 2 mg per day of 17-β-oestradiol, norethisterone, or placebo in a double-blind study. Eight of the women receiving hormone replacement therapy (HRT) had recurrent VTE, compared to only one woman in the placebo group. In this study, which was interrupted for safety reasons, FV_{Leiden} carriers did not experience more frequent VTE than noncarriers, suggesting that this thrombophilic risk factor was not predictive of recurrence during HRT. In another study, 77 postmenopausal women who had had VTE were compared to 163 controls. The ORs (95% CI) were 3.9 (1.3 to 11.2), 3.2 (1.7 to 6.0), and 15.5 (3.1 to 76.7) in FV_{Leiden} carriers, in HRT users, and in FV_{Leiden} carriers taking HRT, respectively, suggesting a synergistic effect.[211]

There is no strong evidence that genetic thrombophilic factors can be used to identify a subpopulation at high risk of VTE. The risk of VTE is high in HRT users with prior VTE, whatever is the underlying mechanism.

Pregnancy

Pregnancy is an acquired risk factor for VTE, multiplying the risk fivefold. In families with inherited thrombophilia, asymptomatic carriers have an increased probability of VTE during pregnancy relative to noncarriers. Women carrying two risk alleles, such as homozygotes for FV_{Leiden} and double heterozygotes for FV_{Leiden} and FII G20210A, have a particularly high risk.[212]

In another approach, women with a history of VTE during pregnancy were compared with age-matched women with a prior pregnancy free of VTE. The results confirm that the probability of pregnancy-related thrombosis is high in women with homozygous FV_{Leiden} or with double FV_{Leiden} and FII G20210A mutations.[213,214] Unequivocal PC deficiency (<50%) and AT deficiency (<60%) were also associated with an increased risk of VTE during pregnancy.

Thrombophilia has also gained attention as a risk factor for pregnancy complications (see Chapter 117), as recently reviewed.[215,216] Despite this association, the conflicting results from literature preclude to give definite recommendations.[217]

THROMBOPHILIA SCREENING

There is no consensus on the subsets of subjects who qualify for thrombophilia screening, the types of laboratory test to use, or the clinical treatment of patients with thrombophilia. The most salient recommendations are summarized here.[19,218–222]

Thrombophilia is mainly associated with VTE rather than arterial thrombosis. Venous thrombosis most commonly occurs as DVT and/or pulmonary embolism, and a given case of thrombosis is not associated with a peculiar genetic thrombophilic state. Less common clinical forms such as upper limb, cerebral, and visceral vein thrombosis (i.e., portal, mesenteric, hepatic, and renal) should be screened because these forms are often associated with thrombophilia.[223,224]

The predictive value of a family history for identifying patients with a thrombophilic state is unreliable, and the absence of a family history does not preclude the need to screen for FV_{Leiden}, FII G20210A, and coagulation inhibitor deficiencies.[206,225–227] The multifactorial nature of VTE explains why thrombotic events in carriers of thrombophilic factors are triggered in half of the cases by acquired risk factors.[12,19] Therefore, VTE associated with an acquired risk factor does not preclude the need for screening. It makes sense to restrict screening after a first thrombotic event to young patients and to patients experiencing recurrences after withdrawal of oral anticoagulants.[228,229] Finally, testing for thrombophilia would be useful if we were able to identify patients who are particularly prone to recurrence, but the risk of recurrence is not markedly increased for FV_{Leiden} or FII G20210A.

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CHAPTER

82

Epidemiology and Risk Factors for Venous Thromboembolism

JOHN A. HEIT

Thrombosis can affect virtually any venous circulation. This chapter focuses on the epidemiology of common deep vein thrombosis (DVT) of the leg, arm, or pelvis, and its complication, pulmonary embolism (PE). Venous thromboembolism (VTE) is a complex (multifactorial) disease, involving both environmental exposures (e.g., clinical risk factors) as well as genetic and environmental interactions. Most epidemiology studies of VTE addressed populations of primarily European origin, and the data discussed in this chapter mainly relate to these populations. Where available, data from populations originating from other continents are presented. This chapter focuses on population-based studies of VTE incidence, survival, recurrence, and risk factors.

INCIDENCE OF DEEP VEIN THROMBOSIS AND PULMONARY EMBOLISM

The estimated average annual incidence rate of VTE among persons of European ancestry ranges from 104 to 183 per 100,000 person-years.[1–3,4,5,6] The incidence is similar or higher among African Americans[7,8] and lower among Asians,[9] and Asian[10] and Native Americans.[11] Unlike atherosclerotic arterial disease, the low incidence of VTE among Asians does not appear to increase after emigration and adoption of a western civilization diet and lifestyle, underscoring the probable role of heritability in the etiology of VTE. Using the age- and sex-specific incidence rates for the 5-year time period, 1991 to 1995, projected to the 2000 United States white population, at least 260,000 new cases of VTE occur among whites in the United States annually.[4] If incidence rates among African Americans are similar, then 27,000 additional incident cases occur among African Americans in the United States annually.

VTE is predominantly a disease of older age.[1–3,4,5,6,12] In the absence of a central venous catheter[13] or thrombophilia,[14] VTE is rare prior to late adolescence.[1,15] Incidence rates increase markedly with age for both men and women (FIGURE 82.1) and for both DVT and PE (FIGURE 82.2).[1,5,6] The overall age-adjusted incidence rate is higher for men (130 per 100,000) than women (110 per 100,000; male:female sex ratio is 1.2:1).[1,6] Incidence rates are somewhat higher in women during the childbearing years, while incidence rates after the age of 45 years are generally higher in men. PE accounts for an increasing proportion of VTE with increasing age for both sexes.[1]

SURVIVAL AFTER DEEP VEIN THROMBOSIS AND PULMONARY EMBOLISM

Overall, survival after VTE is worse than expected, and survival after PE is much worse than after DVT alone (Table 82.1).[16–19] The risk of early death among PE patients is 18-fold higher compared with DVT patients alone.[16] PE is an independent predictor of reduced survival for up to 3 months after onset. After 3 months, observed survival after PE is similar to expected survival.[6,16,17] For almost one-quarter of PE patients, the initial clinical presentation is sudden death. Independent predictors of reduced early survival after VTE include increasing age, male sex, lower body mass index, confinement to a hospital or nursing home at VTE onset, congestive heart failure, chronic lung disease, serious neurologic disease, and active malignancy.[16,17,20] Additional clinical predictors of poor early survival after PE include syncope and arterial hypotension.[20,21] Evidence of right heart failure based on clinical examination, plasma markers (e.g., cardiac troponin T, brain natriuretic peptide), or echocardiography predicts poor survival among normotensive PE patients.[20] Survival over time may be improving for those PE patients living sufficiently long to be diagnosed and treated.[16,22,23] The risk of a diagnosis of subsequent cancer is significantly increased within the first 6 to 12 months after an incident of DVT or PE.[24,25]

VENOUS THROMBOEMBOLISM RECURRENCE

VTE recurs frequently; about 30% of patients develop recurrence within the next 10 years.[26] The hazard of recurrence varies with the time since the incident event and is highest within the first 6 to 12 months but never falls to zero (FIGURE 82.3). The aim of acute anticoagulation treatment for acute VTE is to prevent DVT extension and embolization. The duration of acute treatment must be individualized but generally ranges from 3 to 6 months. After this period of time, the acute thrombus is either lysed or organized and unlikely to extend or embolize. From this time forward, any continued anticoagulation is to prevent recurrent VTE (i.e., secondary prophylaxis). The duration of acute treatment does not affect the risk of recurrence after anticoagulation is stopped,[27–33] suggesting that VTE is a chronic disease with episodic recurrence.[26,34–36] Independent predictors of recurrence include increasing patient age and body mass index,[26,37] neurologic disease with leg paresis,[26] and active cancer.[3,26,34,35,38,39] Among cancer patients, the hazard of VTE recurrence is significantly increased with lung, gastrointestinal, or genitourinary (uterus, kidney, ovary, testicle, bladder, prostate) cancer, and with extensive or moderately extensive cancer disease.[35] While men have been reported to have a higher risk of recurrence compared to women,[40–42] sex is not an independent predictor of recurrence after adjusting for sex-specific risk factors.[26,43] Additional predictors of VTE recurrence include "idiopathic" VTE,[29,30,44,45] a lupus anticoagulant or antiphospholipid antibody,[28,46] antithrombin, protein C or protein S deficiency,[47–49] hyperhomocysteinemia,[50] inflammatory bowel disease,[51] a persistently increased plasma

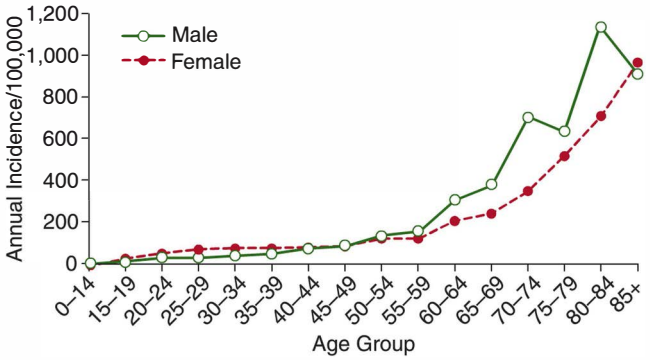


FIGURE 82.1 Annual incidence of VTE by age and sex. (From Silverstein M, Heit J, Mohr D, et al. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based, cohort study. *Arch Intern Med* 1998;158:585–593.)

Table 82.1

Survival (%) after DVT versus PE

Time	DVT Alone	PE
0 d	97.0	76.5
7 d	96.2	71.1
14 d	95.7	68.7
30 d	94.5	66.8
90 d	91.9	62.8
1 y	85.4	57.4
2 y	81.4	53.6
5 y	72.6	47.4
8 y	65.2	41.5

From Heit J, Silverstein M, Mohr D, et al. Predictors of survival after deep vein thrombosis and pulmonary embolism: a population-based, cohort study. *Arch Intern Med* 1999;159:445–453.

D-dimer among patients with idiopathic VTE,[52,53,54] and possibly persistent residual DVT.[55] Anticoagulant-based secondary prophylaxis should be considered for patients with these characteristics.

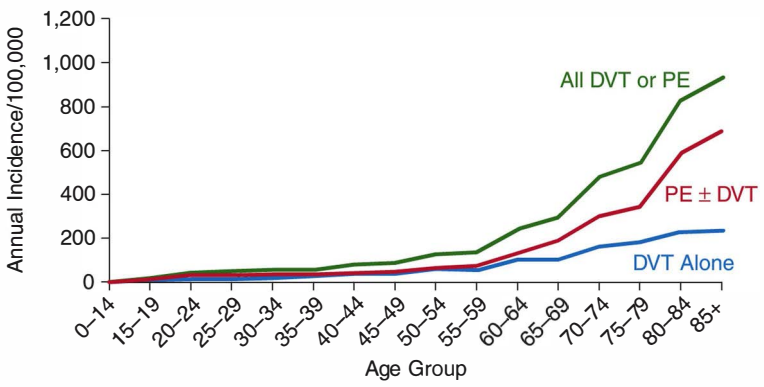
Equally important is the fact that several baseline characteristics predict either a reduced risk of recurrence or are not predictive of recurrence.[26,30,34,38,45,56] For women, pregnancy or the postpartum state,[26,57] oral contraceptive use,[26] hormone therapy,[58] and gynecologic surgery[26] are associated with a reduced risk of recurrence. Recent surgery and trauma/fracture either have no effect[26] or predict a reduced risk of recurrence.[38] Additional characteristics having no significant effect on recurrence risk include recent immobilization, tamoxifen therapy, and failed prophylaxis.[26] For these patients, and for patients with isolated calf vein thrombosis, a shorter duration of acute therapy likely is adequate.[30,44,59] While the reported risk of recurrence by incident event type (DVT alone vs. PE) varies with one report suggesting a higher recurrence risk associated with incident PE,[19,26,59] patients with recurrence are significantly more likely to experience recurrence of the same event type as the incident event type.[60] Because the 7-day case fatality rate is significantly higher for recurrent PE (34%) compared to recurrent DVT alone (4%),[61] secondary prophylaxis should be considered for patients with incident PE, especially those with chronic heart and/or lung disease causing reduced cardiopulmonary functional reserve; it is these patients who are most likely to die from recurrent PE.

Complications of VTE include venous stasis syndrome (post thrombotic syndrome) and chronic thromboembolic pulmonary hypertension, which are discussed in Chapter 85.

RISK FACTORS FOR VENOUS THROMBOEMBOLISM

In order to improve survival, avoid recurrence, prevent complications, and reduce health care costs, the occurrence of VTE must be reduced. However, the incidence of VTE has remained virtually unchanged since about 1981.[1,4,5,6,62] The main independent risk factors for VTE are shown in Table 82.2. Additional risk factors include central vein catheterization or transvenous pacemaker, prior superficial vein thrombosis, varicose veins, urinary tract infection, and family history of VTE; patients with chronic liver disease have a reduced risk.[63–67] Compared to residents in the community, hospitalized residents have over a 100-fold increased incidence of acute VTE.[62] Hospitalization and nursing home residence together account for almost 60% of incident VTE events occurring in the community.[68,69] Thus, hospital confinement provides an important opportunity to significantly reduce VTE incidence. Of note, hospitalization for medical illness and hospitalization for surgery account for

FIGURE 82.2 Annual incidence of all VTE, DVT alone, and pulmonary embolism with or without DVT (PE ± DVT) by age. (From Silverstein M, Heit J, Mohr D, et al. Trends in the incidence of deep vein thrombosis and pulmonary embolism: a 25-year population-based, cohort study. *Arch Intern Med* 1998;158:585–593).



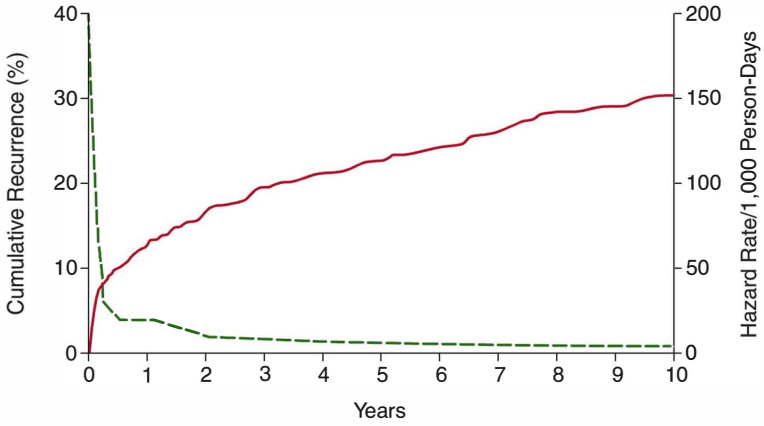


FIGURE 82.3 Cumulative incidence of first VTE recurrence (*solid line*), and the hazard of first recurrence per 1,000 person-days (*dashed line*). (From Heit J, Mohr D, Silverstein M, et al. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. *Arch Intern Med* 2000;160:761–768).

almost equal proportions of VTE (22% and 24%, respectively), emphasizing the need to provide prophylaxis to both of these risk groups. Nursing home residence independently accounts for over one-tenth of all VTE disease in the community.[68,69]

The risk among surgery patients can be further stratified based on patient age, type of surgery, and the presence of active cancer.[70,71] The incidence of postoperative VTE is higher for surgery patients who are 65 years of age or older.[72] High-risk surgical procedures include neurosurgery, major orthopedic surgery of the leg, thoracic, abdominal, or pelvic surgery for cancer, renal transplantation, and cardiovascular surgery.[65,72,73] Obesity[74–76] and poor American Society of Anesthesiology physical status[77] are risk factors for VTE after total hip arthroplasty.

Active cancer accounts for almost 20% of incident VTE events occurring in the community.[68,69] The risk appears to be higher for patients with malignant brain tumors and cancer of the pancreas, ovary, colon, stomach, lung, kidney, and bone,[78,79] and in patients with distant metastases.[79] Cancer patients receiving immunosuppressive or cytotoxic chemotherapy are at even higher risk for VTE,[63,79] including therapy with L-asparaginase,[80,81] thalidomide,[82] lenalidomide[83] bevacizumab,[84] or tamoxifen,[85] or epoetin or darbepoetin.[86,87] A routine examination for occult malignancy is warranted for patients presenting with idiopathic VTE, especially among patients with abdominal vein or bilateral leg vein thrombosis,[88,89] or in whom VTE recurs.[90] Among cancer patients, the risk of chemotherapy-associated VTE is higher in patients with pancreatic or gastric cancer, platelet count ≥350 × 10^9/L, hemoglobin <100 g/L or use of red cell growth factors, leukocyte count ≥11 × 10^9/L, or body mass index ≥35 kg/m^2;[91] biomarkers (plasma soluble P-selectin and D-dimer) add further predictive value.[92]

A central venous catheter or transvenous pacemaker accounts for 9% of incident VTE occurring in the community.[68] Central venous access via femoral vein catheters is associated

Table 82.2

Independent risk factors for DVT or PE

Baseline Characteristic	Odds Ratio	95% CI
Body mass index (kg/m^2)	1.08	1.05, 1.11
Major surgery	18.95	9.22, 38.97
Hospitalization for acute medical illness	5.07	3.12, 8.23
Nursing home confinement	4.63	2.77, 7.74
Trauma/fracture	4.56	2.46, 8.46
Active cancer	14.64	7.73, 27.73
Neurologic disease with leg paresis	6.10	1.97, 18.89
Pregnancy or postpartum	4.24	1.30, 13.84
Oral contraceptives	4.03	1.83, 8.89
Estrogen alone	1.81	1.06, 3.09
Noncontraceptive estrogen plus progestin	2.53	1.38, 4.63

CI, Confidence interval.
From Barsoum MK, Heit JA, Ashrani AA, et al. Is progestin an independent risk factor for incident venous thromboembolism? A population-based case-control study. *Thromb Res* 2010;126:373–378.

with a higher incidence of venous thrombosis compared to subclavian vein catheterization.[93] Prior superficial vein thrombosis is an independent risk factor for subsequent DVT or PE, remote from the episode of superficial thrombophlebitis.[63,94] The risk of DVT imparted by varicose veins is uncertain and appears to vary by patient age.[63,95,96]

Long haul (>4 to 6 hours) air travel is associated with a slightly increased risk for VTE (~1 per 4,656 flights)[97,98,99] that is preventable with elastic stockings.[100] Hypertriglyceridemia doubles the risk of VTE in postmenopausal women.[101] HMG-Coenzyme A reductase inhibitor (statin) therapy may provide a 20% to 50% risk reduction for VTE.[102–104] However, the risk associated with atherosclerosis, or other risk factors for atherosclerosis, remains uncertain.[105–108] Diabetes mellitus,[67] current or past tobacco smoking, high-density lipoprotein cholesterol, lipoprotein (a) and chronic obstructive pulmonary disease are not independent risk factors for VTE.[63,109,110] The risk associated with congestive heart failure, independent of hospitalization, is low.[63,70]

Among women, additional risk factors for VTE include oral contraceptive use,[67,70,111,112,113] hormone therapy,[67,114,115] pregnancy and the postpartum period,[70,112,116,117] and therapy with the selective estrogen receptor modulator, raloxifene.[118] First and third generation oral contraceptives convey higher risk than second generation oral contraceptives.[113] Oral contraceptives containing drospirenone convey two- to threefold increased risk for VTE compared to oral contraceptives containing levonorgestrel.[119,120] Injectable depot medroxyprogesterone acetate for contraception is associated with a threefold increased risk for VTE, while a levonorgestrel intrauterine device imparts no risk.[121] Hormone therapy is associated with a two- to fourfold increased risk of VTE,[67,114] but the risk may vary by type of estrogen.[122] The overall incidence of pregnancy-associated VTE is about 200 per 100,000 woman-years; compared to nonpregnant women of childbearing age, the relative risk is increased about fourfold.[117,123] The risk during the postpartum period is about fivefold higher than the risk during pregnancy.[117] Prior superficial vein thrombosis is an independent risk factor for venous thrombosis during pregnancy or postpartum.[124]

Other conditions associated with VTE include Bechet syndrome, celiac disease,[125] heparin-induced thrombocytopenia,[126] homocystinuria and hyperhomocysteinemia,[127,128] hyperthyroidism,[129] immune thrombocytopenia,[130] inflammatory bowel disease,[131] intravascular coagulation and fibrinolysis/disseminated intravascular coagulation, myeloproliferative disorders (especially polycythemia rubra vera and essential thrombocythemia),[132,133] chronic kidney disease with severely reduced glomerular filtration rate,[134] nephrotic syndrome,[135] paroxysmal nocturnal hemoglobinuria,[136] thromboangiitis obliterans (Buerger disease), thrombotic thrombocytopenic purpura, sickle cell disease,[137] systemic lupus erythematosus, and Wegener granulomatosis.[138]

Family-based studies indicate that VTE is highly heritable and follows a complex mode of inheritance involving environmental interaction.[139–141] Genetic risk factors for VTE are discussed in Chapter 81. While the clinical utility of diagnostic testing for an inherited or acquired thrombophilia remains controversial, such studies hold the potential for further stratifying individual patients into high and low risk for incident and recurrent VTE, targeting prophylaxis to those who would benefit most, and ultimately, reducing the occurrence of VTE.

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CHAPTER

83

Clinical Manifestations and Diagnosis of Venous Thromboembolism

HENRI BOUNAMEAUX AND ARNAUD PERRIER

Pulmonary embolism (PE), the source of which is predominantly located in a thrombosis of the deep veins of the legs, is a major health concern. It is the third cause of mortality by cardiovascular disease after coronary artery disease and stroke. In Western countries, it remains a leading cause of death in the puerperium and the postoperative period. In addition, late consequences of deep vein thrombosis (DVT) may produce disabling leg symptoms including venous ulcers in a substantial proportion of patients, resulting in a considerable economic burden. Also, chronic pulmonary hypertension can develop in a small percentage of patients with PE within a few years and require pulmonary thrombendarterectomy, a difficult procedure performed in few dedicated surgical centers.

During the past two decades, the approach to diagnosing DVT and PE has changed in several major ways. First, it has been recognized that these two conditions are manifestations of a single entity, venous thromboembolism (VTE). Second, novel noninvasive diagnostic tools such as D-dimer (DD) measurement, venous compression ultrasonography (CUS), and multidetector computed tomography angiography (MDCTA) have become available. These tools have drastically reduced the need for invasive procedures such as phlebography and pulmonary angiography. Third, the clinical index of suspicion has progressively become lower, so the vast majority (80% or even more) of patients with suspected VTE do not have the disease. Fourth, efficient, mainly noninvasive strategies have been validated in large-scale outcome studies, and more attention has been paid to their cost implications.[1,2]

Finally, the appropriate approach to the diagnosis of VTE differs depending on the nature of the clinical setting: clinical suspicion of a first episode of DVT or PE, clinical suspicion of a recurrent episode of DVT or PE, or screening of asymptomatic DVT or PE in high-risk patients or in patients participating in clinical trials on prophylaxis or therapy of VTE.

CLINICAL MANIFESTATIONS

Clinical Classification of DVT and PE

DVT is defined as the presence of a thrombus in the deep vein system. Although it may occur in all veins (lower and upper limbs, renal, mesenteric, splenic, portal or cerebral veins), it mainly affects the deep veins of the lower limbs (FIGURE 83.1). It must be distinguished from superficial thrombophlebitis, a more benign condition[3] that usually does not require therapeutic anticoagulant therapy, except in selected patients.[4] In general, deep vein thrombi arise in the calf veins (peroneal and posterior tibial veins, almost never anterior tibial veins) and may progress proximally, the so-called *ascending thrombosis*. In some cases (pregnancy, Cockett or May-Thurner syndrome, tumor), DVT may develop proximally, for example, in the common or external iliac vein, and progress distally, the so-called *descending thrombosis*.

Lower limb DVT may be *proximal* (from and including the popliteal vein and upward) or *distal* (in the calf below the popliteal veins), an important distinction because the embolic potential of proximal vein thrombosis is definitely higher than that of distal vein thrombosis.[5] Calf muscle vein thrombi are considered as distal vein thrombosis though their clinical relevance is not well established.

DVT can be *symptomatic* or *asymptomatic*. Asymptomatic thromboses occur more frequently in bedridden patients, especially in the postoperative period, because symptoms (mainly pain and swelling) that are essentially due to venous stasis are usually more prominent in the upright position and thus in outpatients. Symptoms do not necessarily parallel clinical relevance: an asymptomatic iliac thrombosis in a bedridden patient carries a higher risk than a painful calf muscle vein thrombosis in an ambulant individual.

PE is defined as the presence of clots in the pulmonary arteries. The origin is from the lower limb deep veins in more than 90% of cases. Other sites of DVT can also result in PE, but this a rarer occurrence mainly because DVT is most often located in the lower limbs. Traditionally, PE was classified as *massive* or *nonmassive* but this classification is ambiguous because it is used alternatively to designate extensive PE on imaging (usually more than 50% amputation of the pulmonary vasculature), a finding only loosely correlated to clinical outcome, and PE accompanied by shock or at least systemic hypotension, which carries a high mortality and indicates thrombolytic treatment. Moreover, this classification does not account for those patients who have no systemic hypotension but present with right ventricular (RV) dysfunction secondary to PE, demonstrated on echocardiography. According to the ESC definitions, PE is now classified into the following categories (Table 83.1):[6]

- High-risk (previously *massive*) PE: PE provoking shock or cardiorespiratory arrest (5% of cases)
- Intermediate-risk (previously *submassive*) PE: PE provoking RV strain despite normal systemic blood pressure (30% of cases)
- Low-risk (previously *nonmassive*) PE: PE associated with normal systemic blood pressure and normal RV function (65% of cases).

Presentation of Deep Vein Thrombosis

Classic symptoms of DVT include swelling and pain in the involved limb, but those symptoms are neither specific nor sensitive.[7,8] Many nonthrombotic conditions can produce

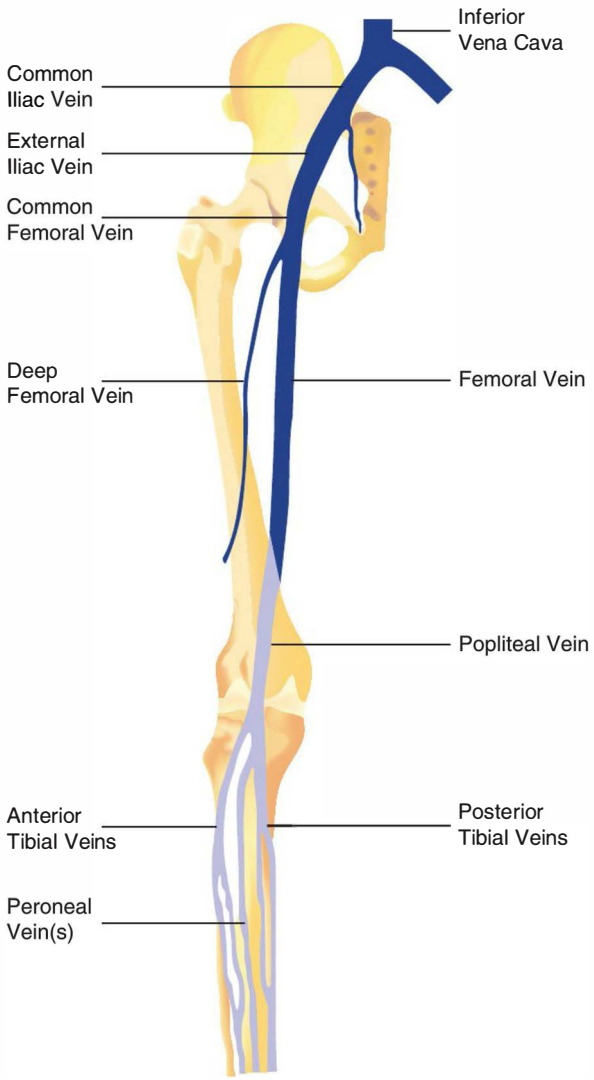

FIGURE 83.1 Anatomy of the deep veins of the lower limb. Note that the femoral vein was previously named "superficial femoral vein," a potentially dangerous misnomer because this vein belongs to the deep vein system.

symptoms similar to those encountered in DVT, such as muscle strain, direct twisting injury to the leg and hematoma, Baker popliteal cyst, cellulitis, and the postthrombotic syndrome. The presence of one or several traditional risk factors (history of immobilization or prolonged hospitalization/bed rest, recent surgery, prior episode of DVT or PE, cancer, trauma, hormonal treatment, pregnancy or postpartum) increases the probability that symptoms are due to DVT. The most informative findings on clinical examination are pain on palpation of the deep veins, significant unilateral edema or swelling, and superficial venous dilation. Warmth, tenderness, and erythema are inconstant and less useful. It is nevertheless possible to assess the clinical probability of DVT in two or three categories based on a limited number of risk factors and clinical signs (see further Section).[9] There is no correlation between clinical presentation and severity, as many thrombi are nonobstructive and are not associated with venous stasis or significant inflammation of the vessel wall or perivascular tissue. Severe forms of proximal vein thrombosis include the *phlegmasia alba dolens* that implies total occlusion of the deep venous system. The leg, then, must rely on the superficial venous system for drainage, which is not effective enough and results in edema, pain, and a white appearance of the leg. The next step in the disease progression is occlusion of the superficial venous system, thereby preventing all venous outflow from the extremity. At this stage it is called *phlegmasia cerulea dolens*. The leg becomes more swollen, cyanotic, and increasingly more painful. Additionally, the edema and loss of venous outflow leads to microcirculatory damage due to the very high venous pressure. Venous gangrene is the ultimate consequence. *Phlegmasia cerulea dolens* is frequently associated with cancer.

Presentation of Pulmonary Embolism

Clinical diagnosis of PE is not possible, due to the lack of sensitivity and specificity of clinical signs and symptoms.[10] Incidental finding of PE is increasingly frequent (2.6% of patients undergoing a CT in a recent meta-analysis[11]), particularly in hospitalized patients or in cancer patients. However, PE is most often symptomatic and usually presents as one of the following three clinical pictures: pleuritic pain associated or not with dyspnea (65% of cases), isolated dyspnea, usually acute but sometimes slowly progressive (20%), or syncope (<10%). These clinical presentations correspond to three syndromes of different pathology and variable severity:[12] (a) pulmonary infarction, (b) isolated dyspnea, and (c) shock, which defines high-risk PE (see next section).

A. Pleuritic pain is usually due to irritation of the visceral pleura by peripheral emboli. Hemoptysis may also be present, although more rarely. Although this syndrome is usually coined as "pulmonary infarction," the histopathologic correlate is in fact an alveolar hemorrhage probably provoked by the efflux of blood from the high-pressure bronchial circulation in the segment obstructed by the embolus.[13] The classic radiologic picture is a wedge-shaped pleural-based infiltrate that affects around 20% of patients.[14] Other common chest x-ray anomalies include plate-like atelectasis and pleural effusion. Tachycardia and dyspnea are less frequent in this clinical syndrome reflecting the peripheral character and lesser hemodynamic repercussions of such pulmonary emboli.[12]

B. In the isolated dyspnea syndrome, the absence of pleuritic pain is probably due to more proximal embolization of the pulmonary vasculature. Patients may complain of retrosternal chest pain oppressive in character evoking the differential diagnosis of angina. In fact, such pain probably reflects true myocardial ischemia due to increased RV wall tension and reduced right coronary artery flow. Tachycardia, although more frequent, is still present in only 45% of patients.[12] The electrocardiogram is rarely normal, but its anomalies are often nonspecific. Although dyspnea is usually of abrupt or rapid onset, in some patients it may progress over several days.

C. Systemic arterial hypotension and shock are the hallmark of high-risk PE causing acute severe pulmonary hypertension and RV failure. It is usually due to large central clots. Although suggestive of PE in patients with obvious risk factors such as recent surgery, syncope may be a misleading presentation.[15] Suspected high-risk PE with shock is a distinct situation requiring a specific diagnostic approach detailed in Section 5.2.2.

Table 83.1

Risk stratification of patients with PE

Risk Category	Mortality	Risk Markers			Clinical Decisions
		Shock or Hypotension	RV Dysfunction[a]	Elevated BNP or Troponin	
High	>15%	+	+	+	Thrombolysis or embolectomy
Nonhigh					
Intermediate	3%–10%	–	+	±	Hospital admission
Low	<1%	–	–	–	Early discharge or outpatient treatment

[a]Echocardiographic signs of RV dysfunction.
Adapted from Torbicki A, Perrier A, Konstantinides S, et al. Guidelines on the diagnosis and management of acute pulmonary embolism: the Task Force for the Diagnosis and Management of Acute Pulmonary Embolism of the European Society of Cardiology (ESC). *Eur Heart J* 2008;29:2276–2315.

DIAGNOSTIC TOOLS

Diagnostic Criterion Standards for DVT and PE

Ascending phlebography is still considered the diagnostic standard for diagnosing DVT but it is invasive, costly, and not devoid of risk. It consists of injection of iodinated contrast dye in a superficial foot vein with sequential radiograms of the leg to follow the dynamic course of the contrast in the veins. Tourniquets can be used to force the dye into the deep veins but their use is controversial. The documentation is adequate when images are obtained in different views, and a filling defect surrounded by contrast is characteristic for a fresh thrombus (FIGURE 83.2, left). It is still used as the standard surrogate endpoint in thromboprophylactic trials for efficacy assessment of new drugs after orthopedic surgery. It can be used for quantification of the thrombotic burden, for example, by Marder score.[16] In clinically suspected individuals, however, it has been largely replaced by CUS. In a classic series by Hull et al.[17] of 160 patients clinically suspected of DVT and left untreated based on a normal phlebogram, the 3-month thromboembolic risk was 1.9% (95% confidence interval [CI] 0.4% to 5.4%), indicating the potential of even the gold standard for false negatives. That figure is the benchmark for comparing the safety of other diagnostic tests to rule out DVT.

Although it is considered the criterion standard for diagnosing PE, pulmonary angiography is difficult to interpret, frequent disagreement occurring even between expert readers,[18] more often on the absence (17% of angiograms) than on the presence of PE (8% of angiograms).[19] The 3-month thromboembolic risk in patients left untreated after an angiogram that was considered normal was 1.7% (95% CI 1.0 to 2.7) in a systematic review pooling the results of eight series totaling 1,050 patients.[20] The test is also costly, invasive, and not devoid of hazards. Moreover, the mortality due to pulmonary angiography was 0.2% (95% CI 0 to 0.3) in a pooled analysis of five series with a total of 5,696 patients.[21] However, the rare deaths attributable to pulmonary angiography occurred in very sick patients with hemodynamic compromise or acute respiratory failure. Nevertheless, pulmonary angiography provides excellent anatomic views of the arterial vasculature of the lungs (FIGURE 83.2, right) and allows quantification of the embolic burden quite precisely, for example, by the Miller et al.[22] index. Because of its invasive character, it should probably be restricted to patients for whom a clinically likely diagnosis could not be confirmed by other means or in those patients for whom endovascular treatment of PE is being considered.

In fact, phlebography and angiography are no longer used routinely in patients with a clinical suspicion of DVT or PE. Nevertheless, they still have a role in patients in whom the clinical likelihood of an acute thromboembolic event is high and in whom noninvasive tests have remained negative or inconclusive. Although valid, that change in clinical practice will probably result in decreasing expertise among radiologists performing these exams, which may decrease their accuracy in the future and further restrict their roles for routine use. Contemporary diagnostic standard equivalents are diagnostic criteria that have been proven safe in outcome studies with clinical 3-month follow-up. Admittedly, this standard is appropriate to ensure that a rule-out criterion has a high negative predictive value and safety, but does not inform on specificity and positive predictive value since positive tests in modern diagnostic strategies are no longer verified by the gold standard.

Obsolete Tests

While venous occlusion plethysmography and continuous wave Doppler flow examination are only used in anecdotal situations nowadays, electrocardiogram, arterial blood gases, and chest x-ray are still part of the so-called prior clinical probability assessment in patients with suspected PE although they no longer belong to the diagnostic tools. The same applies to the ^{125}I Fibrinogen uptake test in the lower legs in suspected DVT. This method has been utilized mainly for screening in clinical studies of thromboprophylactic regimens in the 70s and 80s.

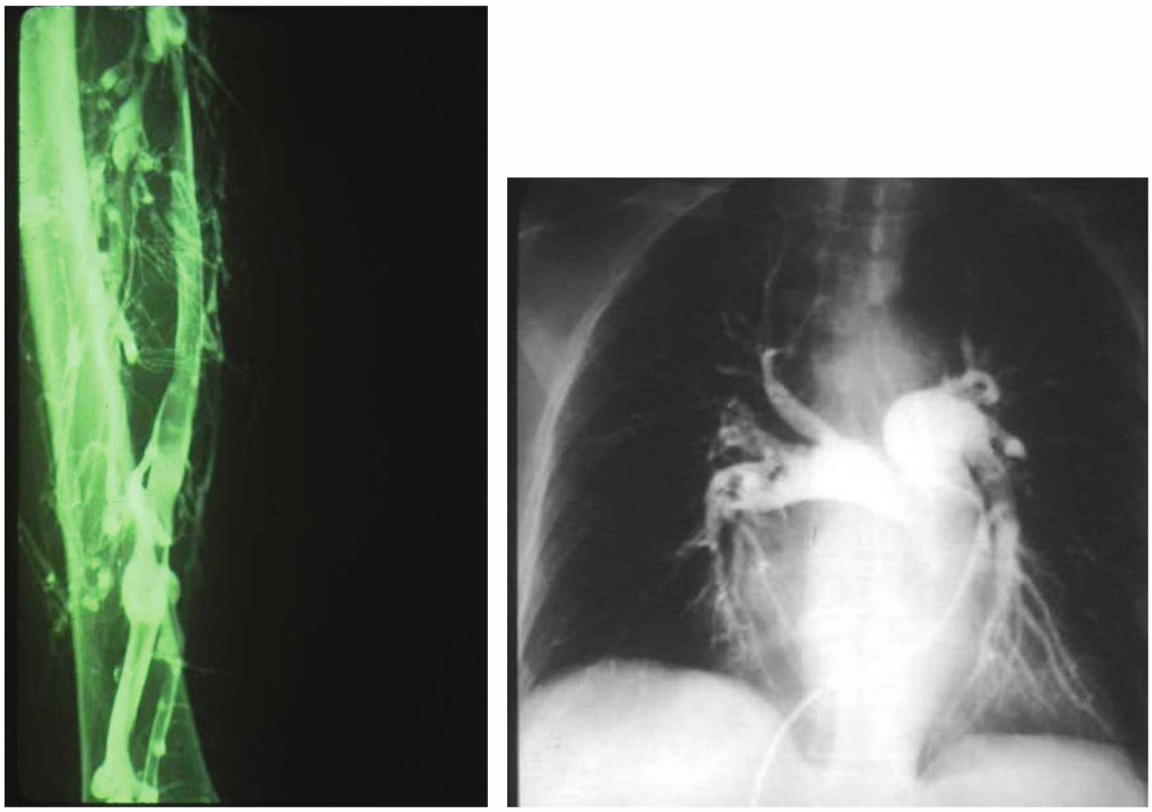

FIGURE 83.2 The historical diagnostic gold standards for DVT (phlebography, **left**) and PE (pulmonary angiography, **right**). The *arrows* point to filling defects surrounded by contrast dye (characteristic for a fresh thrombus) in the femoral vein (**left**), in the inferior lobar artery left, and in all lobar arteries right (**right**).

Clinical Probability Assessment

Sensitivity and specificity of clinical symptoms and signs, and abnormalities of blood gases, chest radiograph, and electrocardiogram in suspected DVT or PE are low when considered singly. The electrocardiogram is usually normal in small peripheral PE. Larger PE may induce modifications such as large P waves in leads II, III, and AVF, the S1Q3T3 pattern, or ST segment depression in leads V1 to V4. A right bundle branch block or right axis deviation is also possible.[23] Hypocapnia and hypoxemia are frequent in PE. However, approximately 20% of patients with proven PE have a normal arterial oxygen pressure and alveoloarterial oxygen gradient.[24] The most frequent anomalies of the chest radiograph are cardiomegaly, pleural effusion, plate-like atelectasis, and elevated hemidiaphragm.[14] Pulmonary artery enlargement and oligemia are rare and nonspecific. The typical infiltrate of so-called pulmonary infarction has already been discussed. Nevertheless, the chest radiograph remains extremely useful in a patient with suspected PE for differential diagnosis with conditions such as left ventricular failure, pneumonia, or pneumothorax.

Clinicians can combine these findings and other signs or symptoms very effectively either implicitly or by prediction rules in order to classify patients according to their probability of having the disease, the so-called prior clinical probability. As a practical tool for assessing prior clinical probability in suspected DVT, the Wells score[25] and its simplified version[26] have gained wide acceptance in spite of its partial subjectivity (Table 83.2). It is probably not more accurate than the simple implicit evaluation. However, it is easier to teach to junior physicians and allows discussing occasional disagreements among experienced clinicians on explicit grounds. Moreover, it has been shown that interobserver reliability was higher when using an explicit clinical model (kappa coefficient of agreement = 0.62 vs. 0.33 for implicit evaluation).[27] Nevertheless, both the implicit assessment and the explicit Wells score allow categorizing usefully patients in low, intermediate, or high clinical probability groups in which the prevalence of DVT is approximately 5%, 20%, and 80%, respectively.

Likewise, the initial step of any diagnostic workup in suspected PE consists of assessing the clinical probability of the patient having the disease. The aim of this step is to (a) identify the patients (probably those with a high or intermediate clinical probability) who require anticoagulant treatment while awaiting the results of diagnostic tests and (b) select the patients (those with a non–high clinical probability) in whom the diagnosis of PE can be excluded on the basis of a negative DD test result, also in relation with the type of DD assay (see also below) (FIGURE 83.3).

Two scores have been widely used (Table 83.3).[28,29] All these means of assessing clinical likelihood of PE allow a fairly accurate classification of patients into three categories corresponding to a prevalence of the disease of 5% to 10% (low clinical probability), 20% to 30% (intermediate clinical probability), and 60%

Table 83.2

Description of the Wells scores[9,26] for patients with clinically suspected DVT

Items, Original Score	Points	Items, Simplified Score	
Cancer	+1	Idem	+1
Paralysis or recent immobilization	+1	Idem	+1
Bedridden >3 d, or surgery/trauma <4 wk	+1	Idem	+1
Pain on palpation of the deep veins	+1	Idem	+1
Entire leg swollen	+1	Idem	+1
Calf swelling	+1	Idem	+1
Pitting edema (symptomatic side only)	+1	Idem	+1
Dilated superficial veins (symptomatic side only)	+1	Idem	+1
		Previously documented DVT	+1
Alternative diagnosis at least as likely as DVT	−2	Idem	−2
Clinical Probability		**Clinical Probability**	
Low	0	Unlikely	0–1
Intermediate	1–2	Likely	≥2
High	≧3		

to 80% (high clinical probability). Most patients with suspected PE have a low or intermediate clinical probability of having the disease. Those with a low or intermediate probability of PE can usually be investigated by entirely noninvasive algorithms. However, these rules also have limitations. The original Geneva score[30] required arterial blood gas values while breathing room air, a variable that was not available in 15% of the patients in the external validation sample. The Canadian score (Table 83.3) includes the clinician's judgment of whether an alternative diagnosis is more likely than that of PE.[29] This criterion has major

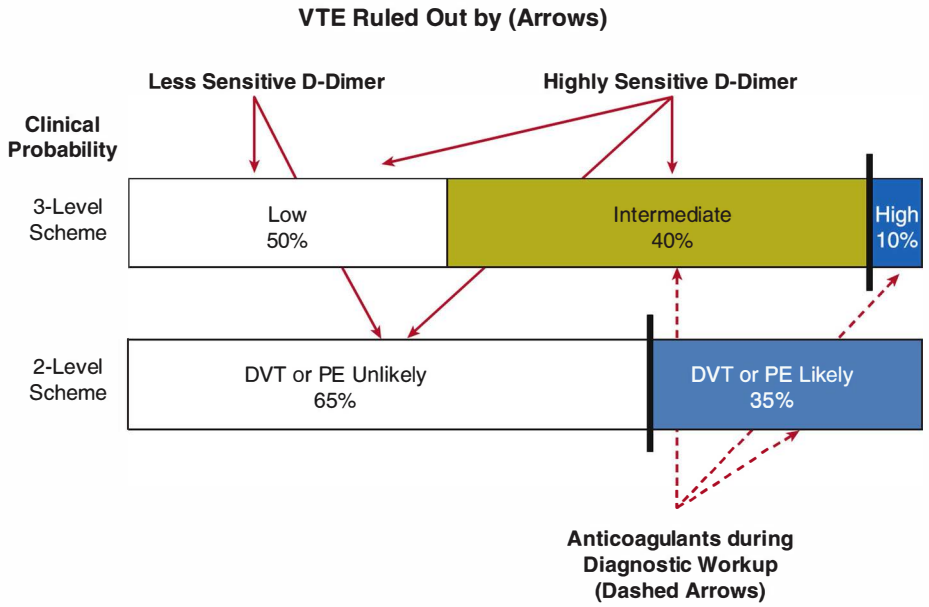

FIGURE 83.3 The objectives of clinical probability assessment. Clinical probability assessment can be expressed by means of 3-level or 2-level categories. It allows to rule out VTE by means of a negative DD test in patients with a low or unlikely assessment (less sensitive DD assays) or low and intermediate or unlikely assessment (highly sensitive DD assays). It also identifies patients who require anticoagulant treatment while awaiting results of diagnostic tests (patients with intermediate or high or likely assessment). The figures in the boxes represent the proportion of patients in the respective category.

weight in this score and can clearly not be standardized, thereby hampering its use by nonclinical health professionals.

The more recent "revised" Geneva score is entirely based on readily available clinical variables and is independent of physicians' implicit judgment (Table 83.3).[28]

In two recent outcome studies, the Wells score for PE has been used with a single cutoff of 4 points instead of two cutoffs.[31,32] This resulted in a classification of patients into two clinical probability categories (PE unlikely or likely) instead of the traditional three levels of probability (low, intermediate or high), which would theoretically allow the use of a less sensitive DD assay in a higher proportion of patients than when using the three-level probability scheme but is further from implicit clinical judgment. Further simplifications of both the Wells rule and the revised Geneva score, in which all items carry the same weight, have also been proposed and have undergone external validation.[33,34]

In a systematic review and meta-analysis of the performance of all available clinical prediction rules for suspected PE,[35] it was concluded that the various prediction rules have a similar accuracy in suspected PE. However, they are not identical and the choice between the various prediction rules and classification schemes must be guided by the local prevalence of PE, the type of patients considered (outpatients or inpatients) and the type of DD assay used. When using a highly sensitive DD assay, a three-level classification scheme is preferred because it mimics more closely clinicians' empirical reasoning and potentially rules out VTE in a higher proportion of patients. Less-sensitive DD assays should be used in combination with the Wells score with two levels of probability (VTE likely or unlikely) as these assays are not able to exclude VTE in 40% of the patients in the intermediate clinical probability category, and the VTE unlikely category includes a higher proportion of patients than the low probability class. The performance of the Wells score for PE is sensitive to the overall prevalence of PE in the population of patients with suspected disease: in a series in which the prevalence of PE was higher than 20%, the prevalence of PE in the low and intermediate probability categories was significantly higher (16%, pooled average) than in series with a lower prevalence of the disease (3.4%). This is not a problem when using a highly sensitive DD assay, but might be with less-sensitive assays. We therefore advise using the revised Geneva score in populations with a prevalence of PE above 20% as that rule has been derived and validated in such settings. Finally, the Wells score for PE (two—or three-level) is the only validated score in inpatients.

Table 83.3

Description of the two most widely used scores for clinical probability assessment in patients with clinically suspected PE

Revised Geneva Prediction Score for PE[28]		**Wells Clinical Prediction Score for PE[29]**	
	Points		**Points**
Age > 65 y	+1	Previous PE or DVT	+1.5
Previous DVT or PE	+3	Heart rate > 100/min	+1.5
Surgery (under general anesthesia) or fracture (of the lower limbs) within 1 mo	+2	Recent surgery or immobilization	+1.5
Active malignancy (solid or hematologic malignancy, currently active or considered as cured since <1 y)	+2	Clinical signs of DVT	+3
Unilateral lower limb pain	+3	Alternative diagnosis less likely than PE	+3
Hemoptysis	+2	Hemoptysis	+1
Heart rate 75–94 bpm	+3	Cancer	+1
Heart rate ≥95 bpm	+5		
Pain on lower limb deep vein palpation and unilateral edema	+4		
Clinical Probability		**Clinical Probability**	
Low	0–3	*Initial rule*	
Intermediate	4–10	Low	0–1
High	≥11	Intermediate	2–6
		High	≥7
		Dichotomized rule	
		Unlikely	≤4
		Likely	>4

Fibrin D-Dimer Measurement

DD is a degradation product of cross-linked fibrin and its blood concentration is increased in patients with acute VTE. It has been used as an adjunct in the diagnostic workup of DVT and PE for 20 years.[36] When assayed using a quantitative enzyme-linked immunosorbent assay (ELISA) or an automated turbidimetric assay, the DD level has been shown to be highly sensitive (more than 95%) in acute DVT or PE, usually with a cutoff value of 500 μg/L. Hence, a DD level below this value reasonably excludes acute VTE, at least in patients with a low or intermediate clinical probability. Their 3-month thromboembolic risk is <1%, as shown in a meta-analysis on the use of the highly sensitive VIDAS D-dimer Exclusion test.[37] According to these data, PE could be ruled out in about one third of clinically suspected outpatients. This proportion, however, varies according to patient categories (Table 83.4).[38–47] The Tinaquant test, an immunoturbimetric assay, has also been largely validated.[32] The result was negative ruling out PE in 41% of patients who had either a low clinical probability of PE or were classified as "PE unlikely" according to the dichotomized Wells rule. The 3-month thromboembolic risk was also <1%. Finally, the SimpliRed assay, a whole blood less-sensitive bedside test has also been well validated[45] but interobserver variability may be a problem, and exclusion of the disease is only possible in patients with low or "unlikely" clinical probability. Conversely, patients with a high clinical probability should not be tested for DD because a concentration below the diagnostic cutoff is rare in this category of patients,[39] and above all because the upper limit of the 95% CI for the 3-month occurrence of PE remains above 3% despite a negative result.

In order to increase the exclusion rate of DD in elderly patients, a European group devised an age-dependent DD exclusion cutoff for PE that was based on 5,132 patients aged 50 or above from four previous prospective cohort studies.[48] The individualized DD cutoff value was defined as patient's age × 10 μg/L in patients aged above 50. In 1,331 patients in the derivation set with an "unlikely" score from clinical probability assessment, PE could be excluded in 42% with the new cutoff value versus 36% with the old cutoff value (<500 μg/L). In the two validation sets, the absolute increase in the proportion of patients with a DD below the new cutoff value compared with the old value was 5% and 6%, respectively. This increase was largest among patients aged above 70 years, ranging from 13% to 16% in the three datasets. The failure rates (all ages) were 0.2% (95% CI, 0 to 1.0) in the derivation set and 0.6% (95% CI, 0.3 to 1.3) and 0.3% (95% CI, 0.1 to 1.1) in the two validation sets, respectively. Thus, it appears that an age-adjusted DD cutoff point, combined with clinical probability, greatly increases

Table 83.4

Diagnostic yield of DD for ruling out PE in specific patient populations

Author	Clinical Conditions	NNT to Rule Out One PE
High-sensitivity, Low-specificity DD tests		
Perrier et al.[38]	Outpatients with suspected PE	3.3
Righini et al.[39]	High clinical probability Nonhigh clinical probability	9.1 2.2
Righini et al.[40]	Cancer No cancer	9.1 3.1
Le Gal et al.[41]	Previous VTE No previous VTE	6.3 3.1
Chabloz et al.[42]	Pregnancy before the 30th wk Pregnancy between wk 30 and 42	2.6 4
Righini et al.[43]	Elderly outpatients of more than 80 y	20
Miron et al.[44]	Inpatients (nonsurgical patients) Inpatients (surgical patients)	30 Infinite[a]
Low-sensitivity, High-specificity DD tests		
Wells et al.[45]	Outpatients with suspected PE	2.1
de Groot et al.[46]	In and outpatients with suspected PE	3.7
Kearon et al.[47]	In and outpatients with suspected PE	3.1

The number needed to test (NNT) reflects the number of patients in whom DD measurement has to be performed to rule out one PE.
[a]No patient with negative DD result in this cohort

the proportion of older patients in whom PE could be safely excluded; this is presently being externally further validated in a prospective clinical outcome study. It is clear that this rule will be valid only for the two tests that were used in the original studies, the VIDAS and the Tinaquant DD tests. A similar approach has been proposed for pregnant women clinically suspected of DVT[49] but the proposed increased cutoffs clearly require validation in prospective management studies.

Although DD is very specific for fibrin, the specificity of fibrin for VTE is poor. Indeed, fibrin is produced in a wide variety of conditions such as cancer, inflammation, infection, and necrosis. Hence, a DD concentration above 500 μg/L (the diagnostic cutoff for many assays) has a poor positive predictive value for VTE, and cannot reliably rule in the disease. Nevertheless, based on the results of a retrospective analysis of a large patient sample, Bosson et al.[50] suggested that a DD level above 2,000 μg/L was predictive of the presence of PE, independently of the clinical score with an odds ratio of 6.9. They noted, however, that this result requires clinical validation in a prospective study, particularly because the PE prevalence in their series was high: 27%, 58%, and 85% in the low, intermediate and high clinical probability categories, respectively. The Geneva group had previously reported that patients with an intermediate or high clinical suspicion of PE and a DD level above 4,000 μg/L had a positive likelihood ratio of five of having the disease, but few clinicians would accept the diagnosis just on this basis.[51] Recent quantitative point-of-care (POC) tests have shown a promising sensitivity of 96% but only in patients suspected of DVT and in a relatively small sample size.[52]

In summary, negative highly sensitive DD assays rule out DVT and PE in outpatients with a low or intermediate clinical probability of PE, while whole-blood agglutination tests only rule out the disease in patients with a low clinical probability. Finally, DD is unlikely to be useful in very elderly patients or in hospitalized patients with suspected VTE, unless specific cutoff levels are well established in these subgroups.

Venous Compression Ultrasonography

In studies using phlebography as the gold standard, lower limb venous CUS, an entirely noninvasive test, has a sensitivity of 97% (95% CI 96% to 98%) and a specificity of 98% for symptomatic proximal DVT.[53] It has become the cornerstone of DVT diagnosis in clinically suspected individuals. The single well validated diagnostic criterion for DVT on CUS is absence of full compressibility of the deep vein when applying pressure through the ultrasound probe (FIGURE 83.4). Intraluminal venous ultrasonography has been reported to have variable accuracy, and neither changes in venous diameter during Valsalva maneuver nor assessment of Doppler flow have been found to improve diagnostic accuracy for DVT. The extensiveness of the exam (particularly the inclusion or noninclusion of calf veins in the diagnostic procedure) is heavily debated among experts and will be discussed in paragraph 5.1. In a patient with clinically suspected PE, finding a DVT (at least a proximal one) by CUS is sufficient evidence to warrant anticoagulant treatment without further lung imaging. CUS shows a DVT in approximately 30% to 50% of patients with proven PE.[53,54]

The observation of a low 3-month thromboembolic risk in patients with a negative CUS of the proximal veins (around 1% in management studies) questions the need for diagnosing the so-called distal DVT, at least in non–high clinical probability patients.[55] Indeed, in such patients, detecting clots in the posterior tibial or peroneal veins or even in calf muscle veins may be double-edged: on one hand, the potential of reducing the 3-month thromboembolic risk is limited (because it is already quite low), and, on the other hand, the risk of false-positive findings and subsequent unnecessary anticoagulant treatment in patients who could be left untreated, is quite high. Nevertheless, several large series suggest that complete examination of the leg deep vein system without any other exam is safe and effective in managing patients with clinically suspected DVT, as evidenced from a meta-analysis.[56] This finding, however, does not solve the problem of

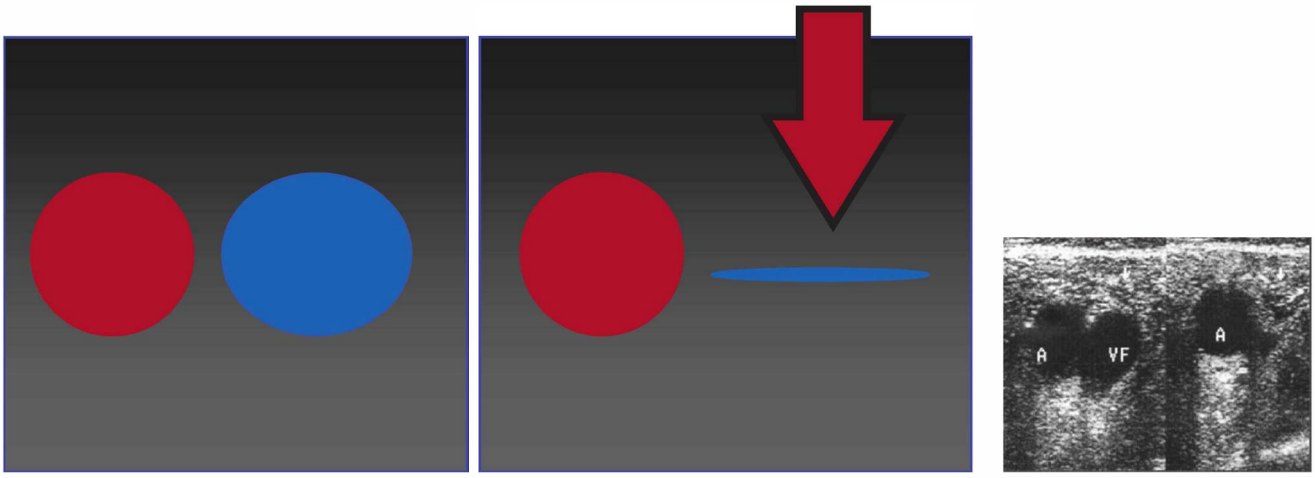

FIGURE 83.4 Ultrasonogram showing from the left to the right, the adjacent femoral artery (A) and vein (VF), the full compressibility of the vein by the transducer, characteristic of the absence of thrombosis (from **left** to **right**) schematic representation (without and with compression) and ultrasonographic representation (without and with compression), and (last image on the **right**) the incompressibility of the vein by the transducer, characteristic for the presence of a totally or partially occluding thrombus. This procedure is referred to as CUS.

the increased population put at risk of anticoagulation-induced bleeding. The ongoing randomized, double-blind CACTUS (NCT00421538) study comparing anticoagulant therapy with placebo should help clarifying this issue in the future.

Ventilation/Perfusion Lung Scintigraphy

Perfusion lung scintigraphy (also called V/Q scan) is a noninvasive technique allowing the visualization of pulmonary perfusion through intravenous injection of albumin macroaggregates labeled by technetium-99m.The macroaggregates are trapped in approximately 0.1% of the pulmonary capillary vessels and may be imaged by a gamma camera. Pulmonary hypoperfusion is not highly specific for an embolus, since any disease that narrows the airways or fills the alveoli with fluid will result in hypoxic pulmonary vasoconstriction, a protective mechanism designed to minimize the shunt effect due to poorly ventilated alveolar units. A perfusion defect corresponding to a segment or a large part of a segment is more specific for PE. The addition of ventilation scintigraphy (by Xenon 133, Krypton 81m or aerosolized technetium-99m) further increases specificity, a so-called mismatched defect (perfusion defect with normal ventilation) usually representing PE. The interpretation of lung scan has long been based on criteria validated in the landmark Prospective Investigation On Pulmonary Embolism Diagnosis (PIOPED) study,[19] and their subsequent revisions. More recently, it has been greatly simplified and lung scan results are now classified into three categories: normal (no PE), high probability (PE), and nondiagnostic.[57] The high negative predictive value of a normal lung scan has been confirmed by several studies, and is recognized as a valid criterion for excluding PE. The positive predictive value of a high probability scan is approximately 90%, and most clinicians consider such a result to rule in PE. Recent evidence suggests that the ventilation scan may be validly replaced by chest x-ray with an overall agreement of 88% and a positive predictive value of 86% for a scintigraphic mismatch.[58] The proportion of diagnostic ventilation–perfusion lung scans (i.e., normal or high probability) varies between 50% and 70% according to the patient population studied and the adopted diagnostic criteria. The combination of clinical probability and CUS allow reaching a clinical decision in most cases. In a series of 1,239 patients, Wells et al.[59] withheld anticoagulant treatment in 702 (57%) patients who had a nondiagnostic scan, a low or intermediate clinical probability of PE, and normal serial CUS, and the 3-month thromboembolic risk was only 0.5% (95% CI 0.1% to 1.3%).

Whether a baseline lung scan should be systematically performed in patients with suspected PE and a proximal DVT shown by CUS remains controversial. Recurrent PE is quite a rare event in treated patients, and the interpretation of a lung scan performed for a suspected recurrence is not always straightforward. In our experience, a baseline lung scan is rarely useful, and is unlikely to be cost-effective (see specific section on recurrent VTE below). Whatsoever, except in some dedicated centers, lung scan has been largely replaced by computed tomography angiography (CTA).

Computed Tomography Angiography

The rapid acquisition of high contrast images by spiral (also called helical) CTA scanning allows an adequate visualization of the pulmonary arteries up to at least the segmental level (FIGURE 83.5).[60] Two systematic overviews on the performance of single-detector computed tomography angiography (SDCTA) in suspected PE reported wide variations regarding both CT sensitivity (53% to 100%) and specificity (73% to 100%).[61,62] Variations in image acquisition protocols, selection bias, and flaws in the design of the older studies may partly account for these differences. Two clinical studies[63,64] have shed new light on the performance of CTA. They included a wide spectrum of patients with suspected PE; the diagnostic criteria for PE were appropriate and both spiral CT and reference diagnostic tests were read in a blinded fashion. They both report a sensitivity around 70% and a specificity of 90%. As in previous series, CTA was technically inadequate in 5% to 8% of patients because of motion artifacts or insufficient opacification of the pulmonary vessels.

Sensitivity of CT is higher in central pulmonary emboli than in segmental and subsegmental arteries. MDCTA, which allows both a thinner collimation (1 to 2 mm collimation) and a better definition without increasing image acquisition time, will likely improve sensitivity.[65] To what extent this will be at the expense of specificity remains to be evaluated. Indeed, in the Geneva series, the positive predictive value of CTA decreased from 100% to 85% at the main pulmonary or lobar artery level, respectively, and to 62% at the segmental level, demonstrating that specificity of CTA is also dependent on the vascular level. Moreover, the clinical significance of isolated segmental and subsegmental emboli is widely debated, and patients with no DVT, a negative CTA, and a low or intermediate clinical probability of PE left untreated may have a very low thromboembolic risk.[66] In summary, CTA showing a thrombus down to the segmental level can be taken as adequate evidence of PE, whereas the necessity to treat isolated subsegmental thrombi in a patient without a DVT is unclear. A negative SDCTA alone does not rule out PE. Finally, the probability of PE is very low in patients with a low or intermediate clinical probability, absence of proximal DVT, and a negative SDCTA. Second generation MDCTA exhibits enhanced sensitivity at the cost of slightly reduced specificity. Thus, in a meta-analysis the negative predictive value of a negative MDCTA was as high as 99.1%.[67]

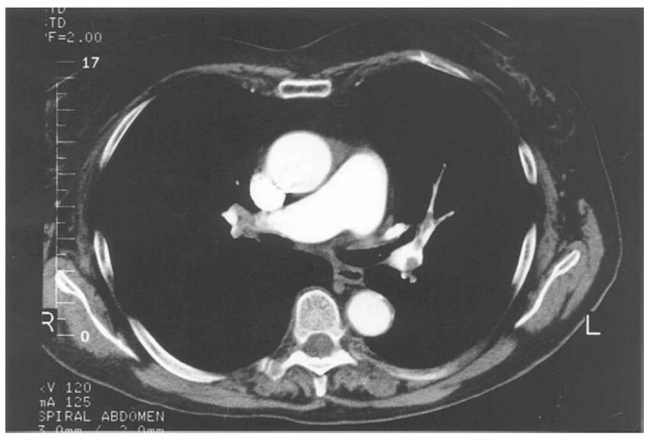

FIGURE 83.5 MDCTA showing filling defects of the contrast dye in the right pulmonary artery and in several segments of the left pulmonary artery tree (*arrows*).

Some groups proposed a combined CT phlebography of lower limbs and abdominal veins together with CTA of the pulmonary arteries, allowing a complete examination of VTE in one session.[68] Although appealing at first glance, the exam is more costly and results in greater irradiation of the patient. Moreover, patients with suspected PE in whom a DVT is found by CUS do not require a CT exam at all and are thus spared the irradiation, contrast dye administration, inconvenience, and costs of CT.

Dual energy CTA provides a morphologic analysis similar to the perfusion scan. Cardiac gated CTA is useful to rule out not only PE but also aortic dissection and coronary artery disease, but it takes longer and the radiation dose is higher.

Magnetic Resonance Pulmonary Angiography

Because it is devoid of irradiation, magnetic resonance imaging (MRI) is a potential candidate for diagnosing PE. Recently, the accuracy of gadolinium-enhanced magnetic resonance pulmonary angiography and magnetic resonance venography (MRV) for diagnosing PE has been studied in a prospective, multicenter accuracy study on 371 adults with diagnosed or excluded PE from seven North American hospitals.[69] The reference standard diagnosis was made by using various tests, including computed tomographic angiography and venography, ventilation–perfusion lung scan, venous ultrasonography, DD assay, and clinical assessment. MRA, averaged across centers, was technically inadequate in 25% of patients (92 of 371). Of note, the proportion of technically inadequate images ranged from 11% to 52% across centers. Including patients with technically inadequate images, magnetic resonance angiography (MRA) identified 57% (59 of 104) of those with PE. Technically adequate MRA had a sensitivity of 78% and a specificity of 99%. Technically adequate MRA and MRV had a sensitivity of 92% and a specificity of 96%, but 52% of the patients (194 of 370) had technically inadequate results. Therefore, MRA should be considered only in centers with an adequate experience of the technique only in patients for whom standard tests are contraindicated, which will limit its use to a small minority of patients at the present stage.

Echocardiography

Doppler echocardiography has several uses in suspected PE, including in risk stratification. In a small subset of patients with PE (4% in a recent registry),[70] transthoracic echocardiography allows direct visualization of the clot in the right heart chambers or in the right main pulmonary artery. Direct imaging of part of the left main pulmonary artery requires transesophageal echocardiography. However, transesophageal echocardiography is uncomfortable for patients, and its sensitivity in suspected PE does not appear to be significantly higher than that of transthoracic echocardiography. In fact, the most frequent echocardiographic manifestations of PE are indirect and reflect the hemodynamic changes caused by an acute increase in pulmonary arterial resistance and pulmonary hypertension.

Pulmonary arterial pressure may be estimated in most patients by the tricuspid regurgitation velocity. A cutoff value of 2.7 m/s for the presence of pulmonary arterial hypertension was adopted in several series. Signs of RV strain include dilation of the right ventricle, RV hypokinesis, and in severe cases, paradoxical motion of the interventricular septum. Several echocardiographic measurements have been proposed to quantify RV dilation, of which the most standardized is the right ventricle over left ventricle diameter ratio. However, a visual estimate appears to be as accurate in the eyes of an experienced observer.[71] A particular pattern of RV hypokinesis characterized by hypokinesis of the mid-free wall and preservation of apex motion appears to be quite specific for acute as opposed to chronic pulmonary hypertension.[72] Conversely, RV hypertrophy and pulmonary artery pressures above 60 mm Hg suggest chronic pulmonary hypertension. The sensitivity of these signs that are often combined lies between 40% and 70% in clinically suspected PE, and their specificity is approximately 90%, provided the patient does not have another disease causing chronic pulmonary hypertension. Echocardiography is the first-line test in suspected massive PE (see Section 5.2.2). Indeed, in patients with shock, it is extremely effective for differential diagnosis with tamponade and cardiogenic shock. Moreover, absence of pulmonary hypertension and/or RV dilation and hypokinesis in that situation renders PE as the cause of shock unlikely.

In patients with suspected nonmassive PE, echocardiography may also represent a useful tool for prognostic stratification of patients,[6] a process that is not followed yet by specific therapeutic measures.

RISK STRATIFICATION

The goal of risk stratification is twofold: identifying low-risk patients who may be managed with a very short hospital stay or even as outpatients, and non–high-risk patients who have an intermediate risk of death and might benefit from more aggressive management than anticoagulants alone, although the superiority of thrombolysis in these patients is still unproved.[6,73] Several instruments allow stratifying patients with PE into various risk categories (Table 83.1).[6] Clinical evaluation is a necessary first step as shock or systemic arterial hypotension carries a very high mortality and requires aggressive treatment, usually thrombolysis, without any further risk stratification. In non–high-risk patients, clinical prognostic scores allow identifying patients with a low risk of mortality due to PE. The intermediate-risk category is defined by the presence of RV dysfunction detected by echocardiography (Table 83.1). More recently, several biomarkers, mainly natriuretic peptides (NT-proBNP and BNP) and troponin have been evaluated as alternatives or additions to echocardiography.

Clinical Prognostic Scores

Clinical prognostic scores fall into two categories: those designed to identify low-risk patients, such as the Geneva risk score[74] or the pulmonary embolism severity index (PESI)[75] and its simplified version[76], and scores aimed at targeting intermediate-risk patients with a mortality high enough to warrant aggressive treatment, such as the PREP index (Table 83.5).[77] The Geneva risk score predicts the 3-month risk of death, recurrent thromboembolic event, or major bleeding, and requires a lower limb venous ultrasound as finding a DVT increases the risk. The prognostic importance of concomitant DVT has been confirmed recently in a registry in which the risk of all-cause mortality in patients with concomitant DVT was 2.0 and PE-related mortality 4.2 compared with patients with no DVT.[78] The Geneva risk score has been externally validated and the 3-month unfavorable

outcome rate was low (5%) in the low-risk category, albeit higher than in the derivation sample (2.2%).[79] The PESI score is entirely based on clinical variables and has been extensively validated,[80–82] but it has the drawback of being complicated to calculate and includes ambiguous items. Recently, a Spanish group proposed a simplified version of the PESI score[76] based on part of the original items that seems to retain a similar accuracy (Table 83.5). Although validated in the RIETE registry,[76] this potentially very useful score requires a prospective validation. Finally, the PREP index[77] that incorporates DD and BNP measurement and echocardiographic assessment of RV function was designed to predict higher-risk patients without shock. It is able to identify patients with a 22.2% 30-day adverse outcome risk, albeit with a wide CI and still requires external validation. Interestingly, only a small proportion of the patients in PREP cohort (9 of 570) had a high risk.

Echocardiography

RV dysfunction on echocardiography appears to be associated with higher mortality in acute PE. In a classic study by Grifoni et al.[83] around 30% of normotensive patients with acute PE had RV dysfunction on echocardiography, defined as RV dilatation, RV/left ventricular end-diastolic diameter ratio >1, paradoxical

Table 83.5

Clinical prognostic scores for stratifying patients with PE

***PESI*[75]**	
Age, per year	Age, in years
Male sex	10
History of cancer	30
History of heart failure	10
History of chronic lung disease	10
Pulse ≥ 110/min	20
Systolic blood pressure < 100 mm Hg	30
Respiratory rate ≥ 30/min	20
Temperature < 36°C	20
Altered mental status	60
Arterial oxygen saturation < 90%[a]	20
Risk Classes	**Outcome (30-d Mortality)**, % (95% CI)[81]
Low risk ≤85	1.1 (0.1–3.8)
High risk 86 or more	11.1 (6.8–16.8)
***Simplified PESI Score*[76]**	
Age > 80 y	1
History of cancer	1
History of heart failure or chronic lung disease	1
Pulse ≥ 110 bpm	1
Systolic blood pressure <100 mm Hg	1
Arterial oxyhemoglobin saturation < 90%	1
Risk Classes	**Outcome (30-d Mortality)**, % (95% CI)
Low risk 0	1.0 (0.0–2.1)
High risk 1 or more	10.9 (8.5–13.2)
***Geneva Risk Score*[74]**	
Cancer	2
Heart failure	1
Previous DVT	1
Systolic blood pressure < 100 mm Hg	2
PaO_2 < 8 kPa	1
DVT shown by ultrasound	1

(Continued)

Table 83.5

Clinical prognostic scores for stratifying patients with PE *(Continued)*

Risk Classes	**Outcome (3-mo Mortality, Recurrent Thromboembolic Event, or Major Bleeding)**, % (95% CI)[79]	
Low risk ≤ 2	5 (2.6–9.6)	
High risk three or more	27.5 (16.1–42.8)	
PREP Score[77]		
Altered mental state	10	
Cardiogenic shock on admission	6	
Cancer	6	
BNP (ng/L)		
100–249	1	
250–499	2	
500–999	4	
≥1,000	8	
RV/LV ratio		
0.5–0.74	3	
0.75–1.0	5	
1.0–1.25	8	
≥1.25	11	
Risk Classes	**Outcome (30-d Mortality, Secondary Cardiogenic Shock as Previously Defined, or Recurrent VTE)**, % (95% CI)[77]	
	All Patients	**Patients Without Shock**
Low risk < 7	2.5 (1.3–4.8)	1.9 (0.9–4.0)
Intermediate risk 7–17	11.6 (7.5–17.6)	11.7 (7.5–18.0)
High risk ≥ 18	43.2 (28.7–59.1)	22.2 (6.3–54.7)

septal systolic motion, or pulmonary hypertension. Six of those 65 patients developed PE-related shock, of whom 3 died compared with none of the patients with normal blood pressure and no signs of RV dysfunction. An analysis of the ICOPER registry[84] yielded similar results, albeit with much higher mortality rates (16.3% 30-day mortality in normotensive patients with RV dysfunction compared with 9.4%). In a monocenter French registry, the odds ratio of dying (2.7) in patients with an RV/LV ratio ≥0.9 was similar even after adjusting for other prognostic factors including shock but mortality rates were much lower (3.3% vs. 1.1%).[85]

Collectively, those results suggest that echocardiography may be an adequate risk stratifier in patients with acute PE but without shock. Nevertheless, mortality rates appear to be lower in series with nonselected patients, approaching the risk if an intracranial bleed due to thrombolytics.[86] Moreover, echocardiography criteria for RV dysfunction are far from uniform, rendering the comparison of those studies difficult.[6]

Biomarkers

Since only 5% to 10% of patients with PE present with shock and only around 30% of the remainder have echocardiographic evidence of RV dysfunction, systematic risk stratification by echocardiography would require performing that test unnecessarily in 60% of patients. Therefore, several biomarkers have been evaluated as first-line tests to identify low-risk or intermediate-risk patients. Brain natriuretic peptide (BNP or NT-proBNP) levels have been shown to be elevated in more severe PE and reflect RV overload.[87] In a recent meta-analysis,[88] patients with an elevated BNP or NT-proBNP concentration had a considerably higher risk of death (OR 7.6, 95% CI 3.4 to 17.0). However, although patients with a high BNP had an absolute mortality risk of 14% compared with 2.2% in those with normal BNP levels (OR 6.5), the odds ratio of dying decreased to 3.3 when the three studies that considered only in-hospital mortality were singled out. Moreover, most of those studies included patients in shock, which certainly had a great influence on the results.

Troponin T or I is a marker of myocardial injury and has been shown to be elevated in severe acute PE, probably due to subendocardial ischemia of the overloaded RV. In one of several recent meta-analyses on troponin in acute PE that focused on normotensive patients,[89] 28% had an elevated troponin level. Their mortality was 15.9% compared with 3.4% in those with a normal troponin. In a recent series of 146 patients with acute PE and without shock, NT-proBNP was elevated in all 18 patients

who had an unfavorable outcome within 3 months, while 55% of patients without complications had abnormal values (OR of complications 15.8, 95% CI 2.1 to 122).[90] The predictive accuracy of NT-proBNP was superior to that of troponin (OR 3.5, 95% CI 1.2 to 9.7) and NT-proBNP was the only independent predictor of an adverse outcome in multivariate analysis. A similar superiority of NT-proBNP was observed in a series of intensive care patients.[91] Highly sensitive troponin assays might have a higher negative predictive value. The 30-day risk for death, shock, or intubation was 0% (0 of 56) in patients with a highly sensitive troponin level <14 pg/mL in a recent series of 156 patients.[92] Finally, in a large series of 1,273 patients with acute PE and no hemodynamic compromise, troponin I and echocardiography were independent predictors of PE-related death and yielded additive information.[93] Mortality ranged from 0.7% in patients with normal troponin and no RV dilatation to 8.3% in those with both a high concentration of troponin and an enlarged RV. Other biomarkers have been assessed, such as fatty acid–binding protein (FABP)[94] or DD,[90] but the evidence is still preliminary, and they suffer from the same shortcomings as BNP and troponin, that is, lack of assay standardization, variable cutoff, low positive predictive value for adverse outcome, variable definition and time frame of adverse outcome, and low specificity.

Computed Tomography Angiography

CTA provides no information in RV function, but measuring the diameter of the heart chambers allows the measurement of the RV/LV ratio in transverse sections. Since CTA is performed in most patients for diagnosing PE, it is an attractive instrument for risk stratification. Preliminary evidence from a retrospective series of 431 patients with acute PE confirmed by CTA was encouraging.[95] Of the 276 (64%) patients with RV/LV ratio >0.9, 15.6% died within 30 days compared with 7.7% of those without RV enlargement. The association between RV enlargement on CT and mortality remained after adjusting for other prognostic variables. However, other reports have been unable to replicate this finding. A large retrospective analysis of a single-center experience including 1,193 patients did not find any association between the RV/LV ratio and mortality, and only a weak association (OR around 2.0) for ventricular septal bowing, another classic sign of RV overload.[96]

Implications for Treatment

Several conditions must be met for risk stratification of acute PE to have practical clinical implications. For identifying low-risk patients, available instruments must be easily available, inexpensive, and have a high negative predictive value for adverse outcome. Clinical scores appear to fit those requirements better than biomarkers, echocardiography, or CTA. Targeting patients with no hemodynamic compromise but a higher risk of recurrence or death who might benefit from more aggressive treatment requires tests with a high positive predictive value. As shown in the previous sections, the in-hospital PE-related mortality in patients with RV dysfunction on echocardiography or elevated biomarkers is in the range of 5% and higher figures relate to all-cause mortality during a longer time span (30 days to 3 months). The only randomized trial on the efficacy of thrombolysis in hemodynamically stable patients with acute PE and RV dysfunction was conducted by Konstantinides et al.[73] Overall mortality was only 2.2% in the control (heparin) group. Alteplase was more effective than heparin alone regarding the primary outcome, which was a composite of in-hospital death or clinical deterioration requiring treatment escalation. The absolute difference in favor of alteplase was 13.6% but was entirely driven by treatment escalation, notably rescue alteplase infusion. Therefore, the benefit of thrombolysis in stable patients with RV dysfunction remains unproved and this controversy will hopefully be settled by the ongoing PEITHO trial (NCT00639743). In the meantime, risk assessment by a clinical score to identify low-risk patients who may benefit of shortened hospital stay or outpatient treatment is warranted, but further risk stratification by biomarkers and/or echocardiography is not routinely recommended. Risk stratification by CT appears too unreliable to be clinically useful.

DIAGNOSTIC STRATEGIES

Systematic evaluation of a new diagnostic test or strategy in suspected VTE should include the following four steps[97]: (a) technical description of the method; (b) systematic comparison with a diagnostic standard to establish the critical cutoff concentration in tests with continuous values such as DD (using Receiver Operating Characteristics curve analysis), and values of sensitivity and specificity of the test for VTE; (c) use of the test in so-called management trials in which anticoagulation is withheld in patients with a diagnostic criterion ruling out VTE; a systematic 3-month follow-up allows detection of delayed events and establishment of the safety of the test; and (d) cost-effectiveness analyses comparing the « new » strategy with other management policies.

Sequential Diagnostic Strategies for Suspected DVT

Several noninvasive strategies have been reported to have a high sensitivity for diagnosing DVT.[98] Some rely on serial proximal CUS of proximal veins (common femoral, superficial femoral, and popliteal veins). The rationale for repeating CUS after 1 week in patients in whom it was initially negative is the potential detection of the rare proximally extending distal DVTs that were not searched for. Because the yield of repeat CUS after 1 week is very low (<1% to a few percent), CUS has been combined with a clinical prediction rule to lower the number of necessary repeat compression sonograms. Thus, repeat CUS was waived in patients with a low clinical probability. In another strategy,[99] repeat CUS was restricted to patients with a DD concentration above a critical cutoff level (500 μg/L), thereby avoiding about 30% of CUS exams. All these strategies are associated with a low (about 2% or less) 3-month thromboembolic risk, which is similar to that observed in suspected patients left untreated following a normal phlebogram.[17] In a randomized study, Wells et al.[26] compared the clinical outcome in 530 patients who underwent proximal CUS (control group) and 566 patients who underwent DD testing followed by proximal CUS unless the DD test was negative and the patient was considered at low clinical probability. The 3-month thromboembolic risk among patients in whom the initial diagnostic strategy had ruled out DVT was 0.4% in the DD group, compared with 1.4% in the control group. The difference did not reach statistical significance but the use of DD

resulted in a highly significant reduction in the use of CUS, since 39% of patients did not require ultrasound imaging. One major issue nowadays is to identify which patients should undergo testing. In the AMUSE study, a simple rule (male gender, use of contraceptive pill, malignancy, recent surgery, absence of leg trauma, vein distension, one point each; calf difference ≥3 cm, 2 points; abnormal POC DD, 6 points; cutoff: DVT ruled out if score ≤3) was able to avoid further testing in half of a primary care population of 1,028 patients (prevalence of DVT 13%), with a 3-month thromboembolic risk of 1.4% (0.6 to 2.9).[100] Finally, complete ultrasound, that is, examination of the proximal and distal leg veins is also an accepted strategy for diagnosing DVT. In a prospective management study, patients clinically suspected of DVT were randomized to CUS limited to the proximal veins or to complete CUS. Thrombosis was diagnosed in 59 of the 257 patients who underwent limited CUS (23%) and in 99 of the 264 patients who underwent complete CUS (38%) with a similar 3-month follow-up (four patients and two patients, respectively, being diagnosed with VTE).[101] This study highlighted that the added costs of complete CUS did not translate into added safety and might even result in added risk due to unnecessary anticoagulant treatment. Therefore, complete CUS is unlikely to be cost-effective, although this has never been formally assessed. FIGURE 83.6 displays the strategy that appeared to be most cost-effective in a formal cost-effectiveness analysis (see also specific section below).[102]

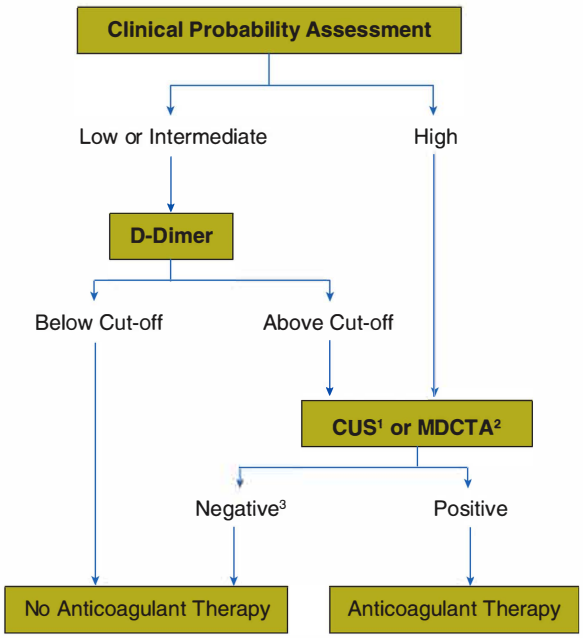

FIGURE 83.6 A contemporary simple diagnostic algorithm for suspected DVT or PE. DD refers to highly sensitive DD assays. If less sensitive assays are used, a negative DD result rules out PE or DVT only in the presence of a low (or unlikely) clinical probability (see also FIGURE 83.1). ¹ CUS (lower limb venous CUS) in case of suspected DVT. ² MDCTA in case of suspected PE.³ In case of negative CUS or MDCTA and high prior clinical probability, consider additional imaging, for example, phlebography (suspected DVT) or lung ventilation/perfusion scintigraphy or pulmonary angiography (suspected PE).

Sequential Diagnostic Strategies for Suspected PE

Diagnosis of Non–high-risk PE

In patients who are not hemodynamically compromised, fully noninvasive strategies have proven effective and safe. One such strategy relies on assessing prior clinical probability, either empirically or by a prediction rule, and on the use of ventilation/perfusion lung scanning. Using CUS at an early stage of the diagnostic workup, given that diagnosing DVT in a patient clinically suspected of PE warrants anticoagulant therapy, should be reserved for patients with contraindication to MDCTA (e.g., with renal insufficiency or allergy to iodine contrast dye), especially if clinical signs or symptoms of DVT are present. In all other situations, the strategy starts with measuring DD and withholding anticoagulants in patients with a concentration below the cutoff level,[32,103] as illustrated in FIGURE 83.6. Of note, it is associated with a very low (<1%) 3-month thromboembolic risk, similar to that associated with a normal lung perfusion scan[104] or a negative pulmonary angiogram.[20] The question of whether a DD concentration below the cutoff level using a highly sensitive assay rules out PE even in patients with a high clinical probability remains controversial, and a conservative approach is appropriate in such patients, that is, skipping the initial DD test. Although SDCTA is probably no longer used, it may be useful to stress that its sensitivity for the presence of PE is only 70%, which precluded its use as the sole diagnostic test.[63] Note that in order to rule out PE, the combination of a negative lower limb CUS and a negative SDCTA was required in patients with a low or intermediate clinical probability of PE,[105,106] and that ventilation–perfusion scintigraphy and/or pulmonary angiography was required in high-probability patients with a negative noninvasive workup.

Thanks to the improved performances of MDCTA, it was hypothesized that lower limb CUS would no longer be required to rule out PE, as suggested in two management studies.[32,107] In the prospective, multicenter Swiss-French study of patients admitted to the emergency department for clinically suspected PE, the proportion of patients with proximal DVT despite negative findings on MDCTA was very low (0.9%; 95% CI, 0.3 to 2.7).[107] Therefore, the improvement of the overall detection rate of PE by CUS was marginal in this series, and the 3-month thromboembolic risk in patients left untreated if PE had been ruled out on the sole basis of a negative MDCTA would have been 1.5% (95% CI, 0.9 to 2.7), similar to that of a negative pulmonary angiography. A strong additional support for the safety of using MDCTA as a single imaging modality arises from the CHRISTOPHER study.[32] In that series, patients with an elevated DD level or in whom PE was considered likely according to the dichotomized Wells rule (n = 1,436) underwent a CTA (multidetector CTA, 88% of the patients). The 3-month thromboembolic risk in patients not treated based on a negative CTA was 1.3% (95% CI, 0.7 to 2.0). Moreover, in the study by Ghanima et al.[108] 432 consecutive outpatients clinically suspected of PE who were sequentially submitted to clinical assessment, DD measurement, and MDCTA, a definitive diagnosis was reached in 96.5% of patients with a 3-month thromboembolic risk of 0.6% (95% CI, 0% to 2.2%). Collectively, those series support the safety of ruling out PE by DD measurement and multidetector CTA in non-–high clinical

probability patients without resorting to lower limb CUS. The final proof was given by a multicenter, randomized, noninferiority study that compared the strategies with and without CUS, and found no difference in outcome between the two arms,[103] which was confirmed in a meta-analysis.[109] Overall, the meta-analysis by Moores et al.[110] that compiled 23 studies with 4,657 patients with a negative CTA (mainly single-detector) who did not receive anticoagulation showed a 3-month rate of subsequent VTE of 1.4% (95% CI 1.1 to 1.8) and a 3-month rate of fatal PE of 0.5 (95% CI, 0.3 to 0.8).

As reviewed by Stein et al.[111] the increasing use of CTA may result in an increased incidence of radiation-related cancer in the not-too-distant future. Dangers of radiation require that protocols for CT angiography be optimized. In addition, the use of combined CT pulmonary angiography and CT venography should be questioned as it was found in PIOPED II study that only a small proportion of patients with PE or DVT would have been undiagnosed if imaging of the pelvic veins had not been done.[68] Moreover, proximal lower limb DVT can be diagnosed by CUS and it has been shown that CT venography and CUS are equivalent for diagnosing proximal DVT of the lower limbs. The radiation dose delivered to the fetus during pregnancy by ventilation/perfusion or perfusion-only lung scintigraphy and CTA are equivalent.[112] Nevertheless, preference between those tests is still debated as the radiation dose to the breast of women of child-bearing age is significantly greater with CTA. As a matter of fact, with CTA the fetus can be shielded with a lead apron, which does not work for isotopes. Of note, a Canadian randomized trial compared a V/Q scan and a CTA-based strategy for investigating suspected PE (mostly MDCTA).[31] Very few patients developed VTE during follow-up and there was no outcome difference between the CTA and the V/Q-based strategy. This is very reassuring, and offers a valuable diagnostic alternative, should CTA be contraindicated.

Diagnosis of High-risk PE

Patients with suspected massive PE have a very high mortality rate and require emergent thrombolytic or surgical treatment in the event PE is confirmed. The clinical presentation requires a differential diagnosis that includes other causes of shock such as pericardial tamponade or myocardial infarction. The clot burden is usually high in that situation, and the diagnostic yield of any imaging study, whether lung scan or CTA, is likely to be high. Therefore, the logical initial test is either MDCTA or transthoracic echocardiography, whichever is more rapidly available (FIGURE 83.7). An echocardiogram showing signs of acute pulmonary hypertension and RV strain in a patient with shock, normal left ventricular contractility, and absence of pericardial effusion is a very strong argument in favor of massive PE. In fact, most clinicians would readily begin thrombolytic treatment in such a highly unstable patient without awaiting further diagnostic information. In a patient temporarily stabilized by vasopressors (such as dopamine or norepinephrine), confirmation should be sought by MDCTA. Angiography should be avoided whenever possible since it carries the highest risk in this patient population[113] and increases the risk of a major bleed at the puncture site due to thrombolytic treatment.[114] The yield of CUS of leg veins has not been studied systematically in this situation, but data collected in patients with nonmassive PE but a high clot burden on V/Q scan suggest that it may be associated with a 20% to 30% false negative rate.[115] FIGURE 83.7 summarizes the proposed diagnostic algorithm.

SPECIAL SITUATIONS

Suspected Recurrent DVT or PE

Diagnosis of recurrent DVT is difficult because acute thrombi cannot be easily distinguished from old thrombi, unless the

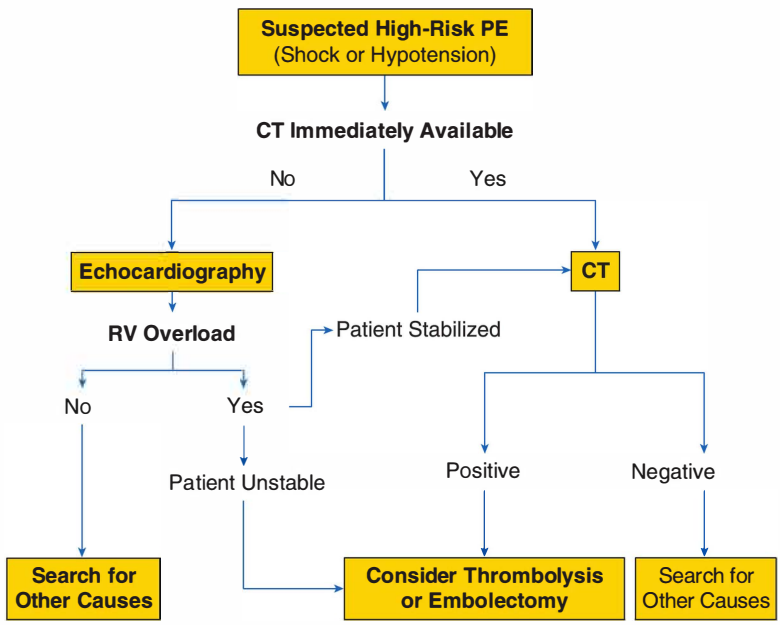

FIGURE 83.7 Diagnostic algorithm for suspected high-risk PE. The term *high-risk PE* has replaced the ambiguous term of *massive PE* and designates patients in shock or with hemodynamic compromise.

suspected recurrent event occurs in a location that is different from that of the initial event. Because initially abnormal plethysmography results return to normal in 90% of patients at 1 year, recurrent DVT in symptomatic patients might be inferred from an abnormal plethysmographic result at least 1 year after the previous event but such a conversion has a positive predictive value of only 80%.[116] Moreover, this test is no longer used. Alternatively, repeated sonographic measurements of the diameter of the common femoral and popliteal veins have been proposed as a tool to discriminate new from old thrombi.[117] The vein diameter has to be measured under compression with the transducer and compared with earlier measurements. Patients with stable or diminished vein diameters can be considered as free of recurrence. The predictive value of a stable or improved diameter to rule out recurrence was 90% (95% CI: 77% to 97%). In a prospective study of 205 consecutive patients, the policy of withholding anticoagulant treatment in patients clinically suspected of recurrent DVT in whom CUS showed stable or improved vein diameters was found to be safe.[118] The main limitation of this method is the need for iterative vein diameter measurements in DVT patients, which is cost- and resource-demanding. Moreover, it does not account for the possibility that asymptomatic events may have occurred between two measurements. In selected cases, phlebography may be the only solution, the presence of an intraluminal defect in at least two views proving the recurrence.

Diagnosis of recurrent PE is also challenging. It can only be confirmed if new emboli can be detected in locations that were previously documented as free of thrombi. For this reason, some opinion leaders strongly advise performing lung imaging in all patients with PE, including patients with proximal DVT and respiratory signs and symptoms, in whom the diagnosis could be made without lung imaging. But again, this suggestion does not account for that asymptomatic emboli may have occurred between the two imaging procedures. In selected cases, pulmonary angiography may be required to settle the case.

Recurrent VTE will be readily ruled out by a DD measurement if the concentration is below the diagnostic cutoff, but this will occur in one third of patients at best when ELISA methods or automated turbidimetric assays are used.[36] In the other cases, the diagnosis will rely upon the subjective combination of clinical findings and imaging modalities, a situation that is far from satisfying.

Suspected VTE during Pregnancy

Pregnancy constitutes an additional challenge in case of suspected DVT or PE. Hemodynamic changes interfere with the interpretation of tests for DVT, and tests using ionizing radiation may theoretically harm the fetus. Widely used noninvasive initial tests such as DD measurement or CUS are of limited value, due to a reduced specificity (DD)[42] or sensitivity (CUS). Nevertheless, a DD concentration below the diagnostic cutoff value rules out VTE. Moreover, using higher thresholds than those used in nonpregnant patients, the specificity of DD assays for the diagnosis of DVT could be improved without compromising sensitivity and safety,[49] which requires however to be validated in prospective management studies.

Many clinicians advocate using phlebography for diagnosing DVT in pregnant women but it is suboptimal under these conditions. The exam should however be performed only if CUS has not demonstrated the presence of DVT. Alternatively, MRI might be used although the experience with this modality is scarce and it lacks availability in many countries. As potential advantages, MRI may diagnose isolated iliac DVT, a condition that is more common during pregnancy, in addition to femoropoliteal DVT, and it is not associated with radiation exposure.

In suspected PE, objective testing is mandatory, and the previously described diagnostic strategy for nonpregnant women should be applied. If DD is above the diagnostic cutoff and no DVT is diagnosed on CUS, imaging should be performed. Because of the minimal radiation of V/Q scan, this exam may be preferred to spiral CT scan, although recent data suggest that CT may not deliver higher radiation doses to the fetus than V/Q lung scintigraphy.[112] Another reason to primarily resort to V/Q lung scanning is that it is the only imaging modality that has been assessed in an outcome study. Albeit retrospectively, Chan et al.[119] reviewed the outcomes of 120 pregnant patients with suspected PE in Ontario over 10 years: 75% of them had a normal V/Q scan ruling out PE, and none had an event during the remaining pregnancy. In fact, whenever possible, in this category of young women, perfusion scan alone should be performed and if perfusion is normal, PE is excluded. In case of abnormal perfusion, ventilation imaging should be performed to categorize the lung scan result as high probability or nondiagnostic lung scan. In the latter case, spiral CT should then be performed unless a priori clinical probability was low. Most experts concur that a certain diagnosis should be obtained before labeling a pregnant woman as having PE. Indeed, the risk of anticoagulant treatment throughout pregnancy and the weight of carrying that label for young women of childbearing age far outweigh the minimal risk of irradiation to the fetus.

Screening Asymptomatic Patients at High Risk of DVT

Screening patients at high risk of DVT is mainly done in the context of thromboprophylactic trials testing the efficacy of new prophylactic drugs or strategies, especially in the postoperative period. In that situation, phlebographically diagnosed DVT is the most commonly employed endpoint. There is a good basis for this policy[120]: In these situations, DVT is probably the only causal pathway leading to PE, and the reduction of adverse clinical events is related to the effect of antithrombotic drugs on asymptomatic thrombi in the legs. In the case of phlebographically diagnosed DVT, fatal PE is unlikely to occur if all phlebographically diagnosed asymptomatic thrombi are prevented. Hence, asymptomatic distal DVT may be considered as a valid surrogate endpoint to clinical events in such trials. Some investigators have advocated the use of phlebography only in symptomatic patients but using nonspecific and insensitive symptoms like leg pain or swelling especially following total hip or knee arthroplasty does not appear very promising. Other screening techniques such as thermography could not confirm initial promises.

Screening to detect and treat postoperative DVT following major orthopedic surgery has been shown not to be cost-effective, compared to systematic prolongation of prophylaxis for 4 weeks in a decision-analysis model[121] and is not recommended by international guidelines.[122] Recently, Tincani et al. screened 850 consecutive patients admitted to the rehabilitation unit for presence of DVT. In this observational study, the cumulative

incidence of DVT was 1.8% when patients were followed up for 6 to 30 months after discharge and routine screening US at the time of admission failed to reduce the rate of subsequent symptomatic VTE in patients given thromboprophylaxis, which further calls into question the practice of screening.[123]

Paradoxical Embolism

The term "paradoxical" has been coined to describe migratory thrombus from the venous circulation to the systemic circulation through a patent foramen ovale, potentially causing devastating damage, especially stroke. The topic will be discussed in Chapter 84, as well as venous thrombosis in unusual sites, for example, upper extremity, cerebral sinuses, and splanchnic veins, with their diagnosis and treatment.

COST-EFFECTIVENESS OF THE DIAGNOSTIC STRATEGIES IN SUSPECTED DVT AND/OR PE

Several diagnostic strategies have demonstrated both efficacy and safety. Selection among them in daily clinical practice is usually guided mainly by test availability and expertise. However, cost-effectiveness issues may also play a role.

Cost-Effectiveness of Diagnostic Strategies in Suspected DVT

A formal cost-effectiveness analysis compared diagnostic strategies for clinically suspected DVT: serial CUS, single CUS in patients with normal DD and second CUS only if DD level was abnormal, and single CUS in patients with an abnormal DD.[102] Under baseline conditions (prevalence of DVT, 24%), the effectiveness of all strategies was similar (4.2 to 4.4 lives saved per 1,000 patients managed). DD as a first test, followed by a single CUS only in case of an abnormal DD level (FIGURE 83.6) was the cheapest option. In a more recent analysis, diagnostic algorithms based on a combination of Wells score, DD, and CUS were the most cost-effective.[124] Use of repeat ultrasound increased incremental costs significantly for only a very small health gain. Finally, in a recent analysis, the office-based strategy evaluated in the AMUSE study (testing only selected patients after application of a prediction rule)[100] was cost-effective compared with the usual hospital-based strategies.[125] It should however be mentioned that the prediction rule incorporated point-of-care DD measurement in the physician's office, which is not very different from usual diagnostic strategies. Finally, as already discussed, complete CUS is probably not cost-effective, although this has not yet been formally assessed.

Cost-Effectiveness of Diagnostic Strategies in Suspected Nonmassive PE

Several cost-effectiveness analyses have compared current strategies for diagnosing PE. A comparison of various test combinations including spiral CTA, DD, lower limb venous CUS, V/Q scan, and/or angiography showed that the strategy including clinical probability assessment, DD and MDCTA described in FIGURE 83.6 had a favorable cost-effectiveness ratio.[126] A strategy based on V/Q scanning in patients with an elevated DD level, angiography being performed only in patients with a non–low clinical probability of PE, a negative CUS and a nondiagnostic V/Q scan was also cost-effective. Another analysis showed that patient age does not seem to influence the cost-effectiveness of the strategies.[2] However, performing proximal CUS in patients with an elevated DD level or a high clinical probability to reduce the proportion of patients undergoing MDCTA (patients with a DVT being treated without further testing) was not cost-effective. This was confirmed in a randomized clinical trial comparing two strategies based on clinical assessment, DD, and MDCTA, with and without CUS.[103] Costs were higher in the CUS arm of the study with no additional clinical benefit.

CONCLUSIONS

Diagnosis of DVT and PE has come a long way during the past two decades. Noninvasive diagnosis is now possible in almost all patients. Numerous diagnostic schemes have been validated in well-designed large-scale outcome studies and may be selected by individual institutions according to test availability and cost-effectiveness issues. Phlebography remains the ultimate diagnostic criterion standard for DVT but is no longer used outside thromboprophylaxis trials. MDCTA has become the cornerstone of PE diagnosis and supplanted pulmonary angiography, which is no longer the gold standard due to invasiveness and interobserver variability, which is increasing due to loss of expertise. However, many surveys on DVT and PE diagnosis show significant variability in clinical practice and frequent use of inappropriate criteria for ruling in or out VTE, with potential dramatic consequences to patients.[127] The most common diagnostic mistakes are the absence of clinical probability assessment, the ruling out VTE with DD in patients with a high clinical probability, or the absence of further diagnostic testing in patients with an elevated DD level. Therefore, continuing efforts to educate physicians on recommended diagnostic approaches are more warranted than ever. Moreover, research must still be pursued in two directions. Selecting patients for testing on clinical grounds is an increasing challenge. Ease of use of tests such as DD and fear of medicolegal consequences of missing DVT or PE have resulted in a dramatic drop in the proportion of patients suspected of VTE in whom the disease is ultimately confirmed. This results in useless resource consumption and, in the case of suspected PE, unnecessary irradiation. Also, increased sensitivity of our diagnostic instruments carries the risk of overdiagnosis, as evidenced by the increasing proportion of patients with isolated subsegmental PE shown by the new generation 16- and 64-detector CT scanners, and the high prevalence of distal DVT in centers routinely using complete CUS, without proof of clinical benefit of anticoagulant treatment. Likewise, diagnosing recurrent VTE remains a daily problem and a true challenge for clinicians.

Resolving those issues will require novel research approaches, such as registry analyses and international collaborations.

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Full Reference Lists can be found online

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CHAPTER

84

Venous Thrombosis in Unusual Sites

VICTOR J. MARDER, HYLTON V. JOFFE, AND SAM SCHULMAN

Venous thromboembolism (VTE) typically presents with lower extremity deep vein thrombosis (DVT) or pulmonary embolism (PE).[1,2] Upper extremity deep vein thrombosis (UEDVT), which usually refers to thrombosis of the brachial, axillary, or subclavian veins, has become increasingly common with widespread use of central venous catheters for chemotherapy and parenteral nutrition.[3–5] Thrombosis in other deep veins, such as the cerebral or mesenteric, may be a clue to an underlying thrombophilia.[6–8] Patients with VTE in these unusual sites have distinct risk factors, signs and symptoms, diagnostic evaluations, and treatment considerations compared to patients with traditional lower extremity DVT or PE and should alert the physician to the need for specialized diagnostic tests, therapeutic approaches, and family studies.

UPPER EXTREMITY DEEP VEIN THROMBOSIS

Historically, UEDVT was considered a benign and self-limited condition.[9–11] However, recent studies have demonstrated that UEDVT may have significant complications, including PE, loss of vascular access, the superior vena cava (SVC) syndrome, and long-term arm pain and swelling.[12,13]

Anatomy

The deep venous system of the upper extremity begins at the brachial vein, which becomes the axillary vein (AV) at the lower edge of the major pectoral muscle. The superficial basilic vein joins either of these deep veins (FIGURE 84.1). The AV extends to the outer border of the first rib where it becomes the subclavian vein. The subclavian vein joins the internal jugular vein (IJV) at the inner end of the clavicle to form the brachiocephalic vein (BCV), which merges with the contralateral BCV to form the SVC.[14] The subclavian artery and vein pass under the clavicle and subclavius muscle and over the first rib. The subclavian artery travels between the anterior and middle scalene muscles, whereas the subclavian vein passes in front of the anterior scalene muscle. The brachial plexus is superior and posterior to the subclavian artery and anterior to the middle scalene muscle. The brachial plexus, subclavian artery, and subclavian vein comprise the neurovascular bundle. Compression of any component of this bundle as it exits the thoracic cavity can cause the thoracic outlet syndrome and involvement of the subclavian vein predisposes to UEDVT.[15]

Etiology

Primary UEDVT refers either to idiopathic disease or to Paget-Schroetter syndrome[7,16,17] and occurs in approximately two cases per 100,000 persons per year.[18,19] Idiopathic UEDVT has no identifiable precipitant but may be associated with occult neoplasms, especially lung cancer and lymphomas.[20,21] Paget-Schroetter syndrome refers to spontaneous UEDVT following strenuous activity, such as pole vaulting, boxing, baseball pitching, weight lifting, or rowing in young and otherwise healthy individuals.[19,22,23] In this setting, repeated injury to the intimal lining of the upper extremity veins activates prothrombotic mechanisms, eventually causing clinical thrombosis.[22] These patients sometimes have coexisting conditions that cause mechanical compression of the vasculature, including the thoracic outlet syndrome, rib anomalies, long transverse processes of the cervical spine, or musculofascial bands.[15,16,24,25]

Secondary UEDVT develops in patients with underlying comorbidities, including cancer,[20,26,27] pacemakers,[28,29] and central venous catheters,[4,30–34] and accounts for most cases of UEDVT (Table 84.1). Other conditions associated with UEDVT include intravenous cocaine use,[35,36] artificial reproductive technology (e.g., *in vitro* fertilization) especially with ovarian hyperstimulation syndrome, [37] and upper extremity surgery or plaster cast.[38] The association of oral contraceptives with UEDVT has in different studies varied from none to a 5.7-fold (95% confidence interval [CI] 2.1 to 15.7) increase of the risk.[39]

Patients with central venous catheters constitute up to one-fourth of cases of UEDVT.[4,30,40] Thrombotic risk may be higher with polyvinyl chloride catheters compared to silicone catheters.[31] Central venous catheters also predispose to UEDVT in patients with heparin-induced thrombocytopenia.[34] Likely, explanations for catheter-associated UEDVT include venous stasis and vessel wall injury[30,41] and the toxic effect of infusate on the vein.[27,42,43] Catheter tips should be positioned in the proximal SVC or at the junction between the SVC and right atrium, where blood flow is most rapid.[4,30,32,44,45] Venous thrombosis and stenosis probably develop in approximately 15% of patients with a permanent transvenous pacemaker, although most of these patients are asymptomatic.[28,29,46,47] Independent predictors of these venous lesions include previous transvenous temporary leads, the number of leads, and a left ventricular ejection fraction of 40% or less.[29]

Thrombophilia

Several studies have evaluated the prevalence of inherited and acquired thrombophilia in patients with UEDVT (Table 84.2).[39,45,48–55,56,57] Antithrombin, protein C, and protein S deficiencies are seldom present in these patients, and the prevalence of factor V_{Leiden}, antiphospholipid antibodies, and anticardiolipin antibodies varies widely so their role in UEDVT is unclear, although several studies have shown an increased prevalence of these antibodies in UEDVT.[7,13] The

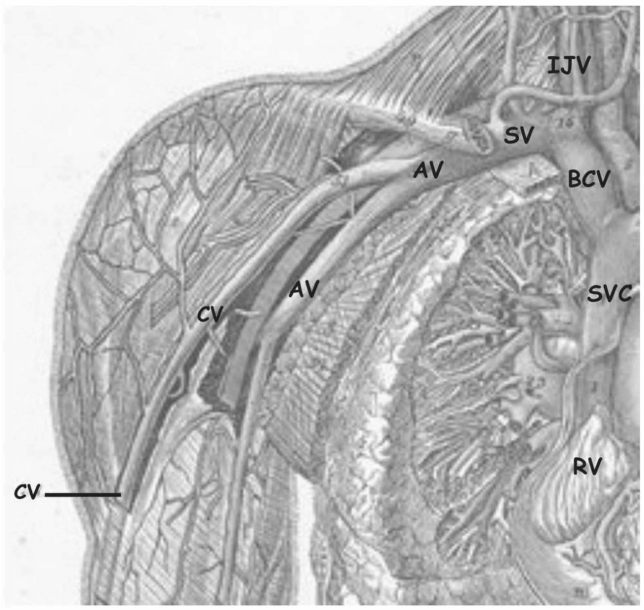


FIGURE 84.1 Veins of the upper extremity. The axillary vein (*AV*) extends to the outer border of the first rib where it becomes the subclavian vein (*SV*). The subclavian vein joins the internal jugular vein (*IJV*) at the inner end of the clavicle to form the brachiocephalic vein (*BCV*), which merges with the contralateral BCV to form the superior vena cava (*SVC*). RV, right ventricle. (Copyright protected material from Anatomy Atlases used with permission of the authors and Dr. Michael P. D'Alessandro, http://www.anatomyatlases.org/atlasofanatomy/plate19/04overview.shtml.)

inconsistency between studies may be partially attributed to small sample sizes, different time points of blood collection, different types of coagulation tests used, and different patient populations.

Table 84.1
Risk factors for UEDVT

Primary UEDVT
Strenuous activity of the upper extremity (Paget-Schroetter syndrome)
Thoracic outlet syndrome
Idiopathic
Secondary UEDVT
Central venous catheters, including the Swan-Ganz catheter
Cancer (perhaps more prevalent in lung neoplasms and lymphoma)
Pacemakers
Implantable cardioverter-defibrillator
Upper extremity surgery or plaster cast
Fibrosing mediastinitis
Heparin-induced thrombocytopenia
Artificial reproductive technology especially with ovarian hyperstimulation syndrome
Intravenous cocaine use
Amyloidosis

Clinical Presentation

Patients with UEDVT have a wide spectrum of presentation, ranging from no symptoms to the catastrophic SVC syndrome, characterized by facial edema, blurred vision, head fullness, vertigo, and dyspnea.[13,58] A coexisting thoracic outlet syndrome may injure the brachial plexus and cause radiation of pain down the medial aspect of the arm into the fourth and fifth fingers.[15] The

Table 84.2
Prevalence (%) of acquired and inherited thrombophilia in patients with UEDVT

Study	Factor V_{Leiden}	Prothrombin Gene Mutation	Antiphospholipid	Antithrombin Deficiency	Protein S Deficiency	Protein C Deficiency
Primary UEDVT						
Heron et al.[52]	10.6	0	22	0	4.3	0
Martinelli et al.[53]	8.3	—	0	0	0	0
Martinelli et al.[51]	8.7	9.6	7.0	—	—	—
Primary or Secondary UEDVT						
Bombeli et al.[6]	25.3	7.3	—	1.0	2.0	2.0
Ellis et al.[54]	50	—	11.1	—	—	—
Leebeek et al.[55]	4.9	0	26.8	2.4	0	0
Linnemann et al.[56]	15.8	4.9	7.7	0.7	0.8	3.3
Prandoni et al.[13]	7.4	—	3.7	3.7	3.7	7.4
Ruggeri et al.[57]	3.7	—	14.8	0	0	3.7
Mayo[58]	3.8	11.4	7.6	0	0	0

Table 84.3

Advantages and disadvantages of imaging modalities used to diagnose UEDVT

	Advantages	Disadvantages
Ultrasonography	• Inexpensive • Noninvasive • Reproducible	• May fail to detect central thrombus that is directly below the clavicle or sternum
Venography or CT scan	• May detect central thrombus • May detect the presence of extrinsic vessel compression	• Contrast dye • Not fully validated
Magnetic resonance angiography	• Accurately detects central thrombus • Provides detailed evaluation of collaterals and blood flow	• Limited availability • Claustrophobia • Not suitable for some patients with implanted metal

usual symptoms of UEDVT are extremity edema, discomfort, and erythema.[13] Patients with coexisting PE may also have dyspnea, chest pain, or cough.[33]

Findings on physical examination may include fever, sinus tachycardia, arm and hand edema, supraclavicular fullness, jugular venous distension, upper extremity cyanosis, a palpable tender cord, dilated cutaneous collateral veins over the chest or upper arm, and a completely or partially occluded central venous catheter.[13,58–60] Low-grade fever may be present, but high fever is likely due to another cause, such as septic thrombophlebitis, coexisting infection, or underlying neoplasm.[61,62] Sinus tachycardia may reflect pain or anxiety but could also suggest compensation for reduced venous return to the heart and may occur in the setting of PE.[63] If thoracic outlet syndrome is suspected, the examiner should palpate the supraclavicular fossa for brachial plexus tenderness and perform provocative tests.[15,64,65]

Diagnosis

Muscle injury, superficial vein thrombosis (SVT), lymphedema, and neoplastic compression of the vasculature can mimic UEDVT.[13] Therefore, the diagnosis of UEDVT cannot be made solely on the basis of the history and physical examination but must be confirmed with objective testing. D-dimer testing may have limited usefulness for excluding UEDVT because the negative predictive value is not reliably known in this setting and a majority of patients are expected to have high D-dimer concentrations because of concomitant disease.[66]

Duplex ultrasonography (US) is recommended as the initial test for diagnosing UEDVT because it is noninvasive and appears to have good sensitivity and specificity for jugular, distal subclavian, and axillary UEDVT.[13,67–72] However, acoustic shadowing from the clavicle and sternum limits the reliability of this technique for assessing thrombosis in the brachiocephalic and proximal subclavian veins.[68,69] Based on a systematic review of published literature, compression US has a sensitivity of 97% and a specificity of 96% for UEDVT compared to venography.[73] Color Doppler does not seem to improve the test performance. However, additional studies are needed because these calculated performance characteristics of US are based on studies with important methodologic limitations such as small sample sizes and large between-study differences.

Contrast venography provides excellent characterization of the venous anatomy[74] but has several important drawbacks, including technical difficulty cannulating the vein in an edematous arm, use of an iodinated contrast agent, and radiation exposure (Table 84.3).[75] The contrast agent may cause renal injury; allergic reactions, including urticaria, bronchospasm, and anaphylaxis; or local extravasation and chemical phlebitis.[75,76] Acetylcysteine and adequate hydration may reduce contrast-induced nephrotoxicity in patients with abnormal renal function; a confirmatory large, randomized, controlled trial of acetylcysteine is underway.[77,78] Although iodinated contrast is rated pregnancy class B and radiation exposure from venography confers minimal risk to the fetus, concerns over teratogenicity limit the use of venography during pregnancy.[79] Despite these disadvantages, venography may be required to confirm or exclude the diagnosis of UEDVT when there is high suspicion for thrombosis despite a negative or inconclusive US study.[67] Venography is also required before interventions, including catheter-directed thrombolysis and angioplasty, and is used to assess the response to these treatments.[7,17,80–82]

Magnetic resonance angiography (MRA) correlates well with venography and provides a more complete evaluation of blood flow, contralateral vessels, and the SVC and the brachiocephalic vessels.[83–88] Therefore, MRA is a reasonable noninvasive alternative when venography is contraindicated or technically impossible (FIGURE 84.2).

Treatment

There are no randomized, controlled trials evaluating the optimal treatment of patients with UEDVT; the general approach used today is based on case series, retrospective reviews, and expert opinion.

Anticoagulation is the cornerstone of therapy for DVT at any site,[89,90] reducing thrombus propagation and maintaining patency of venous collaterals.[4] Usually, intravenous unfractionated heparin (UFH) is promptly started at the time of diagnosis unless there is an absolute contraindication to anticoagulation.

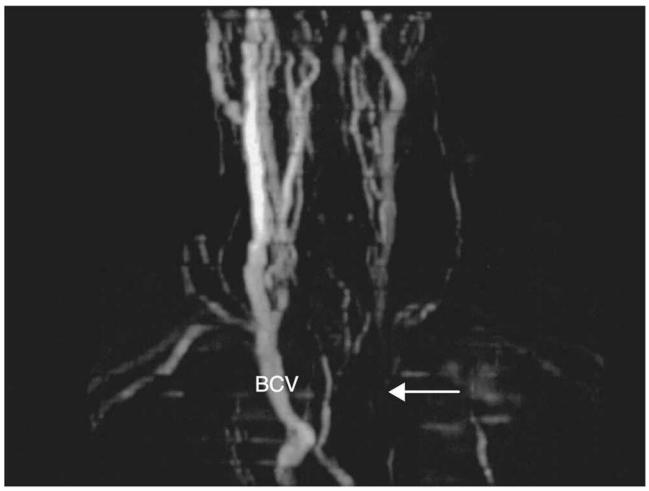


FIGURE 84.2 MRA demonstrating left BCV (*arrow*). (Adapted from Joffe HV, Goldhaber SZ. Upper-extremity deep vein thrombosis. *Circulation* 2002;106(14):1874–1880.)

Low-molecular-weight heparin (LMWH) is a safe and effective alternative and may reduce the duration of hospitalization.[91] Typically, heparin is used as a "bridge" to warfarin therapy, which is continued for a minimum of 3 months with an international normalized ratio of 2.0 to 3.0.[9,59,92] A longer duration of anticoagulation may be appropriate if there is an underlying hypercoagulable state.[63,93,94]

Thrombolysis. Several case series of UEDVT catheter-directed thrombolysis in carefully selected patients have reported excellent outcomes with only minor bleeding complications, such as occasional hematomas or oozing at catheter sites.[17,80,81,83] Although these small studies are underpowered to assess the risk of intracranial or gastrointestinal hemorrhage, the frequency of these serious events is expected to be similar to that of catheter-directed thrombolysis of lower extremity DVT.[95–97]

The best thrombolysis candidates are young, otherwise healthy patients with acute, primary UEDVT because these individuals may have significant long-term morbidity if treated only with conventional anticoagulation.[7,98–100] Other appropriate candidates for thrombolysis include patients with symptomatic SVC syndrome and those who require preservation of a mandatory central venous catheter.[81,101–103] Catheter-directed recombinant tissue-type plasminogen activator (tPA) may be administered as a continuous infusion of 1 to 2 mg/h, for at least 8 hours with serial venography used to assess treatment response (FIGURE 84.3). Percutaneous mechanical thrombectomy with devices such as the AngioJet (MEDRAD Interventional/Possis; Minneapolis, Minnesota) can be used to rapidly extract large quantities of thrombus with the goal of reducing the dose and duration of thrombolytic therapy.[104] Unlike conventional anticoagulation, thrombolysis restores venous patency early, which may minimize injury to the vessel endothelium and potentially reduce the risk of long-term complications, including the morbid postthrombotic syndrome.[59,98]

Several thrombolytic agents have been used to treat DVT. Urokinase is effective[80,81,103,105,106] but is now only approved by the Food and Drug Administration for lysis of pulmonary emboli that are massive or accompanied by unstable hemodynamics. Streptokinase has also been used successfully[107–110] but may be ineffective if previously administered. tPA is a popular choice in the United States.[111] Catheter-directed thrombolysis is preferred over systemic thrombolysis because local delivery of therapy achieves higher rates of complete clot resolution with lower doses of medication.[81,82,112,113] Heparin is usually given concurrently to prevent pericatheter thrombus formation.[59] Thrombolysis is most effective when used within several weeks of the onset of symptoms before progressive thrombus organization has occurred.[81,82,101,106]

Surgery. Although thrombolysis may successfully restore vessel patency, persistent vein compression may predispose to recurrent thrombosis and long-term morbidity.[17,59] Therefore, after successful thrombolysis for primary UEDVT, vascular surgeons may recommend early assessment for vein compression

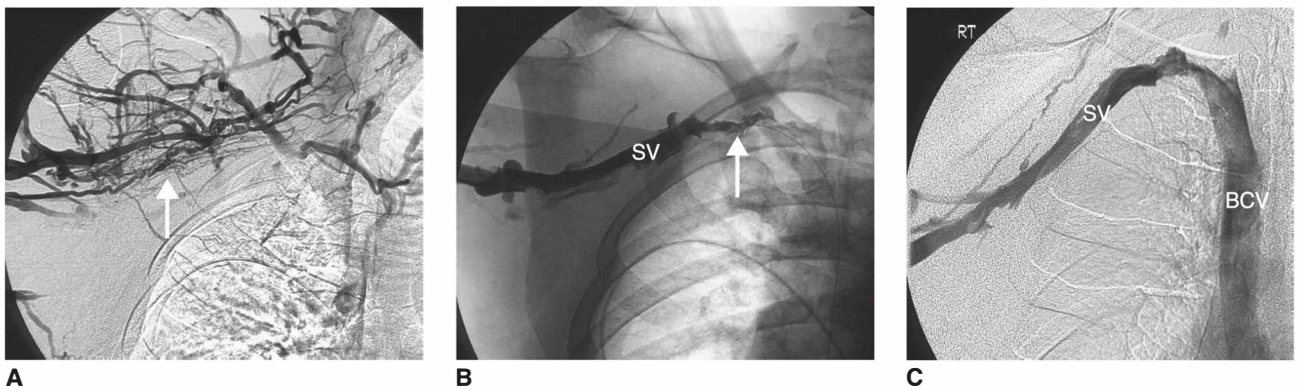


FIGURE 84.3 Multimodal therapy for UEDVT: 51-year-old weight lifter complaining of right arm pain. Initial venogram **(A)** shows occluded right axillary and subclavian veins with flow through collateral vessels (*arrow*). After percutaneous thrombectomy **(B)**, there is persistent occlusion of the proximal subclavian vein (*arrow*). After thrombolysis **(C)**, the subclavian vein (*SV*) is fully patent with flow into the brachiocephalic vein (*BCV*). (Adapted from Joffe HV, Goldhaber SZ. Upper-extremity deep vein thrombosis. *Circulation* 2002;106(14):1874–1880.)

followed by prompt surgical correction, such as resection of bone or lysis of dense, perivascular adhesions.[12,17,59,104,114–118] Persistent strictures after surgery could be treated with venoplasty and possibly vein stenting.[119,120] This multimodal approach can successfully achieve long-term vessel patency.[7,16,17,99,120–123] Although surgical thrombectomy restores venous patency, this approach is considered as a last resort because it is invasive, requires general anesthesia, and may be complicated by pneumothorax and brachial plexus damage.[59] Conservative therapy rather than prompt surgical intervention may be preferable for selected patients with the thoracic outlet syndrome because physical therapy, weight loss, and nonsteroidal anti-inflammatory medications may avert the need for surgery.[15]

SVC filters may protect against clinical PE, but there are limited data supporting their safety and efficacy.[124,125] There is concern that the benefits of SVC filters may be outweighed by their risks, including filter migration, dislodgment, fracture, and precipitation of the SVC syndrome. Potential candidates for SVC filter placement include patients with UEDVT who have an absolute contraindication to anticoagulation or those who develop PE despite adequate anticoagulation.

Prophylaxis. Early small trials suggested that the incidence of UEDVT can be reduced in patients with cancer and an inserted central venous catheter by using pharmacologic prophylaxis with either minidose warfarin (1 mg daily) or the LMWH dalteparin.[126–128] However, several subsequent trials have failed to show a benefit of pharmacologic prophylaxis in this patient population.[44,47–49,129] Similarly, it is unclear whether pharmacologic prophylaxis protects against catheter-associated UEDVT in patients without cancer.[130,131] Adding heparin to parenteral nutrition does not significantly reduce the risk of catheter-associated UEDVT, but these studies are small and underpowered.[130] These limited data may explain why pharmacologic prophylaxis of catheter-associated UEDVT is not commonly used in the United States. In a DVT registry,[33] less than one-fourth of patients with catheter-associated UEDVT and no obvious contraindication to anticoagulation were receiving prophylaxis at the time of diagnosis.

Complications

UEDVT may cause significant morbidity.[10,11,13] PE is present in up to one-third of patients with UEDVT, although fatal PE arising from UEDVT is uncommon.[13,132–134] PE may occur during removal of a central venous catheter because fibrin sheaths may peel off, break loose, and embolize.[58] Therapeutic heparinization for at least 24 hours before removal of the catheter may be a reasonable approach.

Other complications include loss of vascular access, the SVC syndrome, septic thrombophlebitis, brachial plexopathy, thoracic duct obstruction, and recurrent UEDVT.[12,50,135] The postthrombotic syndrome, secondary to venous hypertension from outflow obstruction and valvular injury, can lead to incapacitating limb pain and swelling.[136] This syndrome occurs in 7% to 44% of patients treated only with conventional anticoagulation[5] and may be less likely to occur following multimodal therapy.[7,98,100,137,138] Therefore, aggressive treatment, including thrombolysis, may be most suitable for patients with primary UEDVT who are young and otherwise healthy and more likely to be bothered by the postthrombotic syndrome than are patients with chronic medical conditions. Patients with secondary UEDVT have high short-term mortality rates compared with patients with lower extremity DVT,[139] most of them dying from underlying medical problems.

CEREBRAL VEIN AND SINUS THROMBOSIS

Anatomy and Pathogenesis

Cerebral vein and sinus thrombosis (CVST) includes thrombosis of the veins and dural sinuses of the brain.[140,141] Most often, thrombosis involves the superior sagittal sinus (FIGURE 84.4), which occupies the superior border of the falx cerebri, draining posteriorly into a "confluence of sinuses," then coursing mostly into one of the transverse sinuses, following the sigmoid sinus as it becomes continuous with the IJV. In the preantibiotic era, CVST was associated with chronic suppurant infections of the inner ear and skull, but most cases today

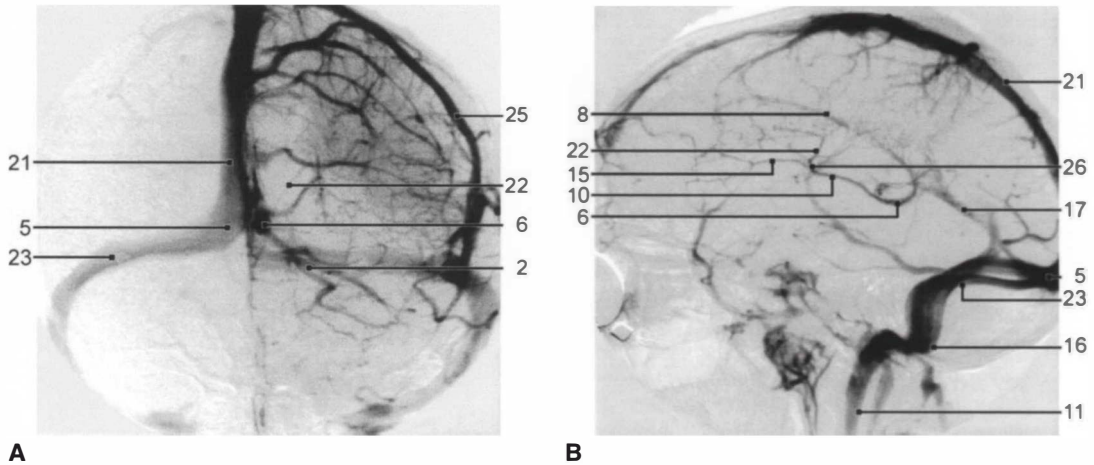
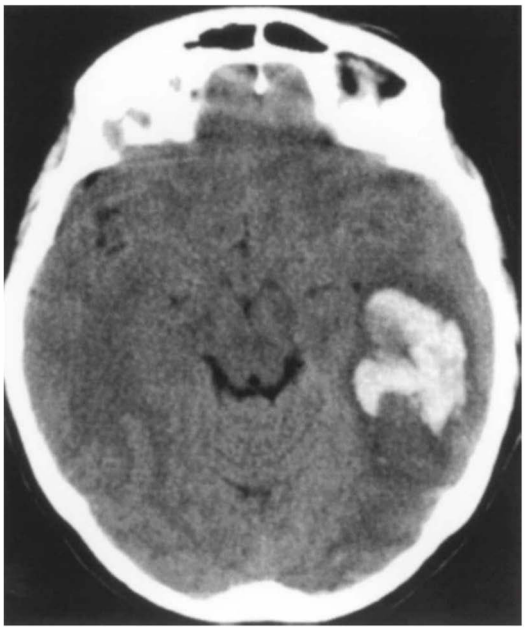


FIGURE 84.4 Venous phase of carotid angiography in the anteroposterior **(left panel)** and lateral **(right panel)** projections. The superior sagittal sinus (*21*), confluence of sinuses (*5*), transverse sinus (*23*), sigmoid sinus (*16*), and IJV (*11*) are as indicated. (Reproduced from the Encyclopedia of Medical Imaging [Medcyclopaedia], with permission.)

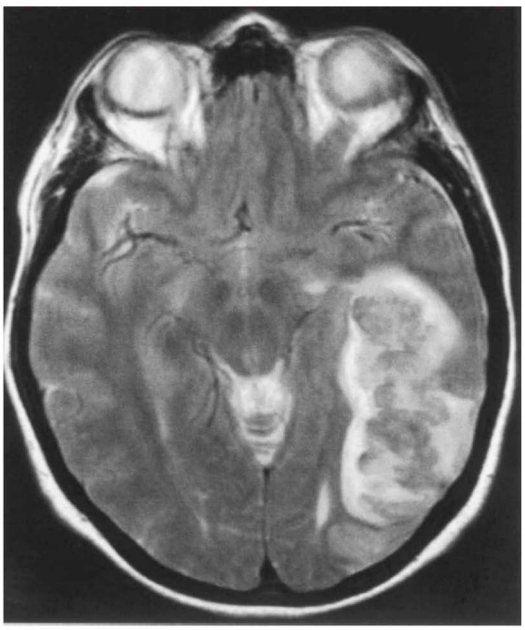
have no identifiable cause or are associated with head trauma, oral contraceptive (OC) usage, pregnancy or the puerperium, cachexia, dehydration, local malignancy, arteriovenous malformations, or other causes of generalized hypercoagulability. CVST is a well-recognized manifestation of the inherited and acquired thrombophilias, complicating virtually all of the known causes.[142–145,146] Of note is the danger of cerebral vein thrombosis in patients with a hereditary predisposition who have been prescribed OC agents[8] and in patients with paroxysmal nocturnal hemoglobinuria (PNH), antiphospholipid syndrome, and myeloproliferative disorders.[147–150] Following thrombus formation in a venous sinus, increased venous pressure leads to cerebral edema and even hemorrhage (FIGURE 84.5), a process that may progress to the development of large and/or multiple venous infarctions, which cross the normal boundaries of arterial supply.[151]

Clinical Aspects

Clinical features of CVST may be nonspecific and are often insidious in onset.[140,141,151] In a series of 32 cases admitted to hospital, the most frequent presenting complaints were headache (81%) and "reduced consciousness" (72%).[152] Among 17 cases initially seen in the emergency department, the presenting symptom was headache in 70% of cases; focal neurologic complaints such as weakness and aphasia were present in 29% and seizures in 24%.[153] In a large observational study, Ferro et al.[154] noted seizures in 39% of 624 cases; 6.9% of patients exhibited



A



B

C

FIGURE 84.5 Intracerebral venous sinus thrombosis. **A:** Shows an axial CT scan with hemorrhagic infarction of the left temporal lobe. **B:** Shows the MR, T2-weighted axial image, with an isointense area of recent hemorrhage and a hyperintense area surrounding the infarcted edematous zone. **C:** Shows an angiographic MR study with occlusion of the left transverse sinus, the sigmoid sinus, and the internal jugular vein. (Reproduced from the Encyclopedia of Medical Imaging [Medcyclopaedia], with permission.)

seizure as an early manifestation, associated with supratentorial thrombotic lesions (OR = 3.0). Headache in combination with papilledema and absent focal neurologic signs can simulate brain tumor ("pseudotumor cerebri").[155] Nausea, vomiting, and mental confusion develop over hours to days, followed in the most severe cases by stupor and coma, but focal sensory or motor losses are not dominant features, and the cerebrospinal fluid and the electroencephalogram may show only nonspecific findings. Fever will usually accompany sinusitis or other infection that leads to direct extension of the process into a contiguous (e.g., cavernous) sinus. Diagnosis is established by magnetic resonance (MR) or computerized tomography (CT) venography,[141,156–159] which documents the important contribution of "cytotoxic edema" in the pathogenesis of cerebral venous infarction.[160] D-Dimer levels have been noted to be elevated, in correlation with the degree of thrombosis, and it is unlikely that an acute thrombotic event exists in the face of a normal D-dimer value.[161] Predictors of adverse outcomes (death, dependency) are age >37 years, coma, intracranial hemorrhage, central nervous system infection, or malignant disease.[162]

Pediatric and Elderly Patients

Neonates

A review of 52 neonates (median gestational age 39 weeks) in Amsterdam indicated an incidence of CVST of 1.4 to 12 cases per 100,000 births.[163] There was an association with assisted or complicated delivery in 62%, and seizures were the primary manifestation (56%), appearing at a median age of 1.5 days (0 to 28 day range). Anticoagulation was administered in 42% of cases, without hemorrhagic complications. Mortality was 19% and moderate to severe neurologic sequelae occurred in 38%. In a prospective study of 90 neonates followed at the Hospital for Sick Children in Toronto,[164] complete recanalization occurred in 90% by 3 months, but a "lack of anticoagulation" predicted thrombus propagation ($P = 0.0007$). Late outcomes at 2.5 years (median) included epilepsy in 17% and neurologic disability (language, sensorimotor, or cognitive/behavioral) in 56%. The importance of anticoagulation is highlighted by a retrospective study in which half of a cohort of 18 patients with CVST managed with hydration only had clot extension.[165]

Children

Presentation of CVST in the postneonatal, pediatric population resembles that seen in adults, with local symptoms reflecting cerebral edema of headache, seizures, and emesis, plus focal neurologic signs or coma and generalized complaints of lethargy and anorexia.[166] Many patients (40%) had prior chronic conditions and the remainder usually had an acute illness (infection or dehydration). Interestingly, iron deficiency or hemolytic anemia was present in 62% of cases, as commonly as was a prothrombotic disorder, which was mostly an elevated factor VIII or homozygous methylene tetrahydrofolate reductase (MTHFR) mutation.[166] A systematic study of prothrombotic associations in neonates and children with CVST [146] noted significant values for hereditary predispositions such as deficiency of antithrombin and protein C (OR 7 to 8), or protein S; factor V_{Leiden} and the prothrombin G20210A mutation (OR 2.4 to 3.2), less so for the MTHFR mutation (OR 1.6); and acquired changes of antiphospholipid antibodies (OR 6.95) and increased lipoprotein (a) (OR 6.27). A separate study of 20 children emphasized the prior occurrence of minor head trauma preceding sigmoid or transverse sinus thrombosis.[167] Of 42 patients with CVST, only 18 were anticoagulated, and follow-up at a median of 1 year showed sequelae in 62%, including pseudotumor cerebri, cognitive disability, epilepsy, and hemiparesis.[166]

Elderly

Not unexpectedly, CVST presents differently and connotes a worse prognosis in elderly patients. In a comparison of 51 patients >65 years of age with 573 who were <65, altered sensorium and mental status changes were more common presenting symptoms (43% vs. 20%, $P = 0.001$), headache (65% vs. 91%, $P = 0.001$) and papilledema (14% vs. 29%, $P = 0.016$) were less common, and seizures occurred equally in the two age groups (45% vs. 39%, $P = 0.37$).[168] The prognosis was much worse in the older patients, complete recovery only 49% versus 82% in younger patients, and death and dependency were higher in the elderly (27% and 22%) versus 7% and 2%, respectively, for younger patients.[168]

Treatment

The principles of management of cerebral CVST are to aggressively treat an identifiable underlying cause such as infection[169] and to initiate anticoagulant or, in select cases, regional thrombolytic therapy, taking care to balance the presence of cerebral hemorrhage with the thrombotic insult.

Heparin and LMWH

Controlled studies of anticoagulant therapy are limited: one in 1991 that compared UFH with placebo in 20 patients and one in 1999 that compared LMWH (nadroparin), with placebo in 60 patients.[170,171] Using an adjusted-dose intravenous UFH regimen, 8 of 10 treated patients had complete clinical recovery at 3 months, compared with 1 of 10 who received placebo. A retrospective comparison of an additional 43 patients with intracerebral hemorrhage secondary to sinus thrombosis showed complete recovery in 14 patients and mortality in 4 (15%), compared with 3 recoveries and 9 deaths (69%) in a similar (but uncontrolled) patient group that was not treated with UFH.[170] Retrospective observation of 37 patients treated with UFH showed recanalization at discharge in 60% and an "excellent" functional outcome in 89% of patients at 3 months.[172]

LMWH has been randomized against placebo for 3 weeks' treatment in a blinded manner, followed by 3 months of warfarin therapy for all patients.[171] Results at 3 weeks showed poor outcome, measured as death or low level of functioning, in both groups (20% vs. 24% for placebo) and a nonsignificant trend that favored LMWH at 12 weeks (13% vs. 21% for placebo). A 1-year follow-up evaluation of 47 patients showed a disappointing number of patients with cognitive impairment (35%), and the authors conclude that the outcome was not significantly influenced by treatment.[173] Using the Cochrane Database, the same group of authors concluded[174] that anticoagulant treatment "appeared to be safe" and was associated with a reduction in the pooled risk of death (0.33) or dependency (0.46) but that such trends were not statistically significant. A similar conclusion in favor of anticoagulant use has been proposed for children, namely, that no data to prove efficacy exists, but the long-term sequelae (death or neurologic deficit in 50% of children) warrant empiric anticoagulation.[175] Clinical outcome of

treatment has been summarized for 624 adults in 21 countries, of whom 520 (83%) received anticoagulation therapy.[162] The prognosis was better than reported earlier,[146] with 356 (57%) having no residue of the illness and 30% with minor or mild impairment. Women with CVST and a gender-specific etiology such as contraceptives, hormonal replacement, or pregnancy have a better prognosis with complete recovery in 81%.[176]

Thrombolytic Therapy

There are numerous reports[177–186] on the use of thrombolytic therapy, usually administered regionally, so this approach can no longer be considered as unduly risky in a seriously ill patient, even in the face of documented intracranial hemorrhage.[187] A Cochrane Database review in 2004 found no randomized, controlled trial of thrombolytic therapy that evaluated efficacy or safety.[188] Still, a search of the literature found 169 patients with CVST who received a plasminogen activator (urokinase in 76%), administered regionally into the thrombus in 88% of cases.[189] The aggregate experience showed death in nine cases (5%), adverse outcome (dependency) in 7%, and intracranial hemorrhage with clinical deterioration in 5%. A recent single institution experience of 20 patients with serious CVST (altered mental status, coma, large space-occupying lesions) reported results with urokinase administered regionally via jugular vein catheter.[190] Of these critically ill patients, 12 recovered, 2 survived with significant handicap, and 6 died. There were five instances of increased intracranial hemorrhage after treatment, three minor and two major. Of the six patients who died, five had herniation secondary to large hemispheric infarcts and cerebral edema prior to thrombolytic treatment, indicating that such patients are best not so-treated.

RETINAL VEIN OCCLUSION

Occlusion of the retinal veins is localized either to the central retinal vein or three to five times more commonly to a branch vein, typically at an arteriovenous crossing,[191,192] with an incidence of 1.6% per decade in an elderly population.[193] The prevalence is similar in populations of different ethnicity.[194]

Pathogenesis

Retinal vein occlusion (RVO) occurs against a backdrop of the systemic vascular complex of diabetes mellitus, hypertension, dyslipidemia, smoking, and atherosclerosis. In a meta-analysis of 21 studies, the odds ratios (ORs) for these associations with RVO were between 1.5 and 3.5.[195] There is also an association with renal dysfunction.

RVO is reported in patients with inherited thrombophilia due to the prothrombin 20210 G/A mutation,[196] familial hyperhomocysteinemia,[197] the MTHFR mutation,[198] and activated protein C resistance.[199] In one survey, the factor V_{Leiden} R506Q mutation was present in 10 of 35 (29%) patients with RVO, similar to that observed in patients with DVT (40 of 209, 19%) but with no preponderance of protein C or protein S deficiency, or lupus inhibitor.[200] Another retrospective study failed to show a factor V_{Leiden} relation.[201] A meta-analysis of 18 studies shows a significant (CI 1.19 to 2.32) but "only moderate" (OR 1.66) relationship of factor V_{Leiden} with RVO, thus explaining the disparate conclusions due to chance variation of individual small studies.[202]

A meta-analysis of 614 patients with RVO showed a significantly higher homocysteine level and lower serum folate level in comparison with those in 762 controls but no increased prevalence of the MTHFR genotype.[203] Correlations of elevated homocysteine and plasminogen activator inhibitor-1, an association with positive antiphospholipid antibody, external forces, such as trauma and increased intraocular pressure from tumor or hemorrhage have been highlighted in review articles.[204–206] Thrombosis has occurred in hematologic diseases with a commonality of hyperviscosity, including hyperleukocytosis, dysproteinemia, erythrocytosis, hemoglobinopathy, and cryofibrinogenemia.[207]

Clinical Aspects and Treatment

The primary clinical manifestation of RVO is painless loss of visual acuity, the acuteness and severity of which depend upon the degree of venous occlusion and whether the central (FIGURE 84.6) or a branch vessel is involved. The natural history of the disorder, assessed in 725 patients by the Central Vein Occlusion Study Group, indicates that 15% (81 of 547) of involved "eyes" evolve to ischemia at 4 months, 34% by 3 years, and that the strongest predictor of visual acuity at 3 years is visual acuity at the onset of disease.[208] Attempts to alter the course of illness have utilized hemodilution, with both negative and positive effect,[209,210] laser photocoagulation without benefit over placebo for visual acuity at 3 months or with protection in some eyes against neovascularization,[211-213] rheologic agents with a suggestion of positive results in preliminary studies,[214,215] thrombolytic therapy with unconvincing results,[216-219] or surgical approaches, including sheathotomy and radial optic neurotomy,[220-222] the latter even combined

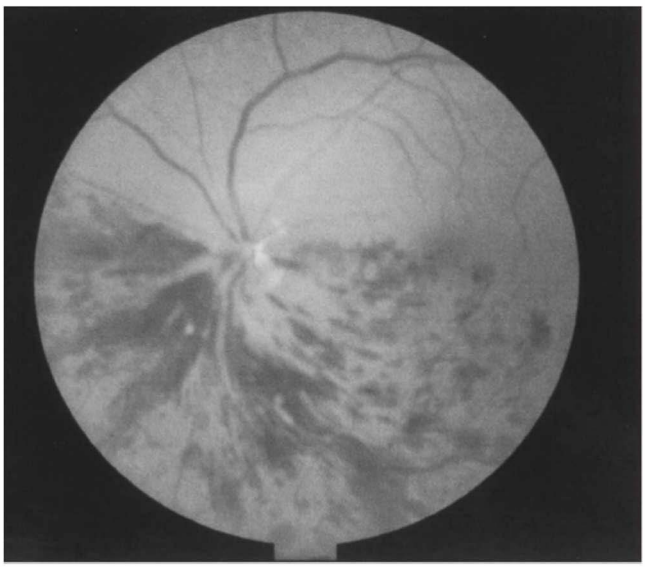


FIGURE 84.6 Funduscopic examination of the retina, showing central RVO. Widespread hemorrhages primarily over the inferior aspect of the retina, with venous engorgement and papilledema. (Reproduced from John A. Moran Eye Center, University of Utah, with permission. Available at: http://insight.med.utah.edu/opatharch/images/retina/22222.jpg.)

with local infusion of rt-PA.[223] LMWH shows variable results in two small trials against aspirin as control treatment.[224,225] Intravitreal administration of steroids (dexamethasone) may speed visual improvement (vs. sham injections),[226] but a similar approach using triamcinolone showed no difference versus "standard" care.[227]

The major new therapeutic direction has utilized intravitreal injections of vascular endothelial growth factor (VEGF) inhibitors,[228] notably, bevacizumab,[229–231] ranibizumab,[232–236] and pegaptanib.[237] All of the trials are prospective but observational, and no comparative studies between VEGF inhibitors have been performed as yet. Results generally show some degree of improvement in visual acuity and decrease in macular edema, without significant tolerance or safety issues. However, a significant proportion of the trials indicate that repeat injections are required to maintain benefit and to prevent recurrence. A Cochrane Database review[228] concludes that ranibizumab and pegaptanib "have shown promise in the short-term" but that larger randomized and controlled trial data are needed, and the agents must still be considered experimental.

PORTOMESENTERIC VEIN THROMBOSIS

Predispositions

Symptoms of portomesenteric vein thrombosis (PVT) are often vague and nonspecific.[238–241] Nonlocalized, colicky pain that is severe and out of proportion to physical findings is nearly universal. Nausea with or without vomiting is seen in half the cases, as is anorexia, but ileus is absent until the condition evolves after days or weeks into a surgical abdomen, the harbinger of bowel infarction.[242] PVT is often not recognized in the acute phase and may present clinically as progressive splenomegaly and ascites without evidence of worsening hepatic function. The stool is often guaiac-positive, but laboratory findings are nonspecific, with mild hemoconcentration and a moderate neutrophilia. Blood-tinged ascites signifies bowel infarction as a late manifestation. In children, the initial manifestation of PVT often is upper gastrointestinal bleeding or cytopenias secondary to splenomegaly that is discovered coincidentally by routine physical examination.[243]

Conditions that predispose to PVT include myeloproliferative syndromes,[244] inflammatory bowel disease,[245] cirrhosis and portal hypertension,[246] intraabdominal neoplasm (especially carcinoma of the pancreas), intraabdominal infection (especially spontaneous bacterial peritonitis), and blunt or penetrating trauma (including abdominal surgery).[247] Local conditions may be especially prevalent with organ removal as, for example, after splenectomy, in which the incidence has been reported to be 7% (4 of 60),[248] and after liver transplantation, which carries a prevalence of 8% to 26%.[249,250] Although not common, portal vein thrombosis has been noted after umbilical vein catheterization[251] and gastric bypass surgery.[252] Relatively less traumatic laparoscopic abdominal surgery can result in PVT, as noted in a series of 18 cases that followed gastric bypass, fundoplication, colectomy, cholecystectomy, and appendectomy.[253] The main presenting symptom was nonspecific abdominal pain appearing at a mean of 14 days after surgery, suggesting that diagnosis was delayed in many cases by the subtlety of clinical findings. Of the 18 cases, 11 had "systemic predisposition" as an added risk factor.

A substantial portion of patients with "primary" PVT have an underlying thrombophilic condition,[254] and it has been reported with deficiencies of antithrombin, protein C, or protein S; mutations of prothrombin G20210A, MTHFR genotype TT677, and factor V_{Leiden}; and acquired diseases such as paroxysmal nocturnal hemoglobinuria, antiphospholipid syndrome, and as a complication of estrogen administration for contraception.[238,255–268] In patients with myeloproliferative syndrome, the presence of the JAK2 V617F mutation indicates a higher risk for vascular events[244] (see Chapter 102). There does seem to be an increased prevalence of the prothrombin mutation (40% vs. 5% in controls),[269] but some questions exist about the involvement of the factor V_{Leiden} mutation in this condition.[270,271] PVT is reported to occur in 4% to 10% and to recur in 63% of patients with antithrombin, protein C, or protein S deficiency.[272] In the pediatric population, PVT has been described in five cases after receiving chemotherapy.[273]

Apparent PVT may in fact represent tumor invasion of the vessels, notably, for pancreatic and hepatocellular malignancies.[274–276] This distinction has obvious therapeutic and prognostic implications, and criteria for establishing that only "benign" PVT exists may often prove inaccurate, as in 9 of 33 such cases in one series.[277]

Diagnosis and Management

Plain abdominal radiography and barium contrast studies are nonspecific and are of little assistance in the diagnosis.[278] Noninvasive imaging studies using US (FIGURE 84.7), MR, or CT angiography are the primary diagnostic modalities.[279–281] Two retrospective surveys reinforce a conservative approach with anticoagulation using surgery for cases of bowel infarction and potential perforation.[282,283] Anticoagulant treatment should be started with UFH intravenously until the patient is stable and

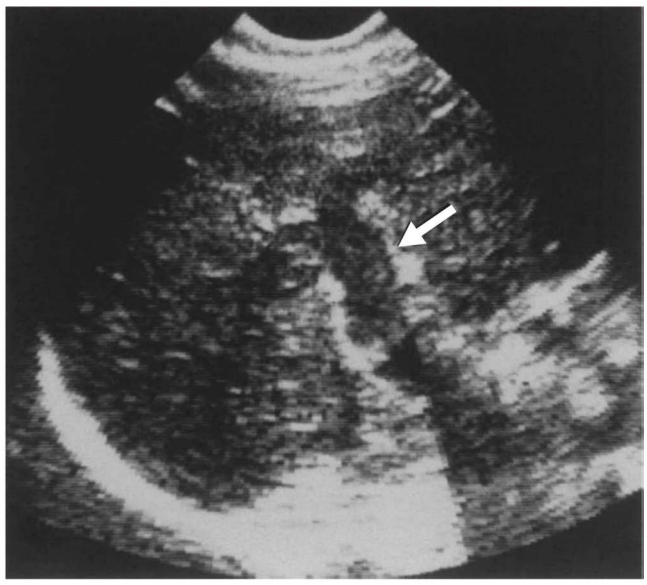


FIGURE 84.7 Ultrasonography image of abdomen showing portal vein thrombus. Echogenic structure in the portal vein (*arrow*), with dilation of a portal vein branch in the right lobe of the liver. [Reproduced with permission from the Encyclopedia of Medical Imaging (Medcyclopaedia).]

surgical intervention becomes unlikely. Then treatment can be switched to LMWH with overlap to a vitamin K antagonist. In case of liver cirrhosis with hypoprothrombinemia or otherwise high bleeding risk, it is probably safer to continue with LMWH with reduction to a prophylactic dose after 1 week. The duration of anticoagulation should be at least 3 to 6 months, longer in case of severe thrombophilia.

Early noninvasive diagnosis has allowed more efficient treatment, decreasing the mortality from 90% for untreated patients and 30% to 50% primarily for surgically treated patients[239,242,284] to more favorable outcomes of 90% or better survival.[283] A role for mechanical thrombectomy or early vascular grafting has been advocated in patients after orthotopic liver transplant.[250] In patients with contraindications to heparin, direct antithrombin agents such as argatroban or hirudin are reasonable options,[285–287] and gradual vascular recanalization is to be expected in patients who are appropriately treated with anticoagulation.[288] In a cohort of 95 (of 102) patients with acute PVT "unrelated to cirrhosis" who were treated with anticoagulation, portal vein patency was established in 39% at about 1 year, whereas failure was related to the presence of ascites and an occluded splenic vein.[289] Under proper imaging guidance, rapid lysis of thrombus can be achieved by regional thrombolytic administration and stent placement,[290–298] even in the neonatal period.[299]

SPLENIC VEIN THROMBOSIS

Splenic vein thrombosis is rarely recognized at the time of the acute event.[301] Typically, the thrombosed vein is discovered during an evaluation of vague abdominal pain with isolated splenomegaly but without ascites or significant hepatic dysfunction, or in relation to pancreatic disease (inflammatory, malignant, or traumatic).[302,303] Splenic vein thrombosis has been reported after "noninvasive" laparoscopic splenectomy[304,305] and is associated with the same acquired disorders that predispose to portal/mesenteric venous thrombosis.[306,307] Portal and splenic vein thrombosis occasionally complicate the nephrotic syndrome, as does renal vein thrombosis.[308] Anticoagulant therapy for the acute thrombosis may not be indicated, unless a significant thrombophilic process is discovered, but invasive approaches of splenic artery embolism[309] or vascular stenting[310] may be needed for complicating bleeding varices.

HEPATIC VEIN THROMBOSIS (BUDD-CHIARI SYNDROME)

Clinical Presentation

In classic hepatic venous outflow obstruction (Budd-Chiari syndrome [BCS]), the major hepatic veins and/or the inferior vena cava (IVC) is occluded. Symptoms may be acute and fulminant, with sudden abdominal pain, enlarging liver, rapid development of massive ascites, and mortality risk of up to 67%.[311,312] More often, the course is chronic and insidious, leading variably to intractable ascites, hepatic insufficiency, and gastrointestinal bleeding due to varices.[302,311,312,313,314] The process may develop so rapidly that liver function tests may be normal or near normal at presentation. Common to all presentations is an absence of hepatic venous flow.[315]

Hepatic vein thrombosis is associated with a variety of conditions. Toxins such as alkaloids found in senecio beans, comfrey tea ("bush tea"), and high-dosage alkylating agents can induce thrombosis or fibrosis of the smallest hepatic veins.[316–318] Other conditions include myeloproliferative disorders, hepatocellular carcinoma, renal neoplasm, trauma, intraabdominal infection, pregnancy, OC use, inflammatory bowel disease, and Behçet disease.[313,319–321]

Many cases are associated with hematologic disease, especially paroxysmal nocturnal hemoglobinuria and myeloproliferative syndromes[262,263,322,323] and antiphospholipid syndrome.[262,324] In a comparison of predispositions, there was a greater association of the myeloproliferative disorders, PNH, and Behçet syndrome with BCS and PVT than with ordinary DVT/PE.[323] There are reports of an association of antithrombin deficiency,[325] heparin-induced thrombocytopenia,[326] and factor V_{Leiden} mutation[270,327] with BCS. Impaired fibrinolysis may explain some cases of BCS, perhaps those occurring in patients with Behçet syndrome.[328]

"Congenital webs" have been noted in the hepatic veins[313,329] and are generally considered as contributory to the disease (FIGURE 84.8). However, the webs may constitute "organized thrombi of varying ages" and fibrous and recanalized material.[330] Therefore, in some cases at least, the "webs" represent a sequelae of thrombi rather than congenital malformations, which are especially prevalent in Asia, presenting as a more gradual obstructive illness than acute hepatic vein thrombosis.[331,332] BCS is rarely diagnosed in children, who show a similar tendency for this complication of myeloproliferative syndromes.[333,334]

Hepatic venography is sensitive and specific for advanced BCS but may be negative in the early stages of the process. US, computed tomography with contrast, and especially

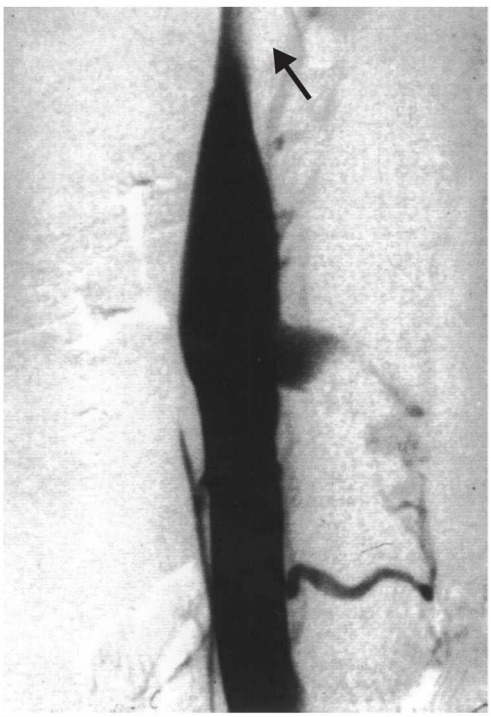


FIGURE 84.8 Inferior vena cavography. There is narrowing and occlusion (*arrow*) of the IVC at the level of the confluence of the hepatic veins caused by an endoluminal "web." (Reproduced with permission from the Encyclopedia of Medical Imaging [Medcyclopaedia].)

magnetic resonance imaging (MRI) provide documentation of pathology,[335–337] including caudate lobe hypertrophy obstructing hepatic vein drainage into the IVC.

Treatment

Anticoagulant treatment, according to principles described above for PVT, diuretics, prevention of portal hypertension, and removal of risk factors constitute the mainstay in the management. Experience with regional thrombolytic therapy has been striking in case reports, and there is sufficient foundation for its application under dire clinical circumstances.[338–342] However, one single center reports striking benefit after systemic or regional thrombolysis and did note its value as adjunct to mechanical interventions, especially with fresh thrombosis.[338] In addition to or following a thrombolysis attempt, transjugular intrahepatic portosystemic shunt is applied widely to patients with BCS, as a less invasive means of achieving hepatic vein to portal vein anastomosis than with surgical side-to-side portocaval shunting.[343,344] The procedure may improve vascular flow[345] and symptomatic aspects, but encephalopathy may worsen, restenosis may occur, and long-term anticoagulation is still required.[346,347,348] Orthotopic liver transplantation (OLT) is indicated for patients with hepatic decompensation and can be lifesaving in select cases.[349,350–352] In a large review of 248 patients with BCS who underwent OLT, survival was 76% at 1 year and 68% at 10 years.[352] Deaths were mostly in the first 3 months, due to infection and multiple organ failure in about half, about 20% secondary to graft failure or hepatic artery thrombosis.

RENAL VEIN THROMBOSIS

RVT is associated with acute illness and severe dehydration in the neonate and may also occur acutely in the adult; however, the more common form in the adult is a chronic presentation caused by the nephrotic syndrome, usually secondary to membranous glomerulonephritis.[353,354] Acute RVT (FIGURE 84.9) presents with a triad of flank pain, hematuria, and sudden deterioration of renal function, whereas the chronic form is usually asymptomatic and undetected until sought by appropriate diagnostic tests.[355,356]

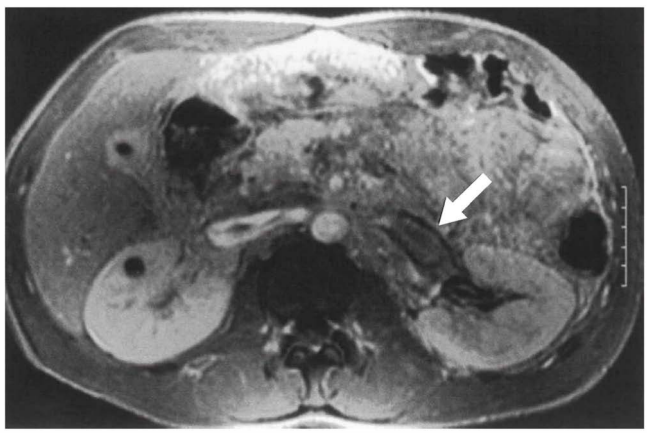


FIGURE 84.9 Renal vein thrombosis. Axial contrast-enhanced T1-weighted MRI section demonstrating thrombosis of the left renal vein (*arrow*). [Reproduced with permission from the Encyclopedia of Medical Imaging (Medcyclopaedia).]

FIGURE 84.10 Abdominal ultrasonography of inferior vena cava (*IVC*). A renal vein thrombus has extended into the IVC. (Reproduced with permission from the Encyclopedia of Medical Imaging [Medcyclopaedia].)

The nephrotic syndrome not only predisposes to RVT but also to extension into the IVC (FIGURE 84.10) and to lower extremity DVT, PE,[353,357] and to involvement of the splenic/mesenteric/portal vein system.[308,310] Several factors may contribute to the thrombotic tendency, including reduced plasma levels of antithrombin and protein C, caused by excessive urinary loss of the protein, and reduced levels of free protein S, perhaps secondary to an increase in C4b-BP.[358]

In a review of 43 patients with neonatal RVT, about half demonstrated renal failure, thrombocytopenia or anemia, and/or a renal mass on presentation,[359] and in a large retrospective survey of 271 patients with RVT, 44% had extension into the IVC.[360] Prenatal RVT has been documented on rare occasions, presenting as hydrops fetalis, fetal distress, or coincidentally upon ultrasound exam.[361]

RVT occurs in patients with inherited thrombophilias, such as deficiency of antithrombin or protein C, or the factor V_{Leiden} mutation,[362–364] and with acquired illnesses such as sickle cell disease, paroxysmal nocturnal hemoglobinuria, antiphospholipid syndrome, and Behçet disease.[149,365–369] RVT also may follow renal transplantation, as reflected by a 4% rate of postgraft thrombosis (7 out of 176 transplants), of which five grafts were lost.[370] As with portal or hepatic vein occlusion, the renal vein may be occluded by retrograde tumor spread of renal cell carcinoma, an occurrence observed in 9 of 115 cases.[371]

Patients with acute RVT are usually treated with anticoagulants. However, renal grafts that are threatened by vascular thrombosis can be salvaged by thrombolytic treatment[370,372,373] by regional or systemic routes.[374–379]

PELVIC VEIN THROMBOSIS

Pelvic vein thrombosis as a distinct entity, unassociated with extension of DVT from the lower extremities, occurs in and around the uterine adnexa and complicates 0.1% or fewer of all deliveries, perhaps as many as 1% of patients with pelvic infection.[380] Ovarian venous thrombosis is considered to be a form of septic thrombophlebitis,[381–383] usually on the right side, and because fever and pain may be present, appendicitis is the differential diagnosis. When limited to the ovarian vein, treatment with antibiotics alone may be all that is required, but if thrombosis also involves the iliac system, heparin treatment is indicated.[384]

LEFT COMMON ILIAC VEIN THROMBOSIS (MAY-THURNER SYNDROME)

Occlusion of the iliofemoral vein occurs more often on the left side than the right. In the description of this "predominantly sinistral occurrence of thrombosis of the pelvic veins," May and Thurner[385] pointed out that this clinical peculiarity had been explained since the time of Virchow[386] by "obstruction of the flow of the left common iliac vein (CIV) by the artery resting upon it." May and Thurner described pathologic "spur-like projections" into the left CIV; it seems likely that these lesions were inflammatory responses to chronic local injury caused by the pulsations of the contiguous right common iliac artery (CIA). They further explained the thrombotic event as the result of the spur, which caused venous stenosis, "hampering the flow of blood" and promoting the formation of thrombosis in the left half of the pelvis."[385] The anatomic relationship of the pelvic vessels involved in May-Thurner syndrome (MTS) (FIGURE 84.11)[387] suggests that compression of the left CIV would be a routine aspect of every person's pelvic vascular anatomy. Indeed, CT arteriograms on 50 asymptomatic patients showed >50% compression of the left CIV in 24% and more than 25% compression in 66% of subjects, most often secondary to overlying right CIA pressure against the vertebral body.[388] Women have greater left CIV compression than men (mean 41% vs. 27%, $P = 0.003$),[388] perhaps reflecting the effect of prior additional pressure on the pelvic vein by a gravid uterus. Left CIV compression has been observed in adolescents,[389,390] preteens,[391] and even in infants.[392] As with other unusual sites of thrombosis, a "second hit" thrombophilic event often precedes the thrombosis, for example, during the postpartum period,[393] with the antiphospholipid syndrome,[394] with OC use,[395] and with other typical thrombophilic predispositions.[396]

In addition to the expected signs of proximal iliofemoral thrombosis, patients with MTS may have unusual (though not unique) manifestations. One study of 470 patients with patent foramen ovale and cryptogenic stroke showed MTS in 6.3% of cases,[396] and a review of 7 patients with phlegmasia

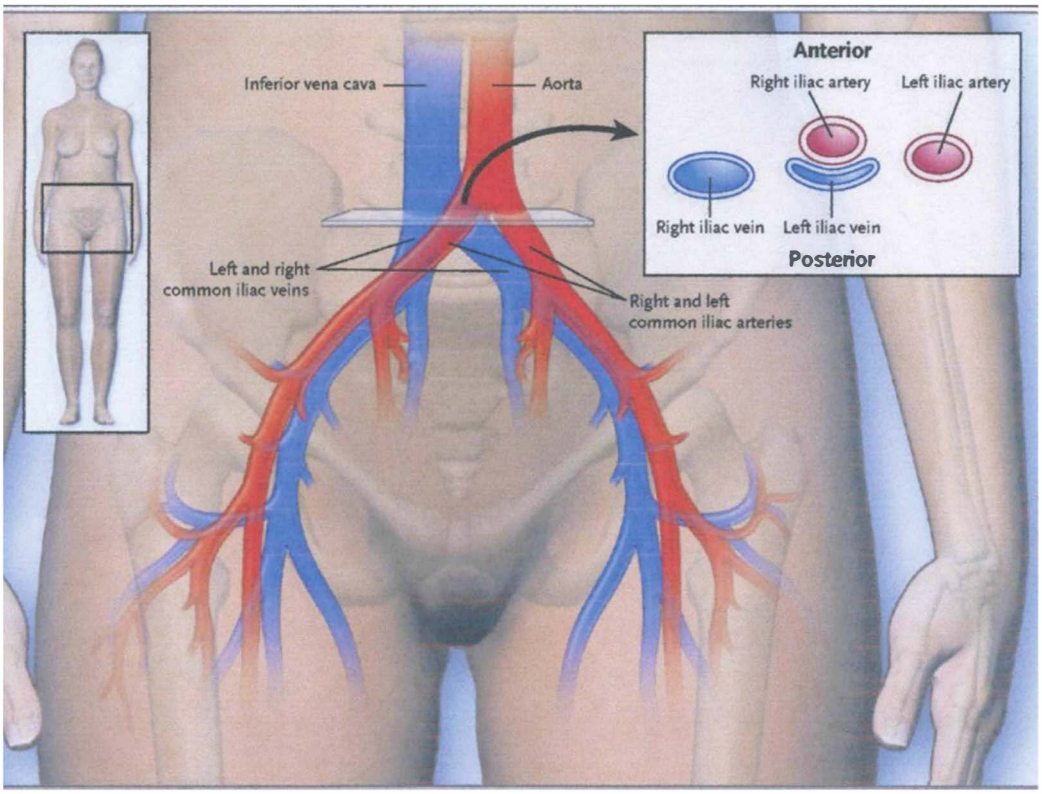


FIGURE 84.11 Relationship of pelvic arteries and veins, as related to MTS. The right common internal iliac artery crosses over and compresses the left common internal iliac vein, providing the anatomic basis for the higher incidence of pelvic venous thrombosis on the left side. (Reproduced with permission from Fazel R, Froelich JB, Williams DM, et al. A sinister development. *N Engl J Med* 2007;357:53–59.)

cerulea dolens showed that 3 had MTS.[397] A dramatic and likely unique consequence of MTS is retroperitoneal hemorrhage, presumably secondary to spontaneous acute rupture of a varix formed in the collateral circulation in response to the massive pelvic venous obstruction.[398–400]

Acute treatment is effective, consisting primarily of regional thrombolytic infusion, thrombus aspiration, and stenting.[390,397,401–403] In a study of 13 adolescents with lower extremity proximal DVT who underwent catheter-directed thrombolytic infusion, MTS was documented in 53%, all of whom had placement of endovascular stents and technical success in 94%.[390] About 40% of successfully treated patients had recurrent DVT within a month, most of whom were effectively treated with thrombolysis again. In 21 patients with venographically verified MTS, initial thrombolysis was routinely followed by balloon angioplasty and/or stent placement, with rapid symptom relief in all but one.[401] Of importance, only 2 of 18 patients who had stent placement had a recurrent thrombosis, whereas 3 of 3 who were treated without stent placement had recurrent thrombosis during the follow-up period of 11 months. An unusual complication of stent placement was embolization to the right ventricle.[404] Long-term management required anticoagulation as with other major venous thromboembolic events.

SUPERFICIAL VEIN THROMBOSIS

SVT usually occurs in a localized and self-limited form, but 5% to 30% of patients also have DVT,[405–407] estimated as a 6.3-fold increased risk.[408] In the study that showed a 29% incidence of concomitant DVT,[407] the notable risk factors were age >75 years, active cancer, inpatient status, and nonvaricose vein location. SVT that involves the greater saphenous vein has a 10% association with pulmonary embolization. The presence of factor V_{Leiden}, the prothrombin G20210A mutation, and protein C, protein S, or antithrombin deficiency can all significantly elevate the OR for formation of SVT.[409–412] In a retrospective assessment of 427 hospitalized patients with symptomatic "isolated" SVT, 19 (4.4%) developed a DVT or PE, the single important predictive factor being severe chronic venous insufficiency.[413] However, in a study of 737 patients with SVT, the prospective risk of subsequent cancer (3.5% vs. 3.9% in controls) or arterial cardiovascular events (4.3% vs. 4.4% in controls) was not increased,[414] and the 3-month follow-up of 788 such patients showed that the rate of death (1.2%) or major bleeds (0.4%) was not adversely affected by SVT.[407]

Treatment of SVT has been assessed in randomized prospective trials. One trial showed a trend (not significant) for decreased incidence of DVT after 12 days of LMWH therapy (40 mg/d or 1.5 mg/kg/d enoxaparin) (1% vs. 3.6% for placebo, $P = 0.37$).[415] A second study of 60 patients with greater saphenous vein thrombosis showed fewer symptomatic or asymptomatic DVT or pulmonary embolic events at 6 months after twice daily subcutaneous injections of either high-dose UFH (12,500 U for 1 week, then 10,000 U for 3 weeks) compared to a prophylactic dose (5,000 U for 4 weeks) (3.3% vs. 20%, $P = 0.05$).[416] However, a comparison of a weight-adjusted (therapeutic) dosage of LMWH (nadroparin) versus a fixed prophylactic dose for 1 month failed to show an advantage for the higher dose (7 of 81 vs. 6 of 83, $P = 0.74$) for SVT progression or venous thromboembolic complications.[417] Addition of a 10-day course of an anti-inflammatory agent (acemetacin) to single-dose nadroparin induced more rapid symptomatic relief of pain and tenderness,[418] but abatement of symptoms clearly does not reflect resolution of the underlying SVT.[419]

An apparently definitive study of anticoagulant treatment in 3,002 patients with isolated, acute, symptomatic SVT was published in 2010.[420] This trial compared 45 days of subcutaneous fondaparinux (2.5 mg/d) with placebo, efficacy outcome being the composite of any cause death, and symptomatic PE or DVT, SVT extension into the saphenofemoral junction or recurrence; safety outcome was primarily major bleeding. The efficacy endpoint clearly favored the active treatment arm (0.9% vs. 5.9%, $P < 0.001$), with no increase in major bleeding. The report notes that 88 patients would need to be treated to prevent one PE or DVT, and a subsequent analysis[421] concluded that the 45-day treatment "did not appear to be cost-effective when used in all patients with isolated SVT of the legs." Clearly, treatment of patients with SVT, especially with a prolonged and expensive course, will depend on concomitant clinical factors.

SUPERIOR VENA CAVA SYNDROME

SVC syndrome usually presents with edema of the upper extremities, torso, neck and face, varying degrees of cyanosis, and engorged cutaneous veins.[422] Most cases result from mediastinal tumor that compresses the SVC, sometimes with concomitant airway compromise.[422–424,425] The SVC syndrome may also be caused by bland thrombosis, almost always secondary to local effects of a foreign body, for example, intravascular devices such as dialysis[426] or central venous access,[427] catheters, and pacemakers,[429] or as a complication of a vascular inflammatory process such as Behçet disease (see Chapter 100).[430,431] Treatment modalities include mechanical interventions by angioplasty and stent placement,[424,428] anticoagulants,[429] local thrombolytic administration,[424,425,427] and bypass or decompressive surgery,[423,425] in addition to treatment of an underlying malignancy when appropriate.[422]

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CHAPTER

85

Sequelae of Venous Thromboembolic Disease: Postthrombotic Syndrome and Chronic Thromboembolic Pulmonary Hypertension

VITTORIO PENGO AND SUSAN R. KAHN

POSTTHROMBOTIC SYNDROME

The postthrombotic syndrome (PTS) is an important, underrecognized, chronic sequela of deep vein thrombosis (DVT). PTS develops in 20% to 50% of patients with DVT,[1,2] even when appropriate anticoagulant therapy is used to treat DVT (see Chapter 87). Clinical manifestations of PTS range from mild symptoms or signs to more severe manifestations such as intractable leg pain, severe edema, and leg ulcers that limit daily activity and ability to work.[3] PTS reduces quality of life and productivity,[4,5,6] and is costly as measured by health resource utilization, direct costs, and indirect costs.[7,8]

DIAGNOSIS OF THE POSTTHROMBOTIC SYNDROME

PTS is a Clinical Diagnosis

PTS is diagnosed on clinical grounds based on the development of characteristic symptoms and signs in a patient with prior DVT (Table 85.1). Symptoms include pain, aching, heaviness, swelling, cramps, itching, or tingling in the affected limb. These may be present in varying combinations and can be persistent or intermittent. Typically, symptoms are made worse by standing or walking and improve with rest and leg elevation. Signs noted on physical examination of the limb include edema, perimalleolar or more extensive telangiectasiae, brown pigmentation, and venous eczema (FIGURE 85.1). Secondary varicose veins may become manifest, and tender thickening of the subcutaneous tissues of the medial lower limb known as lipodermatosclerosis may occur. Venous leg ulcers may develop, which require careful medical attention and often recur. Leg ulcers can be precipitated by minor trauma and are typically chronic, painful, and slow to heal.[3,9]

The clinical picture of PTS can be nonspecific, as conditions other than DVT (e.g., primary venous insufficiency, chronic congestive heart failure) may produce similar symptoms or signs in the lower extremities. However, PTS is usually the correct diagnosis in a patient with objectively confirmed prior DVT who has typical PTS symptoms and signs in that limb. In some patients, it may take up to 3 to 6 months for the initial symptoms of acute DVT to resolve, hence a diagnosis of established PTS should be deferred until after the acute phase has passed.[10]

In the absence of clinical features of PTS in a patient with previous DVT, demonstrating the presence of venous abnormalities such as valvular reflux, persistent venous obstruction, or venous hypertension on invasive or noninvasive imaging or testing does not indicate a diagnosis of PTS. This is because while many patients with symptomatic PTS have detectable venous abnormalities, many DVT patients who do not have symptoms of PTS can also be shown to have such abnormalities.[11–14] Therefore, there is no indication to routinely perform invasive or noninvasive venous testing in patients with a clear clinical diagnosis of PTS.

Objective confirmation can be useful in patients who have a characteristic presentation of PTS but who do not have a history of previous DVT. Compression ultrasonography can assess for lack of compressibility of the popliteal or common femoral veins, along with continuous-wave Doppler to assess for reflux of their venous valves. In one study, the combination of a standardized clinical evaluation, compression ultrasonography, and continuous-wave Doppler analysis reliably diagnosed or excluded previous proximal vein thrombosis in almost 90% of patients.[15]

Clinical Tools to Diagnose PTS

Three clinical tools to measure PTS are the Villalta PTS scale,[16] Ginsberg PTS measure,[17] and the CEAP (Clinical, Etiological, Anatomical, Pathophysiological) classification for chronic venous disease.[18] The Villalta scale is a clinical scoring system that is based on the severity ratings of PTS symptoms and signs. The Ginsberg measure diagnoses PTS if both pain and swelling of the limb of more than 1 month's duration are present, are of typical character (worse at the end of the day or with prolonged sitting or standing, better after a night's rest or with leg elevation), and occur at least 6 months after acute DVT. As well, objective evidence of valvular incompetence is required. The CEAP measure combines clinical and objective findings into seven classes of chronic venous disease with subcategories to denote etiologic, anatomic, and pathophysiologic attributes. Other, less frequently used scales have been proposed as well.[19]

In a step toward harmonizing the definition and measurement of PTS in clinical studies and with a view to encouraging PTS research, a subcommittee of the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis has recently recommended that the Villalta scale be adopted as a standardized measure to diagnose and grade the severity of PTS.[20] This scale is a reliable and valid measure of PTS and shows responsiveness to clinical change.[21]

Table 85.1

Clinical manifestations of the PTS

Limb Symptoms	Signs on Physical Examination
Heaviness	Edema
Pain	Telangiectasiae
Swelling	Venous ectasia, secondary varicose veins
Itching	Hyperpigmentation, venous eczema
Cramping	Redness
Tingling	Dependent cyanosis
Bursting sensation	Lipodermatosclerosis Healed ulcer, open ulcer

The individual components of the Villalta scale and its scoring algorithm are shown in Table 85.2.

Distinguishing Postthrombotic Syndrome from Recurrent Acute Deep Vein Thrombosis

It is not uncommon for PTS symptoms to wax and wane over time. If new symptoms in the affected leg develop or chronic symptoms in the leg become worse and there are no associated

FIGURE 85.1 The postthrombotic syndrome.

Table 85.2

Components of Villalta PTS scale

Symptoms	None	Mild	Moderate	Severe
	0 Point	1 Point	2 Points	3 Points
Pain	❑	❑	❑	❑
Cramps	❑	❑	❑	❑
Heaviness	❑	❑	❑	❑
Paresthesiae	❑	❑	❑	❑
Pruritis	❑	❑	❑	❑
Clinical Signs	**None**	**Mild**	**Moderate**	**Severe**
	0 Point	1 Point	2 Points	3 Points
Pretibial edema	❑	❑	❑	❑
Skin induration	❑	❑	❑	❑
Hyperpigmentation	❑	❑	❑	❑
Redness	❑	❑	❑	❑
Venous ectasia	❑	❑	❑	❑
Pain on calf compression	❑	❑	❑	❑
Venous ulcer	Absent ❑	Present ❑		

Each symptom is self-rated by the patient and each clinical sign is rated by the clinician as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), except ulcer, which is marked as present or absent. All numeric points are summed to yield a total score: a score of 0–4 indicates the absence of PTS and a score of ≥5 indicates PTS (5–9, mild PTS, 10–14, moderate PTS and ≥15 or the presence of an ulcer, severe PTS).
Modified from Villalta S, Bagatella P, Piccioli A, et al. Assessment of validity and reproducibility of a clinical scale for the post-thrombotic syndrome. *Haemostasis* 1994;24:158a.

symptoms that could suggest acute pulmonary embolism (PE), patients can be advised to rest their leg and wait a day, as in most cases the symptoms are likely to be due to PTS and will settle down. However, patients with PTS are at increased risk of recurrent venous thromboembolism.[22] Hence, if leg symptoms persist or progress, especially if new clinical risk factors for DVT are present, investigation is warranted to rule out recurrent DVT. The general approach to diagnosing recurrent DVT is the same as in patients without PTS (see Chapter 83). However, diagnosis of recurrent ipsilateral DVT can be challenging, as finding a noncompressible venous segment in the same area as the initial DVT can represent new or residual thrombus. Compared with the previous ultrasonogram, an unambiguous change in the extent of thrombosis or an increase in clot diameter of 4 mm or more on the current ultrasonogram indicates new ipsilateral DVT.[23–25] Studies suggest that strategies that use D-dimer testing alone[26] or in combination with clinical probability testing[27] can reduce the need to perform ultrasonography in patients with suspected recurrent DVT.

PATHOPHYSIOLOGY OF THE POSTTHROMBOTIC SYNDROME

PTS occurs primarily as a consequence of venous hypertension, which in turn leads to reduced venous return, diminished calf muscle perfusion, impaired microvasculature function, and clinical manifestations of PTS.[9,28]

DVT leads to chronic venous hypertension by two principal mechanisms, persistent venous obstruction, and venous valvular reflux. Follow-up studies of patients with DVT who were treated with standard anticoagulants have shown that in many cases, there is only partial clearance of the thrombus.[29–32] Damage to venous valves with consequent valvular reflux is frequent after DVT, and is likely caused by thrombus-induced activation of inflammation, fibrous scarring associated with acute and resolving thrombosis, or venous dilation distal to the obstructed venous segment.[33–37] Residual venous obstruction 6 months after DVT, alone or in combination with valvular reflux, predicts the development of PTS, whereas reflux alone does not.[31,38]

Elevated levels of markers of inflammation (e.g., ICAM-1, interleukin-6, and C-reactive protein),[37,39] D-dimer,[40,41] and vein wall response to acute thrombosis[42] early after diagnosis or within a few months of DVT were associated with the development of PTS. A large, multicenter study is underway to prospectively assess the relationship between markers of inflammation and vessel injury and the development of PTS.[43]

EPIDEMIOLOGY OF POSTTHROMBOTIC SYNDROME: INCIDENCE, RISK FACTORS AND PROGNOSIS

Data from contemporary prospective studies with 1 year or longer follow-up demonstrate that 20% to 50% of DVT patients will develop PTS and 5% to 10% will develop severe PTS, which may include venous ulcers.[1,2,14,41,44–47] In most cases, PTS develops within the first 1 to 2 years after DVT.[1,2]

It is difficult to predict which DVT patients will develop PTS. A key finding from a recent prospective cohort study of 387 patients with acute symptomatic DVT was that incomplete resolution of leg symptoms and signs by 1 month after DVT strongly predicted the development of PTS during the subsequent 2 years.[2] In addition, venous thrombosis of the common femoral or iliac vein (compared with distal or popliteal vein DVT),[2,41,47,48] previous or recurrent ipsilateral venous thrombosis,[1,2,41,45,49–51] higher body mass index,[2,46,47,52,53] and older age[2,45,46] are associated with a greater risk of developing PTS. One study reported that DVT patients who had subtherapeutic International Normalized Ratios (INRs) more than half of the time during the initial 3 months treatment with vitamin K antagonists had a threefold higher risk of developing PTS,[46] thereby reinforcing the importance of close monitoring of the intensity of oral anticoagulant therapy following the initial thrombotic episode. The risk of PTS is not influenced by the patient's sex,[2] presence of the factor V Leiden, or the prothrombin mutation[2,47] or by the context of the initial DVT (i.e., unprovoked, secondary, or cancer related).[2,41,45,46]

In summary, extensive DVT, prior ipsilateral DVT, persistent venous symptoms 1 month after DVT, obesity, and older age appear to be robust clinical predictors of PTS. However, DVT patients without these features can also develop PTS.

Despite the common belief that patients with PTS have a uniformly poor prognosis, outcomes might be better than previously reported, with more than half of patients remaining stable or improving during long-term follow-up.[2,54–56] Currently, it is not possible to predict the course of PTS in individual patients.

PREVENTION OF THE POSTTHROMBOTIC SYNDROME

Preventing First and Recurrent Deep Vein Thrombosis

Preventing DVT with the systematic use of thromboprophylaxis in high-risk hospitalized patients as recommended in evidence-based consensus guidelines[57,58] will prevent cases of PTS. Furthermore, as recurrent ipsilateral DVT is an important risk factor for PTS, providing anticoagulation of appropriate intensity and duration to treat the initial DVT so as to reduce the risk of recurrent DVT is a key means of preventing cases of PTS.[59,60]

Elastic Compression Stockings to Prevent the Postthrombotic Syndrome

Graduated elastic compression stockings (ECS) decrease venous hypertension, reduce edema, and improve tissue perfusion.[61] Two trials have evaluated the effectiveness of long-term use of ECS for the prevention of PTS, started within days of diagnosis of symptomatic proximal DVT. Among 194 patients who were randomly assigned a made-to-measure 30 to 40 mm Hg knee-length ECS to be worn daily for at least 2 years or no stockings, ECS led to a decrease from 47% to 20% of mild PTS and from 23% to 11% of severe PTS.[62] A subsequent study evaluated the effectiveness of the "off the rack" knee length 30 to 40 mm Hg ECS, worn for 2 years, versus no stockings in 180 proximal DVT patients. Use of ECS reduced the risk of PTS from 49% to 26% (adjusted hazard ratio 0.5 [95% confidence interval [CI] 0.3 to 0.8] in favor of ECS).[45] Conversely, in a randomized trial of patients with proximal DVT who had not developed PTS by 1 year after the initial DVT, there was no benefit of daily use of 20 to 30 mm Hg ECS for up to 4 years, compared with use of sham stockings (1 or 2 sizes too large); PTS developed in 0/24 patients in the ECS group versus 1/23 patients in the sham

stockings group ($P = 0.49$).[13] A meta-analysis that pooled the three studies concluded that use of ECS after DVT significantly reduces the risk of any PTS (odds ratio [OR] 0.3; 95% CI 0.20 to 0.48) and of severe PTS (OR 0.39; 95% CI 0.20 to 0.76).[63] Notwithstanding these results, questions remain regarding the effectiveness, optimal duration, and feasibility of use of ECS to prevent PTS.

The above-noted trials had certain limitations, which could impact the validity of the results including modest sample size, recruitment at a single center, absence of placebo control, and lack of blinding of assessors. It is not clear if ECS actually prevent PTS or rather improve PTS symptoms, in which case it may be as effective and more convenient for patients to begin wearing ECS when symptoms develop. It is not known if lighter ECS (20 to 30 mm Hg) are as effective as 30 to 40 mm Hg ECS to prevent PTS, as the former are easier to apply and the two strengths appear to have similar effectiveness in preventing recurrent venous ulcer.[64] Finally, the use of ECS to prevent PTS after distal (calf vein) DVT has not been studied.

Interestingly, a recent trial reported that beyond an initial 6 months period of use, there was no incremental benefit in wearing ECS for an additional 18 months.[65] This finding was corroborated by a prospective management study of individually tailored duration of use of ECS.[14] Among 125 patients with proximal DVT who were advised to wear stockings for 6 months then subsequently asked to continue stockings only if PTS symptoms had developed, 17% were able to stop using stockings at 6 months, 50% were able to stop use at 1 year, and the rate of PTS at the end of 2 years was similar to that of the ECS (i.e., active treatment) arms of two previous randomized trials.[45,62] It is anticipated that an ongoing large multicenter, blinded trial that is comparing active versus placebo stockings to prevent PTS in 803 patients with proximal DVT will provide important additional information on the effectiveness of ECS.[43]

ECS are unlikely to cause harm but are difficult to apply, uncomfortable, expensive, and require replacement every few months. Based on the available published evidence and pending the results of ongoing trials in this area, a reasonable approach is to prescribe below knee, 30 to 40 mm Hg ECS to patients who have persistent leg pain or swelling after proximal or distal DVT, and if well tolerated, to continue their use for as long as the patient obtains clinical benefit.

Thrombolysis of Acute Deep Vein Thrombosis to Prevent the Postthrombotic Syndrome

Some studies support the tenet that early thrombolysis of acute DVT may reduce the occurrence of PTS, presumably by early reestablishment of venous patency and preservation of venous valve function.[66,67] A meta-analysis of 12 trials reported that thrombolysis appears to offer advantages in terms of reducing PTS.[68] However, studies of thrombolysis to date have enrolled small numbers of patients and have had various methodologic limitations including that in many cases, the primary study outcome was a surrogate measure of PTS such as vein patency or valvular reflux. Therefore, the long-term impact of thrombolysis on PTS and potential risks associated with these procedures are uncertain. The safety, effectiveness, and cost effectiveness of newer endovascular strategies such as catheter-directed thrombolysis, including pharmacomechanical catheter-directed thrombolysis (i.e., lysis in addition to thrombus fragmentation and/or aspiration), are currently being evaluated in well designed, randomized controlled trials in which PTS is the primary study outcome.[28,69,70]

TREATMENT OF ESTABLISHED POSTTHROMBOTIC SYNDROME

Elastic Compression Stockings

Compression therapy and frequent leg elevation are helpful to reduce leg swelling, heaviness, and aching in patients with established PTS.[59,71] ECS should be applied in the morning and removed in the evening or at bedtime. The main contraindication to use of ECS is symptomatic peripheral arterial disease (see Chapter 94), as the compressive effects of ECS may worsen claudication.[72] Practical limitations to using ECS include poor hand or arm strength or lack of finger dexterity to apply ECS. In these cases, assistance with ECS application can be provided by a family member of caregiver, or by using a stocking application aid (rubber gloves, fabric sleeve, plastic or metal frame).

Patients should be prescribed a knee length 30 to 40 mm Hg ECS. If this is too constricting or difficult to apply, a 20 to 30 mm Hg stocking can be tried. Some patients need greater compression strengths (e.g., 40 to 50 mm Hg) to reduce edema. Knee-length and thigh-length stockings appear to have similar physiologic benefits, but knee-length ones are easier to apply.[73]

Other Forms of Compression Therapy

In a small, randomized crossover trial, the use of an intermittent compression extremity pump twice daily for 2 months (20 minutes per session, 50 mm Hg pressure) reduced edema and improved symptoms in patients with severe PTS.[74] Recently, a randomized crossover trial in 32 patients with severe PTS evaluated a portable, battery-operated lower limb venous return assist device that is worn on the calf throughout the day. Use of the device alone or in combination with ECS for an 8-week period was associated with improvement in quality of life and reduced severity of PTS.[75]

Medications

There is limited evidence that "venoactive" or "phlebotonic" remedies such as aescin (derivative of horse chestnut seed) or rutosides may reduce symptoms of PTS. In a clinical trial of 120 patients, administration of oral hydroxyethylrutosides for 1 year improved PTS symptoms (defined by reduction in Villalta PTS score to <5, or decrease in score by at least 30% from baseline score) to a similar extent as ECS.[76] A Cochrane review of 17 trials that compared horse chestnut seed extract to placebo, compression stockings, or other medications in patients with chronic venous insufficiency reported that horse chestnut seed extract appears to be efficacious for short-term relief of symptoms such as leg pain and edema, and side effects were mild and infrequent, and included stomach complaints, dizziness, nausea, headache, and itching. However, the authors concluded that larger, methodologically rigorous trials are required to assess long-term effectiveness and safety of these medications.[77] Diuretics or nonsteroidal anti-inflammatory drugs for the treatment of PTS-related edema have not been studied and are not recommended.

Venous Ulcer Management

The goals of postthrombotic ulcer management are to reduce symptoms, promote healing, and prevent recurrence. Postthrombotic venous ulcers are primarily treated with leg elevation, compression bandages or stockings applied by a trained practitioner, and topical dressings. Multicomponent compression systems are more effective than single component ones.[78] No specific wound dressing has been shown to be superior.[79] Drugs such as oral zinc have not been shown to be effective for the treatment of venous leg ulcers.[80] The use of systemic antibiotics to promote ulcer healing is not supported by evidence, and the usefulness of topical antiseptics is unclear.[79] Surgery such as superficial and perforating vein ablation and deep vein reconstruction, and subfascial endoscopic perforating vein surgery combined with superficial vein ligation, may be of benefit in select patients when ulcers do not respond to conservative treatment,[81] but their benefit to harm profile in patients with postthrombotic ulcers has not been specifically evaluated. Recent reviews have addressed venous ulcers in more detail.[81,82]

Noninvasive Endovascular Procedures or Surgical Treatment of PTS

A few case reports or small case series published by vascular interventional radiologists or venous vascular surgeons at highly specialized referral centers suggest that procedures designed to correct obstruction or reflux such as vein dilation and stent placement, venous bypass grafting, endophlebectomy with reconstruction, valve reconstruction, and interruption of perforating veins may be of benefit to selected patients with moderate or severe PTS.[28,83] To date, however, there have been no methodologically rigorous comparative trials that have addressed the effectiveness of such procedures at improving the clinical manifestations of PTS, or their safety profile.

Prevention and management of PTS are summarized in Table 85.3.

UPPER EXTREMITY POSTTHROMBOTIC SYNDROME

PTS of the arm occurs in 15% to 25% of patients after upper extremity DVT (see Chapter 84).[84,85] Upper extremity PTS is a potentially disabling condition that has an adverse impact on quality of life, especially if the dominant arm is involved.[86] Symptoms of PTS in the arm include swelling, heaviness, and fatigue with exertion.[85,86] Dilation of the superficial veins of the upper arm and chest wall and dependent cyanosis of the arm may be noted on clinical exam. To date, there have been no controlled studies of the effectiveness of elastic bandages, compression sleeves (as are used for lymphedema), or venoactive drugs to prevent or treat PTS after upper extremity DVT. However, anecdotal evidence suggests that patients with persistent arm swelling and pain after upper extremity DVT may have relief of their symptoms with the use of elastic bandages or compression sleeves.[59]

Table 85.3

Strategies for prevention and management of PTS

Prevention
• Prevent initial DVT by administering thromboprophylaxis in high-risk patients as recommended in evidence-based consensus guidelines
• Prevent recurrent ipsilateral DVT by providing anticoagulation of appropriate intensity and duration for the initial DVT and by using appropriate thromboprophylaxis if long-term anticoagulation is discontinued
• Use knee-length, 30–40 mm Hg elastic compression stockings for up to 2 years after DVT; optimal duration of stocking use is uncertain
• The role of thrombolysis for the prevention of PTS is not yet established. Catheter-directed thrombolytic techniques require further evaluation in properly designed trials before being endorsed as effective and safe in reducing the risk of PTS.
Management
• Elastic compression stockings reduce edema and improve PTS symptoms
• Intermittent pneumatic compression units and the VenoWave device are effective for severe symptomatic PTS
• Venoactive agents such as aescin (horse chestnut extract) or rutosides may offer short-term improvement of PTS symptoms; large, controlled trials addressing long-term effectiveness and safety are needed.
• Leg elevation and compression therapy are primarily used to treat venous ulcers
• Providing support and ongoing follow-up to patients with PTS is a key component of management

CHRONIC THROMBOEMBOLIC PULMONARY HYPERTENSION

Definition

Chronic thromboembolic pulmonary hypertension (CTPH) is considered a consequence of previous PE and is characterized by an increase in the mean pulmonary artery pressure (≥25 mm Hg) associated with persistence of thrombi and vascular remodeling in the pulmonary circulation.

Epidemiology

In the early 1990s this condition was deemed rare accounting for only 0.1% of patients who survived an episode of PE.[87] Subsequent data showed that this condition is not so rare, with an incidence varying from 0.4% to 8%.[88,89,90–92,93,94,95,96–97] Variability in the incidence of CTPH may depend on patient characteristics, method of incidence calculation, severity of disease, and the referral institution.

Natural History of CTPH

After an episode of acute PE the fate of emboli may be that of an early complete resolution in most cases[98] or a complete reperfusion at lung scanning already within 1 month.[99] However, in the THESEE study,[100] residual obstruction was demonstrated 3 months after acute PE in 19% of patients and in a study by Sanchez et al.,[101] 29% of patients with PE had significant perfusion defects after a median follow-up of 12 months. It was at this point widely recognized that an incomplete reperfusion due to the persistence of thromboembolic material in pulmonary circulation might predispose to CTPH.[102] For this reason, after a course of oral anticoagulant treatment the follow-up of patients with PE is important and the evaluation should ascertain the presence of residual or new (recurrent) thrombi, the indirect measurement of pulmonary artery pressure, and complete screening for the presence of thrombophilia in selected cases. Because many cases of CTPH have no antecedent history of PE,[89] it is possible that previous PE was not diagnosed or silent (FIGURE 85.2A). Thus, it may happen that a patient first diagnosed with PE is actually affected by a recurrent PE or even by CTPH, especially when the pulmonary artery pressure is high already at the first diagnosis.[95] A pulmonary artery pressure of 50 mm Hg at the time of acute PE is associated with a threefold higher risk of persistent pulmonary hypertension after 1 year. Cases of previous PE may not have been diagnosed, if it occurred in a patient with good respiratory reserve, or in one who did not receive antithrombotic therapy. This might explain why CTPH is relatively frequent at young age as seen in a prospective study, where the mean age at the time of acute PE was 48.6 years among those who later developed CTPH versus 62.0 among those who did not.[93]

Another possibility is that after an episode of acute PE, the PE recurrence was silent or unrecognized (FIGURE 85.2B). Recurrence may be easily missed if patients are not followed up or if the recurrence is mild, for example, in patients on suboptimal oral anticoagulation. The diagnosis of recurrence might be challenging. In general, the same method used for the first diagnosis should be used again. For example, perfusion defect(s) in previously perfused areas or any perfusion defects in case a perfusion lung scintigram became negative after the acute event, make the diagnosis of recurrence very likely.[103] How the persistence of thrombi in the pulmonary circulation translates into CTPH remains to be explained. Residual thrombi undergo organization in proximal pulmonary arteries and the vascular bed subsequently undergoes peripheral vasculopathy indistinguishable from that seen in idiopathic pulmonary arterial hypertension.

Risk Factors

In an inception cohort study of patients who underwent a standardized diagnostic workup for PE, 223 patients with a first episode of PE and 82 patients with PE and previous VTE, 18 patients were diagnosed with CTPH (cases) and 287 were not (controls).[93] At multivariate analysis younger age (per decade), a previous PE, a larger perfusion defect (per decile decrement in perfusion), and idiopathic presentation were significantly associated with an increased risk of CTPH. Confirmation that a large perfusion defect is a risk factor for CTPH comes from a study by Liu et al.,[97] in which only patients with massive PE (vascular obstruction >50%) treated with thrombolytic agents were included. In contrast with data from Pengo et al.,[93] Ribeiro et al.[95] reported a higher risk of CTPH in patients older than 70 years of age at presentation. Other medical conditions increasing the risk of CTPH are splenectomy,[104,105] ventriculoatrial shunt for hydrocephalus, and chronic inflammatory disorders (such as osteomyelitis and inflammatory bowel disease).[104] A European database study confirmed previous risk factors and identified thyroid replacement therapy and a history of malignancy as additional medical conditions associated with CTPH.[106] As far as thrombotic risk factors are concerned, the rate of hereditary thrombophilia is low but elevated levels of factor VIII (>230 IU/dL) have been reported in 41% of CTPH patients compared to 22% in pulmonary arterial hypertension of other causes and 5% in

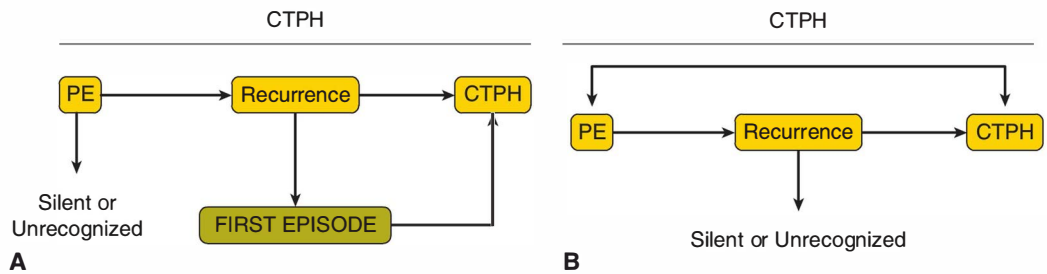


FIGURE 85.2 Possible natural history of CTPH. **A:** The first diagnosed episode of PE is a PE recurrence that predisposes to CTPH. **B:** Recurrence of PE, an important risk factor for CTPH, may be missed or silent.

controls,[107] and a high frequency of phospholipid-dependent antibodies (20%), a clear risk factor for venous thromboembolism, was consistently observed in various studies.[106,108,109]

Pathogenesis

It is not clear why in some patients thrombi persist in pulmonary vasculature, and lead to vessel restriction with increased vascular resistance and pulmonary hypertension. One possibility might be that endogenous fibrinolysis is impaired; the other might be that fibrin is resistant to fibrinolysis. In 1994, Lang et al.[110] reported that there is no endothelial cell-mediated fibrinolytic imbalance in pulmonary arteries of patients with CTPH. It was then noted that fibrin derived from patients with CTPH is resistant to "*in vitro*" lysis[111] and in some patients this may be related to the presence of a dysfibrinogen.[112] However, fibrin from patients with pulmonary hypertension other than CTPH was similarly resistant to *in vitro* lysis.[113] Another proposed mechanism is staphylococcal infection of the thrombus that delays its resolution. In fact, staphylococcal DNA was found in six out of seven thrombi obtained during endarterectomy in patients with CTPH.[114] Furthermore, genetic factors were considered to be involved in the etiology in a Japanese study.[115] The persistence of thrombi in the pulmonary arteries determines a progressive pulmonary hypertension. In 1973 on the basis of unchanged lung perfusion scintigram and increasing pulmonary hypertension over time in a patient with CTPH, Moser and Braunwald[116] proposed that the "open" pulmonary arteries had advanced changes associated with pulmonary hypertension and that the "closed" vascular bed, which had never been subjected to high pressures, had retained a normal structure. The small vessel arteriopathy is characterized by medial hypertrophy and intimal proliferation, microvascular thrombosis, and plexiform lesions resembling the alterations found in primary pulmonary hypertension. Thrombosis and vasoconstriction mediated by neurohumoral factors such as endothelin-1 promote further reduction of cross-sectional area and remodeling of vascular bed. As in nonthromboembolic pulmonary hypertension, endothelin-1 levels in CTPH closely correlated with the hemodynamic and clinical severity of disease.[117]

Clinical Presentation and Preliminary Evaluation

Signs and symptoms of CTPH are related to the stage of the disease and can be absent, mild, or severe according to the hemodynamic impairment of right ventricle secondary to the degree of pulmonary vascular resistance (PVR). Classical symptoms of effort dyspnea and fatigue correlate with the limited capacity to increase cardiac output and with the defective blood oxygenation as a consequence of increased pulmonary dead space. Less frequently, patients complain of episodes of palpitations, lightheadedness or presyncope, chest pain, or they report an episode of hemoptysis. Interpretation of symptoms may soon translate to clinical suspicion of CTPH in a patient with previous PE. However, because many patients do not provide a history of PE, CTPH diagnosis is delayed as symptoms are attributed to other diseases.[118,119] On physical examination, jugular vein distension is an important finding as it may be the marker of right heart pressure. At auscultation, an accentuation of pulmonary closure sound, a reduced splitting of the second heart sound as well as a systolic murmur related to tricuspid incompetence

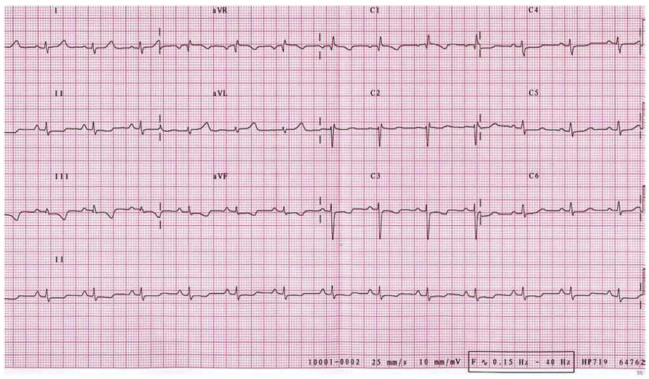


FIGURE 85.3 EKG of a patient with moderate CTPH. Negative T wave in II, III, aVF. ST depression in C3-C4-C5.

can be found. Occasionally, pulmonary auscultation reveals a systolic bruit, which is generated by the turbulence of blood flow through a restricted pulmonary artery.[120]

Electrocardiogram in the advanced stage of the disease typically shows negative T waves in precordial V1–V5 leads, and in II, III, aVF (FIGURE 85.3), pulmonary P wave and right axis deviation >90 degrees. Chest x-ray may show dilatation of main pulmonary arteries[121] and increased cardiothoracic ratio with left second and right second arc protrusions, that result from left main pulmonary artery and right atrium enlargements, respectively (FIGURE 85.4). Avascular areas and pleural thickening may be evident in the lungs. In one study comparing

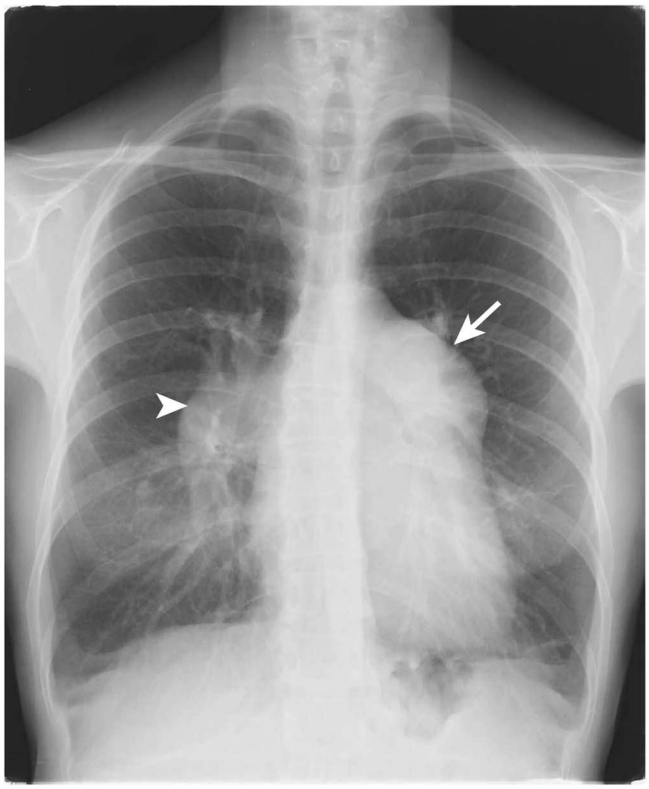


FIGURE 85.4 Chest x-ray of a patient with severe CTPH. A left second arc protrusion (*arrow*) and increased right descending pulmonary artery diameter (*arrow head*) are clearly evident.

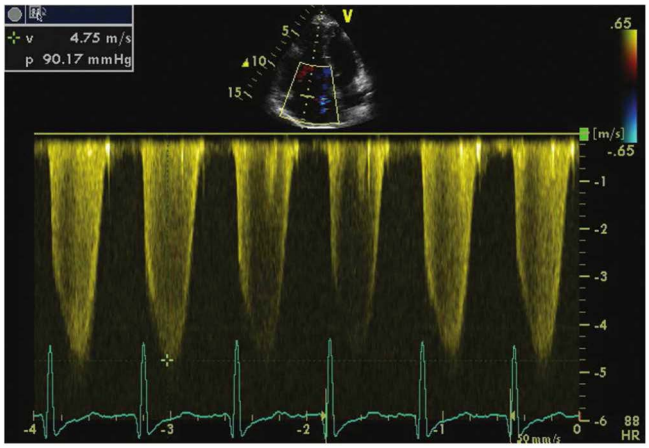


FIGURE 85.5 A patient with CTPH and severe tricuspid regurgitant jet (4.7 m/s) indicative of high systolic pulmonary artery pressure.

chest radiographs of patients with severe CTPH with those of patients with primary pulmonary hypertension,[122] the diagnostic pattern of CTPH was the presence of one of two findings, an avascular area or marked increase of the right descending pulmonary artery diameter (>20 mm) together with pleuritic change. Blood oxygen saturation may be normal at rest but impaired during exercise when taken during the 6-minute walking test. Although not specific due to the inability to distinguish between pre- and postcapillary pulmonary hypertension, echocardiography is an essential tool in the diagnostic path of CTPH. Dilatation of right ventricular (RV) is present when the diastolic diameter exceeds 30 mm. RV dysfunction, paradoxical septal motion, and a D-shaped left ventricle can be appreciated visually. Quantitative report of RV dysfunction is based on a tricuspid annular plane systolic excursion of <18 mm. A tricuspid regurgitant jet is indicative of pulmonary hypertension when its velocity exceeds 2.8 m/s[123] (FIGURE 85.5).

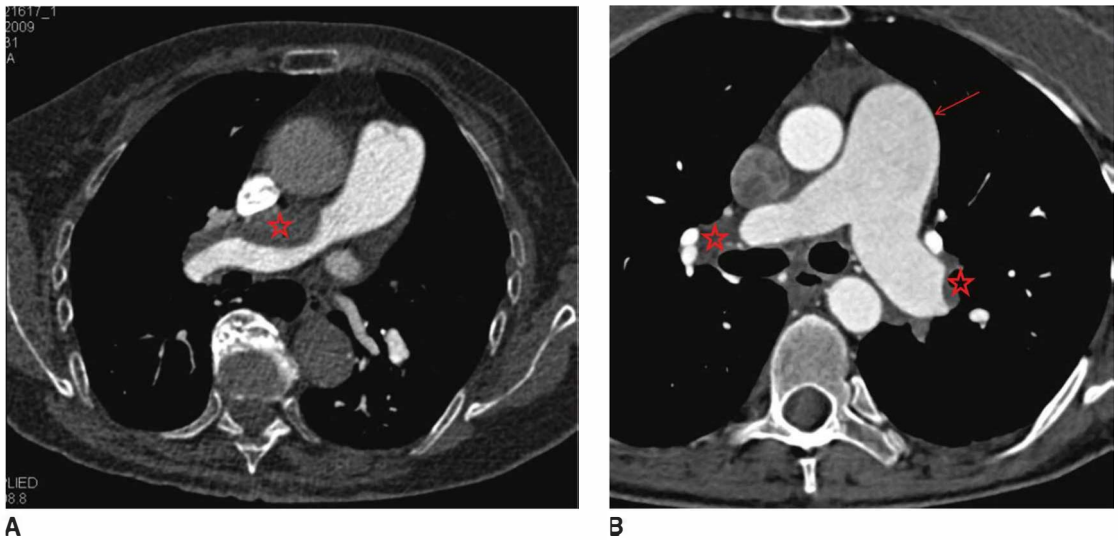
Diagnosis

After preliminary evaluation and the detection of pulmonary hypertension by echocardiography the next step is to document the thrombotic obstruction in pulmonary arteries by means of ventilation/perfusion (V/Q) lung scanning or by computed tomography (CT). If V/Q lung scanning is negative, CTPH is highly unlikely[124] but in case of high clinical suspicion a CT should also be performed. The current generation of high resolution CT scanners better defines sites of vascular obstruction distinguishing thrombi lining pulmonary vessels in either a concentric or eccentric manner (FIGURE 85.6), and also provides information on areas of mosaic pulmonary parenchyma that are often present in CTPH. If thrombotic obstructions are demonstrated, then prior to possible surgical intervention, multiplanar pulmonary angiography must be performed.

Vessel pockets, ligaments, increments, sudden changes of the vessel size, and vessel occlusion are typical angiographic findings.[125] Abrupt changes of pulmonary vessel size, with bands, webs, pouches, and dilated central vessels with irregular tapering of the peripheral vasculature, and segmental complete vessel obstruction, are characteristics of CTPH (Table 85.4).

Therapy

After CTPH is diagnosed, all the patients must receive vitamin K antagonists to maintain an INR between 2.0 and 3.0. CTPH is the only form of pulmonary hypertension amenable with surgical intervention. This procedure is not a simple embolectomy with removal of thrombi from the lumen of pulmonary vessels but implies a true endarterectomy to remove thrombi inside the vessel wall. The decision to proceed with pulmonary endarterectomy (PEA) must take into account the cardiovascular performance, site of thromboembolic obstruction, PVR, and willingness of the patient to be operated. The San Diego surgical classification[126] is based on operative findings of pulmonary artery obstruction, where patients with type 3 (disease within



A B

FIGURE 85.6 CT scan of two patients with severe CTPH. In **(A)** the main right pulmonary artery contains a large protruding thrombus remodeling the vessel wall (*star*). In **(B)** the pulmonary artery truncus is extremely dilated (*arrow*) and thrombi completely occlude right and left pulmonary arteries (*stars*).

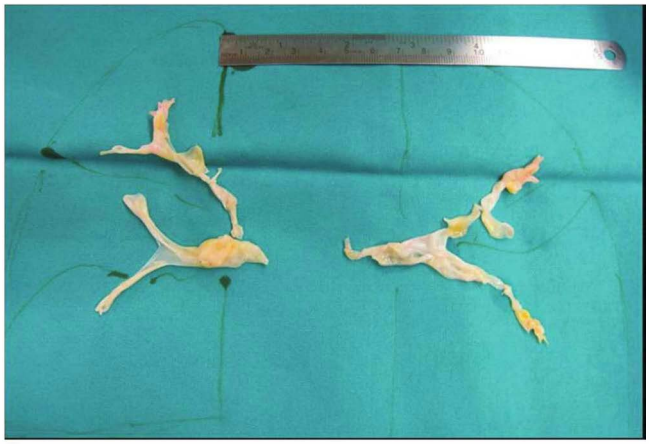
Table 85.4
Differences between idiopathic and CTPH

	Idiopathic PH	CTPH
Previous thromboembolism	Unusual	Possible
Ventilation-perfusion lung scan	Normal or subsegmental defects	Segmental perfusion defects
Contrast CT	No obstruction	Obstructions, bands, and webs
Pulmonary angiography	No vessel occlusion	Vessel occlusion, vessel pockets, ligaments, increments, sudden changes of the vessel size
Thrombophilia	Unusual	Possible

distal segmental arteries only) or type 4 disease (distal pulmonary vasculopathy) had higher postoperative pulmonary artery systolic pressure and greater postoperative PVR compared with that seen in patients with type 1 (fresh thrombus in the main-lobar pulmonary arteries) or type 2 disease (proximal pulmonary vasculopathy). Currently there is no preoperative classification system that might allow for better risk stratification.[127] Patients considered for PEA are usually those with hemodynamic imbalance and symptoms at rest or with minimal exercise (New York Heart Association [NYHA] class III and IV). If not surgically treated, prognosis in such patients is poor.[128,129] On the other hand, clinical course of patients in NYHA class II is not fully known and some of them are stable for years[93]; surgery in these patients is considered very low risk and decision to operate is largely based on patient willingness. Distal disease (segmental and subsegmental pulmonary vessels) with very high PVR implies a small benefit of PEA, while proximal large vessel disease with reasonable PVR is amenable for surgical approach. Associated diseases may increase the risk of PEA although the only absolute contraindication is the presence of severe underlying lung disease, either obstructive or restrictive. Current best practice results in operative mortality rates of 4% to 7% and the best outcomes are associated with (a) surgeon and center experience; (b) concordance between PVR and anatomic disease; (c) preoperative PVR <1,000 to 1,200 dynes/s/cm^{-5}; (d) absence of select comorbidities (e.g., splenectomy, ventriculoatrial shunts); and (e) postoperative PVR <500 dynes/cm/s^{-5}.[125]

Benefits of surgery may take ≥6 months to achieve. Postoperative patient care is crucial and includes optimized ventilation and fluid management, prone positioning, inhaled nitric oxide, prostanoid agents, and pharmacologic therapy for right heart function.[130] If this support fails in case of severe postoperative reperfusion edema, venovenous extracorporeal life support is a good therapeutic option.[131] In cases where it is only possible to perform a thromboendarterectomy in proximal vessels (FIGURE 85.7A) and the improvement of hemodynamic parameters after 12 months is poor (FIGURE 85.7B), pulmonary vessel angioplasty (FIGURE 85.7C) may be an option. Lung transplantation might be the final effective treatment in selected patients.[132]

Medical treatment may be used in CTPH patients who are not considered candidates for surgery or with persistent/recurrent pulmonary hypertension after PEA,[133] the goal being reduction of pulmonary hypertension, increased cardiac output, and alleviation of symptoms. Drugs used in this setting are endothelin-1 inhibitors, phosphodiesterase type-5 inhibitors, and prostanoids. The only randomized, placebo-controlled clinical trial that has addressed the safety and efficacy of medical treatment was the BENEFIT study.[134] Primary end points were change in PVR as a percentage of the baseline value and change from baseline in 6-minute walk distance after 16 weeks of treatment with bosentan or placebo. This study revealed a significant drop in PVR in

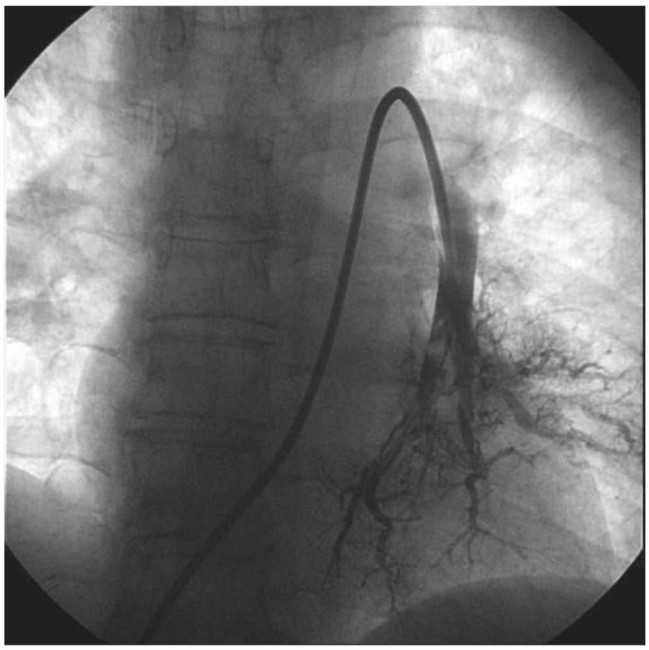


A

	Before-PEA	After-PEA(1 Year Later)
C.I. (L/min/mq)	2,08	2,88
PVR (UW/mq)	15,8	12
PAPm (mm Hg)	38	35
6MWT	200	400
NYHA Class	III-IV	II

B

FIGURE 85.7 PEA in a patient in whom only a partial removal of thrombi from proximal vessels was achieved **(A)**. One year later there was a significant clinical but modest hemodynamic improvement **(B)**. It was then decided to treat the patient with pulmonary angioplasty.



C

FIGURE 85.7 *(Continued)* **(C)** and endothelin-1 inhibitors.

the bosentan group but no change in the 6-minute walk test, functional class, or time to clinical worsening. In a recent meta-analysis it was shown that bosentan therapy is associated with an improvement of hemodynamics and probably exercise capacity[135] in patients with CTPH. Some authors have recently suggested a role for the medical therapy of patients awaiting PEA.[136]

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CHAPTER

86 Prevention of Venous Thromboembolic Disease

MENNO V. HUISMAN, SHARIF PASHA, PATRICK MISMETTI, AND AJAY KAKKAR

Venous thromboembolic disease frequently complicates the course of patients undergoing a variety of surgical interventions. Indeed, it was in the determination of the frequency of thrombosis in general surgical patients using I^{125} labeled fibrinogen leg scanning that Kakkar et al. in 1969[1] were able to describe the natural history of postoperative deep vein thrombosis (DVT). The understanding of the natural history of this disease, and, subsequently, risk factors associated with its occurrence in surgical patients and its wider epidemiology have allowed the development of strategies to both identify patient populations at risk for the development of venous thromboembolism (VTE) in the perioperative period, quantify that risk, and thus provide methods for prophylaxis against VTE.

Beyond recognising that thrombosis commonly occurs in surgical populations, the establishment of evidence that demonstrates the adverse consequences if thromboembolic disease is not prevented has been important. Not only would patients suffer symptomatic DVT or pulmonary embolism (PE), but they may also experience, as a first manifestation of their thrombosis, a fatal PE.[2] Approximately 80% of patients who develop PE will have no evidence of peripheral venous thrombosis before their presentation with PE.[3] Because PE is frequently fatal, even if diagnosed and treated in hospital,[4] strategies based on a policy of treating massive PE or DVT as a way of preventing death from fatal PE expose surgical patients to an unacceptable risk of fatal thromboembolic complications. Beyond the consequences of the acute thrombotic episode are the long-term implications. In the intermediate term there is the need for prolonged anticoagulant therapy for a thrombotic episode. This treatment is associated with serious, if not fatal, bleeding complications.[5] Furthermore, patients with cancer or impaired inhibition of coagulation are at greater risk for the development of recurrent thromboembolism, which in some cases may be fatal.[6] Beyond this intermediate risk, patients who have suffered either symptomatic or asymptomatic DVT or PE are at greater risk for the development of the postphlebitic syndrome, the symptoms of which include leg swelling, varicose veins, venous ulceration, and other trophic skin changes, which are disabling and have impact on the patients quality of life.[7]

RISK OF VENOUS THROMBOEMBOLIC DISEASE IN GENERAL SURGICAL PROCEDURES

More than three decades of research have identified risk factors frequently associated with the development of postoperative VTE in general surgical patients. Most important amongst these are advancing age, a history of VTE, long periods of immobility, the presence of malignancy, obesity, the use of estrogen-containing oral contraception or hormone replacement therapy (HRT), and the type of surgical intervention.[8,9] The way that these risk factors interact in an individual patient to create a specific risk for the development of thrombosis is unpredictable. Therefore, although it would be possible to assign an individual surgical patient a score with regard to their risk for the development of thromboembolic episodes in the perioperative period, the use of such models has yet to be fully validated. A second approach on which decisions about thromboprophylaxis can be made is to define specific surgical risk groups in whom decisions about the use of thromboprophylaxis can be made[10] (see Table 86.1).

CLINICAL TRIALS IN VENOUS THROMBOEMBOLISM—CONSIDERATIONS ABOUT EFFICACY AND SAFETY

The adoption of any specific measure for the prevention of VTE in surgical patients must be based upon evidence for both the efficacy of a particular intervention with regard to preventing thrombosis in patients at differing risks, and the safety of the intervention, in particular for pharmacologic prophylaxis, this being the rate of the bleeding complications associated with anticoagulant therapy.

In terms of assessing the efficacy of any given method of thromboprophylaxis, a number of different efficacy endpoints have been identified. Clearly, the most important endpoint is that of the prevention of fatal PE.[11] Although this complication is rare, and trials designed with this endpoint are the most challenging, landmark studies in the field of thrombosis research have used autopsy-proven fatal PE as a primary clinical trial endpoint.[11] Demonstration that an individual method of prophylaxis is able to reduce the frequency of PE provides strong confirmation of its utility and relevance in routine clinical practice for the given patient population.

Beyond the endpoint of fatal PE, symptomatic VTE, whether it is nonfatal PE or DVT, represents an important clinical complication that occurs more frequently than that of fatal PE. The use of those clinical endpoints, objectively confirmed using appropriate diagnostic tests, is crucial in trials assessing the efficacy of various thromboprophylactic strategies.[12–14]

Most cases of thromboembolic disease are, however, asymptomatic. Therefore clinical trials comparing different methods of thromboprophylaxis that use asymptomatic DVT as an endpoint require fewer patients. Studies using either fibrinogen leg scanning or more recently venography for the detection of asymptomatic DVT have provided the foundations of our understanding of the efficacy of various methods for preventing

Table 86.1

Risk stratification for postoperative VTE and frequency of VTE without prophylaxis

	Venographic DVT		Pulmonary Embolism	
	Calf (%)	Proximal (%)	Symptomatic (%)	Fatal (%)
Low risk: <40 y and uncomplicated surgery and no additional risk factors	2	0.4	0.2	<0.01
Moderate risk: >40 y or prolonged or complicated surgery or additional "minor" risk factors	20	5	2	0.5
High risk: major surgery for malignancy or previous VTE or knee/hip surgery or heparin-included thrombocytopenia	50	15	5	2

DVT, deep-vein thrombosis; VTE, venous thromboembolism.

DVT in surgical patients. These studies form the basis of many of the guidelines on the use of antithrombotic therapy in the perioperative period.[10,15–17] It is essential, however, when using surrogate efficacy endpoints such as venographically proven DVT, that the consistency of the natural history of the thrombotic episode between the asymptomatic DVT and the potential fatal PE has been defined for the population in question. It is also essential that the study design and the antithrombotic intervention does not change that natural history, thereby potentially providing an erroneous result with the use of this surrogate endpoint.

The balance between the efficacy in preventing a thromboembolic episode versus perioperative bleeding complications must be addressed in the use of any antithrombotic agent. This balance must be considered in the specific clinical setting in which the patients find themselves The potential risk for development of thrombosis or bleeding is dependent upon the individual clinical scenario (type and site of operation, patient comorbidities, and concomitant medication).

Studies over the past three decades have established that pharmacologic prophylaxis is not only effective in preventing thrombosis but is associated with an increase in perioperative bleeding complications.[18] Thus the balance between efficacy and safety for any recommended pharmacologic thromboprophylaxis should demonstrate a desirable benefit/risk ratio and have the additional benefit of proven cost efficacy, which provides an economic rationale for routine protection against thromboembolic disease in moderate- and high-risk surgical populations.

METHODS OF PROPHYLAXIS

Mechanical Methods of Prophylaxis

Mechanical methods of prophylaxis are designed either to eliminate stasis or to interrupt thrombus progression from the deep venous system to the pulmonary circulation.

None of the currently available mechanical methods of thromboprophylaxis have been as extensively investigated as the pharmacologic methods.[10] Of those available methods, one of the most popular is graduated compression stockings. These stockings apply a decreasing pressure from ankle to thigh across the lower limb, reducing the venous cross-sectional area and thus enhancing venous flow, and reducing stasis. Other mechanical methods increase pulsatile flow such as pneumatic calf compression, electrical calf stimulation, and venous foot pumps. All of these methods enhance venous flow and can be used intraoperatively and in the postoperative period, although after surgery they are cumbersome to use to maximum effect. Another approach to the prevention of PE is with either venous ligation[19] or inferior vena cava (IVC) filters, which interrupt the transition of thrombus from venous system to pulmonary circulation. No randomized trials have been done to evaluate IVC filters for primary prophylaxis in surgical patients. They have been evaluated in patients with an established DVT for the prevention of recurrent PE, where they have been shown to be effective in reducing the frequency of PE initially, but in the longer term having been associated with an excess of VTE.[20]

Pharmacologic Methods of Prophylaxis

A more popular approach to the prevention of VTE in the perioperative period has been the use of pharmacologic methods.

Aspirin

Aspirin and other antiplatelet drugs have become the mainstay of antithrombotic therapy for patients with atherosclerosis. Aspirin is highly effective in the prevention of arterial thrombotic episodes in patients with established vascular disease. The mechanism of action is through the inhibition of prostaglandin H synthetase 1 and thus a resultant loss of platelet cyclooxygenase activity.[21]

Interestingly, although aspirin is effective in the prevention of arterial thromboembolic events, its role in the prevention of venous thrombosis remains controversial. Despite the fact that aspirin is delivered orally, studies that evaluated its use in the prevention of VTE have often been of limited size and attended by important methodologic flaws that make their interpretation very difficult.[22,23] When compared with studies using other methods of thromboprophylaxis, the studies with aspirin do not justify its routine use, as a sole agent, for VTE prevention in medium- or high-risk surgical patients. Aspirin alone is therefore not recommended as thromboprophylaxis in any patients at risk of VTE.[10]

Dextrans

The polysaccharide dextrans have been evaluated as potential thromboprophylactic agents.[24] Their mode of action appears to be that of hemodilution, combined with an inhibitory effect on fibrin polymerization and an enhancement of fibrinolysis with some antiplatelet effect. They must be given intravenously and run the risk of severe anaphylactic reaction and volume overload. They are therefore not routinely used for thromboprophylaxis in most centers.

Unfractionated Heparin

Low-dose unfractionated heparin (LDUH) has remained the mainstay of thromboprophylaxis in general surgical patients.[9,25] Indeed, it is in the evaluation of this agent that so much of our understanding of the natural history of VTE in surgical patients has been generated. Unfractionated heparin (UFH) binds and potentiates the activity of circulating antithrombin with the resultant inhibition of activated clotting factors X and II (thrombin). The agent can be given in a fixed dose of 5,000 anti-Xa units subcutaneously twice daily, commenced preoperatively, and continued in the postoperative period without need for monitoring of anticoagulant activity.

Low- and Ultralow-Molecular-Weight Heparins

Low-molecular-weight heparins (LMWHs) are derived from UFH through depolymerization.[26] A variety of different LMWHs are available for clinical practice, each of which has a different molecular weight and a different ratio of anti-Xa to anti-IIa inhibitory activity. The mechanism of action of LMWHs is the same as that of UFH, that is, the potentiation of antithrombin activity and the ability to release tissue factor (TF) pathway inhibitor and therefore inhibiting TF:VIIa activity. LMWHs achieve a lesser degree of inhibition of thrombin activity compared with UFH but a greater inhibition factor Xa activity. Advantages of the LMWHs include a longer plasma half life, the ability to dose once or twice daily subcutaneously without the need for dose monitoring, a limited effect on platelet activation, and a reduced incidence of heparin-induced thrombocytopenia[27] and osteoporosis.

A new generation of LMWHs and ultra-LMWHs is currently being investigated for the prevention of VTE.[28] Among these are bemiparin[29,30] and semuloparin,[28,31] both of which are being evaluated in major general and orthopedic surgery. These drugs have higher anti–factor Xa and only minimal anti–factor IIa activities compared with first-generation LMWHs, and may in the future offer an alternative to currently approved drugs.

Indirect Factor Xa Inhibitors

The pentasaccharide fondaparinux—the first in a new class of indirect-acting selective inhibitors of activated factor X—became available in the early 2000s. Fondaparinux has a short 5-saccharide sequence, and can bind antithrombin and potentiate its inhibition of activated factor X. The molecular length is not sufficiently long to bind and inhibit thrombin. This drug has been evaluated primarily in the prevention of thromboembolic disease in orthopedic patients undergoing high-risk hip or knee replacement procedures[32,33,34,35] and in the treatment of DVT and PE.[36]

A single study has been undertaken to evaluate its use in the prevention of thromboembolic disease in general surgical patients.[37] This study compared fondaparinux at a dose of 2.5 mg once daily with the LMWH dalteparin at a dose of 5,000 I U once daily. There were no significant differences in efficacy or safety between fondaparinux and the LMWH.

Direct Thrombin Inhibitors

Another interesting class of antithrombotic agents is the direct thrombin inhibitors. These agents, either naturally occurring or synthetic, are able to bind directly to and inhibit thrombin. They include hirudin, bivalirudin, desirudin, argatroban, melagatran–ximelagatran, and dabigatran etexilate. Most of these agents require parental administration, apart from ximelagatran and dabigatran etexilate, which are prodrugs and orally bioavailable. Development of ximelagatran ceased in 2006 following reports of hepatotoxicity.

These direct thrombin inhibitors are primarily being evaluated in either prophylaxis against thromboembolic disease in high-risk orthopedic patients undergoing joint arthroplasty,[38–40] or as a rescue antithrombotic strategy in patients with heparin-induced thrombocytopenia.[41] Desirudin has been approved in the United States for VTE prophylaxis in patients undergoing elective hip replacement surgery.[42]

New Oral Antithrombotic Agents

The limitations of UFH, LMWH as well as of vitamin K antagonists (VKAs) have led to the development of new oral antithrombotic drugs, of which specific and direct inhibitors of either thrombin or FXa are the most advanced evaluated. In contrast to LWMH and UFH, direct thrombin inhibitors inactivate both fibrin-bound thrombin and free thrombin, while direct FXa inhibitors inhibit FXa within the prothrombinase complex. In addition, both classes of new drugs have a rapid onset and offset of action, a low propensity for food and drug interactions and produce very predictable anticoagulant responses after fixed-dose administration so that routine coagulation monitoring is unnecessary.

The direct thrombin inhibitor dabigatran has been approved in many countries and is administered as the prodrug dabigatran etexilate. It has a longer half-life than the anti-Xa inhibitors (14 to 17 hours) and is eliminated to 80% via renal excretion. Two FXa inhibitors, rivaroxaban and apixaban, have been approved. They are given as active drugs and have multiple elimination pathways. All three drugs reach maximum plasma concentration after 2 to 4 hours. They are reviewed in detail in Chapter 110.

INTERPRETING DATA FROM CLINICAL TRIALS THAT HAVE ASSESSED ANTITHROMBOTIC AGENTS FOR THROMBOPROPHYLAXIS IN SURGICAL PATIENTS

Thromboprophylaxis in surgical patients presents a major challenge. Surgical trauma makes patients prone to perioperative bleeding complications. The delicate balance between efficacy (in terms of preventing VTE) and safety (in terms of not inducing excessive bleeding complications) of thromboprophylaxis has been discussed above.

Only a few antithrombotic agents have been assessed to the extent that there is a clear demonstration that their use is associated with a significant reduction in fatal PE, in well-designed randomized clinical trials.[11,43] However, in meta-analyses of methodologically sound clinical trials, where PE has been

included as one of the endpoints in the study, a number of agents have been shown to possess antithrombotic efficacy. With the use of symptomatic VTE as an endpoint, fewer patients, other than those with fatal PE, are required to demonstrate efficacy. Asymptomatic endpoints are only relevant when they predict both symptomatic DVT and potentially fatal PE.[25,44,45]

Consistent assessment of bleeding in surgical patients is difficult. Bleeding in the perioperative period is determined not only by factors inherent to the patient, but to the nature and extent of operation, the experience of the surgeon, and any other concomitant drug therapy that the patient may be receiving. These factors interact in an unpredictable way with the administration of any particular antithrombotic therapy in the intra- and postoperative period. Since bleeding is an expected complication of operation, blood loss *per se*, both intraoperatively and in the early postoperative period, and consequent requirement for transfusion are difficult surrogate markers for assessment of bleeding risk for an antithrombotic agent in the perioperative setting. This situation is contrary to that seen, for instance, in patients who are receiving antithrombotic therapy for treatment of established thrombosis where bleeding would not be expected.[46] Differences between baseline risk and risk attributed to an antithrombotic agent are necessary to identify as their occurrence outside the controlled setting of a clinical trial may have important adverse consequences.

Few perioperative pharmacologic prophylaxis trials have used endpoints that assess the potential impact of excessive bleeding on surgical outcome.[47] However, such endpoints, which include need for reoperation secondary to bleeding, wound hematoma, and in particular, the need for surgical evacuation of wound hematomas, or consequences of bleeding, resulting in either anastomotic dehiscence, wound dehiscence, or arthroplasty joint sepsis, may represent more important endpoints, when considering the potential adverse consequences. However, when looking across the board at results of clinical trials that assessed antithrombotic agents in the perioperative period, it is clear that routine antithrombotic therapy with LDUH, LMWH, or fondaparinux is not associated with a significant adverse impact in terms of bleeding on surgical outcome in the general surgical population. With any drugs that are cleared by the kidneys, however, caution must be exercised in patients with reduced renal function, particularly in elderly patients, in those with diabetes mellitus, and in patients at high risk of bleeding.[10]

GENERAL SURGERY

Frequency of Thromboembolic Events

The rate of thrombosis reported in patients without prophylaxis undergoing general surgical procedures ranges from 15% to 30%, with rates of PE ranging between 0.2% and 5% and fatal PE < 0.012%[1,9,11,25,48] (see Table 86.1). These historic rates of thrombosis may overestimate the magnitude of the current problem in surgical practice. Earlier mobilization, shorter length hospital stay, improved anesthetic techniques, greater attention to a reduction in perioperative sepsis through antibiotic prophylaxis, and greater use of thromboprophylaxis itself, have had an important impact on reducing the frequency of postoperative DVT in general surgical patients. However, against this is the clear trend for surgical procedures to be undertaken in far sicker patients, who spend longer time severely immobilized in the intensive care unit, and that procedures are now undertaken in older patients and far greater numbers of patients with malignant disease. Thus, in an era where studies using control or placebo against novel antithrombotic agents in surgical practice are no longer ethically justified, it is not possible to know the current rate of thromboembolic disease in patients who do not receive any form of thromboprophylaxis, but it would be wrong to assume that VTE is no longer a serious complication in contemporary surgical patients.[49] Indeed, the Million Women Study highlighted the ongoing risk of VTE among women undergoing inpatient surgery in the United Kingdom over the period 1996 to 2001, with an estimated 1 in 140 middle-aged women being admitted with VTE over the 3 months after surgery.[50] A U.S. study, based on data from the Nationwide Inpatient Sample, also reported a doubling between 1998 and 2005 in the number of hospitalized patients with a clinically recognized episode of acute PE among hospitalized surgical and medical patients. At the same time, the rates of bleeding and heparin-induced thrombocytopenia related to anticoagulant prophylaxis remained stable or declined.[51]

Methods for prophylaxis against VTE in general surgical patients include general measures and specific interventions with antithrombotic strategies. General measures include careful history taking to assess thrombosis risk and in particular to identify important risk factors such as increasing age, obesity, the presence of malignancy, and a history of VTE[52,53] (see Table 86.2). Careful attention to the prevention of perioperative sepsis, maintenance of fluid balance, and early mobilization also play an important role in the reduction of thrombosis risk.

Table 86.2

Risk factors for venous thromboembolic disease

Factors	Severity
General Risk Factors	
Age	Moderate
Obesity	Mild
Ethnicity	Mild
Immobilization	Moderate
Previous VTE	Severe
Estrogen therapy	Mild
Inherited thrombophilia	Severe
Malignancy	Moderate
Heparin-induced thrombocytopenia	Severe
Surgical Factors	
Orthopedic surgery	Severe
Tourniquet application	Moderate
General anesthesia >30 min	Moderate
Venous catheterization	Moderate
Major trauma	Severe
Paralysis	Moderation

Cancer chemotherapy, antiphospholipid antibodies, and hyperhomocystinemia, all recognized risk factors for venous thromboembolism (VTE) in nonsurgical patients, are also likely to increase the risk of postoperative VTE.

Patients who are taking either oral contraceptives or HRT should have this therapy discontinued, ideally 1 month before surgery.

The most popular methods for the prevention of thromboembolic disease in moderate- or high-risk general surgical patients are LDUH, LMWH, and fondaparinux. Indeed the highest quality of evidence in terms of both efficacy and safety with regard to thromboprophylaxis in the perioperative period is available with these agents.

The International Multicentre Trial published in 1975 randomized 4,121 patients aged over 40 years undergoing major surgical intervention to a control group or to receive LDUH in a dose of 5,000 U started 1 to 2 hours before surgery and continued three times daily in the postoperative period until the patient was fully mobile.[11] The primary endpoint in this study was autopsy-proven fatal PE. Nearly 70% of patients in this study who died underwent autopsy. The trial demonstrated a significant reduction in the frequency of fatal PE (16 patients in the control group vs. two in the lose-dose heparin group; $P < 0.005$) and provided the first evidence that heparin-based thromboprophylaxis not only prevented DVT, but was able to reduce the frequency of fatal PE and to have a significant impact on improving surgical outcome. The International Multicentre Trial hailed the modern era of thromboprophylaxis and has formed the basis of recommendations for thromboprophylaxis adopted throughout the world. The only safety concern was an increase in wound hematoma rates, associated with low-dose prophylactic heparin therapy ($P < 0.01$), also seen in other studies.

Numerous studies have since been completed that have assessed the use of LDUH with endpoints including PE, symptomatic VTE, and asymptomatic DVT.[25] These studies all confirm that when compared with control groups, LDUH therapy is associated with a >60% reduction in the frequency of both DVT and symptomatic thromboembolic disease.[25]

The striking feature of LDUH prophylaxis is the associated reduction in perioperative mortality, with the frequency of PE-associated mortality falling from eight to one per 1,000 operated patients, thus profoundly impacting on surgical outcome. However, concerns about potential bleeding complications as manifest by wound hematoma formation drove the quest for the development of safer antithrombotic agents in surgical practice.[11,25]

As a result, LMWH has been investigated extensively[47,48,54–58] since the first report of its use for the thromboprophylaxis in man in general surgical patients by Kakkar et al. in 1982.[59] In this study, patients were randomized to receive one of two doses of a LMWH. The study confirmed that LMWH given in a fixed dose once daily was capable of providing thromboprophylatic efficacy and was safe.

A recent meta-analysis has reviewed studies that randomized over 44,000 general surgical patients to trials comparing LMWH against LDUH.[48] In this meta-analysis, both agents provided equal efficacy and safety. The advantage of LMWH was that it may in general be administered once daily versus twice or three times daily with LDUH to achieve antithrombotic efficacy in surgical patients. LMWHs have broadly replaced LDUH for thromboprophylaxis in general surgical patients.

A recent multinational randomized study—SAVE-ABDO—compared enoxaparin 40 mg/d started preoperatively with semuloparin, an ultra-LMWH, 20 mg/d started postoperatively for the prevention of VTE in 4,413 patients with risk factors for VTE who were undergoing major abdominal surgery.[31] While semuloparin failed to achieve noninferiority over enoxaparin for the prevention of VTE and all-cause mortality, the rates of major bleeding (odds ratio [OR] 0.63; 95% confidence interval [CI], 0.46 to 0.87) and the composite of clinically relevant nonmajor and major bleeding (OR 0.71; 95% CI, 0.54 to 0.93) were lower with semuloparin. This potential benefit in terms of safety is worthy of further investigation.

The selective inhibitor of factor Xa, fondaparinux, has been evaluated in patients undergoing high-risk abdominal surgery.[37] In a trial of some 3,000 general surgical patients, most of whom underwent operation for malignant disease, there was no significant difference in either efficacy with regard to thromboprophylaxis, major bleeding complications, or death in patients who received fondaparinux commenced postoperatively with LMWH (dalteparin) commenced preoperatively. In a study involving 1,309 patients undergoing major abdominal surgery, all of whom had intermittent pneumatic compression (IPC), postoperative fondaparinux reduced the VTE rate by 70% versus mechanical prophylaxis alone, at a low bleeding risk.[60]

Mechanical Methods of Prophylaxis in General Surgery

Mechanical methods of prophylaxis have been assessed in general surgical patients.[24] Although they have no risk of bleeding complications, few trials of methodologic quality have evaluated their efficacy. In particular, no trials have demonstrated that mechanical methods of thromboprophylaxis, alone, are able to reduce the frequency of fatal PE in general surgical patients.

The use of graduated compression stockings alone is not advocated, with the exception of patients at high risk for bleeding, although recent analyses suggested a 50% reduction in the rate of DVT with their use compared with control.[61,62] However, when used in combination with LDUH, they appear beneficial. Studies have demonstrated a 75% reduction in the rate of DVT identified for patients using LDUH and graduated compression stockings compared with those who received LDUH alone.[62] There is no evidence that this method of prophylaxis alone can reduce the frequency of PE. In addition, graduated compression stockings that are used inappropriately may cause a tourniquet effect on the lower limb, resulting in a potential enhancement of the risk of thromboembolic disease.[63]

Intermittent pneumatic calf compression has been assessed for the prevention of DVT in general surgical patients.[64,65] It is not possible, however, on the basis of these small studies to make recommendations about its routine use. The results suggest either a similar efficacy to LDUH or no definite thromboprophylactic effect for the use of IPC alone. In a randomized trial of 2,551 cardiac surgical patients, the combination of pneumatic calf compression with LDUH reduced the rate of symptomatic PE to 1.5% compared to 4.0% for LDUH alone ($P < 0.001$).[66] In general surgical patients with multiple risk factors and at very high risk for VTE, a combination of pharmacologic and mechanical prophylaxis with graduated compression stockings and/or IPC is advocated.[10]

Laparoscopic Surgery

Laparoscopic surgery is a major advance in general surgical practice, providing opportunities for reducing surgical trauma, patient discomfort associated with intra-abdominal and pelvic

surgical procedures, and hospital length of stay. The problem of VTE has not been investigated extensively in patients undergoing minimal access surgical procedures.[67] From the point of view of the pathogenesis of VTE, minimal-access surgical procedures such as cholecystectomy are associated with varying reports of activation of blood coagulation, ranging from only minimal[68–70] to the same degree of coagulopathy as that observed in the open cholecystectomy procedure.[71,72] Often, laparoscopic procedures may take longer than the open surgical procedure, and during operation both the pneumoperitoneum and the reverse Trendelenburg position used in operations such as cholecystectomy or fundoplication result in profound venous stasis of the lower limbs.[73] Laparoscopic surgical procedures appear associated with a shorter hospital stay and therefore a potentially more rapid postoperative mobilization. The outcome of patients once discharged from hospital in terms of their mobility after laparoscopic procedures, compared to those who remain in hospital, has not been investigated extensively.[67]

A number of surveys have determined current surgical practice of thromboprophylaxis after laparoscopic cholecystectomy and reported rates of thromboembolic complications. In a UK study of 417 surgeons, 91% reported never having encountered a thromboembolic episode after laparoscopic cholecystectomy.[74] Most surgeons use LDUH heparin in these patients. In a similar study undertaken in Denmark, 80% of surveyed surgical departments reported no problems with postoperative VTE in patients undergoing laparoscopic cholecystectomy.[75]

Beyond these surveys, there have been a few studies using objective screening techniques that have attempted to determine the rates of VTE associated with various laparoscopic procedures. In a small study, with the screening endpoint of contrast venography 6 to 10 days after operation, no case of DVT was identified in 25 patients who underwent laparoscopic cholecystectomy without thromboprophylaxis.[76] Similarly, low rates in the absence of thromboprophylaxis have been identified in a series of laparoscopic surgical patients screened with lower limb Duplex ultrasonography.[77,78]

Registries of surgical outcome also indicate a low frequency for thromboembolic complications in laparoscopic procedures. In a North American analysis of more than 100,000 laparoscopic cholecystectomies, the rate of symptomatic VTE was 0.2% up to 3 months after operation.[78] Similar findings were identified in a further literature review including over 150,000 laparoscopic cholecystectomies where various thromboprophylactic strategies were used and where the rates of DVT, PE, and fatal PE were 0.03%, 0.06%, and 0.02%, respectively.[79] Finally, the Swedish registry of laparoscopic cholecystectomy reported a rate of VTE of 0.2% in more than 11,000 cases.[80]

As a consequence of these very low reported rates of VTE, there have been few randomized trials to determine the potential benefit of routine thromboprophylaxis in minimal-access surgical patients. In one study, patients were randomized to placebo or to the LMWH dalteparin in a dose of 2,500 IU once daily for up to 10 days after operation. There was no evidence of DVT screened venographically in either group of patients.[76] In a second trial, graduated compression stockings alone or combined with the LMWH reviparin were compared. The rate of DVT, as screened with ultrasound 5 to 7 days after operation, was below 1%.[77]

In conclusion, for simple laparoscopic procedures such as cholecystectomy or tubal ligation, VTE prophylaxis is not generally recommended. However, for patients with a history of VTE or active cancer or with more complicated procedures such as colectomy, it is indicated.

Cancer Surgery

Cancer is an important risk factor for VTE.[9,48] The frequency of thromboembolic complications in patients undergoing major cancer surgery is thought to be about twice that of patients undergoing the equivalent operation without malignant disease.[9,81,82] In general, LDUH[11] and LMWH[48] have been validated for the prevention of thromboembolic disease in patients undergoing major abdominal or pelvic procedures for cancer. In a recent subgroup analysis of more than 6,000 patients with malignant disease receiving perioperative LDUH or LMWH, compared with 17,000 patients without malignancy, fatal PE was three times as common in cancer than in noncancer surgical patients despite use of prophylaxis.[82]

In a study of more than 2,000 patients undergoing laparotomy for cancer, the hypothesis that a higher dose of LMWH would be associated with a low incidence of postoperative thromboembolic complications in cancer patients was tested. In this trial, in the two-thirds of patients undergoing operation for malignant disease, increasing the dose of the LMWH dalteparin from 2,500 to 5,000 IU once daily was associated with a reduction in the frequency of postoperative DVT from 14.9% to 8.5%. Among the patients with cancer in this trial, there was no significant increase in bleeding complications associated with increasing the dose of LMWH. For the one-third of patients without malignant disease, although there was a reduction in the frequency of postoperative DVT with the higher dose of LMWH, this was associated with a significant increase in perioperative bleeding complications.[83]

Beyond the intensity of perioperative antithrombotic therapy has been the suggestion that prolonging the duration of prophylaxis into the postdischarge period might be associated with a lower frequency of late thromboembolic complications in patients with malignant disease. This hypothesis has been tested in two recent randomized clinical trials involving LMWH.[84,85] Patients undergoing laparotomy for cancer were randomized to either 1 week of in-hospital prophylaxis or 4 weeks of in-hospital and postdischarge prophylaxis with the LMWH enoxaparin 40 mg once daily. Prolonged prophylaxis was associated with a reduction in the frequency of postoperative DVT as screened by venography at the end of the 4-week treatment period.[84] A similar study including patients with or without malignant disease demonstrated the benefit for prolonged thromboprophylaxis in cancer surgical patients randomized to in-hospital or in-hospital and postdischarge thromboprophylaxis with the LMWH dalteparin 5,000 IU once daily for up to 4 weeks.[85] In another trial of patients undergoing major general surgery, some of whom had a history of cancer, 4 weeks of therapy with dalteparin 5,000 IU once daily compared with 1 week of therapy reduced the risk of VTE with no increase in risk of bleeding.[86]

Studies involving the LMWH bemiparin have been conducted recently in patients with cancer, with the focus on identifying the optimal dosing and duration of thromboprophylaxis.[29,30] In a Spanish study, Balibrea et al.[29] reported that patients with cancer were still inadequately assessed for risk of VTE, and that bemiparin 3,500 IU/d was more effective than 2,500 IU/d, with no increase in risk of complications. Kakkar et al.[30] compared

the efficacy and safety of 1 versus 4 weeks of treatment with bemiparin 3,500 IU in patients undergoing abdominal or pelvic cancer surgery; while extended treatment did not reduce significantly or change the incidence of the composite of DVT, nonfatal PE, and all-cause mortality, the incidence of major VTE (proximal DVT, nonfatal PE, and VTE-related deaths) was reduced (relative risk reduction [RRR] 82.4%; 95% CI, 21.5 to 96.1), with no increase in the rate of hemorrhagic events. These data suggest that for certain cancer surgical patients at high risk for the development of postoperative DVT, prolonged thromboprophylaxis may be indicated, although no definite recommendations about routine use for postdischarge prophylaxis can be made on the basis of currently available studies.

SUMMARY

VTE remains an important potential complication in patients undergoing major general surgical procedures and operation for malignant disease. Routine pharmacologic thromboprophylaxis with LDUH, LMWH, or fondaparinux has been validated both in terms of safety and efficacy with or without combination with mechanical methods and should be considered for all such patients, provided they are not at high risk of bleeding complications.

ORTHOPEDIC SURGERY

Patients undergoing orthopedic surgery are at an increased risk of developing VTE,[10,87] especially in case of major procedures like hip and knee replacement or hip fracture surgery.

The risk for any DVT, proximal DVT, any PE or fatal PE after hip or knee replacement or hip fracture surgery in absence of prophylaxis is summarized in Table 86.3.[10] PE was the cause of death in 14% of the patients who died after hip fracture surgery.[88] The standardized and routine use of thromboprophylaxis has rendered fatal PE after orthopedic surgery an uncommon event. However, symptomatic VTE remains present in 1.3% to 10% of the patients.[10]

The pathologic basis for postdischarge thrombosis is still unclear. The thrombosis could be asymptomatic on discharge and become clinically overt later or could be newly developed after discharge. A study in patients undergoing elective hip replacement with a negative bilateral venography at discharge, showed a remarkable high incidence of thrombosis over the succeeding 3 weeks.[89,90] This suggests a late development of thrombosis after hospital discharge.

Table 86.3

Prevalence of VTE after major orthopedic surgery without prophylaxis

	DVT (%)		PE (%)	
Procedure	**Total**	**Proximal**	**Total**	**Fatal**
Hip replacement	42–57	18–36	0.9–28	0.1–2.0
Knee replacement	41–85	5–22	1.5–10	0.1–1.7
Hip fracture surgery	46–60	23–30	3–11	0.3–7.5

The use of antithrombotics for the prevention of VTE has been assessed in multiple randomized controlled trials in patients undergoing elective knee or hip replacement therapy and to a lesser extent in patients after hip fracture therapy. However, data on arthroscopic knee surgery, elective spine surgery, and injury of the lower extremities remain scarce. In addition, issues including the optimal timing and prolongation of prophylaxis remain debatable.

Prevention of Venous Thromboembolism in Elective Hip Replacement

Prevention of VTE is recommended in all patients undergoing elective hip replacement[10] due to the high risk for proximal DVT, and nonfatal and fatal PE (Table 86.3).[10] It could be argued that these numbers are an overestimation of current orthopedic surgery due to shorter hospitalization, earlier mobilization, improved anesthetic techniques, and the standardized use of antithrombotic agents. However, surgical procedures are undertaken in older patients, elevating the risk for VTE. Other risk factors are obesity, the presence of malignancies, and a history of VTE.[91]

Mechanical prophylaxis methods that have been studied in elective hip replacement surgery are graduated compression stockings,[92–97] IPC[98–103] and a venous foot pump.[96,104] These nonpharmacologic methods of prophylaxis give a RRR of 20% to 70% for the development of DVT. However, mechanical prophylaxis is less effective in primary prevention than currently available anticoagulant drugs.[97,99,103] Therefore, primary prevention by mechanical prophylaxis is only recommended in patients with a high risk of bleeding.[10]

In a meta-analysis, aspirin gives a reduction in fatal and nonfatal PE of 25% compared to placebo and no prophylaxis.[105] Since alternative prophylactic therapies are more effective,[23,106,107] the use of aspirin as thromboprophylaxis in elective hip replacement is not recommended.[10]

LDUH is more effective than placebo or no prophylaxis[25] and is considered safe in the preoperative and postoperative setting. However, it is considered to be less effective than other regimens available in routine clinical practice.[107–109]

The most commonly used thromboprophylaxis for total hip replacement in North America is adjusted-dose oral VKAs like warfarin.[110] The primary advantage of VKA is the delayed onset of action, hereby allowing surgical hemostasis. However, VKAs are hardly used in Europe due to the variable response between patients, the lower efficacy compared to LMWH,[108,111] interaction with other drugs, and due to the need for frequent monitoring. The dosage of VKA is adjusted to adhere to the recommended International Normalized Ratio (INR) range of 2.0 to 3.0.[10]

In Europe, the most frequently used thromboprophylaxis is LMWH, which proved to be highly effective and safe for the primary prevention of VTE. LMWH is more effective than LDUH[12,112–116] and when compared to adjusted-dose warfarin no difference in the incidence of total and proximal DVT was found.[117–119] However, in one study[120] LMWH prophylaxis started preoperatively was found to be more effective than warfarin for the prevention of thrombosis but the use of LMWH was associated with a significantly increased incidence of bleeding at the operative site and of blood product transfusion. Another study[121] found a significant reduction in total, proximal, and objectively confirmed DVT in patients using

LMWH < 2 hours preoperatively or at least 4 hours after surgery, compared to warfarin started postoperatively (2.2% vs. 4.4%). Combining the results of the five studies in which LMWHs were compared with adjusted dose warfarin,[117–121] the rates of DVT were significantly lower in the LMWH group (13.7 vs. 20.7%, $P \leq 0.01$). However, the proximal DVT rates were 4.8% and 3.4% ($P = 0.08$), respectively. Major bleeding occurred more frequently in the LMWH-treated patients (5.3% and 3.3%, $P < 0.01$). In a meta-analysis[122] in patients undergoing hip or knee replacement surgery, VKAs were less effective in the prevention of total and proximal DVT compared to LMWHs ($P < 0.01$). Subgroup analysis for patients undergoing elective hip surgery did not significantly modify the results ($P = 0.02$). In conclusion, LMWH are more effective in the prevention of DVT but cause more major bleeding than dose-adjusted VKAs.

The synthetic pentasaccharide fondaparinux has been proven to be highly effective in the prevention of DVT among patients with elective hip replacement surgery.[33,123] In one study,[123] fondaparinux 2.5 mg sc (subcutaneously) once daily 4 to 8 hours after surgery was compared to enoxaparin 40 mg sc once daily started 12 hours before surgery. The rates of VTE were 4% in the fondaparinux group compared to 9% in the enoxaparin group ($P < 0.01$). The rate of proximal DVT was lower in the patients randomized to fondaparinux (1%) compared to LMWH (2%; $P < 0.01$). In another study,[33] the identical fondaparinux regimen 2.5 mg once daily was compared to enoxaparin 30 mg bid (twice a day) 12 to 24 hours after surgery. There was no significant difference between the overall risk of VTE (6% in the fondaparinux group compared to 8% in LMWH patients), nor in the rate of proximal DVT (2% vs. 1%, respectively). In both trials fondaparinux was associated with a nonsignificant increase in bleeding ($P = 0.11$). These data indicate, by indirect comparison, that LMWH and probably fondaparinux are more effective than VKAs in the prevention of VTE in patients undergoing elective hip replacement. However, there is a slight increase in bleedings in the surgical site. The higher efficacy and higher bleeding risks could be attributable to the more rapid onset of anticoagulant effects compared to VKAs.

A number of new anticoagulant agents that block either factor Xa or thrombin have been evaluated in phase III trials and are approved for thromboprophylaxis in elective hip surgery.

In the RECORD 1 study,[124] rivaroxaban 10 mg once daily, beginning 6 to 8 hours after surgery, was compared to enoxaparin 40 mg sc once daily, given 12 hours before surgery and restarted 6 to 8 hours after wound closure. The study medication was administered for 35 days. The primary endpoint, DVT, nonfatal PE, or death from any cause occurred in 1.1% of the patients in the rivaroxaban group and 3.7% in the enoxaparin group ($P < 0.01$). Major bleeding occurred in 0.3% and 0.1% of the rivaroxaban and enoxaparin group, respectively ($P = 0.18$). In the RECORD 2 trial,[125] 10 mg rivaroxaban once daily for 35 days was compared to short-term (10 to 14 days) of enoxaparin 40 mg sc once daily. VTE occurred in 2.0% of the patients in the rivaroxaban group and 9.3% in the enoxaparin group ($P < 0.01$). Major bleeding occurred in one patient in each group (Table 86.4).

In the RE-NOVATE I study,[126] the oral thrombin inhibitor dabigatran 150 or 220 mg once daily (starting 1 to 4 hours after surgery with half the dose) was compared with enoxaparin 40 mg sc (given the evening before surgery). Study treatment was

Table 86.4

Incidence of VTE plus all cause mortality and major bleedings with new oral antithrombotic agents in orthopedic surgery

		Treatment		VTE and Death		Major Bleeding	
Study	Surgery	Control = Enox[a]	Experimental	Control (%)	Experimental (%)	Control (%)	Experimental (%)
RECORD-I	THR	40 mg daily	Rivaroxaban, 10 mg daily	3.7	1.1	0.1	0.3
RECORD-II	THR	30 mg bid	Rivaroxaban, 10 mg daily	9.3	2.0	<0.1	<0.1
RECORD-III	TKR	40 mg daily	Rivaroxaban, 10 mg daily	18.9	9.6	0.5	0.6
RECORD-IV	TKR	30 mg bid	Rivaroxaban, 10 mg daily	10.1	6.9	0.3	0.7
RE-NOVATE I	THR	40 mg daily	Dabigatran, 150 mg daily Dabigatran, 220 mg daily	6.7	8.6 6.0	1.6	1.3 2.0
RE-NOVATE II	THR	40 mg daily	Dabigatran, 220 mg daily	8.8	7.7	0.9	1.4
RE-MODEL	TKR	40 mg daily	Dabigatran, 150 mg daily Dabigatran, 220 mg daily	37.7	40.5 36.4	1.3	1.3 1.5
RE-MOBILIZE	TKR	30 mg bid	Dabigatran, 150 mg daily Dabigatran, 220 mg daily	25.3	33.7 31.1	1.4	0.6 0.6
ADVANCE-I	TKR	30 mg bid	Apixaban, 2.5 mg bid	8.8	9.0	1.4	0.7
ADVANCE-II	TKR	40 mg daily	Apixaban, 2.5 mg bid	24	15	4	5
ADVANCE-III	THR	40 mg daily	Apixaban, 2.5 mg bid	3.9	1.4	0.7	0.8

[a]In all studies enoxaparin was the comparator.
THR, total hip replacement; TKR, total knee replacement.

continued for 28 to 35 days. VTE occurred in 8.6% and 6.0% for the dabigatran 150 and 220 mg group, respectively, and in 6.7% of the patients with enoxaparin. Both dabigatran doses met the noninferiority criteria compared to enoxaparin. Major bleeding occurred in 2.0% in the dabigatran 220 mg group, 1.3% of the dabigatran 150 mg group, and in 1.6% of the enoxaparin group (P = ns). In the RE-NOVATE II trial, dabigatran 220 mg once daily, with a half dose 1 to 4 hours after surgery, for 28 to 35 days was compared with enoxaparin 40 mg once daily, initiated the evening before surgery. The primary outcome, total VTE, and all cause mortality, occurred in 7.7% of the dabigatran group and 8.8% of the enoxaparin group ($P < 0.01$). Major bleeding was not significantly different between the dabigatran and the enoxaparin group (1.4% vs. 0.9% respectively, $P = 0.40$)[127] (Table 86.4).

Prevention of Venous Thromboembolism in Elective Knee Replacement

Most of the prophylaxis regimens discussed above are also used, although some of them with lower efficacy, for elective knee replacement.

Mechanical thromboprophylaxis is common in patients undergoing elective knee replacement surgery. However, compared to the data available for LMWH and warfarin, data about safety and efficacy of mechanical thromboprophylaxis is limited. Four small studies[102,128–130] suggest that IPC devices are efficient in this type of surgery. When applied intra-operatively or directly postoperatively and worn continuously until the patient is fully ambulant, IPC devices are most effective. Poor compliance, patient intolerability, improper use of the devices, and inability to continue the use of the device after discharge limits the efficacy of IPC. A venous foot pump was shown to be efficacious in two small clinical trials[131,132] but has been shown to be considerably less effective than LMWH in two other studies.[133,134]

Due to their limited efficacy in elective knee replacement surgery, aspirin[23,128,130,131,135,136] and LDUH[32,137] are not recommended as thromboprophylaxis in this group of patients.

Multiple randomized clinical trials have evaluated the efficacy of adjusted-dose VKAs using routine venography to screen for DVT.[23,128,130,131,135,136] The rate of venography-detected DVT, in patients receiving VKAs for 10 days after surgery, ranges between 25% and 50% and the rate of symptomatic DVT after 3 months is approximately 1%.[138,139]

LMWH is safe and effective in the prevention of thrombosis in patients undergoing elective knee replacement.[117–119,137,140–147] Considering the results of the six randomized clinical trials that compared the prophylactic use of different LMWHs with VKAs for the prevention of VTE in patients with this surgery, the rate of DVT was 48% with VKAs and 33% with LMWHs (OR 0.56; 95% CI, 0.47 to 0.67).[117–119,143–145] The rates of proximal DVT were 10.4% and 7.1%, respectively (OR 0.67; 95% CI, 0.44 to 1.00). Two of these studies observed a higher rate of bleeding in the LMWH group, but this increased risk was limited to minor bleeding.[118,145] However, two meta-analyses confirmed the superiority in efficacy of LMWH compared to LDUH and VKAs in reducing venography-detected DVT, without an increase in bleeding.[148,149]

Fondaparinux 2.5 mg sc once daily, starting 6 hours after elective knee replacement was compared to enoxaparin 30 mg bid starting 12 to 24 hours after surgery.[34] Venography-detected DVT was present in 12.5% of the patients receiving fondaparinux and 27.8% of the patients receiving enoxaparin ($P < 0.01$). Major bleeding occurred in significantly more in the fondaparinux group compared to enoxaparin (2.1% vs. 0.2%, $P < 0.01$).

In the RECORD 3 study,[150] rivaroxaban 10 mg once daily, initiated 6 to 8 hours after wound closure, was compared to enoxaparin 40 mg sc once daily, started 12 hours before surgery and given again 6 to 8 hours after surgery. Study medication was continued until day 10 to 14. The total number of VTE 13 to 17 days after surgery was 9.6% in the rivaroxaban group and 18.9% in the enoxaparin group ($P < 0.01$). Major bleeding occurred in 0.6% and 0.5% in the rivaroxaban and enoxaparin patient group, respectively. In the RECORD 4 trial,[151] rivaroxaban 10 mg once daily was compared to enoxaparin 30 mg sc bid initiated 12 to 24 hours after wound closure. In 6.9% of the rivaroxaban patient group and 10.1% of the enoxaparin patient group a DVT, nonfatal PE, or death from any cause occurred ($P = 0.012$). Major bleeding occurred in 0.7% of the rivaroxaban group and 0.3% of the enoxaparin group (P = ns). The number of major bleedings in the RECORD trials is relatively low compared to the major bleeding rates in the trials with apixaban and dabigatran (see Table 86.4). This is related to the difference in classification of major bleeding. In the trials comparing enoxaparin with rivaroxaban, rebleeding at the surgical site was excluded from the definition of major bleeding, except if the bleeding event resulted in reoperation or death.[152]

In the ADVANCE 1 trial,[153] apixaban 2.5 mg orally bid was compared to enoxaparin 30 mg sc bid. Study medication was initiated 12 to 24 hours after surgery and continued for 10 to 14 days. VTE was present in 9.0% of the patients in the apixaban group and 8.8% in the enoxaparin group ($P = 0.06$ for noninferiority). Major bleeding occurred in 0.7% of the patients who received apixaban and 1.4% in those who received enoxaparin ($P = 0.05$). In the ADVANCE 2 trial,[153b] apixaban 2.5 mg twice daily was compared to subcutaneous enoxaparin 40 mg once a day. Apixaban was found to be superior to enoxaparin for the prevention of VTE and all-cause death (15% vs. 24%, $P < 0.01$). Major or clinically relevant nonmajor bleeding occurred in 4% with apixaban and 5% with enoxaparin (P = ns).

In the RE-MODEL trial,[154] oral dabigatran 150 or 220 mg once daily was compared to enoxaparin 40 mg sc once daily. VTE occurred in 40.5% and 36.4% of the dabigatran 150 and 220 mg group, respectively, and in 37.7% of the enoxaparin group. Major bleeding occurred in 1.3%, 1.5%, and 1.3% of the groups, respectively. In the RE-MOBILIZE trial,[155] dabigatran 150 and 220 mg was compared to the North-American enoxaparin 30 mg sc bid regimen. Both dabigatran regimens were inferior to enoxaparin in the occurrence of VTE (34%, 31% and 25%, respectively). Bleeding occurred in 0.6% of the patients in both dabigatran groups and 1.4% of the patients in the enoxaparin group (P = ns) (Table 86.4).

Prevention of Venous Thromboembolism in Hip Fracture Surgery

Primary prophylaxis for VTE should be provided in all patients undergoing hip fracture surgery unless firmly contraindicated. However, less data are available in the prevention of VTE in comparison with elective hip and knee replacement surgery.

There is insufficient data available to support the use of mechanical prophylaxis to prevent DVT in patients with

hip fracture.[156] There is no research comparing mechanical prophylaxis with other regimens in patients undergoing hip fracture surgery using contrast venography to detect DVT. In a single study, IPC has been shown to be effective in comparison with no prophylaxis.[157] However, data on efficacy and safety of graduated compression stockings remain insufficient to establish a reliable recommendation and a reliable alternative to pharmacologic prophylaxis.[23,158]

The role of aspirin for prophylaxis of VTE in hip surgery patients is controversial. A meta-analysis indicated that aspirin and other antiplatelet agents are effective in the prevention of VTE.[105] In one trial more than 17,000 patients were randomized to receive aspirin 160 mg daily or placebo. Study medication was started before surgery and continued for another 35 days.[23] In approximately 24% of the patients, additional thromboprophylaxis with graduated elastic stockings, LMWHs, or LDUH was used. Aspirin showed a significant reduction in risk of fatal PE (hazard ratio [HR] 0.42; 95% CI, 0.24 to 0.73) and of clinically overt DVT (HR 0.71; 95% CI, 0.52 to 0.97). The patients administered aspirin as prophylaxis had a significant increase in wound-related and gastrointestinal bleeding. When aspirin plus LMWH/LDUH were administered the proportional reduction of VTE was similar to that with aspirin alone.

In a randomized trial in approximately 60 patients, LDUH was compared with the LMWH dalteparin.[159] In this study, LDUH of 5,000 U three times a day was more effective than dalteparin 5,000 IU once daily. However, a review showed that both LDUH and LMWH are effective in the prevention of VTE in patients with hip fracture without increasing the risk of bleeding, but was unable to determine superiority of one agent over the other.[156]

In five studies, of which four had a small sample size, LMWHs were evaluated for prevention of VTE in patients with hip fracture.[159–162] The placebo-controlled trial did not show a significant benefit from LMWH.[160] None of the trials have evaluated the effect of LMWH in comparison to VKAs in patients undergoing hip fracture surgery. No excessive risk of bleeding is associated with the use of LMWH in comparison with placebo[160] or LDUH.[159]

Use of warfarin as prophylaxis is safe and effective in patients undergoing hip fracture surgery. Postoperatively started warfarin (INR 2.0 to 2.7) was found to be more effective than aspirin, 650 mg twice daily, or no prophylaxis.[163] The rates of DVT were 20%, 41%, and 46%, respectively, and for proximal DVT 9%, 11%, and 30%. Bleeding rates were identical in the three groups. With the three studies comparing adjusted-dose VKAs with no prophylaxis taken together, a RRR of 66% for DVT and 66% reduction for proximal DVT were found.[163–165] The bleeding rates observed with VKAs ranged from 0% to 47%[163–165] and in the largest study there was no difference in bleeding compared to placebo.[163]

Fondaparinux has been investigated in patients undergoing hip fracture surgery.[35,166,167] In one of these studies,[35] patients were randomized to either fondaparinux 2.5 mg daily starting 4 to 8 hours after surgery or enoxaparin 40 mg once daily starting 12 to 24 hours after wound closure. Rates of any VTE by postoperative day 11 were 8.3% in the fondaparinux group and 19.1% in the enoxaparin group ($P < 0.01$). Major bleeding was present in 2.2% of both groups; however, minor bleeding was encountered significantly ($P = 0.02$) more in the fondaparinux group (4.1%) in comparison to the enoxaparin group (2.1%).

The efficacy of anti-Xa and antithrombin agents has not been evaluated in hip fracture surgery. However, a study compared the efficacy and safety of the factor IXa inhibitor TTP889, administered 300 mg once daily, to placebo. The occurrence of VTE did not differ significantly (32.1% vs. 28.2% respectively, $P = 0.58$) and no major bleeding was reported. This suggests a lack of antithrombotic potential for TTP889 in hip surgery patients.[167]

Prevention of Venous Thromboembolism in Elective Spine Surgery

The data available on the incidence and prevention of VTE in patients undergoing elective spine surgery is limited. Venography-detected DVT in the absence of prophylaxis is present in approximately 18% of the patients.[168,169] A review including patients undergoing lumbar spine fusion found a 3.7% incidence of symptomatic DVT and 2.2% of PE.[170]

In an observational study, the incidence of VTE in patients using IPC was 6% in comparison to 21% in the patients receiving no prophylaxis.[171] In 11 small prospective trials,[172–182] DVT was identified by ultrasound in 2% of the patients undergoing spine surgery with mechanical prophylaxis. Since a control group was absent, validity of the degree of prevention is limited.

In a study in patients undergoing laminectomy,[183] LDUH was found to be more effective than no prophylaxis. Enoxaparin was also shown to be promising in the prevention of VTE in patients undergoing elective spine surgery.[184,185] Due to the limited data available, prophylaxis using LMWH, LDUH, or perioperative IPC is only recommended in patients with an additional risk factors for VTE such as advanced age, malignancy, previous VTE, presence of a neurologic deficit, and an anterior surgical approach.[10] In patients with multiple risk factors for VTE, pharmacologic thromboprophylaxis (LMWH or LDUH) is recommended with or without a mechanical method.

Prevention of Venous Thromboembolism in Isolated Lower Extremity Injury

Injuries of the lower extremities are defined as fractures of below-knee bones, injuries of ligament and cartilages of the knee and ankle, and rupture of the Achilles tendon. These injuries are in most cases treated surgically, by plaster cast or braces. Risk factors for VTE after isolated lower extremity injury are advanced age,[186–189] bone fracture[190] rather than soft tissue rupture, and obesity.[189] Venography-proven DVT is present in 19% to 45% of the patients with these fractures not receiving any prophylaxis and the incidence of proximal DVT is 5%.[121,190–192]

The data available on the role of prophylaxis in patients with fractures of the lower extremity is limited. Five studies[188,190,191,193,194] evaluated the effect of LMWH in comparison to no thromboprophylaxis or placebo. In two[188,190] studies ultrasonography was used to assess the presence of DVT after plaster cast removal. In these studies LMWH reduced the occurrence of distal DVT significantly but not the incidence of proximal DVT. In a multicenter trial,[194] all patients underwent ultrasonography after 14 ± 2 days to detect the presence of asymptomatic DVT. In the LMWH group, two patients had thrombosis compared to three patients in the control group (P = ns). In the pooled data of two other trials,[191,193] the presence of DVT was screened by ultrasonography after ≥5 weeks in patients with below-knee

fractures. LMWH did not significantly reduce the risk of DVT (16.5% vs. 10.6%, P = ns). Based on the uncertainty of evidence, no routine thromboprophylaxis is recommended for patients with isolated lower-extremity injuries distal to the knee.[10]

Prevention of Venous Thromboembolism After Knee Arthroscopy

One of the most common orthopedic procedures is arthroscopy of the knee and arthroscopic-assisted knee surgery, including meniscectomy, synovectomy, and reconstruction of the cruciate ligaments. Data available on the risk of VTE after arthroscopy is limited and controversial.[195,196] In three large studies for arthroscopy, the incidence of VTE was up to 0.6% in patients undergoing knee arthroscopy without prophylaxis.[197–199] However, asymptomatic DVT detected by mandatory testing ranged from 3% to18%.[200] Known risk factors for VTE are therapeutic arthroscopy and the duration of tourniquet use.[201,202]

In one trial, 4% of the patients without prophylaxis had DVT on compression ultrasonography (CUS) compared to 1% of the patients with reviparin, 1,750 IU once daily for 7 to 10 days.[203] In a second study, prophylactic dalteparin for 30 days was compared to no prophylaxis in patients undergoing diagnostic and therapeutic arthroscopy. The use of prophylaxis reduced the incidence of DVT, detected by ultrasonography on day 12 and 31, from 16% to 2%.[204] No excess of bleeding was observed in patients who received prophylaxis in these studies.[203,204]

The use of thromboprophylaxis with LMWH in knee arthroscopy is recommended in patients with additional risk factors or following a complicated procedure. In the absence of risk factors only early mobilization is recommended.[10]

Optimal Initiation Time for Pharmacologic Prophylaxis

Two important facts should be taken into account to assess the optimal time of prophylaxis for patients undergoing major orthopedic surgery. The first issue is whether initiation of prophylaxis should be initiated preoperative of postoperative. The second relates to how early after wound closure the prophylaxis should be started.[205]

In the United States, LMWH prophylaxis is initiated 12 to 24 hours after surgery. In Europe, LMWH is started 10 to 12 hours before surgery. In two reviews, conflicting results on the relative value of preoperative and postoperative start of prophylaxis were provided.[121,206] To assess this issue, a study was done in patients undergoing hip replacement surgery. The patients were randomized to either preoperative dalteparin, 2,500 IU started 1 hour before surgery and continued with a dose of 2,500 IU 7 hours after surgery and then 5,000 IU once daily, or postoperative dalteparin 2,500 IU started 7 hours after surgery and then continued 5,000 IU once daily, or postoperative adjusted-dose warfarin.[121,207] Both preoperative and postoperative initiation of thromboprophylaxis was significantly better than warfarin, with rates of DVT of 10.7%, 13.1%, and 24%, respectively. Major bleeding was significantly higher in the preoperative dalteparin group than in the warfarin group. Postoperative start of dalteparin was not associated with an elevated risk of bleeding in comparison to warfarin.

Multiple systematic reviews have compared the preoperative start of LMWH prophylaxis to the postoperative regimen. One systematic review found that postoperative start of LMWH prophylaxis is comparable to preoperative initiation.[208] A second review showed that LMWH is only beneficial in comparison to warfarin in patients undergoing hip replacement surgery when LMWH is initiated in close relation to the surgery (either <2 hours before or 6 to 8 hours after surgery). However, starting LMWH just before surgery is associated with an elevated risk of bleeding.[209] A third review showed that starting LMWH preoperatively is more efficient than postoperative, but this benefit is diminished by an increased risk of bleeding.[210] In conclusion, the American College of Chest Physicians (ACCP) guidelines recommends starting either preoperatively or postoperatively in patients receiving LMWH as thromboprophylaxis. For patients receiving fondaparinux, it is recommended to start either 6 to 8 hours after surgery or the next day. [10]

Optimal Duration of Prophylaxis

The optimal duration of thromboprophylaxis after orthopedic surgery is debatable.[211] Multiple studies have shown that the risk for VTE prevails for at least 4 weeks after hip surgery.[90,197,212–217,218,219] In addition, nearly three-quarters of all symptomatic DVT occur after discharge.[90]

Multiple meta-analyses showed that prophylaxis after discharge is safe and reduces the rate of VTE. [44,45,220] No excess bleeding was observed in patients with extended prophylaxis suggesting that the risk–benefit ratio favors the use of extended prophylaxis. In a systematic review of elective hip replacement surgery, the VTE rate was 1.1% in patients with extended LMWH prophylaxis compared to 2.7% in patients only given in-hospital prophylaxis (absolute risk reduction 1.56%; 95% CI, −0.21% to 3.3%; number needed to treat 64). [221]

Six randomized controlled trials have evaluated extended prophylaxis with LMWH for up to 35 days in patients undergoing elective hip replacement.[89,207,214,218,222,223] All study results were in favor of extended thromboprophylaxis. A systematic review showed a significant decrease in DVT and symptomatic VTE in patients with extended LMWH.[224] The incidence of VTE in extended prophylaxis was 1.4% compared to 4.2% in the group constricted to in-hospital prophylaxis. One randomized controlled trial (RCT) comparing in-hospital LMWH to extended use of LMWH in total hip- or knee replacement did not show a benefit in the prevention of VTE.[225]

Postdischarge prophylaxis with VKAs in patients undergoing hip surgery had been shown to be effective and safe in the prevention of VTE.[226] However, in a trial comparing 6 weeks of LMWH with 6 weeks of VKA prophylaxis in patients undergoing hip surgery, LMWH was more effective and safer than prophylaxis with VKAs.[227]

In a trial including patients undergoing knee replacement surgery, extended LMWH prophylaxis for 4 weeks did not significantly reduce the rate of screened DVT and symptomatic VTE versus in-hospital prophylaxis.[222]

The benefit of extended thromboprophylaxis in patients undergoing surgery for hip fracture has also been assessed. In a randomized controlled trial,[35] patients received 3 weeks posthospital continuation of fondaparinux 2.5 mg once daily or placebo. At follow-up, 1.4% of the patients with extended fondaparinux had objectively proven DVT compared to 35.0% in the placebo group. Bleeding rates did not differ significantly between fondaparinux and placebo.

The 8th edition of the ACCP guidelines recommend that thromboprophylaxis in elective hip replacement and hip fracture surgery be extended beyond 10 days and up to 35 days.[10] The recommended options of prophylaxis are LMWH, fondaparinux, or a VKA.

Predischarge Screening for Deep Vein Thrombosis

Routine screening for asymptomatic DVT has been assessed in order to predict the postdischarge occurrence of VTE in patients after orthopedic surgery.[210] However, screening for asymptomatic DVT has not been shown to be effective in three studies.[138,140,228] Only 0.15% of the patients had asymptomatic DVT at discharge.[140] In the 2nd study, patients were randomized to receive predischarge CUS or a sham CUS.[138] Active CUS detected DVT in 2.5% of the patients, but did not influence the occurrence of VTE on follow-up. In a last study, patients were randomized to 10 days LMWH and predischarge ultrasonography or extension of LMWH prophylaxis for another 3 weeks.[228] CUS did not reduce the incidence of symptomatic VTE. Therefore, routine predischarge screening is not warranted.

MEDICALLY ILL PATIENTS

VTE remains a major cause of morbidity and mortality in patients who are hospitalized.[4,229–234] Despite the availability of effective treatment of DVT and PE, clinically overt VTE increases morbidity and cost, and may lead to an increase in preventable death.[235] Therefore, thromboprophylaxis has been advocated in high-risk patients.

Venous thromboprophylaxis is widely practiced in surgical patients, but, in contrast, many medical patients still do not receive it despite recommendations and guidelines.[236,237–239] There is a need for a constant reappraisal of the burden of VTE in medical patients and of the evidence from prophylactic randomized studies.

RISK FACTORS IN MEDICAL PATIENTS

Over the last few years, the incidence and prognosis of VTE appears unchanged despite prophylaxis being provided to an increasing number of patients; thus other factors may account for the observed trend.[240]

The development of noninvasive and reliable methods for the diagnosis of DVT and PE has facilitated its recognition. Widespread imaging is increasing the apparent incidence of VTE. During the last decade, several authors assessed independent risk factors for VTE in cohorts of medical patients by using multivariate analysis.[241] A systematic review of these independent risk factors for VTE in hospitalized medical patients was recently published, proposing to retain only risk factors found more than once in the literature (Table 86.5). In many countries, these factors are increasing as the population grows. The prevalence of risk factors such as heart failure or malignancy will increase because of the availability of treatments that effectively increase life expectancy.

Institutionalization (current or recent hospitalization or nursing home residence) account for 61% of cases of VTE in a community, of which 24% following medical illnesses, 23% following surgical procedures, and 14% during nursing home residence stays.[242] Thus, medical patients who are hospitalized are at particular risk of developing VTE and its complications because of the combination of chronic risk factors (e.g., age, heart failure, and prior VTE) and an acute, transient increased risk associated with conditions leading to the hospitalization and immobilization (e.g., aggravation of heart failure or respiratory failure, infectious disease, stroke, or acute myocardial infarction [MI]). These acute medical conditions are each independent risk factors for VTE[240,242] and often a cause of

Table 86.5

Independent predisposing risk factors for VTE in hospitalized medical patients found more than once

1 < OR < 1.5	1.5 < OR < 3	OR > 3
Black race	Respiratory failure	Pharmacologic sedation
COPD	Diabetes mellitus	Family history of VTE
HRT	Hypertension	Prolonged immobility
Smoking	Advanced age	Previous SVT
Obesity	History of VTE	Neurologic disease with extremity paresis
Thrombophilia (factor V Leiden)	Cancer and chemotherapy Congestive heart failure CVC or pacemaker Ischemic stroke Transfusion Renal failure Varicose veins	

OR, odds ratio; CVC, central venous catheter; HRT, hormone replacement therapy; COPD, chronic obstructive pulmonary disease; SVT, superficial vein thrombosis; VTE, venous thromboembolism.

prolonged immobilization, which is also an independent risk factor (Table 86.5).

Outpatients are also at a risk of VTE. The same chronic and triggering risk factors in medical patients who are hospitalized have been identified in a general population of outpatients.[243]

ISSUES IN THE ACCEPTANCE OF THROMBOPROPHYLAXIS STUDIES

Surveys show that many medical patients who may benefit from thromboprophylaxis do not receive it [244,245] despite clear and strong recommendations. This discrepancy is mainly explained by the idea that clinically relevant benefits of thromboprophylaxis in patients who are hospitalized with medical illness have not been demonstrated and possibly by the fear of major bleedings.

Asymptomatic Deep Vein Thrombosis as Primary Endpoint in Prophylactic Studies

The main endpoint used in most prophylactic clinical trials in medical, as well as in surgical patients, is a DVT (in most cases asymptomatic) diagnosed by imaging (e.g., venography or ultrasonography). Asymptomatic DVT has been used as a surrogate endpoint for symptomatic DVT and PE, which constitute the burden of VTE described in epidemiologic studies. Although there is no perfect surrogate endpoint,[246] asymptomatic DVT (combined with symptomatic events) has been adopted in most prophylactic studies as the main endpoint. Most thromboprophylactic anticoagulants have been approved by health authorities on the basis of this endpoint. In meta-analyses of placebo-controlled studies, there are good correlations between the incidence of asymptomatic DVT and that of symptomatic PE, and between the reduction of asymptomatic DVT and that of PE. However, in studies comparing LDUH and LMWH, the reduction of PE cannot be predicted as accurately by the reduction of asymptomatic DVT, mainly because of the wide CI around the estimate of the reduction of PE.

To overcome the limitation of using all (including *isolated calf*) asymptomatic DVT as the main endpoint, recent studies have used only asymptomatic *proximal* DVT as the major component of their primary endpoint: Proximal DVT correlates more closely than isolated calf DVT with the risk of PE and death.

Venography or Ultrasonography to Assess Asymptomatic Deep Vein Thrombosis

Venography was used in most studies in medical patients to evaluate the primary endpoint, although in one study,[247] the major component of the primary endpoint, asymptomatic proximal DVT, was assessed by systematic screening with CUS. There is, however, no convincing evidence that the technique of CUS is at least as reliable as venography for the diagnosis of asymptomatic proximal DVT.[248] CUS is more practical because it has almost no contraindication, thereby improving compliance, and it is now accepted as a diagnostic method for the evaluation of proximal DVT in prophylaxis studies by drug agencies.[249] In addition, dynamic CUS examinations can be fully videotaped and be read centrally by a core lab[247–250] as is done with venograms in other studies.

REVIEW OF EVIDENCE FOR THROMBOPROPHYLAXIS IN MEDICAL PATIENTS

Thromboprophylaxis in medical patients was mainly evaluated in hospitalized patients with an acute medical illness inducing a restricted mobility. Patients living in a nursing home with limited mobility or those needing long-term hospitalization for chronic conditions, such as paraplegia or the need for permanent respiratory assistance, may also be candidates for prophylaxis. However, no studies have been performed to evaluate the benefit of prophylaxis with the appropriate length of treatment.

Patients with cancer are at risk of VTE not only because of their disease but because anticancer drugs and central venous lines for anticancer treatments are additional risk factors. A clinically overt VTE may be the occasion for diagnosing a previously unknown cancer. The prognosis of VTE is more severe in patients with cancer than in patients without cancer.[251] A recent review of the literature showed there is no strong evidence to support the routine use of thromboprophylaxis to decrease the risk of VTE associated with central venous catheters (CVCs) or to improve survival in patients.[252] Regarding cancer patients receiving chemotherapy, the PROTECHT study recently suggested that LMWH compared with placebo in 1,150 patients could reduce the incidence of VTE from 3.9% to 2.0% in ambulatory patients with metastatic or locally advanced cancer.[253] Cancer patients suffering from an acute complication such as sepsis, heart, or respiratory failure are at very high risk of VTE.

Prophylaxis in Medical Patients with Acute Disease at High Risk of Venous Thromboembolism

Patients hospitalized for acute MI are at high risk of VTE. In a meta-analysis[254] reviewing placebo-controlled studies of antithrombotic drugs, the rate of PE in the placebo group was 3.9%. Prevention of recurrent MI rather than of VTE is the primary therapeutic objective in patients with acute MI. For this objective, patients with acute MI already receive combinations of high doses of antithrombotic agents, including fibrinolytics, antiplatelet agents (e.g., aspirin, clopidogrel, and glycoprotein IIb/IIIa antagonists) and heparin. Heparin confers incremental benefit in addition to other antithrombotic treatment for the prevention of VTE.[254]

Patients with acute hemiplegic stroke have an incidence of DVT of approximately 50% within 2 weeks in the absence of prophylaxis. Pulmonary emboli are frequent in patients who have a stroke; 13% to 25% of early deaths from stroke have been attributed to PE, most often between the 2nd and 4th weeks.[255] Aspirin given as soon as the diagnosis of ischemic stroke is confirmed can reduce the risk of recurrent stroke, improve survival, and help prevent PE (one event of PE avoided for 1,000 patients treated).[256,257] UFH, LMWH, and heparinoids have been evaluated in patients who have acute stroke. Although anticoagulants did not improve survival, LMWH was shown to reduce the risk of PE by 66% (OR, 0.34; 95% CI, 0.17 to 0.69) and DVT by 73% (OR, 0.27; 95% CI, 0.08 to 0.96). However, there was increased risk of major bleeding (OR, 2.17; 95% 1.10 to 4.28).[258] A review of studies, comparing UFH and LMWH, or heparinoids, which included five trials involving 705 patients, showed a statistically significant reduction of DVT in favor of LMWH (OR, 0.52;

95% CI, 0.56 to 0.79).[259] The recent PREVAIL study, comparing enoxaparin 40 mg once daily to UFH 5,000 U twice daily in 1,762 stroke patients, confirmed these results with a significant DVT risk reduction (RR 0.59; 95% CI, 0.46 to 0.76) in favor of enoxaparin but with a nonsignificant increase in major bleeding risk (RR = 1.66; 95% CI, 0.60 to 4.53).[260]

Prophylaxis in Other Medical Patients

There is a diversity of other acute medical conditions that may increase the risk of VTE. Thromboprophylaxis was evaluated in a number of trials with a broad spectrum of patients. Eligibility for these studies was mainly driven by the patient's global risk of developing VTE.

Mechanical Prevention

A few randomized studies performed in a limited number of patients, mainly surgical patients and patients with neurologic disorders, evaluated graduated compression stockings and IPC. The meta-analysis[62] of the studies performed in the absence of other prophylactic treatments showed an OR of 0.34 (95% CI, 0.25 to 0.46), favoring compression stockings over control for the reduction of asymptomatic DVT. There are limitations for the application of these studies in medical patients. To our knowledge, no randomized clinical trials have evaluated mechanical methods of prophylaxis in general medical patients. None of the two meta-analyses were powered to demonstrate a beneficial effect on the reduction of PE. Because of their safety, the use of mechanical prophylaxis could be recommended in medical patients with risk factors for VTE in whom there is a strict contraindication to prophylaxis with anticoagulants or in patients already receiving anticoagulant for a potential additive benefit.

Antiplatelet Agents

Antiplatelet agents, aspirin in particular, may have some benefit in preventing VTE,[23,261] but only eight randomized studies, with <600 patients, have compared antiplatelet agents versus no prophylaxis in medical patients. A meta-analysis of these clinical trials has shown a statistically nonsignificant 47% ± 17% odds reduction in asymptomatic DVT with antiplatelet agents (39 out of 261 patients vs. 61 out of 266 patients), whereas there was no effect on the bleeding risk (major event rate of <1% in the two groups).[261]

Short-Term Prophylaxis with Heparin and Low-Molecular-Weight Heparin

A meta-analysis of 15,095 patients concluded that in hospitalized medical patients heparin showed a statistically significant reduction in the risk of asymptomatic DVT by 56% (relative risk [RR], 0.44; 95% CI, 0.29 to 0.64; $P < 0.001$) and the risk of PE by 52% (RR, 0.48; 95% CI, 0.34 to 0.68; $P < 0.001$).[262] There was a nonsignificant ($P = 0.08$) adverse trend for major bleeding and a neutral effect for total mortality. There were limitations for the interpretation of the results of the meta-analysis such as the heterogeneity of the different studied populations and the relative small number of patients in most of them. Since these meta-analyses, three placebo-controlled studies have been designed with more solid methodology to evaluate the benefit of thromboprophylaxis in acutely ill medical patients.[250,263,264] The three studies included patients hospitalized for acute medical illness requiring temporary immobilization and sufficient VTE risk factors, to ensure a moderate level of VTE risk (see Table 86.6). Higher-risk patients, such as patients with acute stroke or acute MI, were not eligible. The three studies showed consistent efficacy results for enoxaparin, dalteparin, and fondaparinux—a reduction of VTE in the range of 50%. The RRs for each study are: MEDENOX[263]: 0.37 (95% CI, 0.22 to 0.63), PREVENT[250]: 0.55 (95% CI, 0.38 to 0.80), and ARTEMIS[264]: 0.53 (95% CI, 0.31 to 0.92). The overall estimate of the RR from these three trials is 0.50 (95% CI, 0.38 to 0.66). When looking at the reduction of asymptomatic proximal DVT or symptomatic VTE in the three studies, there is again a consistent approximate 50% reduction in favor of the prophylaxis (see Table 86.7). The previous meta-analysis was then updated with the results of these three studies and showed that during prophylaxis there is a significant risk reduction in symptomatic PE: RR = 0.43 (95% CI, 0.26 to 0.71), in fatal PE: RR = 0.38 (95% CI, 0.21 to 0.69), and without any significant increase in major bleeding: RR = 1.32 (95% CI, 0.73 to 2.33).[265]

The internal comparison of LMWH to LDUH in this setting was addressed in meta-analysis.[262] Two additional studies were published in 2010 with 3,564 patients.[266,267] Combining these new data with the previous meta-analysis, LMWH appears to be as effective as LDUH but safer in terms of major bleeding risk: RR = 0.54 (95% CI, 0.31 to 0.94), $P = 0.03$ (unpublished data).

Long-Term Prophylaxis with Heparin and Low-Molecular-Weight Heparin

In all the previous trials, the active pharmacologic prophylaxis was given for a mean duration of 2 weeks. A more prolonged VTE prophylaxis was recently assessed in the EXCLAIM study in 5,963 acutely ill medical patients.[268] In this double-blind study, a long term-prophylaxis (28 ± 4 days) with enoxaparin 40 mg once daily was compared to a short-term duration (10 ± 4 days) with the same drug. The overall results showed a significant risk reduction in VTE from 2.5% to 4.0%: absolute risk difference = −1.53 (95% CI, −2.54 to 0.52) counterbalanced by a significant increase in major bleeding risk from 0.3% for the shorter duration to 0.8% for the longer duration; absolute difference risk = 0.51 (95% CI, 0.12 to 0.89).[268] The interpretation of this study is complicated because the protocol had to be amended during the study in order to increase the VTE risk profile of the studied population.

Thromboprophylaxis with New Oral Anticoagulants

Two direct anti-Xa inhibitors have been tested for VTE prophylaxis in acutely ill medical patients with two similar studies comparing long-term prophylaxis with rivaroxaban or apixaban, respectively, to short-term prophylaxis with enoxaparin (MAGELLAN study: ClinicalTrials.gov Identifier NCT00571649) and long-term Apixaban to short-term enoxaparin (ADOPT study: ClinicalTrials.gov Identifier NCT00457002). In the Magellan study, 8,101 patients were randomized to rivaroxaban 10 mg once daily for 35 days or the standard approved dose of enoxaparin (40 mg once daily by subcutaneous injection for 10 days). Patients included had one or more acute medical conditions, including infectious disease, heart failure, respiratory insufficiency, ischemic stroke, active cancer, or inflammatory/rheumatic diseases; 30% had two or more such conditions. The primary efficacy outcome, a composite of asymptomatic proximal DVT (detected by ultrasonography), symptomatic DVT, symptomatic nonfatal PE, and VTE-related death was reached at 35 days in 4.4% of patients on rivaroxaban versus 5.7% in patients on enoxaparin (HR 0.77; 95% CI, 0.62% to 0.96). The primary safety outcome, a composite of treatment-related major bleeding and clinically

Table 86.6

Main design features of MEDENOX, PREVENT, and ARTEMIS

MEDENOX	PREVENT	ARTEMIS
Treatments	**Treatments**	**Treatments**
Enoxaparin 20 or 40 mg, or placebo q.d. 14 d	Dalteparin 5,000 IU, placebo q.d. 14 d	Fondaparinux 2.5 mg, placebo q.d. 14 d
Eligibility Criteria		
Age ≥40 y Expected hospital stay ≥6 d Recent immobilization (≤3 d) and CHF (NYHA III/IV) or acute respiratory illness or infection or bone/joint or inflamed bowel if ≥1 added risk for VTE (i.e., >75 y; cancer, previous VTE, obesity, varicose veins, hormones, or chronic heart or lung failure)	Age ≥40 y Expected hospital stay ≥4 d Recent immobilization (≤3 d) and CHF (NYHA III/IV) or acute respiratory illness or infection or bone/joint or inflamed bowel if ≥1 added risk for VTE (i.e., >75 y; cancer, previous VTE, obesity, varicose veins, hormones, or chronic heart or lung failure)	Age ≥60 y and expected bed rest ≥4 d and CHF (NYHA class III/IV) or acute or chronic lung disease or acute infectious or inflammatory disease; no other risk factor analysis required
Primary Efficacy		
At d 14	At d 21	At d 14
• Distal and proximal	• Proximal ultrasonographic DVT	• Distal and proximal
• Venographic DVT	• Symptomatic VTE	• Venographic DVT
• Symptomatic VTE	• Fatal PE	• Symptomatic VTE
• Fatal PE	• Sudden death	• Fatal PE
Safety		
• Major bleeding	• Major bleeding	• Major bleeding
• Death at d 90	• Death at d 90	• Death at d 90

VTE, venous thromboembolism; DVT, deep vein thrombosis; PE, pulmonary embolism; CHF, congestive heart failure; NYHA, New York Heart Association.

relevant nonmajor bleeding, was increased with rivaroxaban at 10 days (2.8% vs. 1.2%; HR 2.3; $P \leq 0.0001$) and at 35 days (1.4% vs. 0.5%; HR 3.0; $P \leq 0.0001$).

APPLICATION OF THROMBOPROPHYLAXIS IN MEDICAL PATIENTS: FROM RANDOMIZED STUDIES TO REAL LIFE

One alleged limitation to these studies was that patients enrolled did not represent real-life patients. In fact, requiring risk assessment in the selection of patients in the latest studies, rather than a specific disease, makes the results more applicable to all patients who present with similar levels of risk. Subgroup analyses have confirmed that the proportional risk reduction in favor of prophylaxis applies to almost all subgroups of patients.[268,269] Patients receiving antiplatelet treatment, for example, for coronary disease, benefit from the addition of heparin or LMWH.[270] Similarly, the effect of mechanical prevention, such as compression stockings,[62] seems to be additive to pharmacologic thromboprophylaxis. On the other hand, the benefit to risk ratio of thromboprophylaxis with anticoagulants deserves to be discussed especially for patients at risk for bleeding, such as stroke patients, renally impaired patients as well as the use of long-term prophylaxis. Relevant to this point is a bleeding score that recently was developed by performing regression analysis in the patients of the EXCLAIM study.[271] The score consists of several items related to bleeding, for example, age, renal insufficiency, current malignancy. When applied, approximately 90% of the total population had a bleeding risk score of at least 7.0 points. More than one-half of all major bleeding (54%), and more than one-third (36%) of any bleeding (major or nonmajor but clinically relevant) occurred in the 10% of acutely ill hospitalized medical patients who had a bleeding risk score of 7.0 points (for the group of patients for whom bleeding contributed to death, the mean score was 8.6). Both major bleeding and any bleeding were uncommon in patients with a bleeding risk score of <7.0. This score has however two disadvantages, that is, it has been

Table 86.7

Proximal DVT or symptomatic VTE at d 14 to 21 in MEDENOX, PREVENT, and ARTEMIS

MEDENOX	PREVENT	ARTEMIS
Enoxaparin 2.1%	Dalteparin 2.6%	Fondaparinux 1.5%
Placebo 6.6%	Placebo 5.0%	Placebo 3.4%
$P = 0.037$	$P = 0.002$	$P = 0.085$

		Bleeding Risk Factor	Points
YES	NO	Active Gastroduodenal Ulcer	4.5
YES	NO	Bleeding in Prior 3 Months	4
YES	NO	Admission Platelets < 50 x 10^9 cells/L	4
YES	NO	Hepatic Failure (INR>1.5)	2.5
YES	NO	Intensive Care Unit/Cardiac Care Unit Stay	2.5
YES	NO	Central Venous Catheter	2
YES	NO	Rheumatic Diseases	2
YES	NO	Current Cancer	2
Male	Female	Sex	1
Years (<40, 40–84, ≥85)		Age	1.5/3.5
mL/min/m^2(≥60, 30–59, <30)		Glomerular Filtration Rate	1/2.5

Probability of Bleeding

Major: 0.1%

Clinically Important: 0.5%

FIGURE 86.1 The International Medical Prevention Registry on Venous Thromboembolism (IMPROVE) bleeding risk tool, a web-based tool for calculation of risk score for bleeding in medically ill patients. This is available at www.outcome-umassmed.org/IMPROVE. This model provides an estimate of the risk of major or clinically important bleeding of in-hospital patients from admission up to 14 days after admission. Answering one or more of the questions with "yes," being male, elevated age and lowered glomerular filtration rate increase probability of bleeding. Probability of bleeding is automatically calculated based on the bleeding risk score. Decousus et al. published a plot in which the bleeding rate could approximately be determined using the risk score. (See Decousus H, Tapson VF, Bergmann JF, et al. Factors at admission associated with bleeding risk in medical patients: Findings from the IMPROVE investigators. *Chest* 2011;139(1);69–79.)

derived post hoc and contains a large number of items, making it difficult to memorize. However, all items can be entered into a web based score calculator. (http://www.outcomes-umassmed.org/IMPROVE/bleeding_risk_score.cfm) (FIGURE 86.1). When proven to be consistent in prospective studies, this score could potentially help clinicians to estimate the bleeding risk for an individual patient.[241] Finally, a systematic assessment of both VTE and bleeding risks could improve the optimal use of thromboprophylaxis.

Questions Still to be Appropriately Addressed

Should outpatients at risk of VTE be given thromboprophylaxis? There is still a substantial number of VTE occurring outside the hospital, and risk factors in outpatients are the same as in patients who are hospitalized. The benefit and feasibility of outpatient prophylactic, as well as curative treatment with anticoagulants, has long been established for a variety of indications. Many patients at risk for VTE remain in nursing homes and may well benefit from thromboprophylaxis. Additionally, patients treated at home with similar diseases and risk factors as those evaluated in hospital studies should probably benefit from thromboprophylaxis. However, the risk-to-benefit ratio should be carefully considered in these patients who may be less compliant.

For how long should treatment be given? Typically, in the clinical trials performed so far, UFH and LMWH prophylaxis did not exceed 2 weeks. The preliminary results provided by the EXCLAIM study[268] should be confirmed with other anticoagulants in order to determine whether extended prophylaxis confers systematically additional benefit.

CONCLUSIONS

VTE remains a public health burden. The results of recent, well-conducted clinical trials reinforce the evidence-based recommendations for more systematic assessment of global risk of VTE in medical patients and for the wider use of thromboprophylaxis. Patients should be carefully selected for high risk of both VTE as well as bleeding.

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Full Reference Lists can be found online

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CHAPTER

87 Treatment of Venous Thromboembolic Disease

SAM SCHULMAN, JEFFREY GINSBERG, AND JAMES DOUKETIS

The management of venous thromboembolic disease has in some respects taken recent giant leaps forward but has in other domains remained stagnant. Whereas the anticoagulant treatment during the past two decades has become simplified and more patient-friendly, the prevention and treatment of long-term sequelae has not improved significantly. Knowledge regarding the molecular biology of thrombosis risk factors and the pharmacogenomics of vitamin K antagonists (VKAs) has undergone a remarkable expansion. However, to date, this has had very limited effect on the practical management of patients with venous thromboembolism (VTE). This chapter focuses on the treatment of deep vein thrombosis (DVT) in the lower extremity and of pulmonary embolism (PE), which are by far the most common locations, often simultaneous, of VTE. Thrombophlebitis will also be discussed herein, whereas Chapter 84 has been devoted to all other types of venous thrombosis.

INITIAL THERAPY

First Anticoagulant Dose in Relation to Diagnosis

As soon as a DVT or PE is suspected, it is crucial to promptly assess the patient to optimize management, which usually includes initiating anticoagulant therapy in those with established DVT or PE and as an interim measure until definitive diagnostic testing is performed. Treatment should start before the diagnosis of DVT or PE has been confirmed in two common scenarios. The first is the patient arriving at the emergency room with symptoms suggestive of DVT and in whom lower limb venous ultrasound is not available and has to be deferred until the next morning. If a standardized clinical prediction rule (see Chapter 83) shows that DVT is likely and the patient has a low risk of bleeding, a dose of low molecular weight heparin (LMWH) should be given while awaiting definitive diagnostic testing.[1] If the bleeding risk is high, it is acceptable to defer therapy until the diagnosis of DVT has been verified; in such patients, best efforts should be made to establish a definitive diagnosis on the day of presentation.

The second scenario occurs in the patient with likely PE according to a clinical prediction rule, in whom it is unacceptable to defer anticoagulant therapy until the diagnosis of PE is confirmed.[1]

Patients with Low Benefit of Therapy

In two randomized controlled trials (RCTs), in which 254 patients with DVT were treated with heparin or placebo and had 3 to 24 weeks of follow-up observation, the risk for disease recurrence was 7% and 21% in the treated and untreated groups, respectively, calculated to a convincing number-needed-to-treat of only seven patients to prevent one recurrent thrombosis.[2,3] However, the need to treat isolated calf DVT has been questioned, based on observational data showing low risk of progression or recurrence without anticoagulation.[4] A comparison of nadroparin versus placebo (CACTUS trial) in patients with isolated calf DVT is ongoing and may resolve this uncertainty (ClinicalTrials.gov NCT00421538). In the meantime, for patients with isolated calf DVT and minimal or no symptoms, it may be reasonable to withhold anticoagulation, perform repeat venous ultrasound testing for 2 weeks, and initiate anticoagulation only if there is thrombus progression into proximal veins. An increasingly prevalent patient group is one with asymptomatic, incidentally identified PE, typically detected by contrast-enhanced computed tomography pulmonary angiography (CTPA) while assessing another disease. On one hand, anticoagulant-treated patients with cancer and incidental PE have similar high risks of recurrent VTE, bleeding, and death as patients with symptomatic PE.[5] On the other hand, the introduction of multiple-detector CTPA has doubled the rate of diagnosis of subsegmental PE compared with single-detector CTPA (9.4% vs. 4.7%) without any change in the 3-month risk of symptomatic VTE.[6] It therefore seems reasonable to withhold anticoagulant treatment of incidental subsegmental PE, particularly if the patient has no active cancer.

Treatment Options for the Initial Phase

The initial therapeutic options are schematically depicted in FIGURE 87.1. The first decision for patients with extensive VTE should be whether thrombolytic therapy (or in rare cases surgical removal) is indicated.

Massive PE, defined as symptomatic PE associated with hemodynamic instability or respiratory failure, is immediately life threatening, with a 90-day overall mortality rate of 53%.[7] Thus, in case of hemodynamic instability (systolic blood pressure <90 mm Hg, tachycardia, elevated jugular venous pressure, clinical evidence of poor tissue perfusion, hypoxemia), there is a need for rapid resolution of the pulmonary artery obstruction. Anticoagulants have too slow an effect and, unless absolutely contraindicated due to active bleeding or recent hemorrhage in the central nervous system, systemic thrombolytic therapy should be administered as soon as the diagnosis has been verified. Thrombolysis is also indicated if the patient deteriorates while receiving anticoagulant therapy. Further details on thrombolytic therapy can be found in Chapter 112.

If thrombolytic therapy fails to improve hemodynamic instability, emergency surgical embolectomy is indicated but requires surgical expertise.

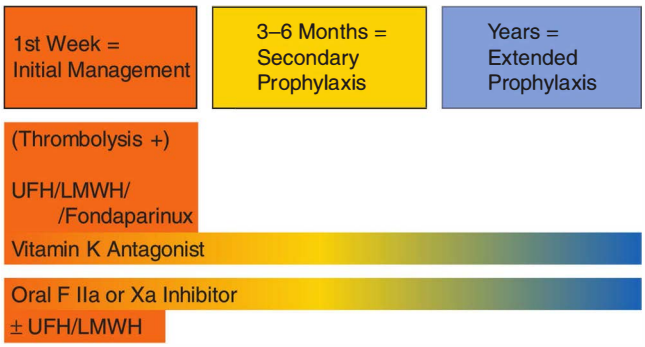

FIGURE 87.1 The pharmaceutical options and phases of treatment of VTE.

For patients with submassive PE, defined as PE without hemodynamic instability or respiratory insufficiency, there is, to date, no compelling evidence to support the use of thrombolytic therapy. In a systematic review, when thrombolytic therapy was compared with heparin for patients with submassive PE, there was no significant difference in the risk for death (odds ratio [OR], 0.89; 95% confidence interval [CI], 0.45 to 1.78), recurrent VTE (OR, 0.63; 95% CI, 0.33 to 1.20), or major hemorrhage (OR, 1.61; 95% CI, 0.91 to 2.86).[8] An RCT (PEITHO) comparing thrombolysis with anticoagulant therapy in patients with PE and acute right ventricular dysfunction or enlargement based on echocardiography or CTPA is ongoing (ClinicalTrials.gov NCT00639743).

Massive DVT, defined as DVT that is associated with limb ischemia or involving the iliofemoral veins, also warrants consideration of thrombolytic therapy. The evidence to support such treatments, however, is weak so far. Systemic thrombolytic therapy has been compared to anticoagulant therapy in several small RCTs, which consistently show more complete and rapid thrombus dissolution, but higher bleeding rates. Further, the studies do not show convincing evidence that the incidence of postthrombotic syndrome (PTS), the important long-term sequel to DVT, is reduced. Two RCTs have assessed catheter-directed thrombolysis for iliofemoral DVT, with the objective of reducing the risk of PTS.[9,10] This treatment is usually performed in a specialized clinical setting such as an intensive care unit and involves catheterization of the popliteal or femoral vein, infusion of a thrombolytic agent over a 1- to 2-day period, and repeat venography to monitor the treatment effect. Such treatment also confers an increased risk of bleeding related to the thrombolytic agent and the invasive procedure. More rapid resolution of massive DVT can be obtained with a combined pharmacomechanic and thrombolytic approach, which also should reduce the dose of the thrombolytic agent, thereby potentially reducing the risk of bleeding. This approach is being evaluated in the ATTRACT trial, which includes patients with DVT that involves the femoral or more proximal veins (ClinicalTrials.gov NCT00790335).

Patients with DVT associated with severe symptoms (*phlegmasia cerulea dolens* or *phlegmasia alba dolens*, see Chapter 83) are at high risk for developing the PTS and, occasionally, limb loss and should be considered for catheter-directed or systemic thrombolytic therapy. Ideally, patients should receive this treatment within 14 days of the onset of symptoms.

The majority of patients, without these potentially life- or limb-threatening conditions, should receive one of the treatment options summarized in Table 87.1.

Table 87.1

Initial treatment of patients with VTE who do not have an indication for thrombolysis or surgery[a]

Treatment	DVT	Nonmassive PE[b]
UFH		
Intravenous[c]	According to aPTT[d]	According to aPTT[d]
Subcutaneous	According to aPTT[d]	According to aPTT[d]
Subcutaneous	Weight-adjusted dose[e]	Weight-adjusted dose[e]
LMWH (All Subcutaneous)		
Dalteparin	200 IU/kg once daily	As DVT or 120 IU/kg b.i.d.
Enoxaparin	1.5 mg/kg once daily	As DVT or 1 mg/kg b.i.d.
Nadroparin	171 IU/kg once daily	85 IU/kg b.i.d.
Tinzaparin	175 IU/kg once daily	As DVT
Fondaparinux (Subcutaneous)		
	7.5 mg once daily[f]	7.5 mg once daily[f]
Direct Xa Inhibitor (Oral)		
Rivaroxaban	15 mg twice-daily (first 3 wk)	15 mg twice-daily (first 3 wk)

[a]The drugs or doses may not be approved in all countries or jurisdictions.
[b]Patients who are hemodynamically stable.
[c]Continuous infusion.
[d]aPTT range corresponding to anti-Xa levels 0.3 to 0.7 IU/mL. Starting dose is with intravenous bolus of 80 U/kg, followed by 18 U/kg/h intravenously or with 17,500 IU/kg b.i.d. subcutaneously.
[e]Given twice daily, first dose 500 IU/kg, then 333 IU/kg, without aPTT monitoring.
[f]5 mg for patients with body weight <50 kg; 10 mg for patients with body weight >100 kg.

Initial Treatment with Unfractionated Heparin

Intravenous unfractionated heparin (UFH), which was the initial treatment standard until 10 to 15 years ago, is currently mainly used for special patient groups summarized in Table 87.2. It is usually recommended to initiate UFH treatment with a bolus dose of 80 IU/kg followed by an infusion of 18 U/kg/h.[1] This UFH regimen is based on three RCTs totaling 292 patients,

Table 87.2

Patients suitable for initial intravenous UFH therapy rather than subcutaneous LMWH

Population	Reason for Intravenous Heparin
High risk of bleeding	Quickly eliminated on discontinuation
	Effective antidote available
Candidates for thrombolysis	Quick elimination before next step or surgery
Very ill patients	Poor subcutaneous absorption

which compared this or a similar regimen with fixed initial dosing.[11–13] The dose is then adjusted according to the results of an activated partial thromboplastin time (aPTT) performed 4 to 6 hours after initiation of UFH to keep the aPTT in a therapeutic range. aPTT reagents vary in their sensitivities to UFH so that an aPTT range that corresponds to an anti-factor Xa heparin level of 0.3 to 0.7 IU/mL should be targeted.[1] Subcutaneous injection of UFH versus intravenous therapy, both adjusted according to the aPTT, was compared in a meta-analysis of six trials totaling 783 patients[14] and demonstrated favorable results with subcutaneous UFH for recurrent VTE (relative risk [RR], 0.62; 95% CI, 0.39 to 98) and major bleeding (RR, 0.79; 95% CI, 0.42 to 1.48). A subcutaneous, weight-adjusted, unmonitored UFH regimen (250 IU/kg twice-daily dosing after a 333 U/kg initial dose) was compared with LMWH therapy in an RCT (FIDO) of almost 700 patients with DVT or PE.[15] There was no statistically significant difference in the incidence of recurrent VTE (3.8% vs. 3.4%) or major hemorrhage during the first 10 days (1.1% vs. 1.4%) with fixed-dose, unmonitored UFH and LMWH. This is the only UFH regimen that allows for convenient outpatient therapy, which actually was achieved entirely for 72% of the patients in this treatment group in the study.

Initial Treatment with LMWH

Over the past 10 to 15 years, the initial treatment of DVT with LMWH has become standard therapy due to its convenience of once-daily subcutaneous dosing, and no requirement for laboratory monitoring. For patients with PE, a twice-daily LMWH regimen is usually recommended (Table 87.1). Several meta-analyses have compared the efficacy and safety of LMWH with UFH. The results of the systematic review by van Dongen[16] are summarized in Table 87.3, showing that in addition to the convenience of administration and the compelling data on the safety without laboratory monitoring, LMWH also has a lower potential for heparin-induced thrombocytopenia (HIT) than UFH. As for individual LMWH regimens, a meta-analysis of five trials totaling 1,508 patients compared once- versus twice-daily LMWH regimens and showed no significant difference in recurrent VTE (OR, 0.82; 95% CI, 0.49 to 1.39), major hemorrhage (OR, 0.77; 95% CI, 0.40 to 1.45), or death (OR, 1.14; 95% CI, 0.62 to 2.08).[17]

Initial Treatment with New Anticoagulants

Fondaparinux is an indirect inhibitor of factor Xa that is produced synthetically, with a lower risk for allergic reactions and a negligible risk for HIT. However, its elimination half-life is longer than that for LMWH (17 hours vs. 4 to 5 hours), which may be a drawback in cases of bleeding or perioperative management. The treatment dose of fondaparinux is 7.5 mg once daily (5 mg for body weight <50 kg; 10 mg for weight >100 kg). This dose regimen was compared in the MATISSE trials against LMWH (enoxaparin 1 mg/kg twice daily) in approximately 2,200 patients with DVT and against intravenous UFH in 2,200 patients with PE.[18,19] The efficacy (recurrent VTE) and safety (major bleeding) were similar for fondaparinux and LMWH (or UFH) in these trials. Fondaparinux is currently the preferred initial anticoagulant in patients with recent HIT, occurring within the preceding 100 days. Rivaroxaban, a direct factor Xa inhibitor, is so far the only available oral anticoagulant shown to be effective for the initial treatment of VTE, although at the time of writing it has not been approved for this indication. In the phase III trial (EINSTEIN DVT), rivaroxaban was given at a dose of 15 mg twice daily for 3 weeks, followed

Table 87.3

Outcomes in meta-analysis of trials comparing fixed-dose LMWH with adjusted-dose UFH for the treatment of VTE

Outcome	Studies	Odds Ratio	95% Confidence Interval
VTE Overall			
Recurrence			
During initial treatment period	15	0.68	0.48–0.97
At 3 mo	13	0.68	0.53–0.88
At 6 mo	6	0.68	0.48–0.96
At end of follow-up	18	0.68	0.55–0.84
Major hemorrhage	19	0.57	0.39–0.83
Death at end of follow-up	18	0.76	0.62–0.92
Proximal DVT Alone			
Recurrence at end of follow-up	9	0.57	0.44–0.75
Major hemorrhage	8	0.50	0.29–0.85
Death	8	0.62	0.46–0.84
PE Alone			
Recurrence at end of follow-up	4	0.88	0.48–1.63

van Dongen CJJ, van der Belt AGM, Prins MH, et al. Fixed dose subcutaneous low molecular weight heparins versus adjusted dose unfractionated heparin for venous thromboembolism. *Cochrane Database Syst Rev* 2004;(4):CD001100.

by a dose of 20 mg once daily, whereas standard therapy with enoxaparin was given for 5 to 10 days followed by a VKA.[20] Rivaroxaban was noninferior to standard therapy for recurrent VTE during the entire study period (how long was the study period?) (2.1% vs. 3.0%; hazard ratio [HR], 0.68; 95% CI, 0.44 to 1.04); moreover, there was no excess in recurrent VTE events during the initial treatment period. Clinically relevant bleeding was seen in 8.1% of patients in each group.

A similar treatment strategy is used in the ongoing phase III trial with the oral factor Xa inhibitor apixaban in the treatment of VTE (AMPLIFY; ClinicalTrials.gov NCT00643201). Apixaban is given at a dose of 10 mg twice daily for the first week, followed by 5 mg twice daily and compared with standard therapy of an LMWH and a VKA.

Studies with the oral factor Xa inhibitor, edoxaban tosylate (NCT00986154), and the oral direct thrombin inhibitor, dabigatran etexilate (RE-COVER trial),[21] assessed treatment only after an initial 5 to 10 days of treatment with an LMWH (or UFH).

Outpatient Treatment of VTE

In the large majority (>80%) of patients with DVT, anticoagulant therapy can be done out-of-hospital by subcutaneous LMWH, which can be self-administered by the patient or with assistance from a family member or a health professional. Most trials supporting outpatient management are designed in a way that makes interpretation of the results difficult since the comparator group was in-hospital treatment with UFH.[1] One study directly compared outpatient versus inpatient treatment with LMWH in 201 patients with VTE and found no difference in clinical outcomes but a 56% cost reduction with outpatient therapy.[22] For patients with PE, a clinical prediction rule for risk stratification such as the Pulmonary Embolism Severity Index (PESI)[23] may be used to assess the safety of outpatient management (see Chapter 83). Several retrospective cohort studies have assessed out-of-hospital management of patients with hemodynamically stable PE who did not require oxygen or narcotic analgesics and were at low risk for bleeding.[24,25] After 3 months of follow-up, there were no cases of fatal PE. A recently published RCT compared inpatient and outpatient treatment for 344 patients with PE at low risk of death, defined as PESI class I or II.[26] The mean stay in hospital was 3.9 and 0.5 days, respectively. During the 90-day follow-up, there was one death but no recurrent VTE or major bleeding among those treated as inpatients compared to one death, one recurrent VTE, and two major bleeding events (within 14 days) among those randomized to outpatient therapy, fulfilling the preset noninferiority criteria. Taken together, these studies suggest that most patients with DVT and at least 40% of patients with PE[23]—after careful risk stratification and documentation—can be treated entirely as outpatients.

Inferior Vena Cava Filter for the Treatment of VTE

An RCT (PREPIC) involving patients with proximal DVT assessed whether, in addition to anticoagulant therapy, placement of an inferior vena cava (IVC) filter, compared with anticoagulation alone, would improve clinical outcomes.[27] After 2 years of follow-up, patients with an IVC filter had a reduction of asymptomatic or symptomatic PE at 12 days (OR, 0.22; 95% CI, 0.05 to 0.90) but were at increased risk for recurrent symptomatic DVT at 2 years (OR, 1.87; 95% CI, 1.10 to 3.20) compared to patients without a filter. Mortality rates were similar in both groups. These results were corroborated in an 8-year follow-up of the same population.[28] Insertion of an IVC filter should considered only if (a) there is an absolute contraindication to anticoagulation therapy, or (b) a treatment failure occurs despite adequate anticoagulation manifest by a new symptomatic DVT or PE (see also Chapter 114).

Initial Supportive Therapy for VTE

Patients with acute DVT who are treated in hospital are sometimes kept in bed due to presumed fear of thrombus detachment and embolization with ambulation. However, a systematic review of five RCTs (3,048 patients) that compared bed rest versus early ambulation after DVT or PE showed that early mobilization did not increase the risk for new PE (RR, 1.03; 95% CI, 0.65 to 1.63) or death (RR, 0.79; 95% CI, 0.40 to 1.56).[29]

Leg edema associated with DVT may be treated with leg elevation and graduated compression stockings. It may be preferable to defer the use of compression stockings until the edema and associated leg pain and inflammation have subsided, which may take 1 to 3 weeks. In one small randomized trial, early start with a compression stocking was associated with decreased thrombus resolution when assessed by venous ultrasound,[30] although opposite findings were reported in another randomized trial.[31]

Treatment of DVT with NSAIDs alone versus UFH has been studied in two small RCTs, and a Cochrane review reported that the poor methodological quality of these studies precluded conclusions about treatment efficacy.[32]

SECONDARY PROPHYLAXIS

It is necessary to continue anticoagulant therapy for at least 3 months in the majority of patients with VTE. In an RCT involving 51 patients with calf DVT, all of whom received compression stockings, treatment with UFH alone was compared with 3 months of treatment consisting of UFH and a VKA.[33] After 3 months of follow-up, there were eight recurrent VTE events in the UFH-only group and no recurrences in the UFH plus VKA group. In another RCT of 68 patients with proximal DVT, treatment with 4 weeks of a VKA was compared with fixed, low-dose UFH (5,000 IU twice daily for 14 days) and recurrent VTE occurred in 0 and 9 patients treated with a VKA and heparin alone, respectively.[34] When coupled with indirect evidence from large RCTs comparing short (4 to 6 weeks) with longer duration VKA therapy,[35,36] there is compelling evidence that a short, 10- to 14-day, course of anticoagulant therapy is not sufficient to protect patients with acute VTE from subsequent recurrent disease.

Vitamin K Antagonists—Initiation

The current standard of practice to manage patients with DVT or PE is anticoagulation with a VKA for 3 to 6 months, which should be followed by an assessment to decide if longer-duration treatment is warranted. Due to the delay until therapeutic international normalized ratio (INR) levels are reached after VKA initiation, such treatment should be started simultaneously with UFH or LMWH therapy. Four RCTs (807 patients)

compared immediate versus delayed start of VKA after VTE and showed no difference in clinical outcomes, but the length of hospitalization was shortened by 4 days with immediate VKA treatment.[37–40]

Warfarin is usually started at a dose of 5 mg daily, with dose adjustments at 2 to 3 days and as needed to attain an INR target of 2.5 (INR range: 2.0 to 3.0) (see Chapter 106). There is no difference in clinical outcomes using a higher ("loading") dose of warfarin, compared with no loading dose.[41–45] Two cohort studies (1,054 outpatients) used a warfarin nomogram with 10 mg daily for the first 2 days and demonstrated a 3-month recurrence rate of VTE of 1.9% and major bleeding of 1.0%,[46,47] results that are clinically acceptable. Irrespective of the warfarin initiation regimen, treatment with LMWH or UFH should continue for at least 5 days, with a purpose of achieving a therapeutic INR for 2 consecutive days.

Vitamin K Antagonists—Maintenance

The intensity of anticoagulation with VKA for patients with VTE is the same as for most other clinical indications, with an INR target of 2.5 (range: 2.0 to 3.0).[48] It was thought that patients with antiphospholipid syndrome-related thrombosis should have a higher intensity INR, but studies failed to show therapeutic benefit with an INR of 3.0 to 4.0 or 3.0 to 4.5 compared with an INR range of 2.0 to 3.0.[49,50]

LMWH for Secondary DVT Prophylaxis

In a meta-analysis of seven RCTs (1,379 patients) of long-term treatment with LMWH compared with VKA therapy,[51] with variable once-daily LMWH dosages,[50–56] there was no significant difference for recurrent VTE (OR, 0.66; 95% CI, 0.41 to 1.07), major hemorrhage (OR, 0.45; 95% CI, 0.18 to 1.11), or death (OR, 1.11; 95% CI, 0.78 to 1.83).[51] Excluding the study with the highest dose of LMWH[52] did not alter the results.

In an RCT (CLOT trial) that compared LMWH with VKA treatment of VTE (672 patients with cancer),[53] LMWH therapy (dalteparin 200 IU/kg/d for 1 month, then 150 IU/kg/d for 5 months) conferred a significant reduction in recurrent VTE (HR, 0.48; 95% CI, 0.30 to 0.77) with no significant difference in major bleeding or death. Another study of 146 patients with cancer and VTE ("CANTHANOX"), which compared LMWH (enoxaparin 1.5 mg/kg/d) with VKA, did not detect any difference in recurrence, but there was a trend to fewer major hemorrhages and deaths with LMWH therapy.[54] Thus, there seems to be a clinical advantage of continuing with LMWH rather than transitioning to a VKA in patients with VTE associated with cancer. LMWH is effective despite chemotherapy-related emesis, and interruption of LMWH for invasive procedures is easier than with VKA therapy. Accordingly, LMWH is considered the drug of choice for patients with cancer-associated symptomatic VTE.[1]

Secondary Prophylaxis with the New Oral Anticoagulants

A phase III trial compared the oral factor Xa inhibitor rivaroxaban (15 mg b.i.d. for 3 weeks followed by 20 mg/d) without initial treatment with LMWH or UFH, with long-term VKA treatment for secondary VTE prophylaxis.[20] Clinical outcome rates were similar to those with VKA therapy, without the need for laboratory monitoring or dose adjustments.

Dabigatran etexilate, a prodrug that is converted after absorption to the active metabolite dabigatran, was compared with warfarin as secondary prophylaxis for 6 months in 2,539 patients with DVT or PE (RE-COVER).[21] Dabigatran (150 mg b.i.d.) was noninferior to warfarin for recurrent VTE or related death (HR, 1.10; 95% CI, 0.65 to 1.84). The risk for major bleeding was similar (HR, 0.82; 95% CI, 0.45 to 1.48), but there was a significant reduction of clinically relevant (major and nonmajor) bleeding (HR, 0.63; 95% CI, 0.47 to 0.84). There was no difference in overall deaths, elevation of liver enzymes, or occurrence of acute coronary syndrome. As with rivaroxaban, dabigatran does not require laboratory monitoring or dose adjustments. These drugs, and other drugs currently under investigation (see Chapter 110), will become valuable alternatives to VKA for this indication.

Shorter Duration of Secondary Prophylaxis

Anticoagulant therapy should continue for at least 3 months to minimize the risk of recurrence. However, patients with distal DVT occurring in association with a reversible risk factor, such as the postoperative state, have a low risk of recurrence, 9% over 6 years.[55] In a Swedish study comparing 6 weeks versus 6 months of VKA after VTE (DURAC I),[36] 6 weeks of treatment was sufficient to prevent VTE recurrence. In a French study assessing different durations of VKA therapy (DOTAVK), there was no difference in VTE recurrence between 6 and 12 weeks of treatment of patients with distal DVT.[56] Thus, select patients with distal, provoked DVT may be treated for only 6 weeks, in which cases it may be easier to simply treat with LMWH rather than a VKA.

Additional Management Options during the Period of Secondary DVT Prophylaxis

Graduated compression stockings (worn for 2 years after DVT) may decrease the overall incidence of PTS by 70% (OR, 0.30; 95% CI, 0.20 to 0.48), with a similar reduction of severe PTS manifestations (OR, 0.39; 95% CI, 0.20 to 0.76).[57] The studies had design flaws, mainly that the investigators and patients were not blinded, but a recently completed double-blind RCT (SOX) that assessed stockings against sham stockings will provide valuable information.[58] At 2 to 4 weeks after the diagnosis of DVT, persistent leg swelling and pain predicts an increased risk for PTS,[59] in which case compression stockings should be used. Additional risk factors for PTS are recurrent DVT, extensive DVT, obesity, and advanced age (see Chapter 85).

Investigation for occult malignancy in patients with unprovoked (or idiopathic) VTE is an often-debated issue. Previously undiagnosed cancer has been found in 6.1% of patients with newly diagnosed VTE (95% CI, 5.0% to 7.1%) and in 10.0% (95% CI, 8.6% to 11.3%) in the next 12 months.[64] Limited screening increased the proportion of patients found to have previously undiagnosed cancer to 49.4% (95% CI, 40.2 to 58.5) and CT of the abdomen and pelvis increased the proportion further to 69.7% (95% CI, 69.1 to 77.8). However, extensive screening is not associated with a reduction of cancer-related mortality.[60] Patients with bilateral DVT, concomitant superficial thrombophlebitis, or VTE progression despite adequate

therapy have a higher risk of malignancy. The SOMIT trial, in which 201 patients with unprovoked VTE were randomized to extensive screening for malignancy or no screening, and CT scanning of the chest, abdomen and pelvis provided the highest yield for occult cancer.[61]

Investigation for thrombophilia in patients with VTE is also widely debated. The yield of the investigation is low in the elderly and for provoked VTE, and in most patients, the results do not alter management. The presence of heterozygous factor V Leiden or prothrombin gene mutation (G20210A) is common but not associated with a clinically important increased risk for disease recurrence (see Chapter 81, Table 81.3). An algorithm for thrombophilia work-up suggests classifying available tests into three levels of priority.[62] We have removed homocysteine from the high-priority tests, based on the HOPE-2 trial. In this study of 5,522 patients with cardiovascular disease or risk factors, folic acid, vitamin B_6, and vitamin B_{12} reduced homocysteine levels by 2.2 μmol/L, in comparison with placebo, but had no effect on VTE (HR, 1.01; 95% CI, 0.66 to 1.53).[63] An algorithm for thrombophilia workup that takes into account the interpretation of protein C and protein S while a patient is receiving VKA therapy is shown in FIGURE 87.2 and Table 87.4.

Table 87.4

Priority of tests for thrombophilia

High-Priority Tests	Low-Priority Tests
Resistance to activated protein C	Fibrinogen level
Factor V Leiden mutation	Thrombin clotting time and further
Prothrombin G20210A	Analyses for dysfibrinogenemia
Factor VIII	Factor IX
Lupus anticoagulant	Factor XI
Cardiolipin/β_2-glycoprotein I antibodies	Homocysteine
Antithrombin activity	JAK2 mutation[a]
Protein C activity	
Protein S antigen, free fraction	

[a]JAK2 mutation is mainly of interest in patients with unexplained visceral vein thrombosis or in patients with features of myeloproliferative disease and unexplained thrombosis.
Modified from Seligsohn U, Lubetsky A. Genetic susceptibility to venous thrombosis. *N Engl J Med* 2001;344:1222–1231.

EXTENDED VTE PROPHYLAXIS

Duration of Anticoagulation After VTE

The risk of VTE recurrence after stopping anticoagulation is lower after distal than proximal DVT, but similar after PE and proximal DVT (Table 87.5).[64] Stopping anticoagulation after 3 months of treatment confers a similar risk of recurrence as discontinuation after 6 months or more (HR, 1.19; 95% CI, 0.86 to 1.65). These trials confirm that continuing anticoagulation in patients with unprovoked VTE prevents recurrent VTE, but only for as long as the patient takes anticoagulants. Consequently, a key management aim is to identify patients at low risk for recurrent VTE in whom long-term (or lifelong)

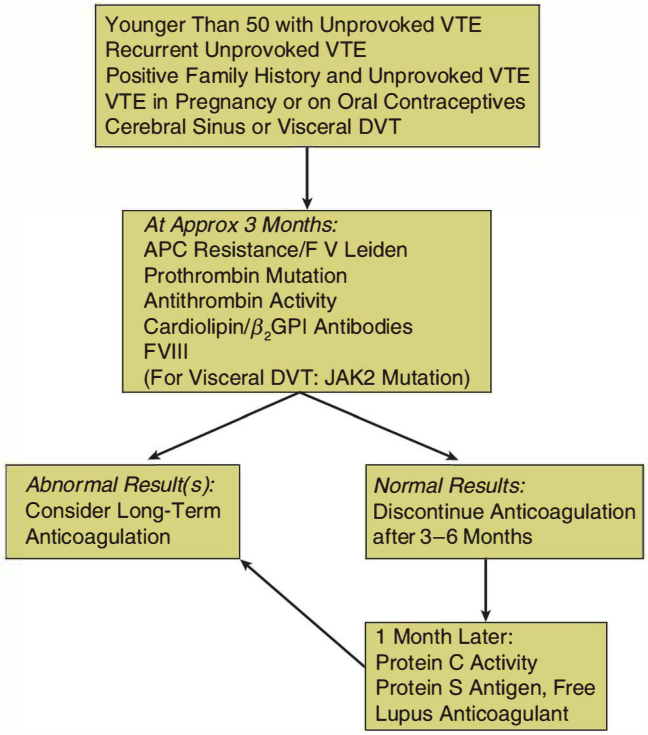

FIGURE 87.2 Algorithm for thrombophilia workup.

Table 87.5

Risk of recurrent VTE after discontinuation of anticoagulation

Variable	Hazard Ratio	95% Confidence Interval
Location of VTE—proximal DVT	1.0	(reference)
Distal DVT	0.49	0.34–0.71
PE	1.19	0.87–1.63
Unprovoked VTE	1.0	(reference)
Provoked VTE	0.55	0.41–0.74
Duration of anticoagulation—3 months	1.0	(reference)
1–1.5 mo	1.28	0.82–2.01
6 mo	0.80	0.51–1.25
12 or 27 mo	0.88	0.57–1.36

Multivariable Cox regression analysis of individual patient data (n = 2,925) from seven trials.
DVT, deep-vein thrombosis; VTE, venous thromboembolism.
Boutitie F, Pinede L, Schulman S, et al. Influence of preceding length of anticoagulant treatment and initial presentation of venous thromboembolism on risk of recurrence after stopping treatment: analysis of individual participants' data from seven trials. *BMJ* 2011;342:d3036.

anticoagulation can be avoided and patients at high risk for recurrence in whom long-term anticoagulation is justified. Identifying patients at low risk for VTE recurrence should consider patient gender, D-dimer levels, select thrombophilic abnormalities, and residual deep vein occlusion (Table 87.6), as well as patient preferences.

The PROLONG trial assessed the clinical utility of using D-dimer levels, when measured about 1 month after stopping anticoagulation to determine risk for recurrence and whether continued anticoagulation for more than 3 months is warranted.[65] Patients with abnormal D-dimer results who were randomized to remain off anticoagulation had a VTE recurrence rate of 15.0% versus 2.9% in patients who resumed anticoagulation (HR, 4.26; 95% CI, 1.23 to 14.6). Patients with a negative D-dimer who did not resume anticoagulation had a VTE recurrence rate of 6.2%. A pooled analysis of 1,818 patients with unprovoked VTE followed for 27 months after anticoagulation was stopped showed an annual recurrence rate of 3.7% (95% CI, 3.4 to 4.3) in patients with a normal D-dimer level and a recurrence rate of 8.8% (95% CI, 6.2 to 11.3) in patients with an elevated D-dimer result.[66] The timing of postanticoagulation D-dimer measurement (<3 weeks, >5 weeks, or 3 to 5 weeks), as well as the D-dimer assay technique used, did not affect the ability of the assay to distinguish patients at low and high risk for VTE recurrence. A 646-patient cohort study that measured D-dimer before stopping anticoagulation found an increased risk for recurrence in those patients with an elevated D-dimer level, although a normal D-dimer value may not predict a low recurrence risk because of the effect of concomitant VKA therapy.[67]

Table 87.6

Facts to consider for the decision on anticoagulation for 3–6 months versus extended prophylaxis

	Limited Duration	Extended Prophylaxis
Risk of recurrent VTE	Distal DVT	Proximal DVT or PE
	Provoked VTE	Unprovoked VTE
	First VTE	Recurrent VTE
	No serious thrombophilia[a]	Serious thrombophilia[b]
Risk of bleeding	History of bleeding	No history of bleeding
	Unstable INRs	Stable INRs
	Need for antiplatelet therapy	No/few other drugs
Patient preferences	Monitoring is inconvenient	Fear of recurrent VTE

[a]Heterozygous form of factor V Leiden or prothrombin mutation increases the risk mildly and does not justify on their own indefinite duration.
[b]Serious thrombophilia consists of homozygosity for factor V Leiden or prothrombin gene mutation; double heterozygosity (factor V Leiden and prothrombin gene mutation); hereditary antithrombin, protein C, or protein S deficiency; persistently positive (on two separate occasions at least 12 weeks apart) lupus anticoagulant or cardiolipin antibodies.

Additional predictors of increased VTE recurrence include male sex (HR, 1.8; 95% CI, 1.2 to 2.5)[68] and residual venous occlusion, although the predictive value of the latter may be less in patients with unprovoked VTE.[69,70] A validated clinical prediction rule is needed to better discriminate patients at low and high risk for recurrence and progress that has been made.[67,71]

Extended VTE Prophylaxis with a VKA

Long-term treatment with warfarin is very effective for the prevention of recurrent VTE, as shown in the DURAC II trial, in which 227 patients with a second VTE event were randomized to 6 months or indefinite duration.[72] Analysis after 4 years showed a risk of recurrence of 20.7% in the 6-month group versus 2.6% in the long-term group (RR, 8.0; 95% CI, 2.5 to 25.9) at the expense of a trend to more (how many more?) major hemorrhages with extended duration.

Low-Intensity VKA Therapy

A reduced intensity VKA regimen (INR goal of 1.5 to 2.0) for extended (6.5 months) prophylaxis, with the intent to reduce the risk of bleeding and the frequency of monitoring, has been investigated in two RCTs (PREVENT trial, 508 patients).[73,74] Compared to placebo, the low-intensity regimen reduced the risk of recurrent VTE (HR, 0.36; 95% CI, 0.19 to 0.67), without a significant increase in major hemorrhage.[74] However, compared to standard-intensity VKA therapy (INR 2 to 3) during a 12-month follow-up evaluation (ELATE trial), the low-intensity regimen was associated with an increased risk of recurrent VTE (HR, 2.8; 95% CI 1.1 to 7.0), without a reduction in major hemorrhage (HR, 1.2; 95% CI, 0.4 to 3.0).[73] Thus, the preferred long-term anticoagulant regimen utilizes an INR goal of 2 to 3. Reduced frequency of monitoring (every 8 weeks in the PREVENT trial) is probably feasible in such patients whose INRs are stable on standard VKA therapy.[75]

Extended Prophylaxis with New Anticoagulants

Rivaroxaban was evaluated for extended prophylaxis after 6 or 12 months of prior anticoagulation in 1,196 patients with VTE (EINSTEIN Extension).[20] Randomization was to rivaroxaban 20 mg once daily or placebo for another 6 or 12 months, and recurrent VTE was significantly less frequent in rivaroxaban-treated patients (1.3% vs. 7.1%, HR, 0.18; 95% CI, 0.09 to 0.39). Clinically relevant bleeding occurred in 5.4% and 1.2%, respectively (OR, 4.14; 95% CI, 1.89 to 9.07), but without any significant difference in the incidence of major hemorrhages.

Dabigatran (150 mg b.i.d.) has also been evaluated for extended prophylaxis after VTE in two trials.[76,77] Compared to warfarin as active control (RE-MEDY), 2,856 patients with 3 to 12 months prior anticoagulation were treated for another 6 to 36 months, and recurrent VTE occurred equally often (1.8% with dabigatran vs. 1.3% with warfarin, HR, 1.44; 95% CI, 0.78 to 2.64), thus fulfilling noninferiority criteria. There was a strong trend for a decrease in major bleeding with dabigatran, 0.9% versus 1.8% (HR, 0.52; 95% CI, 0.27 to 1.01), and a significant reduction of any bleeding (HR, 0.71; 95% CI, 0.61 to 0.83). Acute

coronary events were more common with dabigatran than with warfarin (0.9% vs. 0.2%), whereas there were no differences in deaths or other adverse events.[77]

In the second trial, dabigatran was compared with placebo (RE-SONATE) in 1,343 patients with 6 to 18 months of prior anticoagulation who were treated for another 6 months.[76] The incidence of recurrent VTE was reduced with dabigatran (HR, 0.08; 95% CI, 0.02 to 0.25) with only two major bleeds versus none on placebo; clinically relevant bleeding occurred more often on dabigatran (HR, 2.9; 95% CI, 1.5 to 5.6), and there was one acute coronary event in each group.

In conclusion, the new oral anticoagulants, which are given at a fixed dose without monitoring, are safe and effective as extended-duration anticoagulant therapy.

SUPERFICIAL THROMBOPHLEBITIS

Migratory and/or recurrent superficial thrombophlebitis (as part of Trousseau syndrome) may occur in the presence of a malignancy and often heralds the development of major DVT and/or PE. More often, thrombophlebitis is a benign manifestation of sluggish blood flow in varicose veins, resulting in an inflammatory reaction and secondary thrombus formation that may obliterate and eliminate the varicosity, only for new ones to subsequently develop. Whereas thrombophlebitis of the calf usually is self-limiting and benign, the prognosis is different for the greater saphenous vein, as concomitant DVT is present in 12% to 44% of patients.[78]

As to anticoagulant management of superficial thrombophlebitis, when low-dose LMWH (nadroparin 2,850 IU/d) was compared with weight-adjusted therapeutic-dose nadroparin in a double-blind RCT of 164 patients[79] there was no difference in thrombotic extension or development of VTE (8.6% vs. 7.2% after 3 months). Another placebo-controlled RCT that assessed 45 days of fondaparinux (2.5 mg/d in 3,002 patients) (CALISTO)[80] showed progression or recurrence of the superficial thrombophlebitis or a diagnosis of DVT/PE in 0.9% versus 5.9% in placebo-treated patients (RR, 85%; 95% CI, 74 to 92). However, it still remains to be shown how patients should be selected for this relatively prolonged parenteral therapy.

Overall, thrombophlebitis that is limited to the calf veins can be effectively treated in most cases with oral or topical NSAIDs, but more extensive involvement of thigh veins, and especially if thrombophlebitis encroaches the adductor canal, with the potential to migrate into the deep veins, warrants anticoagulant therapy.

CONCLUSIONS

Substantial improvements in the treatment of VTE have occurred in the past decades due to the introduction of LMWHs, which have changed management from a 1-week hospitalization with intravenous UFH to outpatient therapy with unmonitored, once-daily subcutaneous injections. New oral anticoagulants are emerging as treatment options and will eliminate most of the laboratory monitoring required for VKA therapy. Patient education will be one of the major challenges to maintain good treatment compliance.

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CHAPTER 88

Overview of Arterial Thrombotic Disorders

SAM SCHULMAN AND VICTOR J. MARDER

In comparison with venous thromboembolism, which has a relatively uniform pathophysiology and standardized treatments, arterial diseases have disparate anatomic origins and pathogenetic mechanisms. Arterial thrombotic disorders may originate in (a) the atherosclerotic plaque (myocardial infarction, carotid stroke, peripheral vascular disease, chronic mesenteric ischemia, retinal artery occlusion); (b) in areas of low shear stress, causing a venous-type clot (cardioembolism, paradoxical embolism); (c) in the arterial wall after fibromuscular degeneration, leading to stenosis and aneurysm (renal artery occlusions); and (d) in the inflamed vessel wall, by often-unknown mechanisms (vasculitides such as Takayasu arteritis).

THE ATHEROSCLEROTIC PLAQUE

In the first stage of the atherosclerotic disease, an abnormal constellation of plasma lipoproteins, carrying an excess of cholesterol, penetrate sites with minute endothelial damages due to high shear stress in the arterial tree. The lipid deposits in the intima (fatty streaks) then start a plethora of reactions aimed at modifying (by oxidation), retaining or removing the lipids. Now the inflammatory orchestra enters the stage with all its members such as monocytes, macrophages,[1] T-helper cells, cytotoxic T cells, regulatory T cells, natural killer T cells,[2] dendritic cells,[3] cytokines, and differentiation factors. Even B-cells have been implicated, although more at a distance than in the plaques. The studies on the roles of these inflammatory components have been marred by the variability between species and also by the changing phenotypes of certain cells with different cytokine environments.[1] Platelets are also involved relatively early and become activated, release platelet-derived growth factor and transforming growth factor β, resulting in proliferation of smooth muscle cells, and they interact with the inflammatory process. The coagulation cascade is participating with thrombin generation that can be beneficial for tissue repair but also stimulate more inflammation. Early thrombin formation occurs at the site of small endothelial denudations that uncover lipid laden foam cells.

A protective fibrous cap over the plaque is generated via several mechanisms, including platelet-induced collagen synthesis, smooth muscle cell migration, and inhibited fibrinolysis by locally deposited plasminogen activator inhibitor type 1.

When the overloaded atherosclerotic plaque has reached a certain size, large endothelial denudations, perhaps due to apoptosis, or fractures of the fibrous cap can occur; thrombi form and may then occlude the artery resulting in infarction. A detailed description of the pathophysiologic process rather than this simplified summary will meet the reader in Chapter 89. It is interesting to note that the large number of participants in this disease process challenges us to find new and very different therapeutic options to complement the antithrombotic agents.

CARDIOEMBOLIC DISEASE

Atherosclerosis in large vessels is the etiology for only 20% of ischemic stroke in the Western world[4] and more commonly in Southeast Asia. Cardioembolism accounts for about 30% of the ischemic strokes,[4] and of those, only a small and rather questionable percentage of events are due to paradoxical embolism from true venous vessels. It has been difficult to establish the causal relationship between an often small patent foramen ovale and ischemic stroke.[5]

The focus has instead been on the intracardiac clot formation, most commonly in the left atrial appendix, sometimes in the left ventricle and rarely on the mitral valve or from an atrial myxoma. Atrial fibrillation has been strongly associated with the generation of left atrial clots, typically ascribed to stasis and accounting for 25% of ischemic strokes in the Western world.[6] In addition to stasis as the etiologic factor, there have been many speculations that other mechanisms are involved in the atrial clot formation. Thrombophilic abnormalities have usually demonstrated an association with stroke in small studies, but whenever large studies or meta-analyses were performed, the results became negative or contradictory. The role of an inflammatory component or the lack of atrial boost with resulting cerebral hypoperfusion is even more speculative.[6] More details on the etiology and risk factors for stroke are provided in Chapter 91.

After excluding atherosclerotic or cardioembolic origin for ischemic stroke, we remain with the lacunar infarcts due to cerebral microangiopathic disease and a number of rare conditions as well as a proportion of cases with unexplained origin. The size of this residual proportion depends on how well the patient has been investigated.

FIBROMUSCULAR DEGENERATION

The pathogenesis of fibromuscular degeneration is still an enigma, but there is no atherosclerosis or inflammation in the lesions. The most common form is medial fibroplasia, which has predominance in women of 20 to 60 years of age.[7] Hence, female hormones might play a pathogenetic role. Fibromuscular degeneration is responsible for 5% to 10% of cases with renal artery stenosis, as described in Chapter 95, and it appears to have an asymptomatic prevalence of 2% in the general Western population.[8] The second most common location is the coronary artery, but the pathology has been reported in almost every artery in

the body. The clinical picture corresponds to the affected site: renal artery—hypertension; coronary artery—angina; carotid or vertebral artery—transient ischemic attacks (TIAs), stroke, or dissection.

INFLAMED VESSEL WALL

The heterogeneous groups of vasculitides reflect the diversity of the immune system with a large variety of clinicopathologic pictures. The diseases can be classified as large, medium-sized, or small-vessel vasculitis, and important members are giant cell arteritis, Takayasu arteritis, rheumatoid vasculitis, IgG4-related aortitis, polyarteritis nodosa, Kawasaki disease, Wegener granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, Henoch-Schönlein purpura, cryoglobulinemic vasculitis, drug-induced vasculitis, infection-related vasculitis, systemic lupus erythematosus-related vasculitis, Behçet disease, and Cogan syndrome. The pathology includes granulomatous—sometimes caseating, giant cell, necrotizing, lymphoplasmacytic, leukocytoclastic, neutrophilic (with microabscesses), eosinophilic, aneurysmatic, and/or arteriovenous shunting components. The vasculitides are described outside of this section in Chapter 100.

POPULATION HEALTH AND RISK FACTORS

Acute Coronary Syndromes and Stroke

This section of the textbook contains chapters dealing with dominating population health problems. Coronary heart disease and stroke are well-recognized major causes of death in the developed world. From a global perspective, over 80% of the burden of cardiovascular disease originates in low- and middle-income countries.[9] The INTERHEART study evaluated associations between nine risk factors and myocardial infarction in 52 countries and different ethnicities by examining about 15,000 cases and as many controls. Most of the risk factors were simple, not requiring any sophisticated equipment to assess, and together they were responsible for 90% of the population attributable risk (PAR) globally and in most of the regions (Table 88.1).

Dyslipoproteinemia, smoking, diabetes, hypertension, abdominal obesity, and psychosocial problems had additive effects and daily intake of fruit and vegetables, exercise, and, to a very small extent, alcohol had protective effects. The contribution of each of these risk factors was relatively consistent although with some differences across the geographical regions and ethnicities. The effect of the risk factors was stronger in the young individuals than in the elderly, indicating the importance of early intervention. Primary prevention with anticoagulants is not justified and primary prevention with antiaggregants, usually aspirin, has only a favorable risk/benefit ratio in individuals at higher risk.

Similar results were recently published regarding risk factors for stroke in 3,000 patients and as many controls from 22 countries.[10]

The entire spectrum of risk factors for atherosclerosis is discussed in Chapter 89, in terms of management in the setting of acute coronary syndromes in Chapter 90, for ischemic stroke in Chapter 91, and for peripheral arterial disease in Chapter 94.

Less Common Sites of Arterial Occlusion

In addition to the coronary, cerebral, or peripheral arteries, we sometimes need to investigate and manage diseases affecting arteries in other anatomical regions, that is, the renal, mesenteric, and retinal arteries (Chapter 95). The etiologies are quite heterogeneous and accordingly also the therapies available. The vast majority of patients with renal artery stenosis have atherosclerotic origin and thus similar risk factor to those discussed above. Due to the relatively low prevalence of these arterial disorders compared to the cardiac and cerebral vascular diseases, there are few well-designed therapeutic trials to base treatment strategies on. For renal artery stenosis, five randomized controlled trials have actually been performed, and it appears that medical therapy alone should be the standard choice for the majority of patients. The situation is very different for acute

Table 88.1

Risk factors, their association with myocardial infarction and PAR in the INTERHEART study

Risk Factor	Prevalence Controls	Prevalence Cases	Adjusted OR (95% CI)	PAR (95% CI)
ApoB/ApoA-1	20.0%	33.5%	3.25 (2.81, 3.76)	49.2 (43.8, 54.5)
Current smoking	26.8%	45.2%	2.87 (2.58, 3.19)	35.7 (32.5,39.1)
Diabetes	7.5%	18.4%	2.37 (2.07, 2.71)	9.9 (8.5, 11.5)
Hypertension	21.9%	39.0%	1.91 (1.74, 2.10)	17.9 (15.7, 20.4)
Abdominal obesity	33.3%	46.3%	1.62 (1.45, 1.80)	20.1 (15.3, 26.0)
Psychosocial	—	—	2.67 (2.21, 3.22)	32.5 (25.1, 40.8)
Vegetables and fruits daily	42.4%	35.8%	0.70 (0.62, 0.79)	13.7 (9.9, 18.6)
Exercise	19.3%	14.3%	0.86 (0.76, 0.97)	12.2 (5.5, 25.1)
Alcohol	24.5%	24.0%	0.91 (0.82, 1.02)	6.7 (2.0, 20.2)
All combined			**129.2 (90.2, 185.0)**	**90.4 (88.1, 92.4)**

Adapted from Yusuf S, Hawken S, Ounpuu S, et al. Effect of potentially modifiable risk factors associated with myocardial infarction in 52 countries (the INTERHEART study): case-control study. *Lancet* 2004;364:937–952.
PAR, population attributable risk.

mesenteric ischemia, which is considered a surgical emergency, and even a "second look" is often recommended. Likewise, chronic mesenteric ischemia is preferably managed by surgical methods, although there is controversy whether open surgery or endovascular procedures should be preferred.

Retinal artery occlusions are usually associated with systemic atherosclerotic disease and thereby again the typical risk factors. Although a few randomized controlled trials have been performed, they were all underpowered (<100 patients). The challenges are the short time until irreversible damage (hours) and the potential harm of thrombolytic therapy or invasive procedures.

ADVANCES IN PHARMACOLOGIC MANAGEMENT

Patients with acute coronary syndromes will inevitably be prescribed a number of medications, partly for risk factor modification and partly for secondary prophylaxis. The statins lower low-density lipoproteins and cholesterol but also decrease expression of tissue factor in atherosclerotic plaques, alter prenylation of G-proteins, and activate a transcription factor. This results in reduced production of endothelial cell-derived microparticles and regulation of inflammatory processes with reduction of C-reactive protein, as discussed in Chapter 89. If not already given as primary prophylaxis, statin therapy should be started early in patients with acute coronary syndromes to reduce the risk for unfavorable clinical outcomes (Chapter 90). A somewhat surprising additional effect of statins is a reduction of the risk for venous thromboembolism.[11]

Disappointingly, concomitant elevation of the high-density lipoprotein levels, particularly with nicotinic acid (niacin)[12] or with fibrates[13,14] has not translated into any clinical benefits.

Hypertension is being treated more vigorously than ever before and, interestingly, old agents have manifested their important role. Thiazides remain the basis for most treatment regimens. Conversely, the novel angiotensin receptor blockers are not better than angiotensin converting enzyme inhibitors.

Platelet inhibition with antiaggregant therapy has seen its ups and downs. The dose of aspirin has come down from 325 to 80 mg without any loss of effect but with fewer side effects. The number of antiaggregants used concomitantly has increased, with the introduction of the P2Y12 inhibitors—initially ticlopidin, now replaced by clopidogrel (both prodrugs) and moving to the even more effective, active drug ticagrelor, which binds reversibly to the receptor—but for which there is so far nothing that can quickly eliminate its effect. After acute coronary syndromes or coronary stenting, it is now routine with dual antiplatelet agents for up to 1 year.

Whereas glycoprotein IIb/IIIa inhibitors turned out effective when given intravenously in the acute setting of percutaneous coronary intervention (PCI), the corresponding oral inhibitors xemilofiban, orbofiban, and sibrafiban failed in phase III clinical trials due to increased risk for bleeding and all-cause death, and they were withdrawn.

Patients with stroke benefit from antiaggregants if the pathology is in the large arteries, typically the carotids, and again, low doses of aspirin should be used. Addition of dipyridamole for patients with TIAs or minor stroke reduces the risk for recurrence, as reviewed in Chapter 93.

Patients with peripheral arterial disease mainly benefit from antiaggregants through their reduction of cardiovascular events. There is, however, also evidence for some improvement of walking distance, as reviewed in Chapter 94.

Anticoagulants have a limited role in the acute coronary syndromes, but the direct thrombin inhibitor bivalirudin is gaining terrain in the setting of PCI. Bivalirudin has similar effect as the combination of unfractionated heparin and a glycoprotein IIb/IIIa inhibitor and with a lower risk for bleeding.[15] The factor Xa-inhibitor fondaparinux given at a prophylactic dose for 1 week after acute coronary syndromes has similar effect as therapeutic dose low molecular weight heparin but with a lower risk for major bleeding or death.[16] Secondary prophylaxis with warfarin is beneficial mainly in patients with anterior myocardial infarction or with left ventricle thrombus for a period of 3 months on top of the antiaggregant therapy. Evaluation of the new oral anticoagulants added to antiaggregants after acute coronary syndromes have provided mixed results with a phase III trial with apixaban prematurely discontinued due to increased bleeding,[17] but a trial with rivaroxaban successfully completed.[18] There is undoubtedly an increased risk of bleeding with any combination therapy, and the challenge is to find the dose that still provides a net clinical benefit.

In stroke prophylaxis, the dominating problem has been the underuse of warfarin for patients with atrial fibrillation due to the fear of bleeding complications combined with inconvenience of anticoagulant management. With meta-analyses demonstrating net clinical benefit of warfarin over aspirin for most patients with atrial fibrillation,[19] there is a slow increase in the use of warfarin for these patients. Well-managed vitamin K antagonist therapy is of benefit even for patients with atrial fibrillation and a single additional risk factor. New oral anticoagulants have now been evaluated against warfarin in large trials, described in Chapter 92, and the advantage of no routine laboratory monitoring or dose adjustment is very appealing. There are, however, still remaining problems with these drugs such as uncertainty regarding optimal management of major bleeding complications, management perioperatively, and the financing of the drugs. Clearly, the very low cost of warfarin is not representative of the total societal cost since the management of warfarin is much more expensive than the drug, but that part is usually included in a different budget. For patients who are doing well on warfarin and thereby monitored with long intervals, there is a favorable cost-benefit relation, and they should not be switched without good reason to the new drugs.

In peripheral arterial disease, evidence does not support the addition of anticoagulants to antiaggregants.[20] In acute limb ischemia due to a thromboembolic event, heparin should be given and followed by warfarin if there is reason to believe that the embolic source remains. For patients with multiple reconstructions for critical limb ischemia, obviously failing on antiaggregants alone, it is hard to resist adding long-term anticoagulant therapy.

Thrombolytic therapy in ST-elevation myocardial infarction is losing ground to primary PCI, although prehospital reperfusion with a fibrinolytic agent reduces all-cause mortality.[21] Still, when primary PCI is compared with fibrinolysis in randomized trials, it performs better regarding most clinical outcomes.[22]

For acute stroke and with careful patient selection, treatment with plasminogen activators within 3 hours increases the chance of neurologic improvement. When given 3 to 4.5 hours after

onset of symptoms, there is also a certain increase of this probability. The fibrinolytic therapy is usually given intravenously, and there is less experience from intra-arterial infusion.

For acute limb ischemia, intra-arterial fibrinolytic therapy is, on the other hand, the preferred route. It reduces the need for open surgery, and it is, for some patients, the only possible option.

NOVEL DEVICES IN THE TREATMENT OF ARTERIAL DISEASE

Coronary stents are effective in reducing symptoms of ischemia and decreasing the risk for restenosis compared to angioplasty alone. Coronary thrombectomy and endarterectomy, performed in association with coronary artery bypass surgery, are evolving tools.

Carotid stenting for patients with TIAs has, on the other hand, not fulfilled its promises and appears to be associated with an increased risk for stroke.[23] Suction thrombectomy and thrombus retrieval devices should be considered experimental for patients with stroke and limited use in the setting of clinical trials is advisable.

In peripheral arterial disease, stenting of iliac artery lesions to maintain vessel patency or of more distal arteries after unsuccessful percutaneous transluminal angioplasty to salvage the leg, is often beneficial.[24] Drug-eluting stents may reduce the risk of restenosis further, similarly to the coronary artery setting, but there are controversial results for peripheral arterial disease as reviewed in Chapter 94.

For patients with renal artery stenosis, angioplasty with or without stenting has almost entirely replaced surgery. A recent randomized controlled trial failed, however, to demonstrate clinical benefits compared to medical therapy. There are remaining questions whether certain subsets of patients benefit from revascularization (Chapter 95).[25]

The ideal device should be nonthrombogenic and not cause leukocyte activation. This has to be combined with exceptional strength and durability in, for example, mechanical heart valves. Stainless steel and nickel-titanium are used for such devices, whereas catheters and vascular implants are made from polymers. To decrease thrombogenicity, binding of heparin or its derivatives to the surface has been tested but without convincing benefits. Another possibility is to seed the biomaterial with endothelial cells. This is reviewed in Chapter 96.

CONCLUSION

Although many novel therapies have become available, there is still need for better alternatives to prevent restenosis of coronary arteries without increased risk for bleeding. The proportion of patients with acute ischemic stroke eligible for fibrinolytic treatment is unsatisfactory low. Neuroprotective agents to salvage the penumbra are sought, but so far, the candidates have not been effective. In peripheral arterial disease, stem cell and progenitor cell therapy as well as gene therapy are being investigated and may bring improved remedy for these unfortunate patients.

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Full Reference Lists can be found online

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CHAPTER

89 Pathophysiology of Atherothrombosis

PETER LIBBY, PAUL M. RIDKER, AND KEVIN CROCE

Advances in contemporary vascular biology have contributed enormously to increased understanding of the pathogenesis of atherosclerosis and its thrombotic complications, and have increased our understanding of the mechanisms by which therapeutic interventions can alter the course of this disease. This chapter reviews some of this progress, highlighting the emerging links between atherogenesis, inflammation, and thrombosis: three processes classically considered separately. Indeed, the tendency to view atherosclerosis and thrombosis as distinct has deep historical roots. During the mid-19th century, Karl von Rokitansky[1] postulated that incorporation of thrombi into the artery wall caused atherosclerosis; the celebrated cellular pathologist Rudolph Virchow[2] ridiculed this view. Virchow considered atherosclerosis a disease predicated on proliferation and death of vascular wall cells, complicated by cholesterol accumulation and inflammation. Virchow's eminence deterred acceptance of the relation between thrombosis and atherogenesis.

The role of thrombosis in atherogenesis has resurfaced periodically since the time of these pioneers. Duguid,[3] in the middle of the 20th century, revived von Rokitansky's view that accorded a key role to the incorporation of thrombi in the formation of atheromatous lesions. Hand and Chandler[4] found that induction of thrombi could induce lesions resembling atheroma in experimental animals. Various investigators who used increasingly sophisticated biochemical methods, including Smith and Bini, localized products of thrombosis such as fibrin in human atheromatous plaques.[5,6]

We now recognize that both views in this polarized debate apply to the pathobiology of atherosclerosis, and that thrombotic and inflammatory cellular pathways are inextricably intertwined. Although the very earliest lesions of atherosclerosis may not involve platelets or coagulation, thrombosis nonetheless contributes to all subsequent stages of atherosclerosis. Indeed, some experimental evidence suggests a role for platelets even in early murine atherosclerosis.[7] Moreover, compelling laboratory and clinical evidence now provides growing support for the role of inflammation in both atherogenesis and the pathophysiology of its thrombotic complications. This discussion highlights the interactions between inflammation, thrombosis, and atherosclerosis during various stages of atherogenesis.

THREE THEMES IN ATHEROTHROMBOSIS

Several themes recur while considering the interfaces between atherogenesis, inflammation, and thrombosis. First, the pathogenic effects of thrombosis in the context of atherogenesis reflect dysregulation of fundamental biologic mechanisms of importance in normal homeostasis. The products of coagulation can alter vascular wall cells function in a manner that promotes fibrosis and healing. Substances released from activated platelets, such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), can augment the synthesis of collagen by smooth muscle cells (SMCs).[8] PDGF is a potent chemoattractant that stimulates the migration of SMCs.[9–12] Thrombin, generated during the clotting cascade, is both a smooth muscle mitogen and a proinflammatory mediator that regulates the expression of many other genes that influence vascular injury responses.[13–16] All of these processes may prove essential during wound healing and tissue repair. Yet, these usually salutary functions can become pathogenic in the context of the artery wall. The interface of atherosclerosis with thrombosis invokes the same conundrum that applies to the inflammatory aspects of atherosclerosis. Normal homeostatic mechanisms expressed inappropriately or in excess actually contribute to the pathogenesis of plaques.

A second recurring theme concerns the balance between stimulatory and inhibitory limbs of regulatory pathways. This concept, pertaining to the opposing poles of proteases versus antiproteases, or thrombosis versus fibrinolysis, is quite familiar to students of thrombosis. We shall see subsequently how altered balances between such pathways contribute to atherogenesis.

A third recurring theme has emerged more recently. We increasingly recognize that inflammation can heighten thrombosis and that mediators of thrombosis can play roles not only in clot formation, but also in augmenting inflammation (FIGURE 89.1). For example, thrombin can elicit inflammatory mediators, notably CD40 ligand (CD154), which can induce tissue factor expression on vascular cells and macrophages (MΦ), the principal source of this potent procoagulant in the atheroma.[17,18] In turn, platelets, when activated, can release CD40 ligand; myeloid-related protein 14 (MRP-14), regulated on activation, normal T cell expressed and secreted (RANTES); and other proinflammatory mediators.[19,20] Inflammatory mediators can sensitize vascular muscle and endothelial cells (ECs), as well as mononuclear phagocytes, to undergo programmed cell death or apoptosis. Dying vascular cells and macrophages can elaborate tissue factor-rich microparticles that can promote thrombosis. Therefore, reciprocal reinforcement of inflammation by thrombosis, and *vice versa*, results in amplification of these pathologic processes.

RISK FACTORS FOR ATHEROSCLEROSIS

Work conducted over the last half century has defined several risk factors for development of atherosclerosis and its complicating events. The concept of risk factors has gained widespread

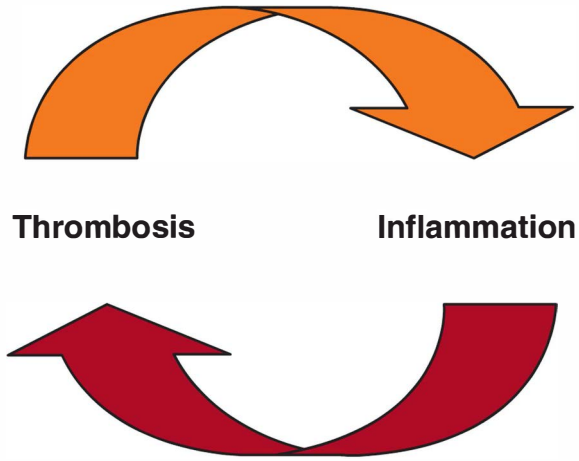

FIGURE 89.1 Accumulating data linking inflammation and thrombosis support the scheme illustrated here, that inflammation begets thrombosis and *vice versa*. An untested corollary of this concept posits that anti-inflammatory therapies may limit thrombosis, and that antithrombotic therapies may reduce vascular inflammation. (From Libby P, Simon DI. Inflammation and thrombosis: the clot thickens. *Circulation* 2001;103:1718–1720, with permission.)

clinical application in generating algorithms for predicting an individual's likelihood of having a future cardiovascular event. The concept of risk factors has led to interventions to reduce cardiovascular events. In the context of the present consideration of pathophysiology, the relationship of certain factors of risk to atherosclerosis and its clinical manifestations suggests hypotheses regarding mechanisms of development of the disease and the mechanisms of its complicating events. A complete exposition of risk factors, risk prediction algorithms, and intervention studies exceeds the scope of this chapter. Many authoritative reviews treat these subjects.[21] This overview of the risk factors for atherosclerosis will consider traditional risk factors, both nonmodifiable and modifiable, as well as emerging risk factors.

Nonmodifiable Risk Factors for Atherosclerosis

Age

Age predicts most potently an individual's risk for an atherosclerotic event.[22] This recognition has important implications, given the anticipated accumulation of the proportion of elderly individuals in many developed countries. From the perspective of thrombosis, the increase in older individuals with atherosclerosis has important implications, as these individuals have higher risk of adverse effects from some therapeutic interventions, including antiplatelet and anticoagulant therapy.

Sex

Sex strongly influences cardiovascular risk. Although women enjoy relative protection from cardiovascular risk premenopausally, their cardiovascular risk increases following menopause. The accumulation of elderly individuals in many populations alluded to above will yield a preponderance of elderly women as an important pool of cardiovascular risk in coming decades. Despite a considerable body of observational data that supported a protective role of hormone therapy against atherosclerotic disease in women, large-scale randomized clinical trials have not affirmed reductions in cardiovascular event rates in women receiving hormonal therapy, particularly in those with established disease.[23] Indeed, several of these studies have shown elevated risk of thrombotic events in women receiving estrogen or estrogen plus progesterone supplementation.[23]

Family History and Genetic Predisposition

A family history of coronary heart disease before age 60 in a parent adds to the other traditional risk factors in estimating the likelihood of a cardiovascular event.[24,25]. Some single-gene mutations clearly contribute to enhanced cardiovascular risk—for example, those that cause loss of function of the low-density lipoprotein (LDL) receptor, such as familial hypercholesterolemia, familial defective apo B, or autosomal dominant hyperlipoproteinemia. Yet these well-understood monogenic causes of atherosclerosis contribute a small portion of population-attributable risk of this disease. Thus, genetic predisposition to atherothrombotic events appears to depend on polygenic inheritance, with each variant contributing a small increment in risk. Most single-nucleotide polymorphisms associated with coronary artery disease have proved poorly reproducible or generalizable.[26]

The introduction of genome-wide association approaches has, in contrast, led to reproducible identification of regions that confer cardiovascular risk in human populations. Notably, the chromosome 9p21 locus consistently associates with augmented cardiovascular event rates in several populations.[27–29] The exploitation of the results of genome-wide association scans for atherosclerosis (and many other chronic diseases) should yield exciting new insights into pathophysiology and potential therapeutic targets in the future. From a practical standpoint at present, however, a simple clinical query regarding family history of premature coronary artery disease appears to confer as much information regarding risk as an assortment of newly emerged genetic markers.[30] Thus from a clinical perspective, genetic testing today adds little to cardiovascular risk assessment over and above the traditional risk factors, including a simple query regarding family history. The 2010 American Heart Association (AHA) guidelines for risk assessment in asymptomatic adults reflect this conclusion.[31]

Modifiable Risk Factors for Atherosclerosis

Lipids

Of the modifiable risk factors, lipids have led the way. The appreciation of links between cardiovascular risk, atherosclerosis, and hypercholesterolemia emerged over the last century. In particular, LDL, a major carrier of cholesterol in humans, participates causally in atherogenesis (FIGURE 89.2). LDL levels correlate with cardiovascular risk in populations.[32] Genetic abnormalities that yield excess LDL correlate with increased individual risk of cardiovascular events.[33] Interventions that lower LDL confer protection from cardiovascular events.[34] A large body of experimental data supports a causal role for LDL in altered cellular events that sow the seeds for atherosclerosis.[35,36] For example, excess LDL correlates with accumulation in the arteries of lipid-laden macrophages, cells that participate importantly in atherothrombosis. Moreover, lowering cholesterol levels by diet alone or by statin treatment can decrease expression of tissue factor within plaques, the procoagulant implicated in triggering many atherothrombotic events.[37]

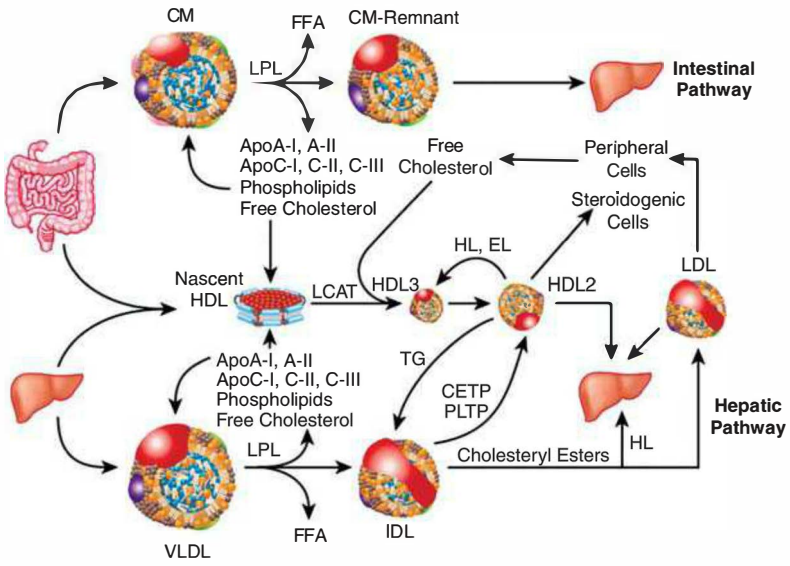

FIGURE 89.2 Schematic diagram of the lipid transport system. EL, endothelial lipase; HL, hepatic lipase. (From Genest J, Libby P. Lipoprotein disorders and cardiovascular disease. In: Bonow RO, Mann DL, Zipes DP, et al., eds. *Braunwald's heart disease: a textbook of cardiovascular medicine*, 9th ed. Philadelphia, PA: Elsevier Saunders, 2011:975–995, copyright Elsevier, with permission.)

Low-Density Lipoprotein

LDL particles exhibit heterogeneity. Small, dense LDL particles confer particular risk and associate with high levels of triglycerides and low levels of high-density lipoprotein (HDL), a triad characteristic of "diabetic dyslipidemia." Larger and less dense LDL particles appear less likely to provoke clinical complications. Several advanced tests can characterize lipoprotein particles. In particular, measuring the levels of LDL's major apolipoprotein, apolipoprotein B (apo B), provides an excellent estimate of particle number, as each LDL bears one apo B moiety. Canadian guidelines recognize apo B measurements as appropriate for clinical use. Yet, for most practitioners, inspection of the lipid profile can provide sufficient information for the management of most patients, without the need for advanced testing.[31]

Inhibitors of the rate-limiting enzyme in cholesterol synthesis, hydroxymethylglutaryl coenzyme A—a class of drugs known as statins—strikingly lower LDL levels. These drugs likely accomplish LDL lowering largely by increasing levels of LDL receptors and enhancing clearance of LDL. Treatment with statins has lowered cardiovascular event rates in broad categories of patients, ranging from those who have already sustained myocardial infarctions (MIs) with high levels of LDL to individuals with no prior history of cardiovascular disease and average or even below-average levels of LDL.[34] Statins undoubtedly reduce cardiovascular events through LDL lowering, but this class of drugs also alters prenylation of small G proteins such as ras, rac, or rho, important in controlling functions of cells involved in atherosclerosis.[38] Statin-mediated reduction of rho function limits the production of prothrombotic microparticles from ECs.[39] Statins also activate the transcription factor Kruppel-like factor 2 (KLF2), which regulates a number of inflammatory functions of ECs, including thrombomodulin gene expression.[40,41] Thus statins may also produce cardiovascular benefit through pathways independent of LDL lowering. Subsequent sections will deal at greater length with putative anti-inflammatory actions of statins.

Triglycerides

Triglycerides have engendered considerable controversy as independent risk factors for atherosclerosis and its complications. Triglyceride-rich lipoproteins include very low-density lipoprotein, a lipoprotein that like LDL, bears apo B as its signature apolipoprotein. Levels of triglycerides typically rise following meals, particularly those rich in fat. Recent studies, however, have indicated that random measurements of triglyceride levels serve well as estimates of future cardiovascular risk, obviating the need for obtaining fasting levels in clinical practice.[42]

Although triglyceride levels correlate well with prospective cardiovascular risk, when adjusted for covariates such as levels of HDL, the incremental risk conferred by triglycerides appears negligible.[43] Moreover, clinical trials have not shown that interventions that lower triglyceride levels, such as fibric acid derivatives, consistently lower cardiovascular events or mortality even in diabetic individuals who have a predisposition to hypertriglyceridemia.[44,45]

High-Density Lipoprotein

Abundant observational data have established low HDL as a consistent and important risk factor for atherosclerosis. From a pathophysiologic perspective, HDL may mitigate atherogenesis through several mechanisms. As proposed originally by Glomset,[46] HDL particles can mediate reverse cholesterol transport, ferrying cholesterol out of cells such as macrophages. Experimental data support the ability of HDL particles to efflux cholesterol from lipid-loaded macrophages. Apolipoprotein A-I (apo A-I) interacts with the ATP-binding cassette transporter ABCA1, a process important in generating nascent HDL particles. Mature HDL particles can efflux cholesterol using a "half" transporter, homologous to ABCA1, known as ABCG1.[47] Animal experiments have documented HDL's function in cholesterol efflux from storage sites to bile acids and ultimately the feces,[48] but only a small fraction of total body reverse cholesterol transport probably occurs from atheromatous lesions in humans. Moreover, HDL efflux from macrophages appears to be independent of HDL itself as a determinant of vascular disease and thus may provide a link between HDL, thrombosis, and inflammation.[49]

In addition to functioning as an acceptor for reverse cholesterol transport, HDL carries many proteins that may help modulate aspects of atherothrombosis, locally in plaques and in innate immunity systemically.[50] In addition to a long appreciated binding capacity for bacterial endotoxin, recent proteomic analyses have demonstrated that HDL associates with modulators of proteinase activity, complement activation, lipid metabolism, and inflammation.[51] Experimental studies both *in vitro* and *in vivo* determined that HDL may exert anti-inflammatory actions relevant to atherothrombosis.[52] Despite this biological plausibility, a large Mendelian randomization study has not supported HDL as a causal risk factor in atherosclerosis.[53]

Although HDL levels indubitably correlate with risk for atherosclerotic events, interventions that raise HDL have not yet proven effective in reducing cardiovascular risk.[54] Clinical trials are evaluating various strategies for raising HDL in patients at risk for cardiovascular disease. Inhibition of cholesteryl ester transfer protein (CETP) raises HDL levels. Yet, one inhibitor of CETP provoked harm rather than benefit in a large clinical trial.[55] The particular agent used in this study, torcetrapib, exerted mineralocorticoid agonist properties that raised blood pressure, lowered potassium, and may have contributed to adverse outcomes. A large clinical endpoint study with another CETP inhibitor, dalcetrapib, halted in 2012 apparently for futility. Two CETP inhibitors that appear not to share the off-target actions of torcetrapib (anacetrapib and evacetrapib) are currently in clinical development. The results of trials with these agents should establish whether CETP inhibition to raise HDL could yield clinical benefit.[56]

Fibric acid derivatives (agents such as fenofibrate, gemfibrozil, or bezafibrate) weakly induce apo A-I transcription through activation of peroxisomal proliferation activating receptor α.[57] This family of drugs can substantially lower triglycerides and slightly raise HDL. A well-understood drug–drug interaction precludes the safe combination of gemfibrozil with statins, agents mandated for use in most individuals eligible for pharmacotherapy of lipid disorders.[58] Several recent studies with fenofibrate have failed to show benefit on cardiovascular outcomes or mortality in people with diabetes, as noted above.[44,45] The clinical role of fibrates in treating individuals with low HDL levels, particularly those already receiving statins, remains unproven.

Nicotinic acid substantially raises HDL levels, and when combined with statins, produces highly favorable, effects on the lipid profile, raising HDL and lowering LDL. While some older clinical trial data show reduction in MI in individuals treated with nicotinic acid, the added benefit of nicotinic acid to the contemporary standard of care remains uncertain. One recent trial (AIM-HIGH) stopped early due to futility [NEW REFERENCE T/K]. Another clinical trial currently in progress is testing whether combinations of statins with sustained release or nicotinic acid and an inhibitor of prostaglandin D intended to reduce flushing may provide added benefit over the current standard of care [NEW REFERENCE T/K].

Hypertension

In addition to lipids, high blood pressure constitutes a major modifiable risk factor. Actuarial data established striking relationships between mortality and blood pressure levels at a time when many physicians thought that hypertension represented an adaptive response to arteriosclerosis that inevitably accompanied aging. Indeed, until the last few decades, many considered hypertension a beneficial adaptation to aging arteries. Consistent cohort studies and cross-sectional data have established the importance of even slight elevations in blood pressure as a contributor to cardiovascular risk.[59] Current thinking accords a primary role to systolic blood pressure as a determinant of cardiovascular risk, although pulse pressure also independently associates with elevated cardiovascular risk. Some elegant analyses of relatively rare genetic abnormalities have inculcated abnormalities in renal electrolyte handling as etiologic in hypertension.[60] Yet the vast majority of patients with hypertension do not have monogenic or well-defined endocrine causes of elevated blood pressure.

Intervention studies have established blood pressure lowering as an important public health goal and an avenue to reducing individual cardiovascular risk.[61] In particular, pharmacologic intervention to reduce blood pressure lowers the risk of cerebrovascular accidents. Although more difficult to establish, current data indicate reduced MI as a benefit of blood pressure control. While dietary measures (as illustrated by the DASH diet) and other lifestyle interventions (such as engaging in regular physical activity) can help control blood pressure,[62] most individuals with established hypertension will also require pharmacotherapy. Several large-scale clinical trials have compared various antihypertensive drugs, and this ensemble of data ultimately suggests that for the most part, effective blood-pressure lowering—regardless of the particular agents used—can lower cardiovascular events. Distilling recent clinical trials for some practical precepts, a thiazide-type diuretic should be part of most antihypertensive regimens. Current practice employs lower doses of thiazides than used previously—12.5 or 25 mg of chlorthalidone or hydrochlorothiazide daily, for example.[63] Beta-adrenergic blocking agents should be used primarily in individuals who have already sustained an MI, and generally should not be first-line antihypertensive agents for others. Black individuals may benefit more from calcium channel blockers than from angiotensin-converting enzyme (ACE) inhibitors. Clinical trials have established no clear-cut benefit of angiotensin receptor blockers (ARBs) over ACE inhibitors in preventing atherothrombotic events, and there is ongoing controversy on whether ARB use associates with increased risk of maliganancy.[64] Studies do not support the combination of ACE inhibitors and ARBs.

Cigarette Smoking

Active cigarette smoking confers considerable cardiovascular risk.[65] Mechanisms by which cigarette smoking augments atherothrombotic events remain uncertain, although observational and experimental data provide some hints in this regard. Cigarette smoking may augment oxidative stress, thought to promote atherogenesis. Cigarette smoking can also lower HDL. Smoking cessation leads to a rapid decline in cardiovascular event rates, yielding benefits in <1 year. As many consider this too short a period to modify atherosclerotic plaques *per se*, some have interpreted this rapid return toward baseline event rates after smoking cessation as an indication that smoking particularly heightens thrombotic diathesis. While the mechanisms remain unproven, smoking cessation should be a priority of practice and public health intervention.

Emerging Risk Factors for Atherosclerosis

In addition to the traditional risk factors enumerated above, several more recently recognized risk markers have come to the fore as potential tools for sharpening risk prediction, and

perhaps shedding mechanistic light on the pathogenesis of atherothrombosis. One such emerging risk factor, hyperhomocysteinemia, has particular relevance to thrombosis.[66] Monogenic defects that lead to hyperhomocysteinemia, such as defects in beta-cystathionine synthase, associate with a thrombotic diathesis in childhood. More modest elevations of homocysteine in adult populations also associate with elevated atherothrombotic risk, although when adjusted for confounders, high levels of homocysteine account for only a modest enrichment in the relative risk of cardiovascular disease.[67,68] Folic acid administration can lower homocysteine levels. Recent trials have evaluated the efficacy of folate supplementation in reducing cardiovascular events. These studies have uniformly shown no benefit from such vitamin therapy, despite lowering of homocysteine.[69–71] As grain and cereal products currently undergo folate enrichment in the United States, as part of a public health intervention to reduce neural tube defects, pharmacologic intervention to lower cardiovascular risk by lowering homocysteine levels with folate or other vitamins appears unwarranted.

Lipoprotein a [Lp(a)]

Lp(a), pronounced "L P little A," denotes a subclass of LDL characterized by an apo B moiety covalently bound to apolipoprotein a. Curiously, Apo(a) bears striking homology to plasminogen, including a variable number of repeats of the kringle structure characteristic of plasminogen, particularly kringle 4. Despite the sequence similarity to plasminogen, Apo(a) appears to lack enzymatic capacity. The distribution of Lp(a) levels in the population is skewed. Those with high levels of Lp(a) have heightened risk of atherothrombotic events. Genome-wide association studies and Mendelian randomization studies support a causal role for Lp(a) in atherothrombosis.[72] Over an entire population, due to the skewed distribution of Lp(a) levels, Lp(a) accounts for a small portion of events (a low population-attributable risk). In individuals with higher levels of Lp(a), however, the incremental risk may substantially elevate the risk of atherothrombotic events. Unfortunately, specific interventions to lower Lp(a) are few, notably nicotinic acid treatment. Studies designed to lower Lp(a) have not been conducted. Current management strategies for those with high levels of Lp(a) include LDL lowering by lifestyle measures and statin therapy.

Oxidative Stress

A great deal of preclinical data and biomarker studies in human populations indicate a role for oxidized low-density lipoprotein in the pathogenesis of atherothrombosis.[73] Yet, a variety of interventions aimed at reducing LDL oxidation have failed to produce clinical benefit. Several studies with antioxidant vitamins have shown no improvement in cardiovascular event rates. A lipophilic antioxidant agent that effectively reduced LDL oxidation experimentally, likewise failed to reduce cardiovascular endpoints in a large clinical trial.[74] Thus, despite the large body of experimental and biomarker data supporting a role for oxidized LDL in the pathogenesis of atherothrombosis, no antioxidant intervention has thus far proven successful in limiting cardiovascular risk.

Other specific oxidant pathways may also contribute to atherothrombosis. Inflammatory cells associated with plaques, notably a subpopulation of macrophages in atheromata, can contain the enzyme myeloperoxidase (MPO), which can generate the potent oxidant species hypochlorous acid (HOCl).[75,76] HOCl can mediate chlorination of tyrosyl residues in proteins. Indeed, the products of HOCl modification accumulate in plaques, indicating the action of MPO *in situ* in human lesions. In particular, the modification of a specific tyrosyl residue in apolipoprotein A-I can impede reverse cholesterol transport mediated by HDL.[77,78]

MPO constitutes a considerable portion of the protein content of granulocytes. Although sparse in undisrupted atherosclerotic lesions, arterial thrombi can trap granulocytes. Thus, MPO-generated HOCl levels may increase selectively at sites of fresh thrombosis. The pathophysiologic implications of this local oxidative stress in aggravating the consequences of arterial thrombosis remain poorly explored.

One of the drivers of hypertension, angiotensin II, not only acts as an arterial vasoconstrictor, but also augments oxidative stress. In particular, angiotensin II can increase the activity of NAD(P)H oxidases in artery wall cells, notably smooth muscle.[79] These oxidases produce superoxide anion, a potential contributor to local oxidative stress in arterial plaques. Whether some of the clinical benefit ascribed to interventions that limit angiotensin II production or signaling arises from reduced superoxide anion production remains speculative.

Infection in Atherosclerosis

Among emerging risk factors, infectious agents, both viral and bacterial, have received periodic attention as promoters of atherogenesis through the decades. The seroepidemiologic associations between infectious agents and atherosclerosis have proved subject to considerable confusion.[80–82] The more recent well-powered and appropriately adjusted seroepidemiologic surveys show little or no consistency in the association of infectious agents with atherothrombotic events. Some have advocated a relationship between burden of exposure to infectious agents and atherosclerotic risk, rather than infection with a specific etiologic virus or microbe.[81]

In situ studies have localized infectious agents or their products to many human atherosclerotic plaques.[83] Whether these invaders or their products promote the disease process remains unproven. In particular, some well-powered intervention trials with antimicrobial agents, including macrolides and a fluoroquinolone, have not shown reduction in recurrent cardiovascular events in individuals who have known coronary heart disease.[84]

While infectious agents may not promote atherogenesis through local actions in the plaque itself, systemic responses to infection could quite reasonably contribute to heightened risk for atherothrombotic events. For example, bacterial products, through ligation of pattern recognition receptors including members of the toll-like receptor family, can augment the expression of proinflammatory cytokines.[85] Ultimately, cytokines produced in response to bacterial products including endotoxins can substantially augment circulating levels of interleukin-6 (IL-6). IL-6 regulates the acute-phase response of hepatocytes and markedly increases production by the liver of fibrinogen and the endogenous inhibitor of fibrinolysis—plasminogen activator inhibitor-1 (PAI-1) (FIGURE 89.3). Thus, infectious agents can tip the homeostatic–hemostatic balance, from one that favors fibrinolysis and limits thrombosis to a pathologic situation characterized by enhanced thrombotic potential and reduced fibrinolytic capacity. These changes

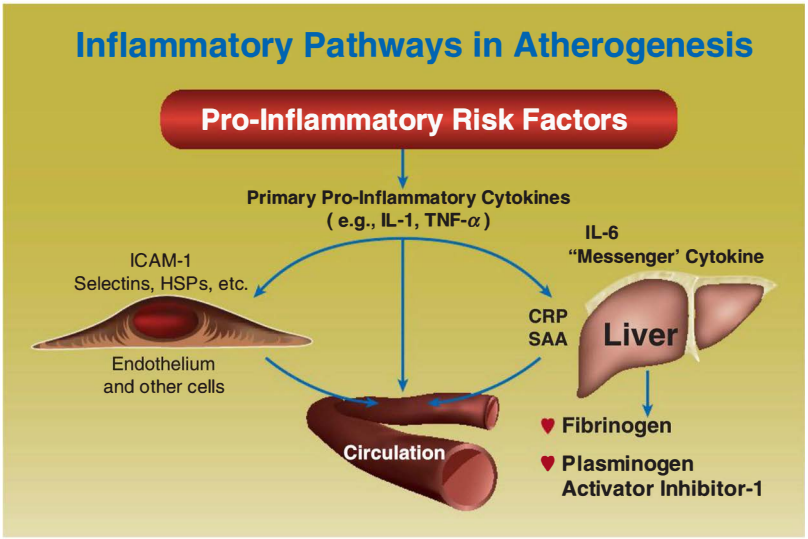

FIGURE 89.3 Pathways by which vascular and extravascular sources of inflammation result in circulating levels of serum markers that provide a reflection of the underlying inflammatory response. Systemic or local inflammation, either in the blood vessel itself or elsewhere, triggers the production of multipotent proinflammatory cytokines, denoted here as "primary" (e.g., IL-1β or TNF-α). These primary cytokines can directly elicit production by endothelial and other cells of adhesion molecules, procoagulants, and other mediators that may be released in soluble form into circulating blood. Primary cytokines also stimulate the production of the "messenger" cytokine IL-6, which induces expression of hepatic genes encoding acute-phase reactants found in blood, including CRP and serum amyloid-A (SAA). These markers in serum therefore provide a window on the inflammatory status of the individual, otherwise inaccessible in the intact subject. (From Libby P, Ridker PM. Novel inflammatory markers of coronary risk: theory versus practice. *Circulation* 1999;100:1148–1150, with permission.)

pertain not only to the "fluid phase" of blood, but also locally at the level of the plaque (FIGURE 89.4). Several cell types in the atherosclerotic plaque can also increase the local production of PAI-1, a function that can promote thrombus accumulation. So infectious agents, even if not driving pathogenesis of atherosclerosis *per se*, can augment the likelihood of thrombotic complications of this disease secondarily.

Inflammation in the Pathogenesis of Atherosclerosis

Considerable current information implicates inflammation as a major participant in the pathogenesis of atherosclerosis and its complications. In particular, many of the traditional risk factors may produce their atherogenic effects by harnessing proinflammatory pathways ordinarily involved in host defenses (FIGURE 89.3). For example, oxidized phospholipids associated with modified LDL particles can augment inflammatory functions in vascular wall cells and macrophages. Angiotensin II can increase levels of proinflammatory mediators, including leukocyte adhesion molecules (such as vascular cell adhesion molecule-1) and proinflammatory cytokines (including IL-6). Thrombin itself not only generates fibrin, but through interaction with protease-activated receptors, also can elicit proinflammatory functions from cells implicated in atherogenesis.

Indeed, platelets not only participate in clot formation, but their granular constituents also include proinflammatory mediators, including RANTES and CD40 ligand (CD154) (FIGURE 89.5). Thus inflammation and thrombosis beget one another, inextricably linking these two branches of host defenses. Numerous experimental studies conducted in cultured cells and in animals, such as genetically altered mice, assert a causal role for inflammatory mediators in the aggravation of atherosclerosis.[86] Biomarker studies in humans support the relevance of these laboratory experiments to the human disease, as discussed in detail below. Of emerging biomarkers, those reflecting inflammation have come most rapidly into clinical practice, as discussed later in this chapter.

INITIATION OF ATHEROSCLEROTIC LESIONS

As noted earlier, the very first steps in the evolution of atheroma may be the only aspects of this disease that do not involve thrombosis. Atherosclerosis usually develops in the context of elevated risk factors. An altered pattern of plasma lipoproteins is a well-established risk factor for lesion initiation.

We have learned much about the earlier stages of atherogenesis by studying experimental animals, which develop atheromatous lesions when they consume diets enriched with cholesterol and saturated fat. Such animal experiments likely represent a caricature of human atherosclerosis, because the achieved levels of lipoproteins exceed by far those found in most human patients with atherosclerosis. Despite the convenience of hypercholesterolemic models of atherogenesis, many other risk factors contribute to this disease. Nonetheless, these well-studied experimental preparations have afforded considerable insight into one pathway for lesion initiation.

Extracellular lipid tends to accumulate in the intima when high levels of plasma lipoproteins bearing cholesterol circulate in plasma (FIGURE 89.6).[87] Part of this accumulation may result from increased permeability of the endothelial layer to lipoproteins at sites of lesion predilection,[88,89] undoubtedly related to hydrodynamic changes at these locations, which typically localize

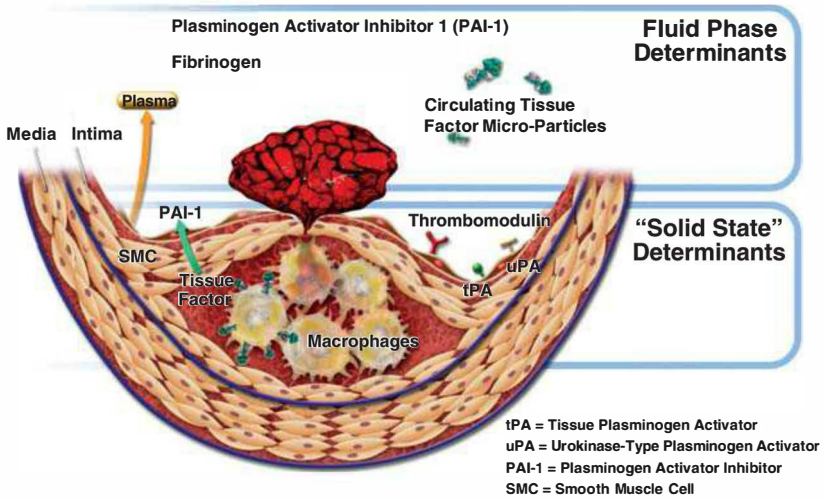

FIGURE 89.4 A two-state model of atherothrombosis. The high-risk atheroma has a thin fibrous cap overlying a large lipid core that contains tissue factor–bearing macrophages. When the fibrous cap fractures, coagulation proteins in the fluid phase of blood gain access to tissue factor–associated macrophages and tissue factor–bearing microparticles derived from apoptotic cells in the solid state of the plaque. These events trigger thrombus formation on the ruptured plaque. The clinical consequences depend on the amount of tissue factor and apoptosis in the plaque's core and on the levels of fibrinogen and PAI-1 in the fluid phase of blood. The interaction of the fluid phase with the solid state will determine whether a given plaque disruption provokes a partial or transient coronary artery occlusion (that can be clinically silent or, less commonly, cause an episode of unstable angina) or a devastating persistent and occlusive thrombus that can precipitate an acute MI. Inflammation regulates the thrombotic/fibrinolytic balance in both the solid state and the fluid phase, as PAI-1 and fibrinogen are both acute-phase reactants and because the inflammatory mediator CD40 ligand (CD154) induces tissue factor expression. EC, endothelial cell; PAI-1, plasminogen activator inhibitor 1; SMC, smooth muscle cell; tPA, tissue plasminogen activator; uPA, urokinase-type plasminogen activator. (From Libby P, Theroux P. Pathophysiology of coronary artery disease. *Circulation* 2005;111:3481–3488, with permission.)

near branch or flow dividers in the arterial tree. In addition to increased penetration into the artery wall, increased retention of lipoprotein particles can contribute to accumulation of lipid.[90] Whatever causes them to accumulate in the intima, lipoproteins provoke biologic reactions that seem to initiate atherogenesis.[91] Lipoprotein particles undergo modification in the arterial intima.[92] In particular, binding of lipoproteins to proteoglycan in the intima may both retard the egress of these particles and render them more susceptible to modification by oxidation.[93,94] These findings have stimulated the formulation of a "response-to-retention" model for the initiation of atherosclerosis.[95] Glycation in the presence of hyperglycemia represents another form of modification of LDL particles in the intima.[96]

Modified lipoproteins can elicit inflammatory mediators, such as proinflammatory cytokines, from the resident cell types in the normal artery: ECs and SMCs.[97–99] Such cytokines, protein mediators of inflammation, can elicit the expression of leukocyte adhesion molecules on the surface of ECs. Leukocytes attached to such "activated" ECs at sites of lesion predilection can enter the intima by diapedesis between EC junctions.[100,101] Chemotactic cytokines, such as macrophage chemoattractant protein-1 (MCP-1) and other chemokines, probably cause such directed migration of leukocytes. Atheromata overexpress these mediators, perhaps partly in response to modified lipoprotein particles. Once resident in the intima, mononuclear phagocytes can imbibe the modified lipoproteins that have accumulated extracellularly. The phagocytic cells bind and internalize these modified lipoprotein particles using "scavenger receptors."[102] The lipid-laden macrophage so produced constitutes the foam cell—the hallmark of the initial lesion of atherosclerosis, the fatty streak (FIGURE 89.6).

We owe much of our knowledge about the initiation of atherosclerosis to studies in well-defined animal preparations. But none of the available animal preparations mimic human atherosclerosis perfectly, not even the power offered by genetically altered mice (discussed later). Hence, the concurrent study of human lesions has proven an important complement to the animal work. With regard to the initiation of lesions, some human studies have furnished insight. Autopsy studies on young Americans, including those killed in armed conflicts in the 1950s and 1960s, showed a high prevalence of fatty streaks and even raised lesions in coronary arteries.[103,104] Systematic study of arteries collected from Americans younger than 35 years and dying of noncardiac causes has affirmed the high prevalence of nascent atheroma in humans in the third and fourth decades of life.[105,106] The general pattern of disease morphology agrees with the inferences made by extrapolation from atherosclerotic animals.

COMPLICATION OF THE ATHEROSCLEROTIC LESION

During formation of the fatty streak, the endothelium generally remains intact, albeit activated, as gauged by the expression of markers such as leukocyte adhesion molecules.[107–110] As macrophages continue to accumulate in the intima and inflammation

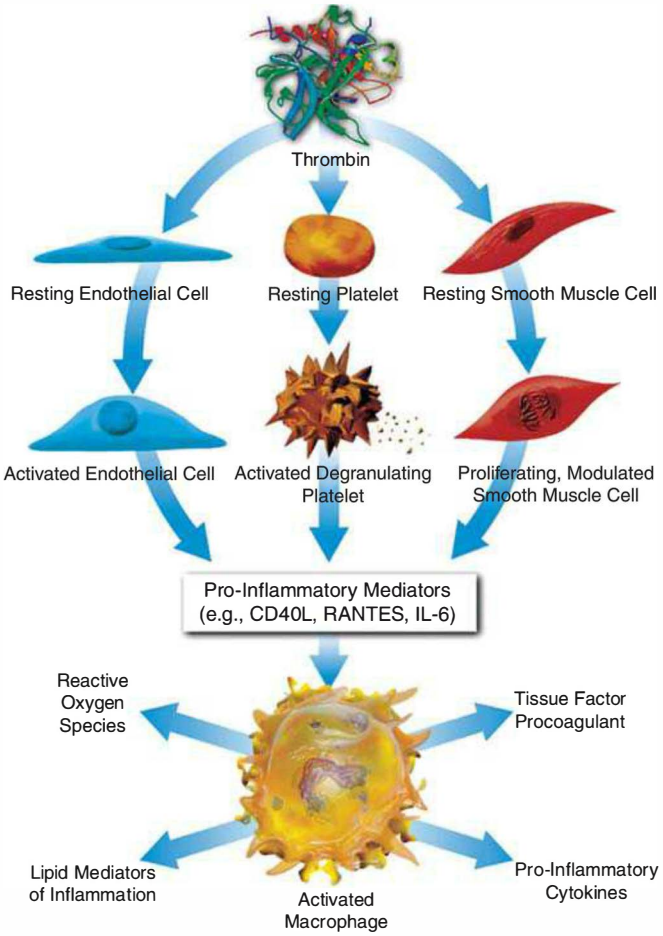

FIGURE 89.5 Thrombin-induced inflammatory processes play a central role in the development and complications of atherosclerotic vascular disease. Thrombin stimulation initiates secretion of platelet, EC, and SMC factors that stimulate macrophage production of tissue factor, proinflammatory cytokines, lipid mediators of inflammation, and reactive oxygen species. By these mechanisms, thrombin stimulates vascular inflammation, and inflammation promotes additional thrombin production through amplification loops that perpetuate atherogenic signals. (From Croce K, Libby P. Intertwining of thrombosis and inflammation in atherosclerosis. *Curr Opin Hematol* 2007;14:55–61.)

persists, small areas of endothelial desquamation occur. Careful examination of the arteries of cholesterol-fed monkeys by scanning electron microscope shows microthrombi occurring at such sites of very focal and limited EC loss.[111] Typically, these microthrombi consist of clusters of platelets adherent to macrophage foam cells uncovered by desquamation of ECs. Scanning electron microscopic studies of human hearts with severe but stable atherosclerosis, removed at the time of cardiac transplantation for ischemic cardiomyopathy and perfusion-fixed immediately, also disclose frequent microthrombi resembling those seen in the cholesterol-fed nonhuman primate.[112–115] The platelets at these sites of microthrombosis, upon degranulation, release mediators that promote SMC migration (e.g., PDGF) and collagen synthesis (e.g., TGF-β), and inhibit fibrinolysis (e.g., PAI-1). Inflammatory activation promotes evolution of the fatty streak toward a more fibrous lesion consisting of an admixture of SMCs, with mononuclear leukocytes, and increased interstitial collagen, a major extracellular protein of the advanced atherosclerotic plaque. Local inhibition of fibrinolysis caused by deposition of PAI-1 may also promote fibrosis of plaques.[116] Observations on advanced human atheroma have documented the presence of each of these platelet-derived mediators in atheroma, although cells within the lesion can also elaborate PDGF, TGF-β, and PAI-1.[117,118]

Initially, the SMCs that migrate and secrete collagen may derive from SMCs resident in the human arterial intima early in life. Other SMCs may migrate into the intima from the tunica media in response to chemoattractant factors, such as PDGF, released from platelets.[9,12] ECs, among other cell types in the artery wall, can also produce PDGF in response to stimuli such as thrombin, which is generated during thrombosis and can present at the sites of early mural microthrombosis.[119] As the lesion progresses, SMCs and the matrix they elaborate form a structure know as the "fibrous cap" near the luminal aspect of the plaque. This cap overlies a central zone known as the "lipid core" or "necrotic core," filled with lipid-laden macrophage foam cells, their debris, and extracellular lipid, including cholesteryl esters and cholesterol monohydrate crystals (FIGURE 89.7). We now recognize that apoptosis contributes to cell death in this region of the plaque.[120,121] Indeed, some of the accumulation of cellular detritus in the lipid core may result from ineffective clearance of apoptotic cells, a phenomenon we referred to as "mummification" in 1995,[122] now deemed due to a defect in "efferocytosis," or efflux or engulfment of dead or dying cells.[123] In the context of thrombosis, the accumulation of debris from macrophages in this region of the plaque has great import, as tissue factor derived from these cells,[124] and associated with cell-derived microparticles,[125] can serve as a major trigger to thrombosis following plaque disruption by mechanisms described in detail below.

Curiously, during much of the early life history of a given atheromatous plaque, atheroma growth proceeds in an abluminal direction. This compensatory enlargement, or "positive remodeling," permits the growth of the atheroma without impinging on the lumen.[126,127] In this manner, the lesion can grow without causing clinical symptoms related to reduced blood flow. This finding may account for the prolonged and usually clinically silent period of asymptomatic progression of atherosclerosis. This phenomenon also may explain why atherosclerosis often presents as an acute thrombotic complication without prior warning because of luminal narrowing. Lesions very likely grow in size and eventually encroach upon the lumen because of bouts of local microhemorrhage with healing and fibrosis.

The growth of new microvessels within the evolving atheroma may contribute to lesion complication. Neovascularization of the atheroma likely occurs in response to angiogenic growth factors, such as acidic fibroblast and vascular endothelial growth factor.[128–130] These microvessels seem to arise by extension of vasa vasorum in the adventitia and the outer third of the tunica media of many arteries. These neovessels may be friable and easily disrupted, resembling those in the diabetic retina. Therefore, the plaque's microvessels may provide a nidus for hemorrhage *in situ* within the plaque. Such breeches in microvascular integrity might well entrain local microthrombosis within

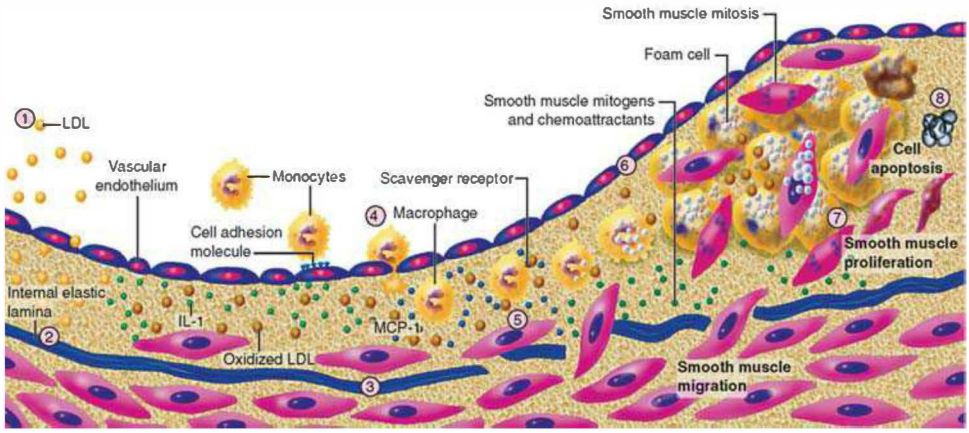

FIGURE 89.6 Schematic of the evolution of the atherosclerotic plaque.

1. Accumulation of lipoprotein particles in the intima. The modification of these lipoproteins is depicted by the darker color. Modifications include oxidation and glycation.
2. Oxidative stress, including products found in modified lipoproteins, can induce local cytokine elaboration.
3. The cytokines, thus induced, increase expression of adhesion molecules for leukocytes that cause their attachment and chemoattractant molecules that direct their migration into the intima.
4. Blood monocytes, on entering the artery wall in response to chemoattractant cytokines such as monocyte chemoattractant protein 1 (MCP-1), encounter stimuli such as macrophage colony-stimulating factor that can augment their expression of scavenger receptors.
5. Scavenger receptors mediate the uptake of modified lipoprotein particles and promote the development of foam cells. Macrophage foam cells are a source of mediators, such as additional cytokines and effector molecules like hypochlorous acid, superoxide anion (O_2^-), and MMPs.
6. SMCs migrate into the intima from the media.
7. SMCs can then divide and elaborate extracellular matrix, promoting extracellular matrix accumulation in the growing atherosclerotic plaque. In this manner, the fatty streak can evolve into a fibrofatty lesion.
8. In later stages, calcification can occur (not depicted) and fibrosis continues, sometimes accompanied by SMC death (including programmed cell death or apoptosis), yielding a relatively acellular fibrous capsule surrounding a lipid-rich core that may also contain dying or dead cells and their detritus. (From Libby P. The vascular biology of atherosclerosis. In: Bonow RO, Mann DL, Zipes DP, et al., eds. *Braunwald's heart disease: a textbook of cardiovascular medicine*, 9th ed. Philadelphia, PA: Elsevier Saunders, 2011:897–913, with permission.)

the atherosclerotic intima, thus potentiating local lesion growth through the aforementioned fibrogenic mechanisms, including PDGF-, TGF-β-, and thrombin-activated proliferation and secretory responses.

As the atherosclerotic lesion evolves, the constituents of the extracellular matrix tend to accumulate. In addition to interstitial collagen, such matrix constituents include proteoglycans, elastin, and microfibrillar proteins.[131] The extracellular matrix of the atheroma may actually constitute most of the volume of certain atherosclerotic plaques, particularly at later or more advanced stages. These matrix constituents interact with the thrombotic and hemostatic mechanisms in several ways. Collagen is a classical platelet activator, and hence may promote thrombosis when exposed to the blood. On the other hand, endogenous heparinoid molecules may bind and activate antithrombin, acting as an anticoagulant.

Complex atherosclerotic lesions often contain calcium. The mechanisms of calcification are currently under intense scrutiny.[132,133] Interestingly, binding of calcium to proteins with carboxyglutamate residues may contribute to lesion calcification. This chemistry resembles that which operates in proteins of the coagulation system. Recent works support a link between inflammation and calcification in atherogenesis.[134]

PLAQUE DISRUPTION AND ATHEROTHROMBOSIS

As lesions evolve, larger patches of endothelial denudation may occur, giving rise to larger mural thrombi. Such superficial erosion of coronary arteries can lead to occlusive thrombosis and sudden death.[135] The degree to which these desquamative lesions arise in bland or inflamed areas remains controversial.[135,136] In a selected population of individuals who have died suddenly and whose cases were then referred to a cardiac pathology group by medical examiners, such superficial erosions with occlusive thrombi account for up to one-third of sudden death cases caused by acute MI. Careful study of the clinical variables in these cases suggests that such superficial erosions occur more frequently in women than in men.[137] Other associations with superficial erosions as the nidus for thrombosis include hypertriglyceridemia, a low HDL level, and diabetes mellitus.[138]

Fracture of the plaque's fibrous cap causes most acute MIs (FIGURE 89.7).[139–141] Sites of fibrous cap fracture invariably bear signs of inflammation.[136,142] The type of atheroma that typically causes thrombosis on the substrate of a fissured fibrous cap includes the presence of numerous mononuclear phagocytes and large lipid pools. The macrophages in the lipid-rich core of

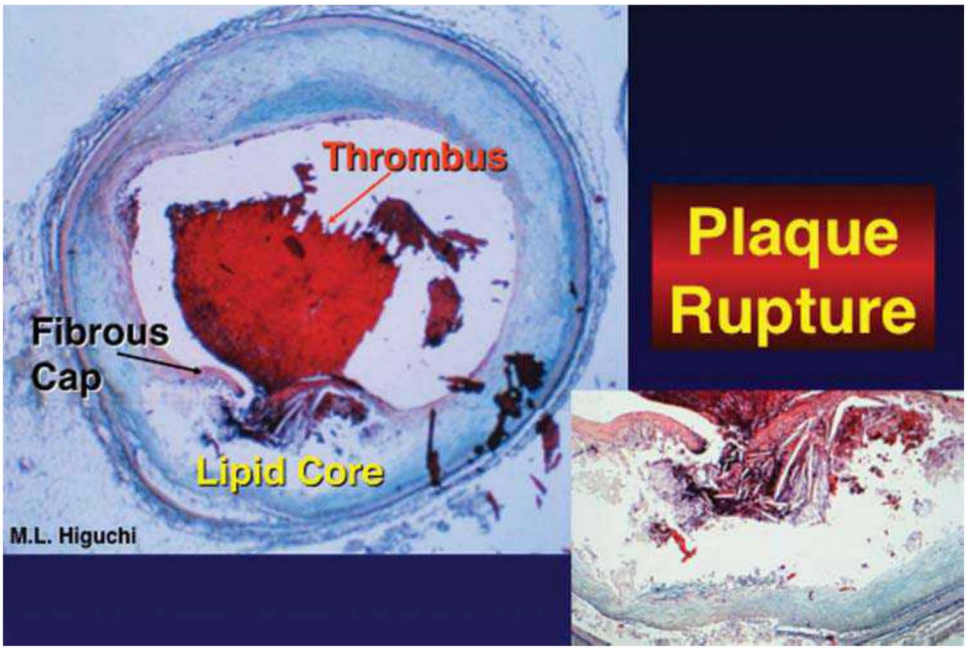

FIGURE 89.7 Plaque rupture: a common cause of coronary thrombosis. The fibrous cap overlying the lipid core of this plaque has ruptured, allowing the blood to contact the plaque's thrombogenic lipid core. Note that the lumen of this plaque is not critically narrowed. The **inset** shows a detail of the site of rupture, illustrating the fracture of the collagenous fibrous cap that caused the thrombus. This is how approximately two-thirds of fatal coronary thrombi form. (Courtesy of Dr. Maria de Lourdes Higuchi, Department of Pathology, University of São Paulo, Brazil.)

such atheroma contains high levels of tissue factor.[143,144] Because it can bind labeled factors VIIa and Xa *in situ*, tissue factor in atheromata appears to retain biologic activity.

When the plaque's protective fibrous cap ruptures, coagulation factors in blood gain access to tissue factor that can trigger thrombus formation. Moreover, as noted earlier, the collagen within the atheroma can activate platelets, further aggravating thrombus formation. Such fibrous cap fractures cause approximately two-thirds of acute MIs, as determined in autopsy studies. As noted earlier, however, plaque disruption appears commonplace in patients with atherosclerosis, and most plaque disruptions do not cause sustained occlusive thrombi, such as those that produce acute MI in the coronary circulation.[145,146]

The balance between procoagulant and anticoagulant factors and fibrinolytic and antifibrinolytic factors likely determines the consequence of any given plaque disruption. In the presence of a robust fibrinolytic system, a mural thrombus might undergo rapid lysis, limiting its clinical consequences. In the presence of elevated prothrombotic factors such as fibrinogen or the fibrinolytic inhibitor PAI-1, however, thrombi would be more likely to grow to the point of arterial occlusion. Nonocclusive mural thrombus, whether produced by superficial erosion or fracture of the fibrous cap, may incorporate into the plaque, as hypothesized by von Rokitansky in the 19th century.[1] Indeed, human atheromata contain a considerable amount of fibrin.[5,6] By means of the fibrogenic mechanisms detailed earlier, mural thrombi can promote smooth muscle migration, proliferation, and matrix secretion. This mechanism could ultimately lead to the evolution of a lipid-rich lesion into one of much more fibrotic character. Such episodes of thrombosis-induced plaque growth may lead to the formation of stenotic lesions capable of limiting blood flow and thereby producing clinical symptoms.

The dichotomy of the so-called vulnerable versus stable plaques is useful conceptually, but it substantially oversimplifies reality (FIGURE 89.8). Indeed, lesions of both morphologies often coexist in a given arterial tree and can even occur serially within a given artery, in close proximity or side by side at branch points.[147] Therefore, the arterial tree of a patient with atherosclerosis probably includes lesions of diverse morphologies: uncomplicated fatty streaks; raised lesions not complicated by thrombosis; lesions that have disrupted, causing acute mural thrombosis; and lesions that have evolved to a more fibrous character, perhaps by healing due to a previous mural thrombosis.

PATHOPHYSIOLOGY OF PLAQUE DISRUPTION

In view of the importance of plaque disruption as a substrate for thrombosis, we consider some current notions regarding the pathophysiology of this process. As noted earlier, two major mechanisms of plaque disruption account for most coronary thromboses: superficial erosion and rupture of the fibrous cap.

Local denudation of ECs characterizes superficial erosion of the arterial intima. Indeed, denuding injury of endothelium was considered primary in the pathogenesis of atherosclerosis for many years.[148] Although we now recognize that the endothelium remains largely intact over atheroma, patchy areas of denudation do occur and represent an important substrate for thrombosis.

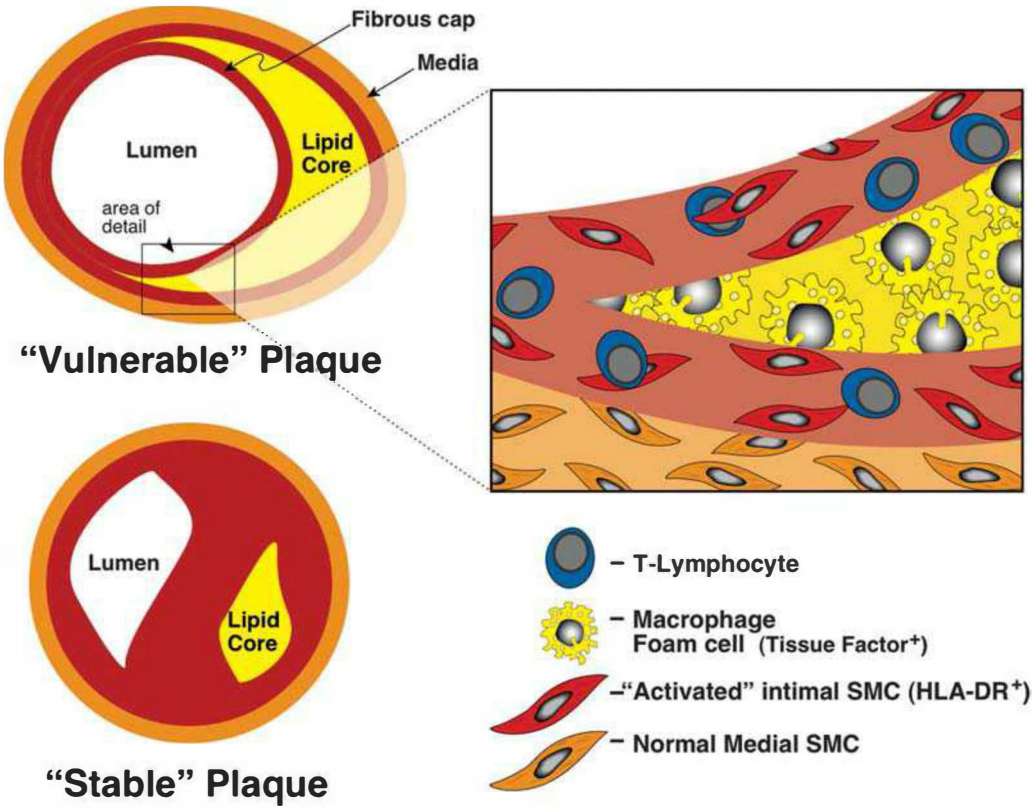

FIGURE 89.8 The characteristics of "vulnerable" plaques and "stable" plaques. Vulnerable plaques often have a well-preserved lumen, because plaques grow outward initially (compensatory enlargement or "positive remodeling"). The vulnerable plaque usually has a large lipid core and a thin fibrous cap separating the tissue factor in macrophages and other cells from circulating coagulation factors. Sites of lesion disruption often contain activated SMCs, detected by their expression of the histocompatibility antigen HLA-DR. In contrast, stable plaques tend to have a relatively thick fibrous cap protecting the lipid core from contact with the blood. (From Libby P. Molecular bases of the acute coronary syndromes. *Circulation* 1995;91:2844–2850, with permission.)

Initially, mechanical injury was considered most important as a cause of denuding endothelial injury. Mechanical denudation certainly applies to atherogenic endothelial injury—produced, for example, by interventions such as balloon angioplasty or stenting. Endothelial desquamation due to hemodynamic stresses was also considered a cause for endothelial denudation. But sites where plaques form are generally considered areas of low shear stress. Although circumferential or "hoop" stresses may be increased in hypertension, the effect of hypertension on endothelial desquamation focally remains uncertain.

Biologic rather than mechanical mechanisms—very likely apoptosis or death of ECs at areas of lesion predilection—might furnish one such pathway to focal endothelial denudation. Although apoptosis is generally considered a noninflammatory process, during atherogenesis, inflammation may actually promote apoptosis.[149] In addition, proteolysis could sever proteins involved in adhesive interactions that anchor ECs to the subendothelial basement membrane. ECs can overexpress matrix-degrading proteinases, including matrix metalloproteinases (MMPs), inducibly in response to inflammatory mediators or oxidized lipids known to localize in atheroma.[150–152] In addition, enzymes generally considered important in fibrinolysis, such as urokinase-type plasminogen activator and tissue-type plasminogen activator (uPA, tPA), may influence matrix degradation in two ways. Plasmin generated by these PAs can directly degrade components of the arterial extracellular matrix.[153,154] Moreover, plasmin can process the inactive zymogen forms of MMPs into their enzymatically active forms. Thrombin can activate latent MMP-2, a matrix-degrading enzyme constitutively produced by vascular cells.[155] The typical basement membrane constituent type IV collagen is a classical substrate for active MMP-2. Therefore, both thrombin and fibrinolytic enzymes may participate in the regulation of proteolysis that could render ECs prone to desquamation.

Proteolysis also likely contributes to fracture of the fibrous cap, the other major mechanism of plaque disruption (FIGURES 89.9 and 89.10). The inflammatory cells that appear quite numerous in rupture-prone atherosclerotic plaques can also produce enzymes capable of degrading structurally important constituents of the extracellular matrix in the plaque's protective fibrous cap.[156] Macrophages in human atheroma, for example, overexpress interstitial collagenases (including MMP-1 and MMP-13).[157–159] These cells can also produce the elastolytic enzymes, cathepsins S and K.[160] Further contributions to elastolysis in atheroma can derive from overexpression of MMP-12, matrix metalloelastase.[161] Additionally, macrophages in

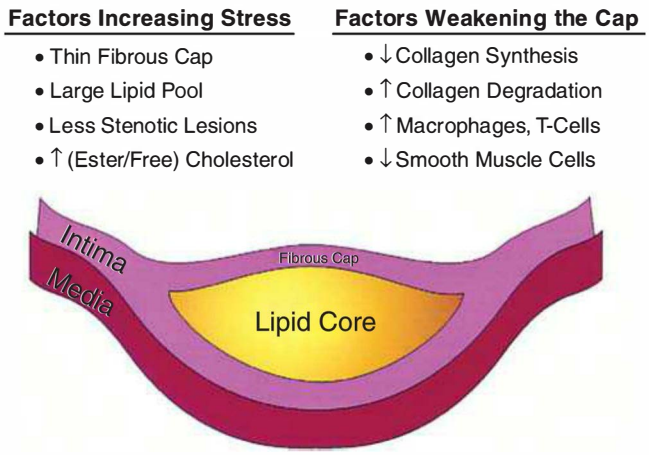

FIGURE 89.9 Biomechanical and biochemical features contribute to disruption of atheromatous plaques and their thrombotic complications. See text for explanation. (From Lee R, Libby P. The unstable atheroma. *Arterioscler Thromb Vasc Biol* 1997;17:1859–1867, with permission.)

atheromatous lesions can elaborate gelatinases, including MMP-2, MMP-9,[157] and MMP-14, among many other proteinases.[162]

The interstitial forms of collagen that lend strength to the plaque's fibrous cap can also diminish in vulnerable plaques because of decreased *de novo* synthesis by SMCs. T lymphocytes comprise an important component of the leukocytic infiltrate in human atheroma. T cells, when activated, can secrete interferon γ (IFN-γ), an inhibitor of SMC collagen synthesis.[8] Considerable evidence supports the activation of T cells in human atheroma and the presence of IFN-γ.[163] Therefore, impaired ability of SMCs to synthesize new collagen to reinforce and maintain the extracellular matrix of the fibrous cap can also lead to thinning and weakening of the structure, characteristic of plaques that tend to rupture and cause thrombosis.[164]

Indeed, SMCs may serve an important function as protectors of the plaque's fibrous cap, due to their ability to synthesize collagen and other structurally important components of the arterial extracellular matrix (FIGURES 89.9 and 89.10). In this regard, SMC death within atheroma may promote plaque disruption.[122] As noted earlier, inflammatory pathways in atheroma likely promote apoptosis. In particular, combinations of inflammatory cytokines can trigger cell death by apoptosis in human SMCs *in vitro*.[165] Moreover, activated T cells within lesions, by expression of Fas ligand, can engage Fas on the surface of SMCs and, in conjunction with inflammatory cytokines including IFN-γ, trigger the deaths of these cells.[149] Therefore, just as in the case of superficial erosion, where ECs are the target, fibrous cap rupture may depend on both proteolysis and cell death. Inflammatory pathways appear to participate decisively in the destabilization of the plaque that triggers thrombosis in both superficial erosion and fibrous cap rupture.

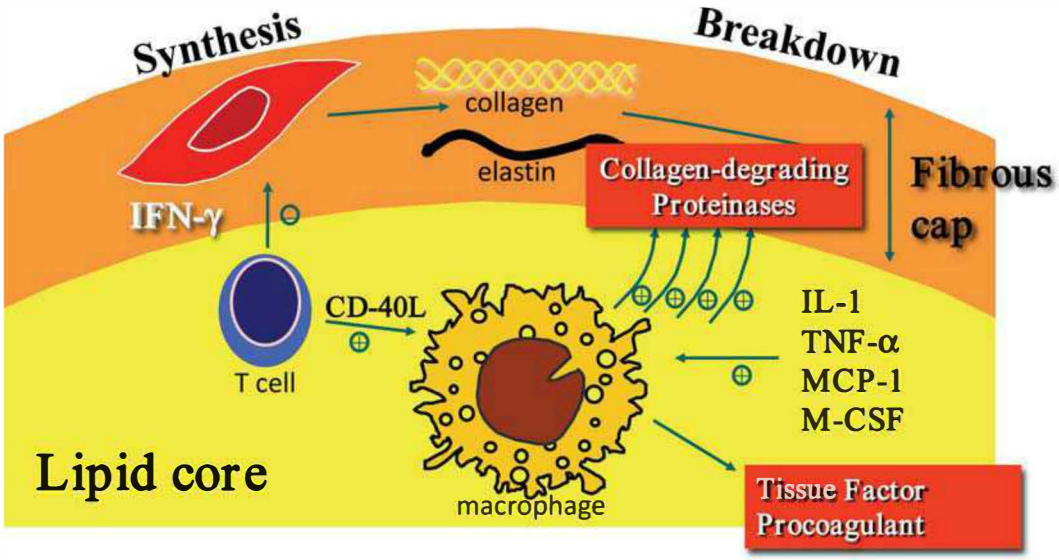

FIGURE 89.10 Molecular mechanisms regulating the stability of atheroma. The vascular SMCs synthesize the collagen and elastin in the atheroma's fibrous cap. When inflammation is present, as characteristic of plaques that cause fatal thromboses, interferon γ (IFN-γ) secreted by activated T cells may inhibit collagen synthesis, impeding the repair and maintenance of the collagenous framework of the plaque's fibrous cap. The activated macrophage secretes proteinases that can degrade both collagen and elastin. The breakdown of these structural molecules of the extracellular matrix can weaken the fibrous cap, rendering it particularly susceptible to rupture and precipitation of the thrombotic complications of atheroma. The macrophage, in turn, undergoes activation by IFN-γ derived from the T lymphocytes and by CD40 ligand (CD154) expressed on the surface of these cells. Platelets also can express active CD40 ligand. Plaques contain other activators of macrophages not derived from T cells, including tumor necrosis factor-α (TNF-α), macrophage-colony–stimulating factor (M-CSF), and macrophage chemoattractant protein-1 (MCP-1), among others. (After Libby P. Molecular bases of the acute coronary syndromes. *Circulation* 1995;91:2844–2850, with permission.)

MECHANISMS BY WHICH THERAPEUTIC INTERVENTIONS CAN ATTENUATE RISK OF ATHEROSCLEROTIC EVENTS

As described above, thrombosis triggered by plaque disruption causes most fatal acute coronary events. The physical integrity of the plaque, notably of the extracellular matrix of the fibrous cap overlying the atheroma's thrombogenic lipid core, thus governs the most important clinical manifestations of atherosclerosis. Atheromata with a "stable" morphology generally have thick fibrous caps and smaller lipid pools than lesions that have ruptured and caused fatal MIs. Considerable evidence points to inflammation as a key regulator of features that govern the biomechanical stability of human atherosclerotic plaques. Experimental studies indicate that lipid lowering reduces inflammation in the atheroma by decreasing the number of plaque macrophages and by decreasing macrophage expression of extracellular matrix-degrading proteases, as outlined above.[166] *In vivo* imaging studies show reduced inflammation in atherosclerotic mice treated with statins, despite only modest decreases in cholesterolemia.[167] Moreover, the collagen content of plaques increases during chronic lipid lowering, rendering plaques less friable and more resistant to rupture. Lipid lowering also reduces tissue factor expression in experimental atheromata.[37] Thus, therapy that treats dyslipidemia may not only render plaques less likely to rupture, but may decrease their thrombogenicity in tandem. Recent clinical studies show prompt decreases in recurrent ischemic events in high-risk patients following aggressive lipid lowering that outstrip the degree and rapidity of atheroma "regression." Biomarkers of inflammation fall in concert with cardiovascular event reduction.[168] Thus, a combination of experimental and clinical trial data indicate that reduction of plaque inflammation partly accounts for the important clinical benefits of lipid lowering.

THE CLINICAL APPLICATION OF INFLAMMATORY BIOMARKERS AND THE ATHEROTHROMBOTIC PROCESS

As described earlier, inflammation participates in plaque initiation, progression, and rupture. In particular, inflammatory cytokines such as IL-1 and tumor necrosis factor-α (TNF-α) trigger increased expression of cell adhesion molecules, including ICAM-1, VCAM-1, and P-selectin, leading to early leukocyte adhesion on the endothelium and enhanced fatty streak formation (FIGURE 89.3). Mononuclear cells within this infiltrate subsequently express growth factors that lead to the proliferation of SMCs, and platelet- and T-cell–derived proinflammatory cytokines such as CD40 ligand induce tissue factor production that in turn can promote thrombosis (FIGURE 89.5). In the aggregate, interactions of multiple inflammatory pathways contribute importantly to plaque growth and instability.

Current approaches to disease detection exploit this role of inflammation in atherothrombosis, and measurement of inflammatory biomarkers has become increasingly common in clinical practice. To date, several inflammatory biomarkers, including TNF-α,[169] ICAM-1,[170,171] P-selectin,[172] macrophage inhibitory cytokine-1 (MIC-1),[173] lipoprotein-associated phospholipase A_2 (Lp-PLA_2),[174,175] IL-1, IL-6, and IL-8,[176–178] MPO,[179,180] pregnancy-associated plasma protein A (PAPP-A),[181] CD40 ligand,[182,183] and MRP-14[20,184] have all shown correlation with events either in the setting of primary disease detection or in acute coronary ischemia. Because of its ease of measurement and long-term stability, the downstream acute-phase reactant C-reactive protein (CRP) (FIGURE 89.3), measured by a highly sensitive assay (hsCRP), has emerged as the most useful of these inflammatory biomarkers and is the only marker endorsed by the Centers for Disease Control and Prevention (CDC) for clinical use.[185,186]

Circulating CRP consists of five 23-kDa subunits and belongs to the pentraxin family of molecules that play major roles in human innate immunity. CRP derives largely from hepatocytes in response to IL-6 stimulation. Although hsCRP serves as a robust and reliable risk marker, Mendelian randomization studies suggest that CRP does not participate crucially in the causal pathway for atherogenesis.[187,188]

C-Reactive Protein in Primary Prevention

To date, more than 50 prospective epidemiologic studies have evaluated CRP with "high-sensitivity" assays. In each of these, CRP levels were increased at baseline among apparently healthy individuals at high risk for future MI, stroke, peripheral arterial disease, and sudden cardiac death.[189] Several large primary prevention studies have also shown that CRP adds important prognostic information at all levels of LDL cholesterol and across the full spectrum of Framingham risk scores (FIGURE 89.11).[175,190–196]

Importantly, CRP levels do not correlate with total or LDL cholesterol, and therefore, the addition of CRP evaluation to cholesterol screening has emerged as a powerful new method for vascular risk detection. Individuals with elevated levels of CRP but low levels of LDL cholesterol have higher absolute and relative risks of developing future vascular disease than those with elevated levels of LDL cholesterol but low levels of CRP (FIGURE 89.12), an observation important to global risk prediction algorithms.[191] CRP levels do track with obesity, hypofibrinolysis, and insulin resistance, and thereby increase with prevalence of the so-called metabolic syndrome.[197] In this regard, several major studies have also shown that CRP levels add prognostic information on risk at all levels of metabolic syndrome (FIGURE 89.13).[197,198] Moreover, CRP levels in healthy individuals predict future development of hypertension[199] and of type 2 diabetes.[200–203]

Recognizing the importance of these data, the CDC and the AHA issued the first clinical guidelines for use of hsCRP, and suggested that this biomarker be used among those identified as "intermediate risk" according to usual measures of global vascular risk.[186] Following analyses from both the Physicians Health Study[190] and the Women's Health Study,[169,178] those initial CDC/AHA guidelines indicated that hsCRP levels of <1, 1 to 3, and >3 mg/L represented lower, moderate, and higher vascular risks, respectively. The AHA guidelines for assessment of cardiovascular risk in asymptomatic adults assigned a IIb level of evidence to the use of hsCRP in cardiovascular risk assessment in asymptomatic intermediate-risk men 50 years of age or younger, or women 60 years of age or younger.[31] Individuals with marked elevations of CRP (values chronically in excess of 10 mg/L) have even higher absolute risk, and therefore false-positive elevations of CRP in people without acute inflammatory states occur rarely.[204] Categorization of hsCRP values provides incremental information on vascular risk in all major studies to date, even

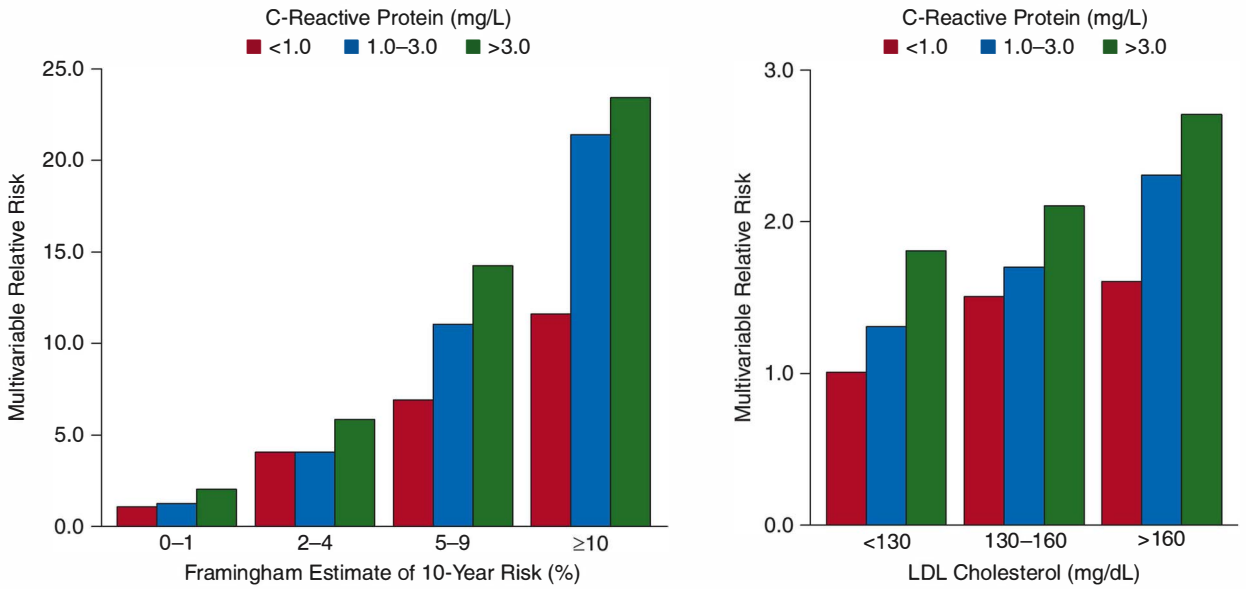

FIGURE 89.11 C-reactive protein levels add prognostic information on risk of future cardiovascular events at all levels of low-density lipoprotein (LDL) cholesterol (**right**) and at all levels of calculated Framingham risk (**left**). (From Ridker PM, Rifai N, Rose L, et al. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* 2002;347:1557–1565, with permission.)

after full adjustment for all components of the Framingham risk score (FIGURE 89.14). Computational approaches have generated risk calculators that include hsCRP in addition to traditional risk factors and family history of premature coronary artery disease: The Reynolds Risk Scores for men and women.[24,25] Application of these risk algorithms correctly reclassifies risk assessment in substantial numbers of individuals.

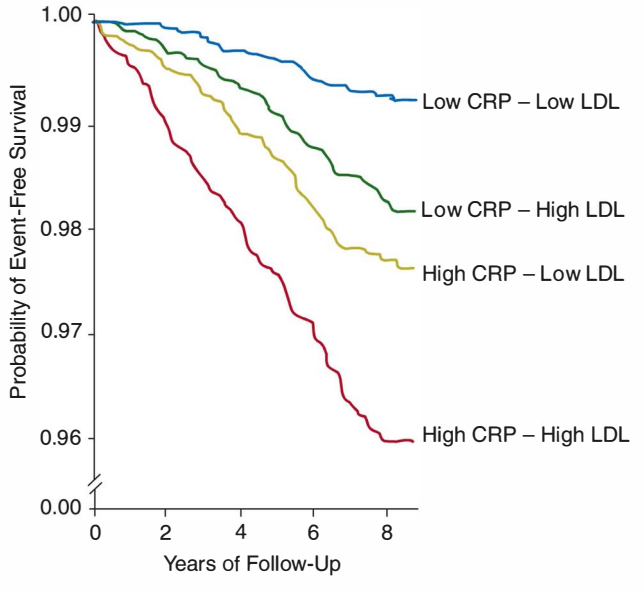

FIGURE 89.12 Cardiovascular event–free survival among healthy individuals, according to baseline levels of LDL cholesterol and CRP. (From Ridker PM, Rifai N, Rose L, et al. Comparison of C-reactive protein and low-density lipoprotein cholesterol levels in the prediction of first cardiovascular events. *N Engl J Med* 2002;347:1557–1565, with permission.)

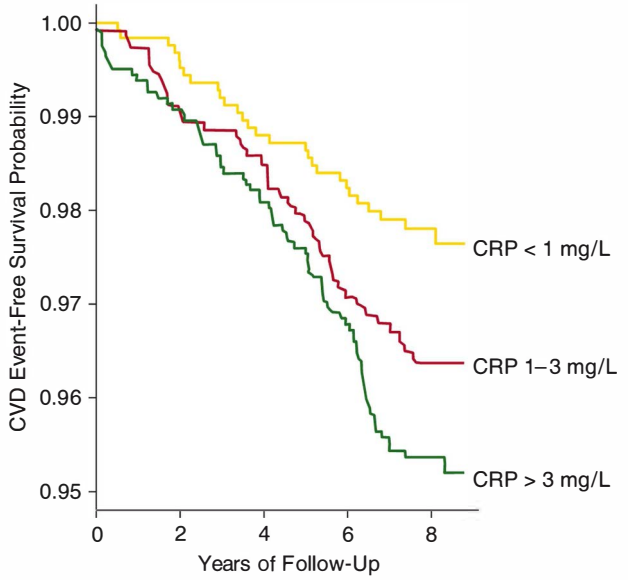

FIGURE 89.13 Cardiovascular event–free survival among individuals with metabolic syndrome by Adult Treatment Panel III criteria, according to plasma C-reactive protein (CRP) levels. CVD, cardiovascular disease. (From Ridker PM, Buring JE, Cook NR, et al. C-reactive protein, the metabolic syndrome, and risk of incident cardiovascular events: an 8-year follow-up of 14,719 initially healthy American women. *Circulation* 2003;107:391–397, with permission.)

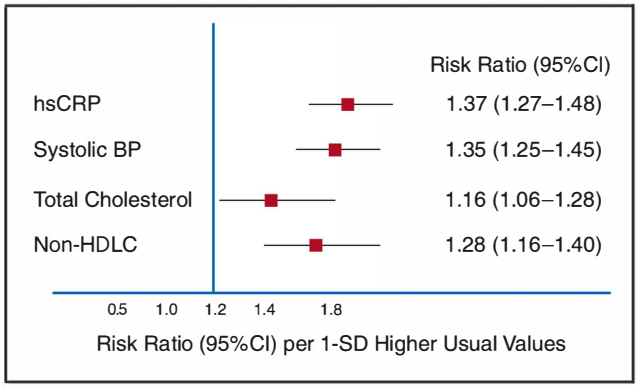

FIGURE 89.14 Direct comparison of hsCRP, systolic blood pressure (BP), total cholesterol, and non-HDLC (high-density lipoprotein cholesterol), adjusted for age, sex, smoking, diabetes, BMI, BP, triglycerides, alcohol, lipid levels, and hsCRP. Comparisons use data from 91,990 participants (5,373 CHD events) from 31 studies. hsCRP, C-reactive protein measured by the high-sensitivity assay. (From Emerging Risk Factors Collaboration. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet* 2010;375:132.)

C-Reactive Protein in Acute Coronary Ischemia, Chronic Stable Angina, and Secondary Prevention

In addition to a predictive role in primary prevention, CRP levels have utility as an adjunct to troponin in the setting of acute coronary ischemia. Multiple studies have shown that those who present with acute ischemia and elevated levels of CRP are at increased short- and long-term risk of vascular death, even when troponin levels are normal.[205] CRP levels may identify those who might benefit more or less from specific therapeutic interventions including aspirin, thiazolidinediones, and glycoprotein IIb/IIIa inhibitors.

C-Reactive Protein Levels and Statin Therapy

Beyond its role in the detection of cardiovascular risk, data have emerged suggesting that the use of CRP levels can improve the targeting of statin therapy. The observation that statins lower CRP levels first emerged from the analysis of the Cholesterol and Recurrent Events trial, in which the long-term use of pravastatin reduced CRP levels in a largely LDL-independent manner.[206] Subsequent studies have shown this to be a class effect, with all statins lowering CRP levels.[207,208]

Post hoc analyses of the Air Force Coronary Atherosclerosis Prevention Study/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) demonstrate that those with elevated levels of CRP but low levels of LDL cholesterol not only have high risk for first vascular events, but also appear to benefit from statin therapy.[209] This observation suggests that statin therapy reduces vascular risk even among those without hyperlipidemia. In contrast, this analysis showed no benefit from statin therapy among those within AFCAPS/TexCAPS who had neither increased lipids nor increased CRP, a group representing approximately 25% of the general population. Moreover, analyses performed within the Pravastatin or Atorvastatin Evaluation and Infection Therapy (PROVE IT-TIMI-22) clinical trial, comparing atorvastatin (80 mg) to pravastatin (40 mg), among 3,745 patients with acute coronary syndrome, showed that levels of both LDL cholesterol and CRP attained after 30 days of therapy correlated with reduction in recurrent adverse events.

This evidence bears directly on the inflammatory hypothesis of atherothrombosis because it demonstrates that CRP reduction *per se* may improve clinical outcomes. Moreover, within the PROVE IT trial, the best cardiovascular event–free survival occurred among those who not only lowered LDL levels below 70 mg/dL, but who also lowered CRP levels below 2 mg/L (FIGURE 89.15).

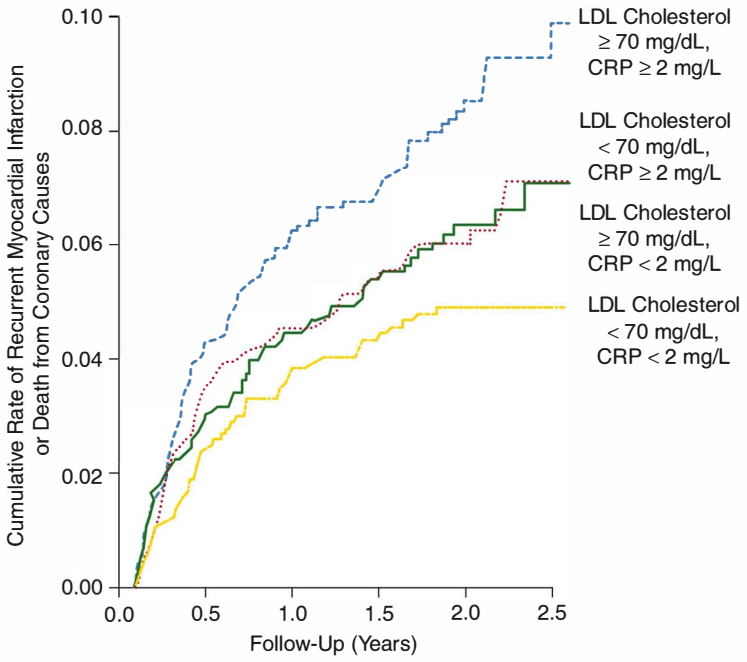

FIGURE 89.15 Incidence rates of recurrent cardiovascular events among patients with acute coronary syndrome, treated with statin therapy according to achieved low-density lipoprotein (LDL) cholesterol and achieved C-reactive protein (CRP) levels. (From Ridker PM, Cannon CP, Morrow D, et al. C-reactive protein levels and outcomes after statin therapy. *N Engl J Med* 2005;352:20–28.)

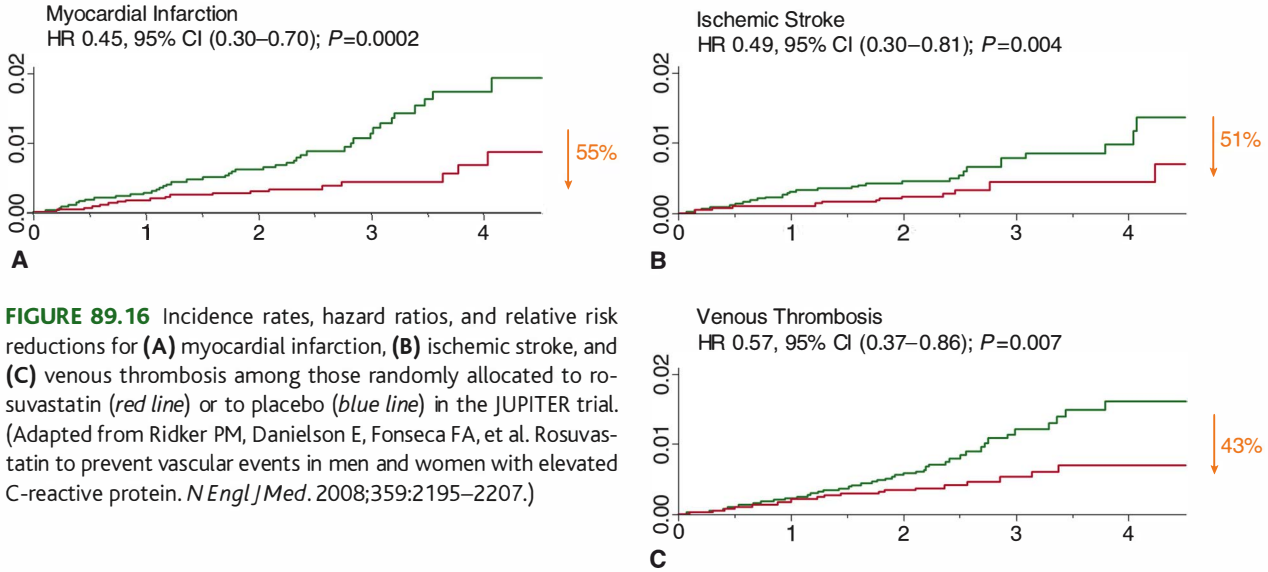

FIGURE 89.16 Incidence rates, hazard ratios, and relative risk reductions for **(A)** myocardial infarction, **(B)** ischemic stroke, and **(C)** venous thrombosis among those randomly allocated to rosuvastatin (*red line*) or to placebo (*blue line*) in the JUPITER trial. (Adapted from Ridker PM, Danielson E, Fonseca FA, et al. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med*. 2008;359:2195–2207.)

The *post hoc* observation from AFCAPS/TexCAPS that individuals with above-median levels of hsCRP but below-median levels of LDL benefitted from statin therapy inspired a prospective study—Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (JUPITER). This trial tested the hypothesis that individuals with low LDL levels (<130 mg/dL) but above-median levels of CRP (>2 mg/L) benefit from statin therapy. Statin treatment markedly reduced events that contributed to the combined endpoint, as well as the prespecified components of this endpoint.[210] In particular, events attributable to arterial thromboses (acute MI and ischemic stroke) fell strikingly (FIGURE 89.16A,B). Based on JUPITER and other evidence, the Canadian guidelines from 2009 and the AHA guidelines for assessment of cardiovascular risk in asymptomatic adults endorse targeting statin treatment according the JUPITER criteria.[31,211]

An additional indication of the antithrombotic action of statins emerged from JUPITER. Observational retrospective analyses of several populations suggested that statin treatment lowered the risk of venous thromboembolic events. No observational data link high levels of LDL with the risk of venous thromboembolism. Yet because of the observational data, the JUPITER protocol prespecified a test of the hypothesis that individuals with above-median CRP levels would have reduced venous thromboembolic events when treated with statins. Indeed, statin treatment reduced both provoked and unprovoked venous thromboembolic events by more than 40% in JUPITER (FIGURE 89.16C).[212] Current mechanistic studies are exploring the pathophysiologic basis of such a reduction in thrombotic events on the venous side due to statin therapy.

Although experimental studies and human biomarker investigations implicate inflammation in atherothrombosis, we lack intervention studies that establish a causal role for inflammation in atherothrombosis, or that prove that anti-inflammatory strategies may improve cardiovascular outcomes. Although aspirin indubitably reduces cardiovascular event rates in individuals with heightened baseline risk, the doses of aspirin that have proven effective in cardiovascular prevention do not produce systemic anti-inflammatory effects, although they effectively inhibit platelet aggregation. Other antiplatelet therapies, however, may mute inflammation, further highlighting the interdependence between inflammatory and thrombotic activation pathways in the pathobiology of atherothrombosis. Specifically, smaller studies show that the platelet ADP receptor antagonist clopidogrel and inhibitors of platelet glycoprotein IIb/IIIa reduce levels of circulating CD40L and the number of circulating leukocyte platelet aggregates, suggesting that these therapies share antithrombotic and anti-inflammatory function.[213,214] We need further studies to validate the efficacy of targeting inflammation directly as a mechanism of atheroprevention.

SUMMARY

Thrombosis causes the most dreaded clinical consequences of atherosclerosis and likely contributes to lesion formation as well, hence the term "atherothrombosis." The interrelationships between lipids, inflammation, thrombosis, and atherosclerosis have recently provided new mechanistic and therapeutic insight into this disease. Current thinking accords a crucial role for thrombosis in the evolution, complication, and acute manifestations of atherosclerosis. In turn, inflammatory signaling pathways appear central in regulating both the formation and thrombotic complications of atherosclerotic lesions. Advances in therapeutics targeting the hemostatic pathways have already permitted reductions in morbidity and mortality in patients with acute MI. The refinements currently under evaluation should provide further inroads to reducing the ravages of late-stage atheroma. Because platelets can elaborate inflammatory mediators and thrombin can stimulate inflammatory functions of cells involved in atherosclerosis, antiplatelet and anticoagulant therapy might even quell aspects of the inflammatory processes that operate during atherogenesis and plaque complication. Loss of myocardium caused by coronary ischemia is a major cause of heart failure. By mitigating the consequences

of plaque thrombosis, and perhaps also by slowing lesion progression, antithrombotic therapy can be viewed as a preventive measure against heart failure, the leading cause for hospitalization in the elderly. The past, studded with controversy about the role of thrombosis in atherosclerosis, has given way to an era in which the intimate links between these processes are not only widely acknowledged but have also given rise to major therapeutic advances. The interdependence of inflammation and atherothrombosis has yielded practical applications that help hone risk stratification and guide and monitor therapy in the clinic. Few areas of medical investigation have witnessed such rapid translation to practice, and the pace of progress in this area promises continued acceleration in the coming years.

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CHAPTER

90

Acute Coronary Syndromes: Manifestations and Management

JOHN PAUL VAVALLE, JOHN H. ALEXANDER, AND MATTHEW T. ROE

Coronary heart disease (CHD) is a global health epidemic that contributes to more than 7 million deaths per year and is the most frequent cause of death worldwide. Currently, more than half of the global burden of CHD is found in developing countries, and this percentage is growing rapidly.[1] Within the United States, there has been a decrease in deaths from CHD over the last three decades due equally to improved therapeutics and risk factor modification. Nevertheless, the prevalence of CHD in the United States remains high with over 1 million hospitalizations for acute coronary syndrome (ACS) annually.[2,3]

ACS is a term that refers to the clinical syndrome of acute myocardial ischemia typically resulting from atherosclerotic plaque rupture that leads to occlusive or subocclusive intracoronary thrombus causing downstream myocardial ischemia or infarction. The term ACS encompasses three conditions: unstable angina (UA), non–ST-elevation myocardial infarction (NSTEMI), and ST-elevation myocardial infarction (STEMI).

The presence of myocardial necrosis, assessed by measurement of cardiac biomarkers such as troponin and creatine kinase MB (CK-MB), differentiates acute NSTEMI and STEMI from UA. STEMI is further distinguished from NSTEMI by the presence of ST-segment elevation on the electrocardiogram (ECG). Rapid identification, triage, and implementation of evidence-based therapies for ACS are essential to reducing the morbidity and mortality associated with this life-threatening condition.

PATHOPHYSIOLOGY OF ACS

The hallmark of ACS is myocardial ischemia that results from insufficient oxygen delivery to meet the oxygen demands of the myocardium. This phenomenon most commonly results from obstructed coronary blood flow due to intracoronary thrombus that has generated on the surface of a ruptured atherosclerotic plaque. However, other mechanisms of ACS have been described, including dynamic coronary obstruction from coronary spasm or vasoconstriction, progressive mechanical obstruction as would be observed with progressive atherosclerosis, coronary artery inflammation, coronary dissection, and secondary angina—where the increased oxygen demands of the myocardium outstrip a preserved supply (Table 90.1).[3,4]

Atherosclerosis

Coronary atherosclerosis develops over decades and is characterized by the progressive deposition of lipids, fibroblasts, and inflammatory cells beneath the endothelial layer within the arterial wall (see Chapter 89). In the coronary arteries, the majority of these atherosclerotic plaques never progress to cause an ACS event. However, the so-called vulnerable plaques that eventually rupture do so as a result of the disruption of the fibrous cap that exposes the thrombogenic lipid core to circulating blood and stimulates the formation of thrombi. A newly utilized term, atherothrombosis, has been developed to describe this process.

Plaque Inflammation and Rupture

Plaques prone to rupture have been termed vulnerable plaques and have been characterized histologically. Typically, these plaques have a lipid-rich or necrotic core with a thin fibrous cap.[5] Activated macrophages and other inflammatory cells are generally present in higher concentrations in vulnerable plaques. Metalloproteinases and other enzymes secreted by these inflammatory cells can lead to thinning of the fibrous cap, making them prone to rupture. Autopsy studies have demonstrated dense infiltration of inflammatory cells into ruptured culprit plaques as compared to stable nonruptured plaques, supporting this notion of the inflamed vulnerable plaque.[6]

Identifying these plaques prone to rupture prior to an ACS event is an area of intense research, but virtual histology using intracoronary ultrasound, intracoronary optical coherence tomography, and molecular imaging with magnetic resonance imaging (MRI) or computed tomography (CT) has shown promise.[7]

Thrombosis

Intracoronary thrombosis is fundamental to the pathophysiology of ACS. At the moment of plaque rupture, platelet and plasma thrombosis transform a stable atherosclerotic plaque into an unstable coronary lesion. Intracoronary thrombi at the time of an ACS event have been demonstrated by angiography, atherectomy, and autopsy studies showing thrombus originating from the site of plaque rupture.[8–10] Markers of coagulation activity, such as elevated plasma fibrinogen levels, prothrombin fragments, fibrinopeptide, and D-dimers, have all been detected in patients with ACS, but are nonspecific markers and cannot be used in isolation.[11,12]

At the core of the thrombotic process is platelet aggregation that begins when plaque rupture exposes collagen and tissue factor to circulating platelets. Platelet activation initiates with the binding of the glycoprotein Ib receptor with von Willebrand factor, resulting in conformational changes of the platelet, as well as release of α-granules, thromboxane A_2 (TXA_2), and other platelet chemoattractants.[13] Mediators such as thrombin, adenosine diphosphate (ADP), and epinephrine amplify the platelet response by recruiting other platelets to the forming thrombus. The final pathway involves the activation of the glycoprotein IIb/IIIa (GP IIb/IIIa) receptor. Once activated, it is responsible for platelet aggregation and stabilization by binding fibrinogen, which cross-links the GP IIb/IIIa integrins between platelets.

Table 90.1

Causes of unstable angina and non–ST-elevation myocardial infarction[a]

Thrombus or thromboembolism, usually arising on disrupted or eroded plaque
• Occlusive thrombus, usually with collateral vessels[b]
• Subtotally occlusive thrombus on preexisting plaque
• Distal microvascular thromboembolism from plaque-associated thrombus
• Thromboembolism from plaque erosion
Non–plaque-associated coronary thromboembolism
Dynamic obstruction (coronary spasm[c] or vasoconstriction) of epicardial and/or microvascular vessels
Progressive mechanical obstruction to coronary flow
Coronary arterial inflammation
Secondary UA
Coronary artery dissection[d]

[a]These causes are not mutually exclusive; some patients have two or more causes.
[b]DeWood MA, Stifter WF, Simpson CS, et al. Coronary arteriographic findings soon after non–Q-wave myocardial infarction. *N Engl J Med* 1986;315:417–423.
[c]May occur on top of an atherosclerotic plaque, producing missed-etiology angina or UA/NSTEMI.
[d]Rare.
From Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304. Modified with permission from Braunwald E. Unstable angina: an etiologic approach to management. *Circulation* 1998;98:2219–2222.

The activated platelet serves as the substrate upon which coagulation occurs. Tissue factor, released at the time of plaque rupture, leads to activation of factor X, converting it to factor Xa. Through multiple positive feedback loops involving both platelets and soluble coagulation factors, factor Xa leads to thrombin generation (factor IIa) that in turn converts fibrinogen to fibrin in the final step of thrombus formation.

As a result of the critical role that platelets and soluble coagulation factors play in ACSs, antiplatelet and anticoagulant therapies have become fundamental to the treatment of ACS. Current proven antiplatelet therapies include aspirin for the inhibition of TXA_2 formation, $P2Y_{12}$ receptor antagonists (clopidogrel, ticlopidine, prasugrel, and ticagrelor) for the inhibition of the ADP receptor pathway, and GP IIb/IIIa receptor antagonists that directly inhibit platelet aggregation. In addition, thrombin receptor antagonists that inhibit thrombin activation of platelets are currently under investigation. Currently proven anticoagulants include unfractionated heparin (UFH) and low molecular weight heparin (LMWH), indirect factor Xa inhibitors such as fondaparinux, and direct thrombin inhibitors (DTIs) such as bivalirudin. A number of additional anticoagulants, including the direct factor Xa inhibitor otamixaban and the RNA aptamer factor IXa inhibitor pegnivacogin, are currently under investigation.[14,15]

Other Mechanisms of ACS

Vasoconstriction

Dynamic coronary obstruction can occur as a result of intense focal vasoconstriction of a segment of an epicardial coronary artery. This is commonly labeled Prinzmetal (variant) angina. This focal spasm is the result of vascular smooth muscle hypercontractility and endothelial dysfunction.

Patients presenting with ACS from vasoconstriction usually have sudden onset of chest pain at rest without any antecedent increase in oxygen demand. Attacks can be precipitated by exposure to cold, emotional stress, adrenergic stimuli, exercise, and cocaine use.[16–18] Variant angina episodes usually do not lead to acute myocardial infarction (MI), but prolonged episodes may result in acute myocardial necrosis, arrhythmias, and even sudden death.[19,20] The diagnosis of variant angina is suspected by transient episodes of chest pain at rest that may be accompanied by dynamic ST-segment changes on ECG and may also be responsive to vasodilators such as nitroglycerin.

Progressive Mechanical Obstruction

Severe narrowing of the epicardial coronary arteries, in the absence of focal spasm or thrombus, may occur as a result of progressive atherosclerosis or may be due to intimal hyperplasia causing restenosis at the site of a previous percutaneous coronary intervention (PCI). In general, these patients present with angina symptoms that have been progressively worsening concurrent with the progression of the myocardial obstruction.

Coronary Inflammation and Dissection

Although much less common than other causes of ACS, it is important to recognize coronary artery inflammation and dissection as causes of coronary obstruction, as they may have devastating consequences. Coronary artery dissection is the most common cause of ACS in peripartal women and often present with acute MI.[21] Inflammatory states such as graft-versus-host disease or infections such as Kawasaki disease may lead

to intense inflammation of the coronary arteries and result in complete coronary occlusion.[22,23] Identification and treatment of these etiologies is dependent upon recognizing and treating the underlying diagnoses.

Secondary Unstable Angina

Secondary UA results from an inability to meet the oxygen demands of the myocardium and usually occurs in the presence of some degree of obstructive coronary artery disease (CAD) that limits the flow reserve of the coronary circulation at times of increased myocardial workload. The causes of secondary angina are varied, but all result in increased myocardial oxygen requirements. Examples include fever, tachycardia, thyrotoxicosis, hyperadrenergic states, hypertensive emergency, and anemia among others. Because of the underlying condition, secondary angina is usually a marker for end-organ dysfunction and may have worse outcomes than other forms of angina.[24]

PRESENTATION, RECOGNITION, AND TRIAGE OF ACS

Presentation

While stable angina typically presents with angina pectoris associated with exertion and is relieved with rest, patients presenting with ACS classically present with rest angina or newly progressive angina. Three principal presentations of UA have been described: (a) rest angina, (b) new-onset angina, and (c) increasing angina (Table 90.2). Patients suffering an STEMI may present with the above symptoms but typically present with the new onset of a prolonged or stuttering episode of pain at rest.

The first step for patients before they can receive possibly life-saving treatment is to recognize their symptoms as being potentially serious and to seek medical attention. Despite attempts to shorten the delay to seeking medical care, the average person with UA/NSTEMI waits approximately 2 hours prior to seeking attention, and in the face of attempts to improve this, there has been little change over the last decade.[25,26] Often times, reasons cited for delay include atypical symptoms such as arm pain, jaw pain, or shortness of breath, or the expectation that the symptoms would be more severe.[27,28] Studies trying to understand reasons for delay in seeking care have demonstrated that older patients, blacks, women, and Medicaid-only recipients are more likely to delay.[25,29]

Patients experiencing an ACS event may not always present with chest discomfort.[30] An analysis of the National Registry of Myocardial Infarction showed that about one-third of the 434,877 patients with MI did not present with chest pain.[31] In this study, this resulted in delays in seeking care, delays to diagnosis by the providers, less implementation of evidence-based therapies, and higher in-hospital mortality. In particular, women, older patients, and those with a history of diabetes or heart failure are more likely to present with silent MIs, and those caring for these patients must be vigilant in their evaluation.

Table 90.2

Presentations of ACS

Class	Presentation
Rest angina[a]	Angina occurring at rest and prolonged, usually >20 min
New-onset angina	New-onset angina of at least CCS class III severity
Increasing angina	Previously diagnosed angina that has become distinctly more frequent, longer in duration, or lower in threshold (i.e., increased by 1 or more CCS class to at least CCS class III severity)

[a]Patients with NSTEMI usually present with angina at rest.
CCS, Canadian Cardiovascular Society.
Adapted from Braunwald E. Unstable angina: a classification. *Circulation* 1989;80:410–414; Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

Evaluation and Diagnosis

Because there is substantial overlap in the presenting symptoms of STEMI, NSTEMI, and UA, the initial evaluation of all patients presenting to a health care facility with symptoms concerning ACS should be the same (FIGURE 90.1). Patients should be evaluated in an acute care setting, with providers and staff equipped to perform critical care.

Because of the possibility of an immediate life-threatening condition, the initial evaluation must focus on rapidly identifying clinical instability and assessing for ST-segment elevation on an ECG if not done prior to arrival by emergency medical personnel. ST elevation in the setting of symptoms compatible with ACS should set the patient on a predefined treatment algorithm for urgent reperfusion therapy with fibrinolytics or primary PCI.[32] The importance of the ECG in the initial evaluation has led the American Heart Association and the American College of Cardiology in its joint treatment guidelines to give its strongest recommendation possible (class I) to obtaining an ECG within 10 minutes of arrival. Rapid evaluation and implementation of treatment for ACS can translate into significant improvements in outcomes and ultimate cost savings.

THE ACUTE MANAGEMENT OF STEMI

Urgent Identification, Triage, and Reperfusion

In acute STEMI, there is complete occlusion of the epicardial coronary artery, leading to transmural myocardial ischemia and infarction. Generally, STEMI results from plaque rupture, leading to an occlusive thrombus composed of fibrin, platelets, and inflammatory cells. Acute STEMI represents a true medical emergency, and management strategies focus on urgent coronary reperfusion therapies to open the occluded coronary vessel and minimize myocardial necrosis. This may involve primary PCIs, fibrinolytic therapy, or a combination of both depending on patient characteristics and available resources and capabilities. The old adage of *time is muscle* holds true for STEMI patients,

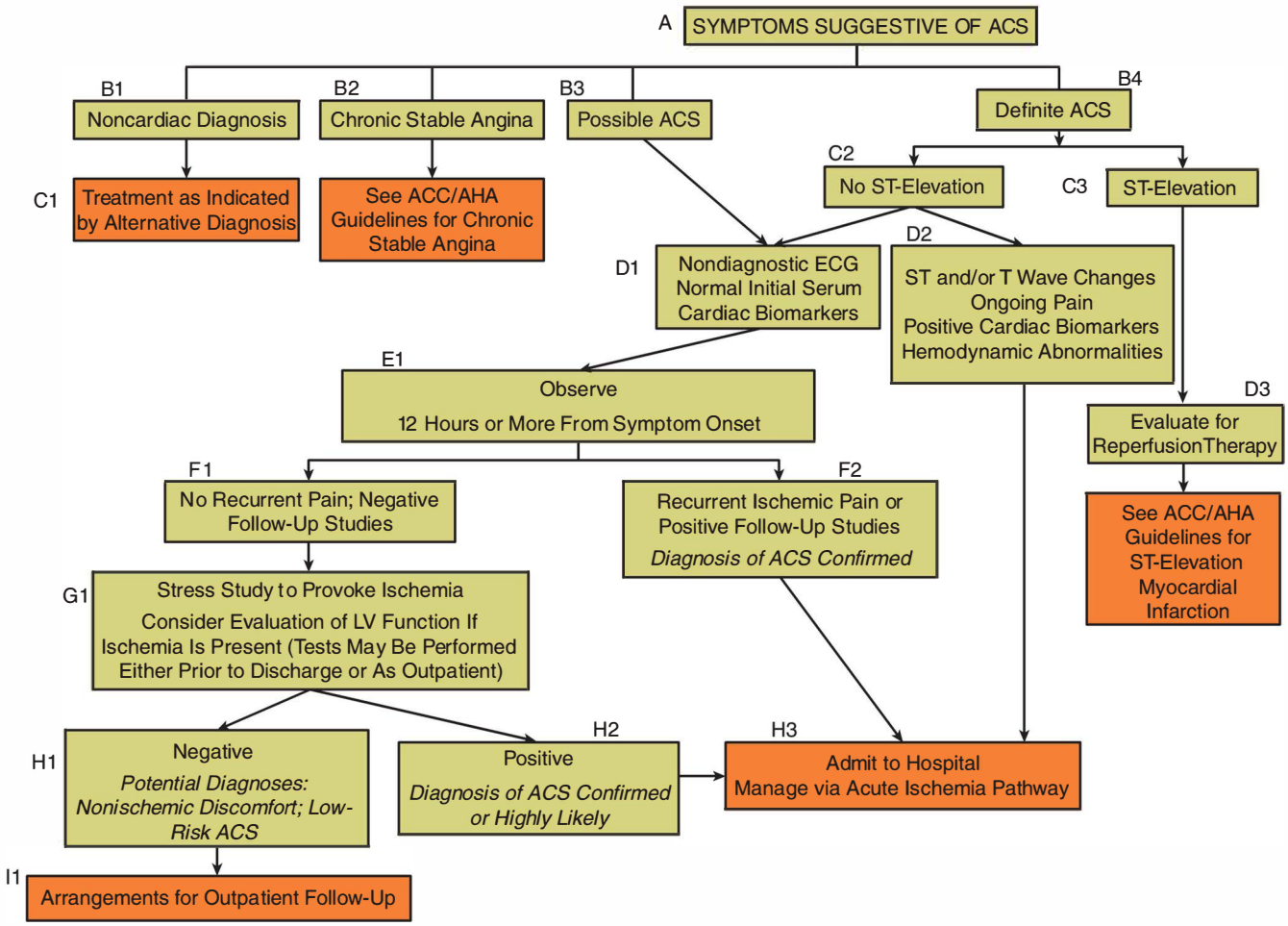


FIGURE 90.1 Treatment algorithm for patients with suspected ACS. ACC/AHA, American College of Cardiology/American Heart Association; ECG, electrocardiogram; LV, left ventricular. (From Anderson JL, Adams CD, Antman EM, et al., ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction. *Circulation* 2007;116(7):e148–e304.)

with each minute of delay associated with an increase in mortality.[33] Reperfusion therapy to open the occluded artery with either fibrinolytics or primary PCI should be implemented urgently.

The diagnosis of STEMI is made in the right clinical context in conjunction with anginal symptoms when at least 1 mm of ST elevation is present in two contiguous leads or a new left bundle branch block is detected on the ECG. Acute ST-segment elevation is associated with the highest risk of early death, and the presence of such findings indicates the need for immediate reperfusion therapy. This diagnosis should trigger a preplanned algorithm to initiate a reperfusion strategy with either fibrinolytics or primary PCI. The initial assessment of a patient with STEMI should focus on evaluating for hemodynamic and arrhythmic complications and on rapidly evaluating for the optimal reperfusion strategy.

Delays in reperfusion can arise from a number of sources. Reasons cited for delay include a lack of initial recognition of the symptoms by the patient, inefficient transportation to medical centers, and delays in initiating reperfusion therapy once the patient arrives. A well-coordinated STEMI response team comprised of cardiologists a emergency departments, and the emergency medical services (EMS) is needed to minimize delays.

Often, the first medical contact with a patient is by EMS. EMS personnel need to be well trained in the recognition and acute management of STEMI. The ability by EMS to perform and interpret 12-lead ECGs can lead to an accelerated diagnosis of STEMI and a more rapid reperfusion.[34] In addition, EMS should be equipped to handle complications that might arise from STEMI and have the ability to perform advanced cardiac life support, defibrillation, and intubation with mechanical ventilation when needed. Although not widely used, prehospital reperfusion with fibrinolytic agents given by appropriately trained personnel has been studied, and in a meta-analysis of six trials, it was shown that prehospital thrombolysis preceded the in-hospital thrombolysis by a mean of 58 minutes and reduced the all-cause hospital mortality with an odds ratio (OR) of 0.83 (95% confidence interval [CI], 0.70 to 0.98).[35]

Given the significant importance of time to reperfusion, benchmark guideline times have been put in place and are used to assess quality outcomes of health systems. Currently,

for fibrinolytic therapy, the recommended door-to-needle time is ≤30 minutes. This means that from the minute the patient arrives in the emergency room to the minute the fibrinolytic infusion is begun, the time elapsed is not >30 minutes. For primary PCI, the established benchmark is a door-to-balloon time of ≤90 minutes.[32]

Reperfusion Therapy for STEMI

Despite strong evidence for improved outcomes with early reperfusion therapy in STEMI, not all eligible patients receive this therapy. Currently, approximately 30% of STEMI patients receive no reperfusion therapy despite a lack of contraindications, and of those treated, <50% receive fibrinolytic agents within the recommended door-to-needle time of 30 minutes and <40% receive PCI within the recommended 90 minutes.[36] However, more recent data in at least one study suggest improvement in this trend, with the percentage of eligible patients in contemporary practice not receiving reperfusion therapy being closer to 10%.[37]

Both fibrinolytic therapy and primary PCI are suitable options for revascularization in STEMI. Choosing between the two therapies is based on a number of factors, but the important common denominator is that the chosen strategy be implemented as rapidly as possible. In general, primary PCI is favored over fibrinolytic therapy when available in a timely fashion, especially in those who are at high risk or presenting late after symptom onset.[38]

A pooled analysis of 23 randomized trials comparing primary PCI to fibrinolysis for STEMI showed that primary PCI was better than fibrinolytic agents for nearly all endpoints, including mortality (OR, 0.73; 95% CI, 0.62 to 0.86), reinfarction (OR, 0.35; 95% CI, 0.27 to 0.45), and stroke (OR, 0.46; 95% CI, 0.30 to 0.72).[39] However, when a patient presents to a hospital where PCI is not immediately available, the decision to transfer to a PCI-capable hospital versus immediate administration of fibrinolytic agents is a complex one.

Factors to consider when deciding between primary PCI and fibrinolysis include such factors as contraindications to fibrinolysis, the presence of cardiogenic shock, time from symptom onset, bleeding risk, diagnostic certainty of STEMI, and the time required to transfer for primary PCI.[32,40] Contraindications to fibrinolytic therapy are listed in Table 90.3 and include both absolute and relative contraindications. Patients meeting any of the absolute contraindications should not receive fibrinolytics and should be considered for primary PCI. The risks of administering fibrinolytics to a patient with a relative contraindication to reperfusion must be weighed carefully.

Primary PCI

One of the most substantial systematic barriers to obtaining primary PCI as the reperfusion modality is the lack of facilities and the time required to transfer a patient from a non-PCI hospital to a PCI-capable hospital. In many rural areas, primary PCI is not readily available and either transfer to a PCI center must be arranged or fibrinolytic therapy administered. In general, if the added time to obtain primary PCI over fibrinolytic therapy is more than 60 minutes, the mortality advantage gained by primary PCI is felt to be largely lost.[38] In many rural hospitals, fibrinolytic agents are the standard of care due to the long distances to the closest primary PCI centers. The focus at these institutions should be on initiating fibrinolytics as quickly as possible.

Table 90.3

Contraindications to fibrinolytic therapy in the management of STEMI

Absolute Contraindications
Any prior cerebral hemorrhage
Known structural central nervous system lesion (arteriovenous malformation, tumor, etc.)
Ischemic stroke within 3 mo UNLESS acute ischemic stroke of <3 h onset
Significant closed head or facial injury within 3 mo
Suspicion of aortic dissection
Active bleeding (excluding menses) or bleeding disorders
Relative Contraindications
History of chronic, severe, and poorly controlled hypertension, or severe hypertension on admission (systolic blood pressure > 180 mmHg or diastolic blood pressure > 100 mmHg)
Traumatic or prolonged (>10 min) CPR or noncompressible vascular punctures
Major surgery or internal bleeding within 3–4 wk
Other central nervous disease (structural or dementia) not noted above
Pregnancy
Active peptic ulcer disease
Current use of anticoagulants (the higher the INR, the higher the risk of bleeding)
Prior exposure to or prior allergic reaction to streptokinase or anistreplase, if using these agents

Adapted from Antman EM, Anbe DT, Armstrong PW, et al. ACC/AHA guidelines for the management of patients with ST-elevation myocardial infarction. *Circulation* 2004;110:588–636.

Fibrinolytic Therapy

Among the first studies to demonstrate the effectiveness of fibrinolytic therapy for the treatment of STEMI was the GISSI trial published in 1986.[41] In this study, streptokinase used to treat STEMI was associated with a significant reduction in mortality at 21 days (10.7% vs. 13%; RR, 0.81; $P = 0.0002$). Early administration of fibrinolytic therapy is critical to maximizing its effectiveness in restoring coronary blood flow. In general, the earlier fibrinolytics are administered after the onset of symptoms, the larger the mortality benefit that is derived from this therapy.[42] Currently, it is recommended that fibrinolytic therapy can be given to patients up to 12 hours after the onset of symptoms. Those receiving fibrinolytic agents more than 12 hours after the onset of symptoms derive little benefit, while those who receive fibrinolytic agents within 1 hour from the onset of symptoms have a significant reduction in mortality.

Several fibrinolytic agents are available and have been studied for the treatment of STEMI (see Chapter 112). These include streptokinase, tissue plasminogen activator (tPA; alteplase), reteplase, and tenecteplase (TNK). All agents can be used for this indication, but tenecteplase and reteplase are generally favored due to their relative ease of bolus dosing.

Overall, the choice of which fibrinolytic agent to use is less important than the early and rapid administration of fibrinolysis to appropriate patients without contraindications suffering from an acute STEMI (Table 90.3). In many parts of the world, fibrinolytics remain the reperfusion therapy of choice, largely due to the unavailability of primary PCI. For those that do not have resolution of anginal symptoms or at least 50% of the ST-segment elevation on ECG 1 hour after administration of fibrinolytics, plans should be made for urgent rescue PCI with a cardiac catheterization team.

THE ACUTE MANAGEMENT OF NSTEMI/UA

Assessing Risk in those with NSTEACS

After the initial triage, patients presenting with ACS without ST-segment elevation (non–ST-elevation ACS [NSTEACS]) or hemodynamic compromise can be evaluated in a more systematic manner, with the intent of determining the probability that the patient's symptoms are due to coronary artery disease (CAD). This involves a careful review of the patient's symptoms and physical findings, analyzing the ECG for ischemic changes such as ST-segment depression or T-wave inversion, reviewing the patient's medical record for a personal history of CAD or risk factors for heart disease, and evaluating serial serum biomarkers such as troponin to assess for MI.

The initial history is critical to assessing the probability of ACS. Typical angina is usually described as a deep substernal chest discomfort that can radiate to the arm and jaw and is associated with shortness of breath or diaphoresis. In ACS, these symptoms may present with rest pain or accelerating angina that occurs with lessening degrees of activity. For older patients, women, and diabetics, these typical features may not be present and are unreliable. Despite the widely held belief to the contrary, the relief of chest pain by sublingual nitroglycerin does not predict the presence of ACS.[43]

The ECG provides important prognostic and diagnostic information at the time of initial triage. It is an essential component of the early triage algorithm. Findings of old Q waves on the ECG are important because they indicate likely prior MI and a higher risk for CAD. A normal ECG does not exclude the diagnosis of ACS, but patients who have an unchanged ECG when compared to a prior one have a low risk of adverse events.[44]

Cardiac Biomarkers

The evaluation of cardiac biomarkers is an essential component to early triage and risk stratification. The clinical diagnosis of MI hinges on the detectable elevation of these cardiac biomarkers in conjunction with other findings of ischemia such as symptoms or ECG changes. Biomarkers are released into the peripheral circulation from infarcted cardiac myocytes when necrosis occurs (Table 90.4). The most widely used cardiac biomarkers currently are CK-MB and the cardiac troponins I and T (TnI and TnT). Myoglobin is rarely used clinically today. These proteins are found in high concentrations within the cardiac myocytes, making them specific for myocardial cell damage, and circulate at low levels within the peripheral circulation in the absence of cardiac myocyte necrosis (FIGURE 90.2). The cardiac troponins are highly sensitive and specific for MI and may be detectable in the blood as soon as 2 hours after the onset of symptoms and may stay elevated for as long as 14 days after an infarct.

The elevation of cardiac biomarkers has important and well-validated prognostic significance beyond that obtainable from the ECG and the patient's clinical parameters. An elevated troponin level is independently associated with an increased risk of death, and there is a direct and proportional relationship between the degree of elevation and this risk of death[45–47] (FIGURE 90.3).

Risk Stratification in NSTEACS Using Risk Scores

Once other causes of the patient's symptoms have been excluded and ACS is felt to be the diagnosis, early risk stratification is important to determining appropriate management. Doing this can aid in selecting the appropriate level of care needed for the patient, guiding the choices of the most appropriate medical therapies, and assisting in deciding between an early invasive strategy in the catheterization laboratory and a more conservative strategy initially with medical therapy.

Early risk stratification can help differentiate patients with NSTEMI and UA who often present with very heterogeneous symptoms. Multiple factors determine the likelihood of obstructive CAD and prognosis. Several risk scores have been developed to help clinicians identify high-risk patients rapidly. These include the Thrombolysis in Myocardial Infarction (TIMI) risk score and the Global Registry of Acute Coronary Events (GRACE) risk score (FIGURE 90.4 and Table 90.5).[48,49]

The TIMI risk score was developed as a simple tool comprising seven questions rating risk on presentation designed to assess patients with suspected UA or NSTEMI. For details on the score, see legend to Table 90.5. The TIMI risk score provides an estimate of death, MI, or recurrent ischemia requiring urgent revascularization within 14 days from presentation. It has been validated in several studies and is a quick and relatively easy bedside tool to help clinicians stratify patients based on risk.

The GRACE prediction score is a model that can be used to predict in-hospital mortality and mortality from discharge to 6 months in patients presenting with ACS.[49,50] The GRACE model for predicting 6-month postdischarge mortality uses factors such as age, personal history of heart failure and MI, initial blood pressure and pulse, ECG, and laboratory findings (FIGURE 90.4). It is a useful clinical tool for patients with NSTEMI, STEMI, and UA to predict 6-month mortality.

Initial Invasive Versus Initial Conservative Therapy

For patients presenting with NSTEMI or UA, one of two treatment pathways is usually decided upon based on patient risk and clinical stability. One pathway is labeled the "early invasive" strategy, while the other pathway for treatment is called the "initial conservative" or "selective invasive" strategy.[3] This terminology refers to the chosen angiographic strategy for the patient and the intended timing of diagnostic angiography with planned PCI. In both pathways, patients receive the usual

Table 90.4

Cardiac biomarkers

Marker	Advantages	Disadvantages	Point-of-Care Test Available?	Comment	Clinical Recommendation
Cardiac troponins	Powerful tool for risk stratification Greater sensitivity and specificity than CK-MB Detection of recent MI up to 2 wk after onset Useful for selection of therapy Detection of reperfusion	Low sensitivity in very early phase of MI (<6 h after symptom onset) and requires repeat measurement at 8 to 12 h, if negative Limited ability to detect late minor reinfarction	Yes	Data on diagnostic performance and potential therapeutic implications increasingly available from clinical trials	Useful as a single test to efficiently diagnose NSTEMI (including minor myocardial damage), with serial measurements. Clinicians should familiarize themselves with diagnostic "cutoffs" used in their local hospital laboratory
CK-MB	Rapid, cost-efficient, accurate assays Ability to detect early reinfarction	Loss of specificity in setting of skeletal muscle disease or injury, including surgery Low sensitivity during very early MI (<6 h after symptom onset) or later after symptom onset (more than 36 h) and for minor myocardial damage (detectable with troponins)	Yes	Familiar to majority of clinicians	Prior standard and still acceptable diagnostic test in most clinical circumstances
Myoglobin	High sensitivity Useful in the early detection of MI Detection of reperfusion Most useful in ruling out MI	Very low specificity in the setting of skeletal muscle injury or disease Rapid return to normal range limits sensitivity for later presentations	Yes	More convenient early marker than CK-MB isoforms because of greater availability of assays for myoglobin; rapid-release kinetics make myoglobin useful for noninvasive monitoring of reperfusion in patients with established MI	

ACS, acute coronary syndrome; CK-MB, MB fraction of creatine kinase; ECG, electrocardiogram; MI, myocardial infarction; NSTEMI, non–ST-elevation MI.
Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

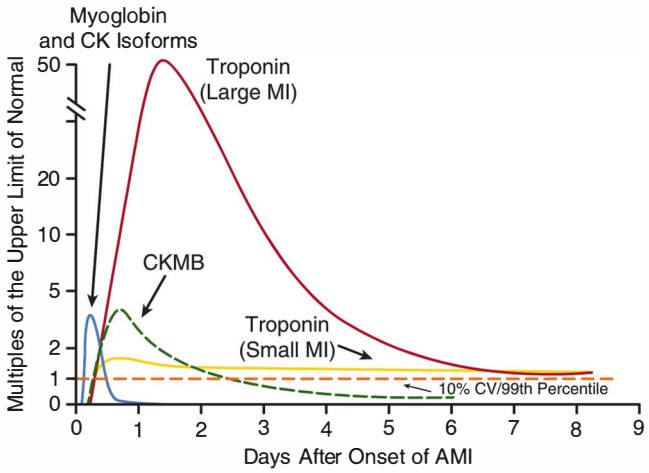


FIGURE 90.2 Release of biomarkers into the serum after acute myocardial infarction. The biomarkers are plotted showing the multiples of the cutoff for acute myocardial infarction (AMI) over time. The *dashed horizontal line* shows the upper limit of normal (ULN; defined as the 99th percentile from a normal reference population without myocardial necrosis; the coefficient of variation of the assay should be 10% or less). The earliest rising biomarkers are myoglobin and CK isoforms (*leftmost curve*). CK-MB (*dashed curve*) rises to a peak of 2 to 5 times the ULN and typically returns to the normal range within 2 to 3 days after AMI. The cardiac-specific troponins show small elevations above the ULN in small infarctions (e.g., as is often the case with NSTEMI) but rise to 20 to 50 times the ULN in the setting of large infarctions (e.g., as is typically the case in STEMI). The troponin levels may stay elevated above the ULN for 7 days or more after AMI. CK, creatine kinase; CKMB, MB fraction of creatine kinase; CV, coefficient of variation; MI, myocardial infarction. (Modified from Shapiro BP, Jaffe AS. Cardiac biomarkers. In: Murphy JG, Lloyd MA, eds. *Mayo clinic cardiology: concise textbook*, 3rd ed. Rochester, MN: Mayo Clinic Scientific Press and New York: Informa Healthcare USA, 2007:773–780. Used with permission of Mayo Foundation for Medical Education and Research. Also adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.)

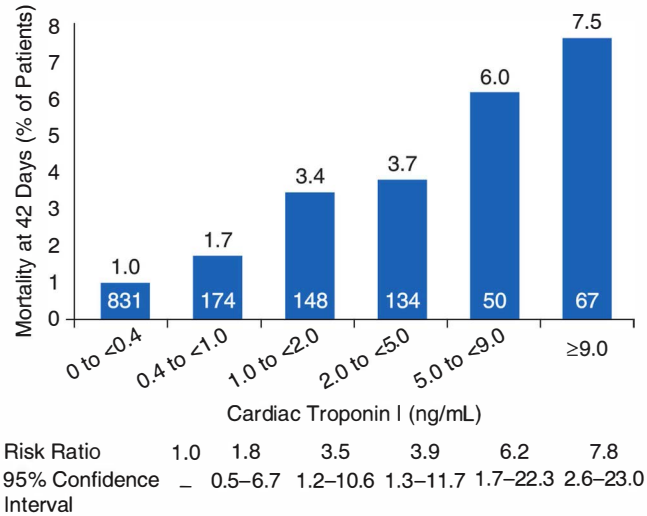


FIGURE 90.3 Stepwise association between elevations in troponin levels and death. (From Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304. Originally from Ohman EM, et al. Cardiac troponin T levels for risk stratification in acute myocardial ischemia. GUSTO IIA Investigators. *N Engl J Med* 1996;335(18):1333–1341.)

medical therapy for NSTEMI and UA that includes antianginal, antiplatelet, and anticoagulant therapy (FIGURES 90.5 and 90.6).

In the early invasive strategy, patients usually undergo coronary angiography within 24 to 48 hours after admission. In the initial conservative strategy, patients do not undergo coronary angiography initially unless they have failed medical therapy or have documented ischemia, such as that demonstrated by a stress test, despite optimal medical therapy.

Deciding between an early invasive and conservative strategy is based largely on patient characteristics. High-risk features favor an early invasive strategy. These may include refractory ischemia, elevated cardiac biomarkers, dynamic ECG changes, hemodynamic or arrhythmic instability, prior revascularization, an elevated risk score (TIMI or GRACE), or left ventricular dysfunction (Table 90.6). In addition, patients treated with an early invasive strategy include those without ST-segment elevation who undergo urgent cardiac catheterization due to clinical instability or refractory symptoms despite medical therapy. Patients at lower risk, or those with extensive comorbidities, where the risks of angiography may be considered to be too high, are typically treated with an initial conservative strategy.

For patients being treated with a conservative strategy, medical therapy is pursued with the intent of delaying the decision to proceed to coronary angiography until further clinical data are obtained. Generally in this group, the decision to go to diagnostic angiography is based upon results of a functional stress test showing objective evidence of ischemia, or upon persistent symptoms despite medical therapy.

The reasoning behind a conservative strategy is to avoid an invasive procedure and its inherent risk until the patient demonstrates objective evidence for ischemia or has not responded to medical therapy. The benefits to this strategy include the fact that many patients will stabilize on medical therapy and not require coronary angiography, thus reducing the use of an invasive and costly procedure. Patients treated in this pathway should have a stress test prior to discharge as well as an assessment for left ventricular function.

The early invasive strategy provides an angiographic method for risk stratification and a definitive evaluation for CAD. This strategy can differentiate those patients with no CAD from

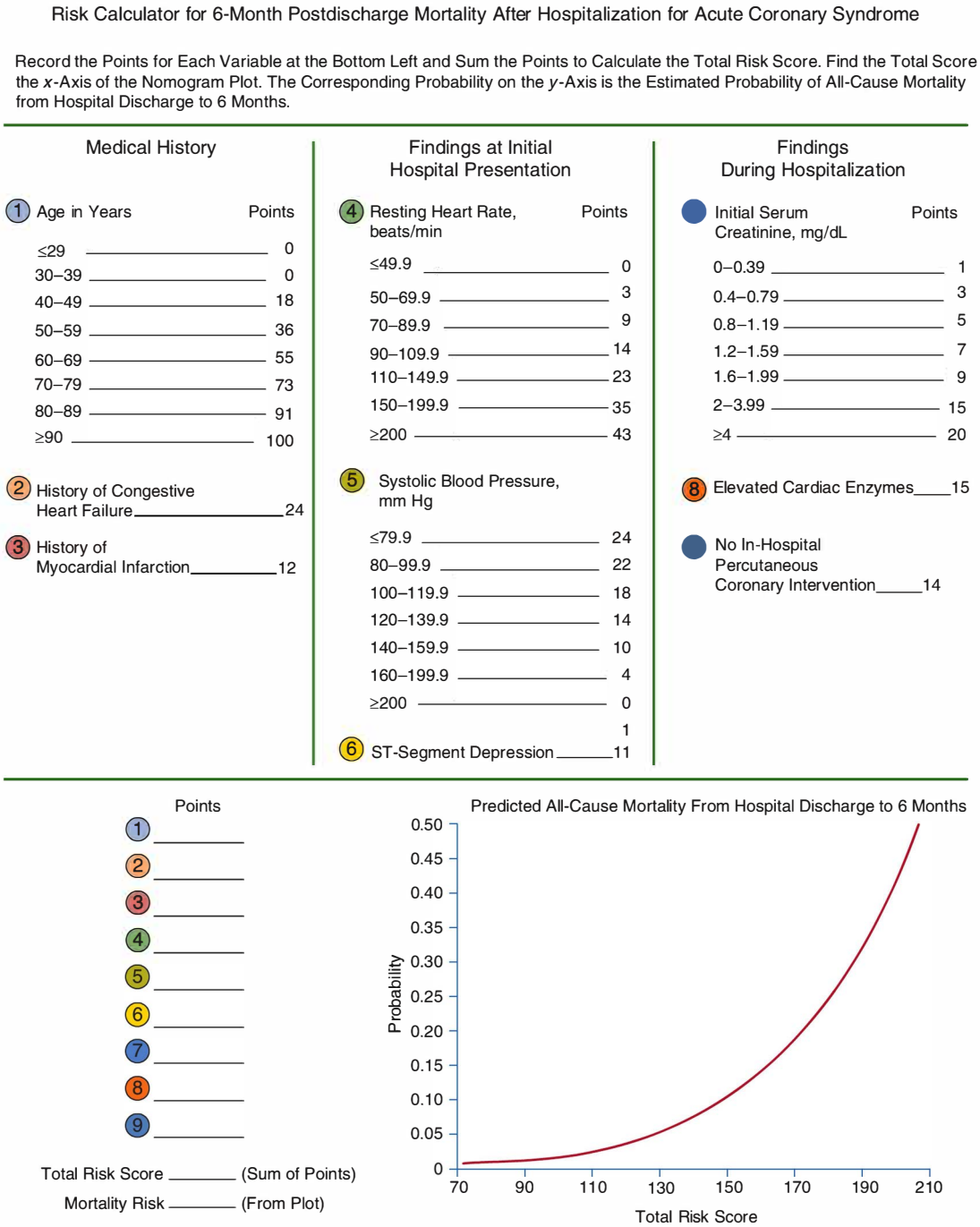


FIGURE 90.4 GRACE risk prediction model. (Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304. Originally from Eagle KA, Lim MJ, Dabbous OH, et al. A validated prediction model for all forms of acute coronary syndrome: estimating the risk of 6-month postdischarge death in an international registry. *JAMA* 2004;291:2727–2733. Copyright © 2004 American Medical Association.)

those with severe CAD who may benefit from revascularization.[51,52] Furthermore, percutaneous revascularization of the culprit lesion may lead to a reduced risk for rehospitalization and reduced angina after discharge.[53]

Several trials have compared the early invasive strategy to the initial conservative approach. A meta-analysis of these trials concluded that a routine invasive approach is superior to a conservative strategy in reducing death or MI (14.4% vs. 12.2%;

Table 90.5

Thrombolysis in myocardial infarction risk score[a]

TIMI Risk Score	All-Cause Mortality, New or Recurrent MI, or Severe Recurrent Ischemia Requiring Urgent Revascularization through 14 d After Randomization (%)
0–1	4.7
2	8.3
3	13.2
4	19.9
5	26.2
6–7	40.9

[a]The TIMI risk score is determined by the sum of the presence of seven variables at admission: 1 point is given for each of the following variables: age 65 years or older; at least three risk factors for CAD; prior coronary stenosis of 50% or more; ST-segment deviation on ECG presentation; at least two anginal events in prior 24 hours; use of aspirin in prior 7 days; elevated serum cardiac biomarkers. Prior coronary stenosis of 50% or more remained relatively insensitive to missing information and remained a significant predictor of events.
MI, myocardial infarction.
Adapted from Antam EM, Cohen M, Bemink PH, et al. The TIMI risk score for unstable angina/non-ST elevation MI: a method for prognostication and therapeutic decision making. *JAMA* 2000;284:835–842; Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

OR = 0.82; 95% CI, 0.72 to 0.93).[54] This analysis showed an 18% relative reduction in death or MI, as well as a sustained reduction in mortality following discharge from the hospital with the invasive approach. In addition, the invasive strategy led to a reduction in angina, fewer hospitalizations, and improvements in quality of life.

A second meta-analysis of more contemporary randomized trials in NSTEMI also showed benefit with an early invasive approach.[55] The results showed a long-term improvement in mortality and morbidity with the invasive strategy, including a reduction in MI and rehospitalizations (FIGURES 90.7 and 90.8). These improvements seem to be sustained for years after the event. An analysis from FRISC-II (FRagmin and fast Revascularization during InStability in Coronary artery disease-II) showed that the reduction in death or nonfatal MI is sustained for up to 5 years, and data from RITA-3 (Third Randomized Intervention Treatment of Angina) also demonstrated a reduction in death and MI at 5 years.[56,57]

Overall, the objective is to identify those patients who are most likely to benefit from an aggressive early invasive approach and minimize the exposure to potential harm in those less likely to benefit. The decision to proceed to early coronary angiography, versus a delayed and more conservative approach, is an individual one that should consider the wishes of the patient and family as well as the patient's comorbidities. It must also be emphasized that ACS is a dynamic process where the clinical stability of a patient and the perceived benefit of an aggressive approach may change rapidly.

Mechanical Reperfusion

Mechanical reperfusion of the coronary arteries can be performed percutaneously or surgically. PCI can restore coronary blood flow through a number of approaches that include balloon angioplasty, intracoronary stenting, or removal of existing thrombus with thrombectomy devices. Used in the appropriate settings, such as for the treatment of acute MI, these therapies can be life saving. Surgical revascularization with coronary artery bypass grafting (CABG) involves the surgical implantation of venous or arterial grafts to the coronary arteries. This procedure is generally reserved for those patients with severe diffuse CAD not easily approached percutaneously.

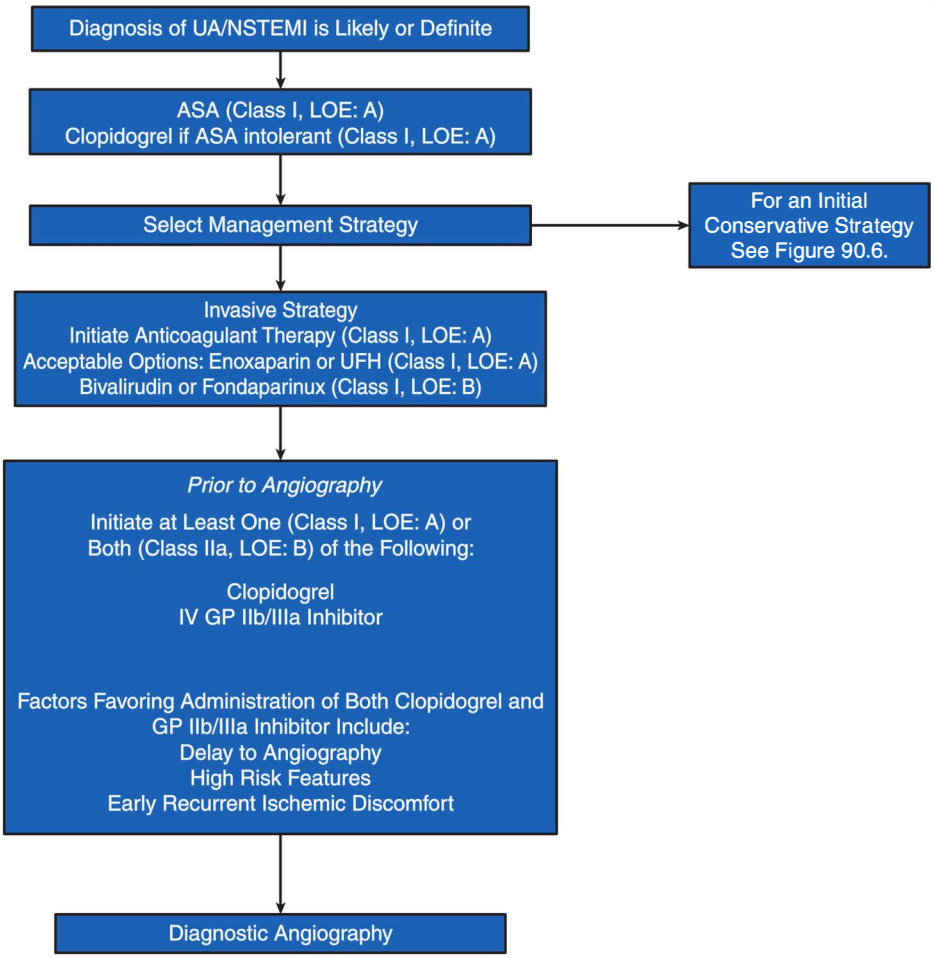
Percutaneous Coronary Interventions

The development of intracoronary stents marked a significant improvement in the ability to revascularize coronary arteries percutaneously. Previously, balloon angioplasty alone was plagued by very high rates of coronary restenosis. The implantation of bare metal stents significantly reduced the rate of restenosis, but the problem is still seen in about 25% of cases where bare metal stents are used. Systemically delivered therapies, except for cilostazol, have not been able to demonstrate any attenuation in the rate of restenosis.[58] The development of drug-eluting stents has been able to address the problem of restenosis due to intimal hyperplasia by the local delivery of drugs that inhibit the proliferative and inflammatory response to vascular injury that occurs with stent implantation. The currently available stents within the United States elute antiproliferative drugs such as paclitaxel, sirolimus, everolimus, and zotarolimus. Early studies of drug-eluting stents showed a marked reduction in the rates of restenosis by about 75% to 90%.[59–61] However, there is a tradeoff with an increased risk of late stent thrombosis that is theorized to be a result of inhibited endothelial coverage of the stent struts from the eluting drug.

An evolving tool in the armamentarium of the interventional cardiologist for the treatment of intracoronary thrombus in the setting of ACS is intracoronary thrombectomy. These devices use a catheter-based system to mechanically remove with suction visible coronary thrombus seen on angiography. The routine use of thrombectomy devices has been shown to improve measures of myocardial reperfusion, reduce infarct size, improve the incidence of LV remodeling, and reduce mortality when performed at the time of PCI in the management of STEMI, although the data supporting this is still somewhat mixed.[62]

Coronary Artery Bypass Grafting

For select patients, there appears to be a survival benefit to surgical revascularization over PCI. A patient registry of consecutive patients with CAD compared 5-year survival rates for medical management, PCI, and CABG between 1984 and 1990.[63–66] All patients with three-vessel disease and those with two-vessel disease with a severe proximal left anterior descending artery stenosis had a survival benefit with CABG over medical therapy or PCI. Another subgroup that appears to derive benefit from CABG over medical or percutaneous therapy for CAD includes those with diabetes. In the BARI (Bypass Angioplasty Revascularization Investigation), survival benefit for CABG was restricted to those with diabetes (76.4% survival after CABG vs. 55.7% after PCI; $P = 0.001$).[67]



LOE: Level of Evidence
UFH: Unfractionated Heparin

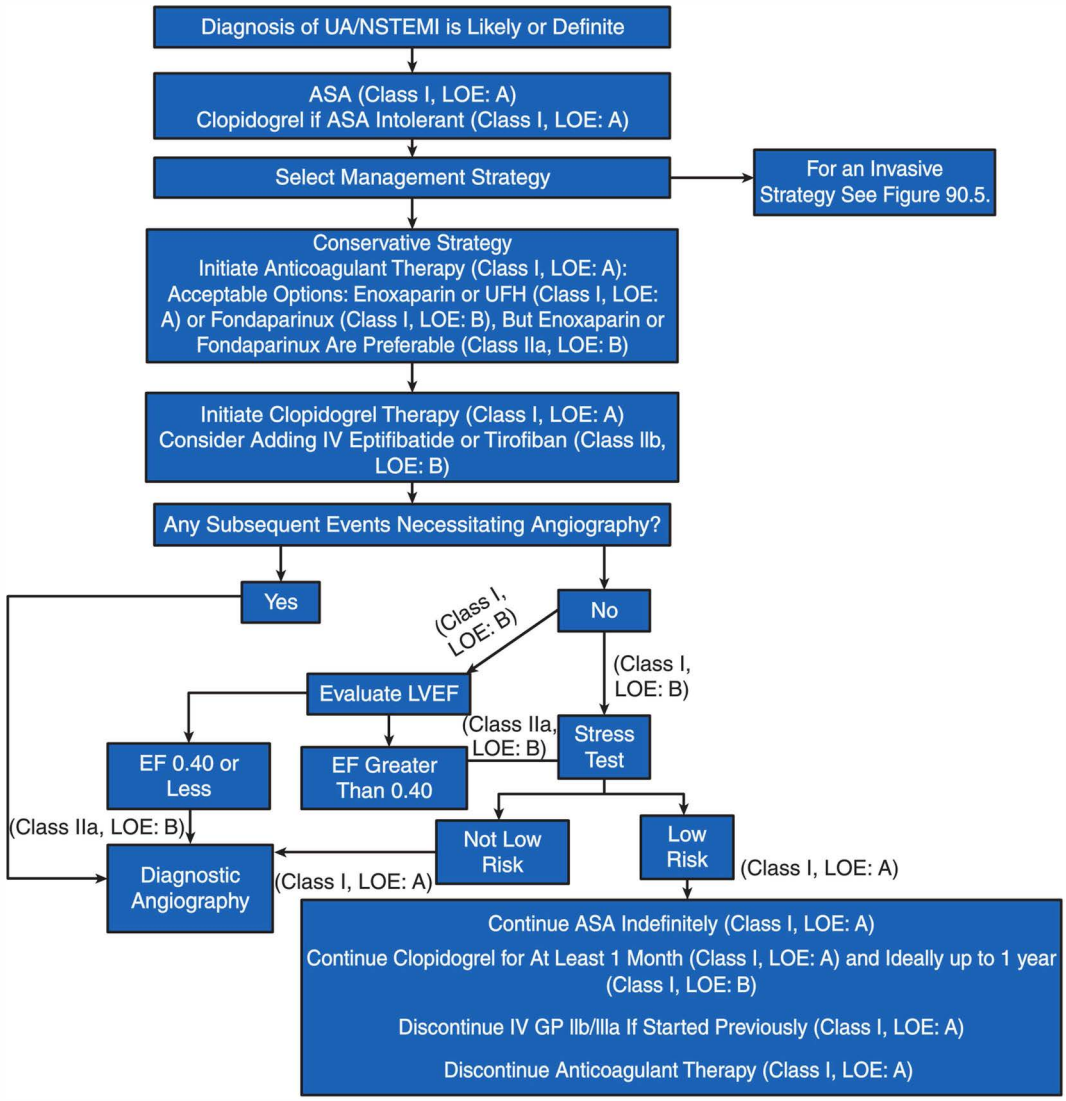
FIGURE 90.5 Treatment algorithm for UA/NSTEMI patients treated with initial invasive strategy. (From Anderson JL, Adams CD, Antman EM, et al., ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.)

The appropriate management of antithrombotic therapy prior to, during, and after CABG surgery is critical to improving the outcome of the patient. Prior to surgery, it is important to assess for the risk of bleeding and thrombotic complications. Historical features such as a history of previous open-heart surgeries, end-stage renal disease, a personal or family history of coagulation disorders, and immobility are all important factors in the risk of both bleeding and thrombotic complications. Table 90.7 lists the timing for discontinuation of antiplatelet and anticoagulant therapies prior to CABG as recommended by the AHA/ACC practice guidelines.

Although many of the risks are not modifiable, they should guide appropriate antithrombotic therapy to minimize risk. Preoperatively, an effort should be made to minimize the operative risk of bleeding by discontinuation of unnecessary antithrombotic agents. Aspirin use in the days leading up to surgery has been associated with greater blood loss, but not clearly linked to other adverse outcomes.[68] However, the use of the ADP receptor antagonists like clopidogrel prior to surgery has been linked to increased bleeding and adverse events like increased chest tube drainage.[69] Currently, it is recommended that clopidogrel be discontinued 5 to 7 days prior to any planned cardiac surgery to reduce the risk of bleeding complications. Additionally, the preoperative use of GP IIb/IIIa inhibitors such as abciximab and the vitamin K antagonists such as warfarin is also linked to an increased risk of bleeding.[70,71] Careful monitoring of anticoagulation at the time of bypass, platelet transfusions, and discontinuation of warfarin allowing for the normalization of the international normalized ratio (INR) are measures that may reduce bleeding risk.

THERAPY—MEDICAL MANAGEMENT OF ACS

Patients being treated for ACS should be admitted to an inpatient unit for observation and continuous telemetry monitoring. Treatment should be carried out in a dedicated cardiac unit with physicians and staff trained in the evaluation and management of acute coronary disease. The evaluation of these patients



LOE: Level of Evidence
UFH: Unfractionated Heparin

FIGURE 90.6 Treatment algorithm for UA/NSTEMI patients treated with an initial conservative strategy. (Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.)

should include monitoring for symptoms of recurrent ischemia, hemodynamic instability, or arrhythmic complications from ACS. For those with continued ischemic symptoms, hemodynamic compromise, or significant rhythm disturbances, admission to a coronary care unit (CCU) is advisable. Admission to the CCU allows for a more frequent assessment of stability, quick defibrillation in the event of ventricular fibrillation or pulseless ventricular tachycardia, and staff who are trained to perform critical care.

The treatment of ACS patients in dedicated cardiac units with physicians and staff knowledgeable of the therapies used to treat CAD promotes the use of evidence-based therapeutics. The acute therapy of ACS can be divided into four groups of medical therapy: (a) antiplatelet therapies, (b) anticoagulants, (c) anti-ischemic therapies, and (d) heart failure and/or secondary prevention therapies.

Antiplatelet Therapy

The currently available classes of antiplatelet therapy that are approved for use in ACS include aspirin, the ADP receptor ($P2Y_{12}$) antagonists, and the GP IIb/IIIa inhibitors (see Chapter 111) (FIGURE 90.8). Aspirin should be initiated with the first medical contact in anyone suspected of having ACS, unless a contraindication exists. A $P2Y_{12}$ inhibitor, such as clopidogrel, should be used in the medical management of ACS for those in

Table 90.6

Clinical features favoring an early invasive strategy versus an initial conservative strategy

Preferred Strategy	Patient Characteristics
Invasive	Recurrent angina or ischemia at rest or with low-level activities despite intensive medical therapy Elevated cardiac biomarkers (TnT or TnI) New or presumably new ST-segment depression Signs or symptoms of HF or new or worsening mitral regurgitation High-risk findings from noninvasive testing Hemodynamic instability Sustained ventricular tachycardia PCI within 6 mo Prior CABG High-risk score (e.g., TIMI, GRACE) Reduced left ventricular function (LVEF < 40%)
Conservative	Low-risk score (e.g., TIMI, GRACE) Patient or physician preference in the absence of high-risk features

CABG, coronary artery bypass graft; GRACE, Global Registry of Acute Coronary Events; HF, heart failure; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; TIMI, Thrombolysis In Myocardial Infarction; TnI, troponin I; TnT, troponin T.
Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

whom a noninvasive strategy is selected and is generally started before diagnostic angiography (upstream) in those undergoing an invasive strategy. The GP IIb/IIIa inhibitors are generally used at the time of PCI for their powerful antiplatelet effect and reduction in acute stent thrombosis. However, they can also be used upstream in conjunction with, or instead of, a $P2Y_{12}$ inhibitor in those at high risk where early cardiac catheterization is anticipated. Table 90.8 lists the AHA/ACC class I recommendations for antiplatelet use in UA or acute MI.

Aspirin

Some of the oldest and strongest evidence for antiplatelet therapy in the treatment of ACS is with aspirin.[72,73–75] Through irreversible inhibition of COX-1, aspirin inhibits the formation of TXA_2 and inhibits platelet aggregation. Aspirin is useful for both the acute treatment of ACS and secondary prevention of cardiovascular events and is one of the most important therapies for ACS.

For patients with acute STEMI, aspirin significantly reduces mortality. The ISIS-2 trial (Second International Study of Infarct

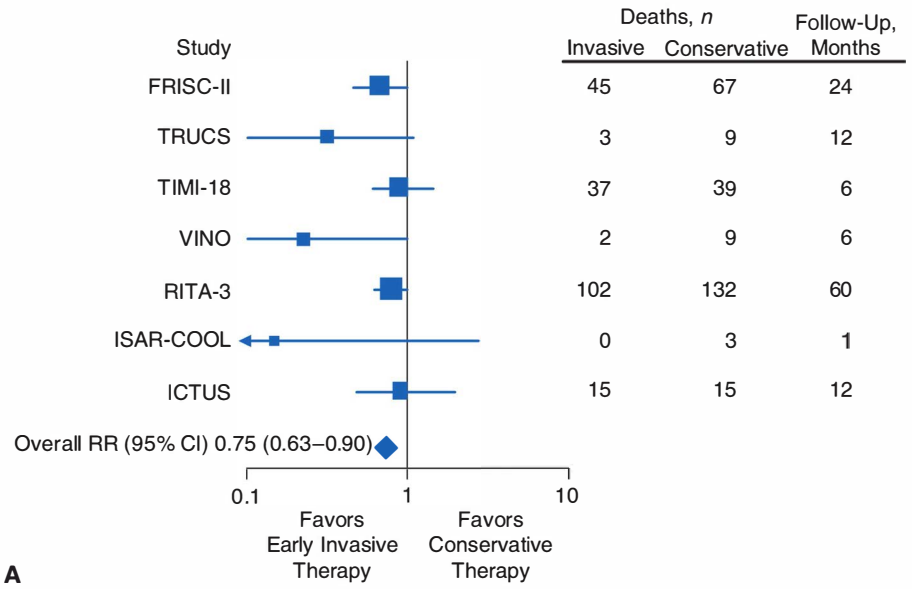
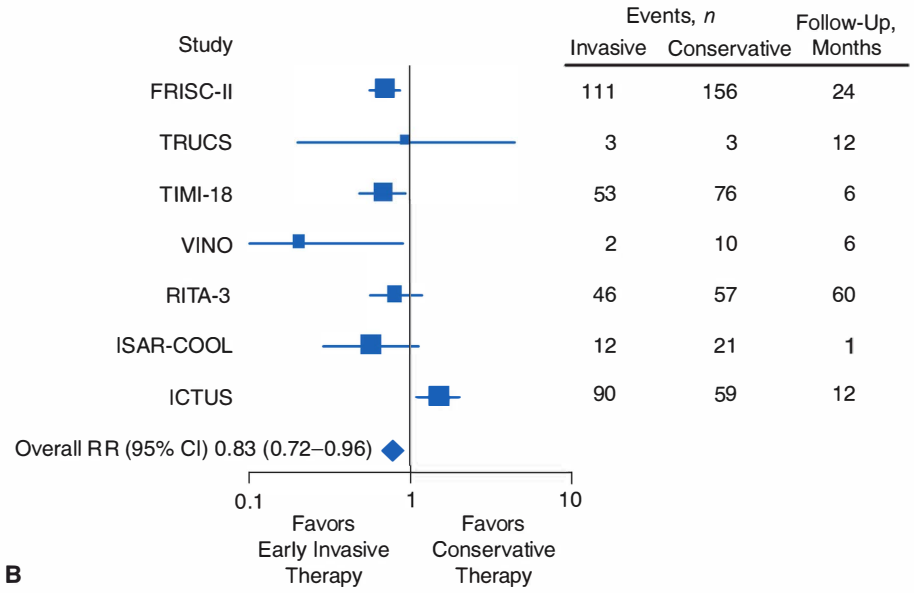
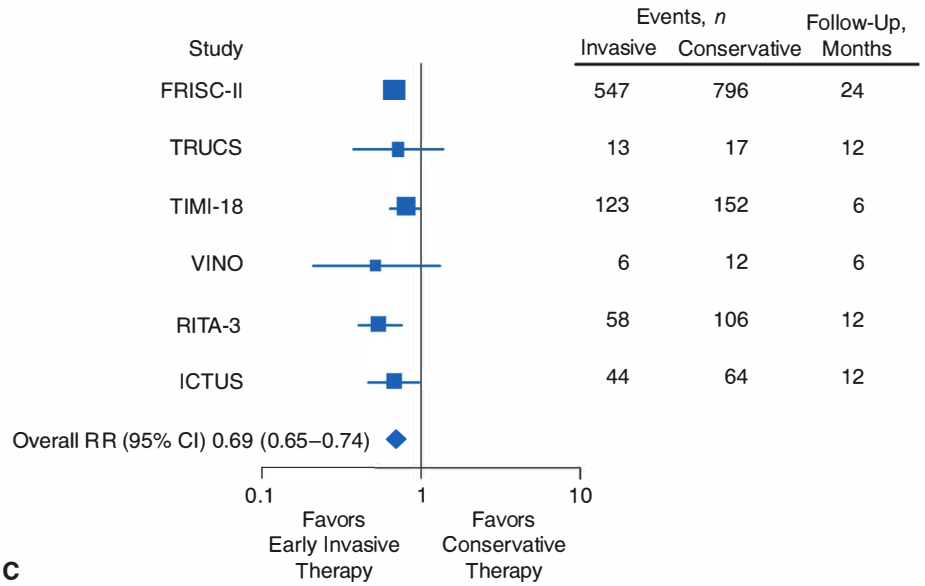


FIGURE 90.7 Summary of trials comparing early invasive versus conservative strategy in NSTEMI. **A**: Relative risk of all-cause mortality for early invasive therapy compared with conservative therapy at a mean follow-up of 2 years.



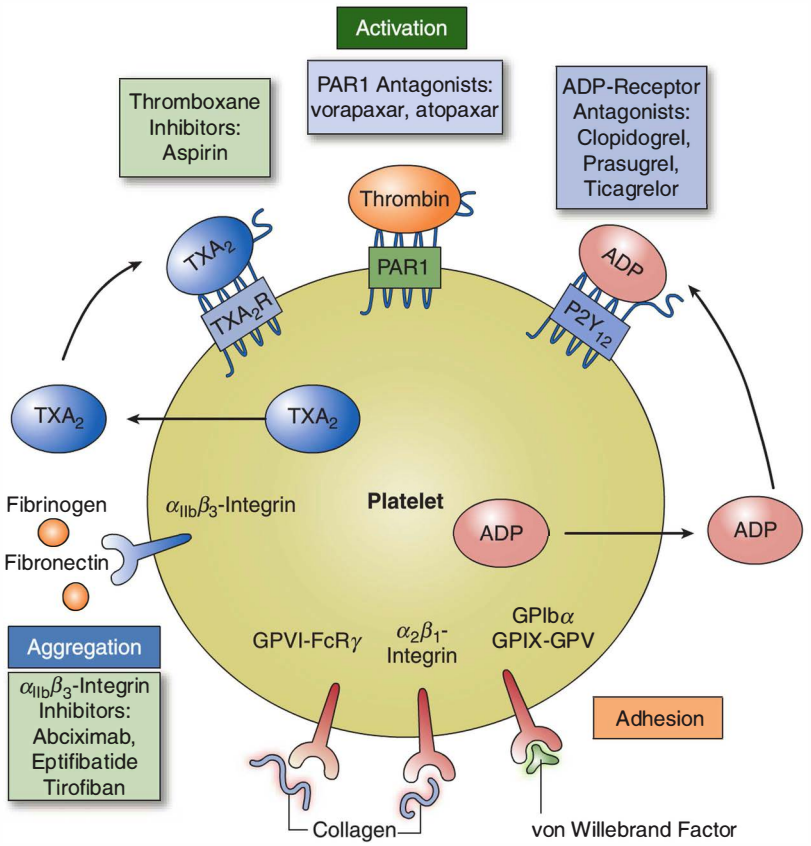
B



C

FIGURE 90.7 (*Continued*) **B**: Relative risk of recurrent nonfatal myocardial infarction for early invasive therapy compared with conservative therapy at a mean follow-up of 2 years. **C**: Relative risk of recurrent UA resulting in rehospitalization for early invasive therapy compared with conservative therapy at a mean follow-up of 13 months. CI, confidence interval; FRISC-II, FRagmin and fast Revascularization during InStability in Coronary artery disease; ICTUS, Invasive versus Conservative Treatment in Unstable coronary Syndromes; ISAR-COOL, Intracoronary Stenting with Antithrombotic Regimen COOLing-off study; RITA-3, third Randomized Intervention Treatment of Angina trial; RR, relative risk; TIMI-18, Thrombolysis In Myocardial Infarction-18; TRUCS, Treatment of Refractory Unstable angina in geographically isolated areas without Cardiac Surgery; UA/NSTEMI, unstable angina/non–ST-elevation myocardial infarction; VINO, Value of first day angiography/angioplasty In evolving Non–ST-segment elevation myocardial infarction: Open multicenter randomized trial. (From Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304; Colombo A, et al. Randomized study to assess the effectiveness of slow- and moderate-release polymer-based paclitaxel-eluting stents for coronary artery lesions. *Circulation* 2003;108(7):788–794; Bavry AA, Kumbhani DJ, Rassi AN, et al. Benefit of early invasive therapy in acute coronary syndromes a meta-analysis of contemporary randomized clinical trials. *J Am Coll Cardiol* 2006;48(7):1319–1325. Copyright 2006, with permission from Elsevier.)

FIGURE 90.8 Targets for antiplatelet therapy.



Survival) demonstrated a significant 23% reduction in 5-week cardiovascular mortality with aspirin—a treatment effect similar to that seen with streptokinase in the same trial.[76] Similarly, powerful results were demonstrated with NSTEMI/UA. Some of the earlier trials with aspirin in NSTEMI/UA showed marked reductions in MI or death by more than 50%.[75,77]

The dose of aspirin used clinically varies widely, and there remains debate as to the optimal dose. A meta-analysis of over 140,000 patients showed similar reductions in outcomes, with doses ranging from 75 to 1,500 mg but a reduction in efficacy with doses <75 mg.[78] Data from the CURE trial (Clopidogrel in Unstable angina to prevent Recurrent Events) showed no difference in ischemic outcomes with different doses of aspirin but an increasing risk of bleeding with escalating doses.[79] The CURRENT OASIS-7 (Clopidogrel Optimal Loading Dose Usage to Reduce Recurrent Events/Optimal Antiplatelet Strategy for

Table 90.7

Timing of discontinuation of antithrombotics prior to CABG

Continue acetylsalicylic acid (ASA). *(Level of Evidence: A)*
Discontinue clopidogrel 5 to 7 d before elective CABG. *(Level of Evidence: B)* More urgent surgery, if necessary, may be performed by experienced surgeons if the incremental bleeding risk is considered acceptable. *(Level of Evidence: C)*
Discontinue intravenous GP IIb/IIIa inhibitor (eptifibatide or tirofiban) 4 h before CABG. *(Level of Evidence: B)*
Anticoagulant therapy should be managed as follows:
1. Continue UFH. *(Level of Evidence: B)*
2. Discontinue enoxaparin 12 to 24 h before CABG and dose with UFH per institutional practice. *(Level of Evidence: B)*
3. Discontinue fondaparinux 24 h before CABG and dose with UFH per institutional practice. *(Level of Evidence: B)*
4. Discontinue bivalirudin 3 h before CABG and dose with UFH per institutional practice. *(Level of Evidence: B)*

Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

Table 90.8

Class I AHA/ACC recommendations for antiplatelet therapy for UA/NSTEMI

1. Aspirin should be administered to UA/NSTEMI patients as soon as possible after hospital presentation and continued indefinitely in patients not known to be intolerant of that medication. *(Level of Evidence: A)* (FIGURES 90.7 and 90.8)
2. Clopidogrel (loading dose followed by daily maintenance dose) should be administered to UA/NSTEMI patients who are unable to take ASA because of hypersensitivity or major gastrointestinal intolerance. *(Level of Evidence: A)* (FIGURES 90.7 and 90.8)
3. In UA/NSTEMI patients with a history of gastrointestinal bleeding, when ASA and clopidogrel are administered alone or in combination, drugs to minimize the risk of recurrent gastrointestinal bleeding (e.g., proton-pump inhibitors) should be prescribed concomitantly. *(Level of Evidence: B)*
4. For UA/NSTEMI patients in whom an initial invasive strategy is selected, antiplatelet therapy in addition to aspirin should be initiated before diagnostic angiography (upstream) with either clopidogrel (loading dose followed by daily maintenance dose) or an intravenous GP IIb/IIIa inhibitor. (*Level of Evidence: A*) Abciximab as the choice for upstream GP IIb/IIIa therapy is indicated only if there is no appreciable delay to angiography and PCI is likely to be performed; otherwise, intravenous eptifibatide or tirofiban is the preferred choice of GP IIb/IIIa inhibitor. (*Level of Evidence: B*)
5. For UA/NSTEMI patients in whom an initial conservative (i.e., noninvasive) strategy is selected, clopidogrel (loading dose followed by daily maintenance dose) should be added to ASA and anticoagulant therapy as soon as possible after admission and administered for at least 1 mo *(Level of Evidence: A)* and ideally up to 1 y. *(Level of Evidence: B)* (FIGURE 90.6)
6. For UA/NSTEMI patients in whom an initial conservative strategy is selected, if recurrent symptoms/ischemia, HF, or serious arrhythmias subsequently appear, then diagnostic angiography should be performed. *(Level of Evidence: A)* (FIGURE 90.6). Either an intravenous GP IIb/IIIa inhibitor (eptifibatide or tirofiban; *Level of Evidence: A*) or clopidogrel (loading dose followed by daily maintenance dose; *Level of Evidence: A)* should be added to ASA and anticoagulant therapy before diagnostic angiography (upstream). *(Level of Evidence: C)*

Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

Interventions) trial was designed, in part, to compare high-dose (300 to 325 mg) to low-dose (75 to 100 mg) aspirin in ACS and showed no difference in its primary outcome of cardiovascular death, MI, or stroke and no difference in major bleeding.[80] Current recommendations from the ACC/AHA guidelines are to use 162 to 325 mg daily after stent implantation, but to use 75 to 162 mg daily for medically treated patients and for those being treated indefinitely on aspirin.[3]

$P2Y_{12}$ Inhibitors

Clopidogrel, ticlopidine, and prasugrel are thienopyridine derivatives that block the binding of ADP to the ADP receptor ($P2Y_{12}$) and inhibit platelet aggregation. Ticagrelor is a nonthienopyridine agent that directly blocks the $P2Y_{12}$ receptor and inhibits platelet aggregation through a similar mechanism. As a group, these four agents can be referred to as the "$P2Y_{12}$ inhibitors." Because the mechanism of action for platelet inhibition is different than that from aspirin, there is felt to be an additive benefit to these drugs when given in conjunction with aspirin.

Clopidogrel

Clopidogrel is currently the most commonly used $P2Y_{12}$ inhibitor. It has largely replaced ticlopidine due to the adverse effects associated with ticlopidine, which include severe neutropenia, gastrointestinal upset, and a risk for a potentially fatal complication known as thrombotic thrombocytopenic purpura (see Chapter 99).[81] When compared to ticlopidine, clopidogrel was better tolerated with similar rates of stent thrombosis and major complications.[82] Table 90.9 lists the major clinical trials evaluating clopidogrel in the management of ACS.

Dual therapy with aspirin plus clopidogrel in NSTEMI/UA was evaluated in the CURE trial.[79] In this study, patients presenting with NSTEMI/UA within 24 hours were randomized to receive aspirin alone or aspirin plus clopidogrel and were followed for 3 to 12 months. The group receiving clopidogrel had a significant reduction in cardiovascular death, stroke, or MI (11.5% vs. 9.3%; $P < 0.001$). The reduction in ischemic events was noted within the first few hours after randomization and was sustained for up to 1 year. This study supports the early administration of clopidogrel to those presenting with NSTEMI/UA, especially in those being treated noninvasively where the risk for CABG-related bleeding is small. Based on this study, treatment with clopidogrel in those medically managed for ACS should continue for at least 1 month, and ideally for up to a year and is given a class I recommendation by the ACC/AHA guidelines (Table 90.8).[3]

A subset of patients from the CURE study who underwent PCI was studied in the PCI-CURE trial.[83] In this analysis, pretreatment with clopidogrel reduced the primary endpoint of cardiovascular death, MI, or urgent target vessel revascularization within 30 days of PCI from 6.4% to 4.5%. In addition, there was a 31% reduction in the endpoint of cardiovascular death or MI. In the CREDO (Clopidogrel for the Reduction of Events During Observation) trial, patients referred for cardiac

Table 90.9

Trials evaluating clopidogrel in ACS

Trial	Setting	Comparator	Reduction in Ischemic Events	Bleeding Risk
CURE	*N* = 12,562 UA/NSTEMI	Placebo	9.3% vs. 11.4%; *P* < 0.001[a]	3.7% vs. 2.7%; *P* = 0.001[b]
CLARITY	*N* = 3,491 STEMI treated with lytics	Placebo	15% vs. 21.7%; *P* < 0.001[c]	1.3% vs. 1.1%; *P* = 0.64[d]
COMMIT	*N* = 45,852 STEMI	Placebo	9.2% vs. 10.1%; *P* = 0.002[e]	0.58% vs. 0.55%; *P* = 0.59[f]
CURRENT	*N* = 25,087 ACS patients	High-dose (600 mg load, 150 mg daily) vs. low-dose (150 mg load, 75 mg daily)	No difference overall, but a reduction seen with high-dose clopidogrel in the subgroup undergoing PCI	No difference in TIMI major bleeding rates

[a]MI, stroke, CV death.
[b]Major bleeding.
[c]Odds of CV death, recurrent MI, recurrent ischemia leading to urgent revascularization.
[d]TIMI major bleeding.
[e]Death, reinfarction, or stroke.
[f]Major noncerebral or cerebral bleeding.

catheterization with a high likelihood to receive PCI (many of whom had ACS) were randomized to receive a loading dose of 300 mg of clopidogrel or placebo prior to PCI, as well as long-term (12 months) or short-term (28 days) clopidogrel after PCI.[84] Long-term therapy with clopidogrel for 1 year reduced events as compared to short-term therapy with a relative reduction in the combined risk of death, MI, or stroke of 26.9% (95% CI, 3.9%–44.4%; p = 0.02).

The use of clopidogrel in the management of ACS should begin with a loading dose of 300 mg, followed by the administration of 75 mg once daily. With a loading dose of 300 mg, the target level of platelet inhibition is reached within 4 to 6 hours; without it, this level is not reached until 3 to 5 days.[85] The use of a 600-mg loading dose results in effective platelet inhibition in about 2 hours.[86]

The use of the 600 mg load for patients undergoing PCI was studied in the ISAR-REACT-2 trial (Intracoronary Stenting with Antithrombotic Regimen-Rapid Early Action for Coronary Treatment).[87] In this study, all patients received 600 mg of clopidogrel at least 2 hours prior to cardiac catheterization, and they were randomized to abciximab (GP IIb/IIIa) plus reduced-dose heparin or placebo plus full-dose heparin. In those with a negative troponin, there was no difference in events, suggesting adequate platelet inhibition with clopidogrel loading alone. For those high-risk patients with positive troponin levels, it appears that the addition of abciximab to clopidogrel is safe and effective at further reducing events.

Clopidogrel resistance has been well described and is an area of intense research. Decreased clopidogrel responsiveness seems to correlate with an increased risk for ischemic events and appears to, at least in part, be related to polymorphisms in the CYP2C19 alleles coding for enzymes responsible for converting the prodrug to its active metabolite.[88,89] Observational studies have also linked the use of proton-pump inhibitors, especially omeprazole, with a decreased antiplatelet effect of clopidogrel—presumably through inhibition of the CYP2C19 enzyme.[90,91] A recently published trial using omeprazole in conjunction with clopidogrel refutes this analysis and does not show an increased risk with the coadministration of omeprazole with clopidogrel.[92] Furthermore, in patients with high platelet residual activity (a marker for clopidogrel resistance) on standard-dose clopidogrel, double-dose clopidogrel does not appear to improve outcomes at 6 months in patients receiving drug-eluting stents.[93]

Prasugrel

Prasugrel was approved for use by the US Food and Drug Administration in July 2009. The phase II trial JUMBO-TIMI 26 established the dosage of 60 mg load and 10 mg daily maintenance.[94] The PRINCIPLE-TIMI 44 trial demonstrated higher platelet inhibition with standard-dose prasugrel, both with loading and maintenance, as compared to high-dose clopidogrel (600 mg load, 150 mg daily maintenance).[95] The TRITON-TIMI 38 trial was a large phase 3 trial that compared prasugrel to clopidogrel in over 13,000 patients with high-risk ACS.[96] Prasugrel (60 mg load, 10 mg daily maintenance dose) showed a 19% relative reduction in the primary endpoint of cardiovascular death, nonfatal MI, or nonfatal stroke; which was primarily driven by a reduction in the rate of nonfatal MI. The components of the composite endpoints that were statistically improved with prasugrel over clopidogrel were nonfatal MI (HR = 0.76), urgent target vessel revascularization (HR = 0.66), and stent thrombosis (HR = 0.48).

The tradeoff for ischemic benefit in the TRITON trial was an increase in the rate of major bleeding, including life-threatening bleeding. Subgroup analyses identified two groups of patients in

whom caution is advised (≥75 years old, <60 kg in weight) and one group in whom the drug should be avoided altogether—those with a history of a transient ischemic attack or stroke. The groups of patients that appear to derive the most benefit from prasugrel are those presenting with STEMI and those with diabetes.

The TRILOGY ACS (Targeted platelet inhibition to clarify the optimal strategy to medically manage ACS) trial evaluates the use of prasugrel for the prevention of ischemic events when started within 10 days of presentation with a UA or NSTEMI event selected for a medical management strategy without revascularization. Using what was learned from the TRITON study, 5 mg, as opposed to 10 mg, of prasugrel is studied in those felt at highest risk for bleeding (≥75 years of age or <60 kg) in an attempt to reduce bleeding risk, while maximizing reductions in ischemic events.[97]

Ticagrelor

The most recently studied $P2Y_{12}$ inhibitor, ticagrelor, appears to have a more potent inhibition of platelet function with a more rapid onset of action and slightly more rapid reversal of action after discontinuation.[98] It was compared to clopidogrel in the PLATO (Platelet Inhibition and Patient Outcomes) trial that enrolled over 18,000 patients with ACS.[99] At 1 year, the primary endpoint of cardiovascular death, MI, or stroke was reduced in the ticagrelor groups as compared to the clopidogrel group (9.8% vs. 11.7%; HR = 0.84; 95%CI, 0.77 to 0.92; $P = 0.0003$). The hazard ratios for MI (HR = 0.84), cardiovascular death (HR = 0.79), or definite stent thrombosis (HR = 0.67) all favored ticagrelor and were statistically significant. When compared on bleeding endpoints, there was no difference in overall bleeding, but a higher rate of non–CABG-related bleeding was seen with ticagrelor as compared to clopidogrel (4.5% vs. 3.8%; $P = 0.03$). Also noted was a higher rate of self-reported dyspnea with ticagrelor. Ticagrelor, unlike clopidogrel and prasugrel, is administered twice daily with a 180-mg loading dose and a 90 mg twice-daily maintenance dose.

Glycoprotein IIb/IIIa Inhibitors

The binding of fibrinogen to the GP IIb/IIIa receptor is a critical component to the formation of platelet thrombi and is the final pathway for platelet aggregation.[100] The GP IIb/IIIa receptor antagonists occupy these receptors and inhibit this binding. The three most commonly used GP IIb/IIIa inhibitors include abciximab, eptifibatide, and tirofiban. They are used for the prevention of ischemic events associated with PCI and as part of the medical therapy for patients with ACS.

Abciximab

Abciximab is an Fab fragment of a humanized murine antibody, which strongly binds to the GP IIb/IIIa receptor. Its role is primarily in the catheterization laboratory, where it is used to reduce the rate of periprocedural MIs at the time of PCI and minimize the need for urgent revascularization. The CAPTURE trial evaluated the use of abciximab in patients with ACS undergoing angioplasty.[101] Abciximab reduced the combined event rate of death, MI, or urgent revascularization at 30 days when compared to placebo (11.3% vs. 15.9%; $P = 0.012$). The GUSTO IV-ACS trial compared a 24-hour and 48-hour infusion of abciximab to placebo in NSTEMI/UA patients for whom an early revascularization was not planned.[102] In this trial, there was harm associated with abciximab with an increased risk of death at 48 hours with an odds ratio of 2.9 for those who received the 48-hour abciximab infusion versus placebo (95% CI, 1.28 to 6.44; $P = 0.008$). As a result, abciximab should not be used for the management of ACS in patients for whom PCI is not planned.

Eptifibatide

Eptifibatide is a cyclic heptapeptide containing the KGD (Lys–Gly–Asp) sequence and has a strong affinity for the GP IIb/IIIa receptor and a short half-life. After discontinuation of the drug, platelet aggregation returns to normal in 4 to 8 hours. Eptifibatide was studied in over 10,000 patients with NSTEMI/UA in the PURSUIT trial.[103] Eptifibatide was here added to the medical management of ACS and continued for 72 hours or longer if PCI was performed. Eptifibatide was shown to reduce death and nonfatal MI up to 30 days after the index event (14.2% vs. 15.7%; $P = 0.04$). As a result, eptifibatide can be used in patients treated medically for ACS or with planned PCI; however, its use is now generally restricted for peri-PCI use in ACS as a result of the EARLY-ACS trial (see below).[104]

Tirofiban

Tirofiban is a nonpeptide mimetic of the RGD (Arg–Gly–Asp) sequence of fibrinogen. It too has a strong affinity for the GP IIb/IIIa receptor and a relatively short half-life. Two of the larger trials evaluating tirofiban are PRISM (Platelet Receptor Inhibition in Ischemic Syndrome Management) and PRISM-PLUS (Platelet Receptor Inhibition in Ischemic Syndrome Management in Patients Limited by Unstable Signs and Symptoms).[105,106]

The PRISM study compared tirofiban to heparin in patients with NSTEMI/UA and showed a reduction in the composite outcome of death, MI, or refractory ischemia at 48 hours with tirofiban that did not persist at 30 days. However, a significant reduction in mortality alone was observed at 30 days in the tirofiban group (2.3% vs. 3.6%; $P = 0.02$), with most of the benefit confined to those with elevated serum troponin levels.

The PRISM-PLUS trial compared tirofiban to UFH and the combination of both in patients with NSTEMI/UA. Excess mortality was seen in the tirofiban alone arm, leading this arm to be discontinued early. The combination of tirofiban and UFH, as compared to UFH alone, significantly reduced ischemic endpoints such as death and MI to 6 months. Tirofiban, in combination with heparin, can be used in the treatment of patients with ACS whether treated medically or with PCI. In general, however, the variable study results seen with tirofiban are felt to be a result of the lack of optimization of dosage.

Summary of GP IIb/IIIa Inhibitors

Overall, a meta-analysis of the trials evaluating GP IIb/IIIa inhibitors found that the ACS patients who benefited the most from these agents were those at higher risk defined by elevations in serum troponin and those patients planned for invasive strategy with PCI.[107] However, the administration of GP IIb/IIIa inhibitors to patients with ACS is not without risk. Most notable is the increased risk of bleeding, which generally occurs in mucocutaneous tissue or vascular access sites. In addition, the risk for profound thrombocytopenia, while small, can be significant and lead to life-threatening situations.

Applying the results of many of these GP IIb/IIIa studies, which were performed before the era of routine clopidogrel loading, to contemporary practice is challenging. The EARLY-ACS trial was designed to help answer this question and compare the early routine administration of the GP IIb/IIIa inhibitor eptifibatide to delayed provisional use in a high-risk cohort of UA/NSTEMI patients with planned PCI.[104] Overall, there was no clinical benefit from the upstream use of eptifibatide in this study, with an increased risk of bleeding. However, consistent with previous observations in earlier trials, in those with elevated troponin, a nonsignificant trend toward benefit was seen with early routine administration of eptifibatide. In those with a negative troponin, a trend toward harm was observed with early eptifibatide.

Anticoagulant Agents

As previously described in this chapter, anticoagulant therapy in addition to antiplatelet therapy is fundamental to the treatment of the underlying pathologic process of intracoronary thrombus formation in ACS. Several different anticoagulant agents have been studied in the treatment of ACS and are now available. These include UFH, LMWH, DTIs, and factor Xa inhibitors. Deciding on the best anticoagulant for an individual patient is complex and hinges on several factors. Among others, these include patient characteristics, ease of dosing, cost, comfort level of the provider with that drug, bleeding risk, and the likelihood of an invasive or surgical procedure. The class I recommendations for the use of anticoagulants from the AHA/ACC guidelines for the management of UA and MI are listed in Table 90.10.

Many clinical trials have been conducted to compare different anticoagulants in the management of ACS. However, because of differences in patients studied, dosing strategies, and antiplatelet use, comparison across trials is difficult. The currently available data suggest that the initial choice of which anticoagulant to use should be guided in part by whether an invasive strategy with early catheterization is chosen as opposed to a conservative strategy with medical therapy. What follows is a brief overview of the available anticoagulants for the treatment of ACS. For a detailed review, please see Chapter 105.

Unfractionated Heparin

UFH is an anticoagulant that works indirectly by binding to and generating a conformational change of antithrombin, which thereafter inactivates factors IIa (thrombin), IXa, and Xa more rapidly. UFH is comprised of polysaccharide chains of varying size. For the management of ACS, it is administered via the intravenous route with frequent laboratory monitoring of its activity.

UFH has been evaluated in the treatment of ACS.[108–111] In these early studies, the use of heparin reduced the risk of death or MI by up to one-half. A meta-analysis of studies evaluating UFH with endpoints up to 12 weeks suggested early benefit, with attenuation of the benefit of anticoagulation over time.[112] This may be due in part to a reactivation of the thrombotic process after discontinuation of the anticoagulant and has been observed with several of the anticoagulants including UFH.[113,114]

One of the biggest limitations of UFH is its marked variability in anticoagulant effect among patients.[115] As a result, the use of UFH must be guided by laboratory monitoring of activated partial thromboplastin time (aPTT). Current guidelines recommend a weight-adjusted regimen of an initial bolus of 60 U/kg (maximum 4,000 U) and an initial infusion of 12 U/kg/h (maximum 1,000 U/h), with titration to an aPTT of 60 to 80 seconds. Another limitation of the heparins, and especially UFH, is the risk for heparin-inducted thrombocytopenia (HIT)—an autoimmune-induced reaction to heparin that results in severe thrombocytopenia and thrombosis (see Chapter 108). This is a life-threatening complication that requires immediate cessation of the heparin drug and should prompt the consultation of a hematologist knowledgeable in the management of HIT.

Low Molecular Weight Heparins

The LMWHs are derived from depolymerization of the larger polysaccharide chains that form the UFH and have a greater anti-Xa effect than anti-IIa effect. They have a longer half-life than UFH and can usually be administered subcutaneously once or twice daily with no laboratory monitoring and less risk for HIT.[116]

Table 90.10

Class I recommendations for anticoagulation use for the management of UA or MI

Anticoagulant therapy should be added to antiplatelet therapy in UA/NSTEMI patients as soon as possible after presentation
For patients in whom an invasive strategy is selected, regimens with established efficacy at a *Level of Evidence: A* include enoxaparin and UFH (FIGURE 90.5), and those with established efficacy at a *Level of Evidence: B* include bivalirudin and fondaparinux (FIGURE 90.5)
For patients in whom a conservative strategy is selected, regimens using either enoxaparin or UFH *(Level of Evidence: A)* or fondaparinux *(Level of Evidence: B)* have established efficacy (FIGURE 90.6).
In patients in whom a conservative strategy is selected and who have an increased risk of bleeding, fondaparinux is preferable *(Level of Evidence: B)* (FIGURE 90.6)

Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

Early evaluation of the use of LMWH in ACS was performed in trials such as the FRISC (Fragmin during Instability in Coronary Artery Disease) study.[117] In this trial, over 1,000 patients with NSTEACS were randomized to dalteparin (a LMWH) or placebo, with therapy continuing for up to 45 days. In the dalteparin group, there was a 63% reduction in MI or death during the first 6 days, and this benefit continued to a lesser degree out to 40 days and was confined only to smokers.

In the ESSENCE (Efficacy and Safety of Subcutaneous Enoxaparin in Non-Q-Wave Coronary Events) and TIMI 11B trials, enoxaparin showed a significant benefit in the occurrence of death, MI, or recurrent ischemia over UFH in patients with NSTEACS.[118,119] Those with higher risk, such as ST-segment changes on ECG or elevated serum troponin levels, seemed to derive the most benefit with enoxaparin.

In the early trials evaluating enoxaparin, the rates of cardiac catheterization and PCI were low. For example, only 13% to 17% of patients in the ESSENCE trial underwent PCI. A more contemporary evaluation of enoxaparin compared to UFH in patients with ACS undergoing an early invasive strategy and a high use of GP IIb/IIIa inhibitors is the SYNERGY (Superior Yield of the New Strategy of Enoxaparin, Revascularization and Glycoprotein IIb/IIIa inhibitors) trial.[120] In this study, enoxaparin was shown to be noninferior to UFH in the prevention of death or MI at 30 days with an increase in bleeding.

A *post hoc* analysis of the SYNERGY data suggested that some of the excess bleeding seen with enoxaparin was due to crossover to UFH at the time of PCI.[121] This suggests that avoiding crossover of anticoagulants at the time of PCI can reduce bleeding risk, but this observation may have in fact limited the adoption of enoxaparin for those being treated with an early invasive strategy as the effect of enoxaparin is more difficult to monitor in the catheterization laboratory than UFH, and it is less effectively reversed with protamine.

In STEMI patients treated with fibrinolytic therapy, the ASSENT-3 (Assessment of the Safety and Efficacy of a New Thrombolytic Regimen), ENTIRE (Enoxaparin and TNK-tPA with or without GP IIb/IIIa Inhibitor as Reperfusion strategy in ST elevation MI)–TIMI 23, and EXTRACT (Enoxaparin and Thrombolysis Reperfusion for Acute Myocardial Infarction Treatment)–TIMI 25 trials evaluated the use of enoxaparin as compared to UFH in conjunction with fibrinolytics.[122–124] In each of these trials, the use of enoxaparin as compared to UFH was associated with a reduction in ischemic outcomes. However, an increase in intracranial hemorrhage was seen in older patients (>75 years old) treated with enoxaparin.[125] As a result, the EXTRACT–TIMI 25 trial used a reduced dose of enoxaparin in patients at least 75 years old. In this study, there was a significant reduction in MI and need for urgent revascularization at 30 days but not death with enoxaparin. This was offset to some degree by an increased risk in major bleeding (2.1% vs. 1.4%; $P < 0.001$) but not intracranial hemorrhage.

Despite favorable outcomes in reducing ischemic outcomes and ease of use, there are several limitations to the use of enoxaparin. In addition to its more cumbersome use in the catheterization laboratory and diminished reversibility, one of the major limitations of enoxaparin is its limited use in patients with renal insufficiency. Because this is a renally cleared drug with a longer half-life than UFH, the use of enoxaparin in patients with renal insufficiency should be closely monitored by measuring anti-Xa activity and avoided in those who are dialysis dependent. Meta-analyses comparing UFH to LMWH in the treatment of ACS have not shown significant differences in either their safety or their efficacy[126,127] for the treatment of NSTEMI, but suggest that LMWH may be more effective than UFH at reducing reinfarction.

Direct Thrombin Inhibitors

The DTIs bind directly to thrombin and have a powerful anticoagulant effect by inhibiting its action. Bivalirudin has emerged as an important therapy in the management of ACS for patients undergoing PCI. It is not recommended for ACS patients undergoing a conservative medically managed strategy. Two pivotal trials that demonstrated the utility of bivalirudin in reducing bleeding complications when compared to the heparin agents while being noninferior on ischemic endpoints were the ACUITY (Acute Catheterization and Urgent Intervention Triage strategY) trial in NSTEACS patients and the HORIZONS-AMI (Harmonizing Outcomes with Revascularization and Stents in Acute Myocardial Infarction) trial in STEMI patients.[128,129]

In the ACUITY trial, patients with NSTEACS who were treated with an early cardiac catheterization were studied. The main comparisons were made between patients randomized to heparin (UFH or enoxaparin) with GP IIb/IIIa inhibition versus bivalirudin with GP IIb/IIIa inhibition versus bivalirudin with provisional GP IIb/IIIa inhibition. Bivalirudin plus GP IIb/IIIa inhibition compared to heparin plus GP IIb/IIIa inhibition was noninferior at 30 days in composite ischemia and major bleeding. Bivalirudin alone when compared to heparin plus a GP IIb/IIIa inhibitor was noninferior with respect to composite ischemia, but significantly reduced major bleeding (3.0% vs. 5.7%; $P < 0.001$).

The HORIZONS-AMI trial was a similar study to ACUITY comparing bivalirudin alone to heparin plus a GP IIb/IIIa inhibitor, but was designed to evaluate patients presenting with STEMI undergoing primary PCI. Again, in this study, there was a significant decrease in major bleeding in the bivalirudin group (4.9% vs. 8.3%; $P < 0.001$) as well as a significant reduction in all-cause mortality at 30 days (2.1% vs. 3.1%; $P = 0.047$), but no significant differences in the composite ischemic events.[129]

Factor Xa Inhibitors

Upstream from thrombin in the coagulation cascade is factor Xa. Inhibition of factor Xa for the treatment of ACS has been extensively studied with the agent fondaparinux. This drug is a synthetic pentasaccharide and is attractive because of its once-daily subcutaneous (SC) administration and lower bleeding risk when compared to the heparins. It too does not require laboratory monitoring of its effect.

The OASIS-5 trial evaluated the use of fondaparinux in the treatment of UA and NSTEMI.[130] In this trial, patients were randomized to fondaparinux 2.5 mg SC daily or enoxaparin and were treated for a mean of 6 days. For the patients in the enoxaparin group, UFH was used at the time of PCI. In the fondaparinux group, additional intravenous fondaparinux was used at the time of PCI depending on the timing from the last SC dose. At 9 days, the number of patients with the primary outcome of death, MI, or refractory ischemia was similar between the two groups. However, there was significantly less major bleeding at 9 days with fondaparinux as compared to enoxaparin (2.2% vs. 4.1%; $P < 0.001$) as well as a reduction in death at

30 and 180 days (HRs and 95% CI, 0.83; 0.71 to 0.97 and 0.89; 0.80 to 1.00, respectively).

The OASIS-6 trial, which evaluated the use of fondaparinux in the treatment of STEMI, demonstrated a reduction in death with fondaparinux when compared to UFH or placebo (HR, 0.88; 95% CI, 0.79 to 0.97) without a reduction in major bleeding.[131] However, the benefit of fondaparinux was seen only in patients *not* treated with primary PCI.

In both trials, the use of fondaparinux alone in patients undergoing PCI was associated with a significantly higher rate of guiding catheter thromboses. As a result, it is recommended that any patient being anticoagulated with fondaparinux needing PCI be given a bolus of UFH at the time of cardiac catheterization. For patients in whom an invasive strategy is planned with likely PCI, fondaparinux is not the recommended anticoagulant for the management of ACS.

Anti-Ischemic Therapies

The American College of Cardiology/American Heart Association (ACC/AHA) class I recommendations for anti-ischemic therapy in ACS are listed in Table 90.11. These include supplemental oxygen, nitroglycerin, beta-blockers, and inhibitors of the renin angiotensin system.

Nitroglycerin

One of the most commonly used medications for the treatment of ischemic symptoms is nitroglycerin, which decreases myocardial ischemia by reducing the oxygen demand of the myocardium and increasing myocardial oxygen delivery. Nitroglycerin is a venous, and to a lesser extent, arterial dilator. It therefore reduces the preload to the heart by reducing venous return and reduces afterload on the heart by reducing systemic resistance. Because of its vasodilatory effects, nitroglycerin cannot be given to patients actively using phosphodiesterase inhibitors such as sildenafil, as this may result in a sudden drop in blood pressure with potentially catastrophic complications including death.[132]

There is little evidence to demonstrate that nitroglycerin causes a reduction in major adverse events. The Fourth International Study of Infarct Survival (ISIS-4) and the Gruppo Italiano per lo Studio della Sopravivenza nell'infarto Miocardico (GISSI-3) trials evaluated the effect of nitroglycerin on mortality in patients with suspected MI and found no reduction in the risk of death.[133,134] However, nitroglycerin has proven to be an important therapy in the treatment of coronary ischemia and is an effective drug for many in the relief of symptoms.

Morphine Sulfate

In those whose symptoms persist in the face of nitroglycerin and other anti-ischemic therapy, morphine sulfate can be used to provide relief to the patient. Although not formally evaluated in clinical trials, in addition to its potent analgesic effect, morphine provides some beneficial hemodynamic changes. These include a reduced heart rate, blood pressure, and preload. Morphine sulfate must be used cautiously as it can cause unsafe drops in blood pressure and suppression of the respiratory drive. Its use in the treatment of ischemic pain was downgraded in the 2007 AHA/ACC guidelines for the management of NSTEMI/UA from class I to IIa as a result of a registry analysis of over

Table 90.11

Class I recommendations for anti-ischemic therapy[a]

Bed/chair rest with continuous electrocardiographic monitoring
Supplemental oxygen with an arterial saturation <90%, respiratory distress, or other high-risk features for hypoxemia. Pulse oximetry can be useful for continuous measurement of SaO_2.
Nitroglycerin 0.4 sublingually every 5 min for a total of three doses; afterward, assess need for intravenous nitroglycerin
Nitroglycerin intravenously for the first 48 h after UA/NSTEMI for treatment of persistent ischemia, heart failure, or hypertension
Decision to administer nitroglycerin intravenously and dose should not preclude therapy with other mortality-reducing interventions such as beta-blockers or ACE inhibitors
Beta-blockers (via oral route) within 24 h without a contraindication (e.g., heart failure) irrespective of concomitant performance of PCI
When beta-blockers are contraindicated, a nondihydropyridine calcium channel blocker (e.g., verapamil or diltiazem) should be given as initial therapy in the absence of severe left ventricular dysfunction or other contraindications
ACE inhibitor (via oral route) within the first 24 hours with pulmonary congestion, or LVEF ≤0.40, in the absence of hypotension (systolic blood pressure <100 mm Hg or <30 mm Hg below baseline) or known contraindications to that class of medications
Angiotensin receptor blockers should be administered to patients with UA/NSTEMI who are intolerant of ACE inhibitors and have either clinical or radiologic signs of heart failure or LVEF ≤0.40

[a]Recurrent angina and/or ischemia-related electrocardiographic changes (0.05 mV or greater ST-segment depression or bundle branch block) at rest or with low level activity; *or* ischemia associated with symptoms of heart failure, S_3 gallop, or new or worsening mitral regurgitation; *or* hemodynamic instability or depressed left ventricular function (LVEF <0.40 on noninvasive study); *or* serious ventricular arrhythmia.

ACE, angiotensin-converting enzyme; LVEF, left ventricular ejection fraction; UA/NSTEMI, unstable angina/non–ST-elevation myocardial infarction.

Adapted from Anderson JL, Adams CD, Antman EM, et al. ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction). *Circulation* 2007;116(7):e148–e304.

50,000 patients with UA or NSTEMI showing an increased odds of death among all subgroups in those receiving morphine.[135]

Beta-Blockers

Beta-blockers are beneficial in the management of ACS. They work to reduce the oxygen demand of the myocardium by slowing the heart rate, reducing contractility, and decreasing systemic blood pressure. Trials mostly conducted in the prethrombolytic era demonstrated a reduction in infarct size, reinfarction, and mortality in both STEMI and NSTEMI patients.[136] For patients with ACS undergoing PCI, beta-blockers have been associated with a reduction in the short-term risk of death.[137] However, more recent studies, and a systematic review of early beta-blocker therapy in STEMI by Freemantle et al.,[138] have questioned the benefit of routine beta-blocker therapy early in the treatment of ACS. In the COMMIT study of over 45,000 patients with STEMI and NSTEMI, early intravenous beta-blockade did not result in a reduction in the composite endpoint of death, reinfarction, or cardiac arrest, but was associated with an increased rate of cardiogenic shock.[139] As a result of these data, it is now recommended that greater caution be used in the early administration of intravenous beta-blockers, and that this be avoided in those with heart failure or high risk for the development of cardiogenic shock. Current guidelines recommend the initiation of oral beta-blockers within the first 24 hours only to patients who are hemodynamically stable and at low risk for developing in-hospital heart failure or cardiogenic shock.

Renin–Angiotensin–Aldosterone System Inhibitors (ACE Inhibitors, Aldosterone Receptor Blockers)

Angiotensin-converting enzyme (ACE) inhibitors have been demonstrated to reduce mortality in a broad range of patients with CAD, MI, or left ventricular dysfunction.[140–143] The beneficial effect of this class of medications appears prolonged, as demonstrated in the TRACE (TRAndolapril Cardiac Evaluation) trial, which showed a continued reduction in mortality and hospitalization rates in patients followed for at least 10 to 12 years.[144]

In STEMI patients, there is substantial evidence for the benefit of early ACE inhibition. In the GISSI-3 trial, a 12% reduction in mortality was seen with the early initiation of lisinopril.[134] In the ISIS-4 study, the administration of captopril was associated with a 7% reduction in mortality.[133] For patients with concomitant left ventricular dysfunction, the benefits of early ACE-inhibitor therapy appear even more pronounced, with considerable benefit evident in the first several days after an MI. In the Survival and Ventricular Enlargement (SAVE), TRACE, and Acute Infarction Ramipril Efficacy (AIRE) trials, mortality was reduced by up to 30% with oral ACE inhibition.[145–147]

The benefit to early ACE inhibition in NSTEMI/UA is less clear. In the ISIS-4, GISSI-3, and CCS-1 (Chinese Cardiac Study) trials, little to no reduction in mortality was observed with ACE inhibition in patients without ST elevation.[133,134,148] Despite the lack of data for early benefit in NSTEMI/UA patients, there still appears to be a significant benefit to the long-term administration of ACE inhibitors in these patients as well as in those with chronic CAD.[140,149]

Consequently, the current recommendations for ACE inhibitors in patients with ACS include the initiation of oral ACE inhibitors within 24 hours for patients with STEMI, pulmonary congestion, or a left ventricular ejection fraction of ≤40%. Even in the absence of these findings, the current data would support the initiation of long-term ACE inhibitors for all patients presenting with ACS, provided they do not have a contraindication such as hypotension.

Angiotensin receptor blockers (ARBs) have been compared to and studied with ACE inhibitors in patients with ACS and heart failure. Because they directly block the angiotensin II (AT II) receptor, they theoretically provide greater protection against AT II than do ACE inhibitors. In the VALIANT (Valsartan in Acute Myocardial Infarction Trail) study, valsartan was compared to captopril as well as the combination of both drugs in patients with left ventricular dysfunction after acute MI.[150] In this study, mortality rates were similar for both drugs, but the combination of the two led to an increase in adverse events without a reduction in mortality. The CHARM (Candesartan in Heart failure Assessment in Reduction of Mortality)-Overall program evaluated candesartan for the treatment of chronic heart failure and demonstrated a reduction in cardiovascular death and hospital admissions.[151]

Aldosterone is another part of the renin–angiotensin–aldosterone system, and inhibition of its receptor with either eplerenone or aldosterone has been shown to reduce mortality in patients with heart failure, both chronically and in the setting of an acute MI. In the EPHESUS (Eplerenone Post–acute myocardial infarction Heart failure Efficacy and SUrvival Study) trial, eplerenone reduced mortality when used in patients with left ventricular dysfunction complicating MI and with diabetes or heart failure.[152] Spironolactone, another aldosterone receptor antagonist studied in the RALES (Randomized ALdactone Evaluation Study) trial, reduced mortality in those with severe heart failure.[153]

STATIN THERAPY IN THE ACUTE SETTING

The 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, have been shown to provide substantial benefit for the long-term prevention of cardiovascular events. In the management of ACSs, they have also demonstrated favorable outcomes. In the Aggrestat to Zocor (A to Z TIMI-21) trial, early aggressive simvastatin reduced cardiovascular events in patients with ACS.[154] The PROVE IT-TIMI 22 (Pravastatin or Atorvastatin Evaluation and Infection Therapy—Thrombolysis in Myocardial Infarction-22) trial studied intensive lipid-lowering therapy (atorvastatin 80 mg daily) versus a standard lipid-lowering strategy (pravastatin 40 mg daily) in patients presenting with ACS.[155] Not only was there a stronger reduction in serum cholesterol levels with intensive statin therapy, but the risk of death, MI, UA, revascularization, or stroke was reduced by almost 20% at 2 years. These data support the use of intensive statin therapy at the time of ACS, with a meta-analysis of four large trials enrolling over 27,000 patients showing that intensive, high-dose, statin therapy leads to a significant 16% odds reduction in coronary death or MI ($P < 0.00001$) and a 16% odds reduction in coronary death or any cardiovascular event ($P < 0.00001$).[156]

SECONDARY PREVENTION OF ACS

When a patient presents with ACS, plans for secondary prevention should begin immediately. Smokers need to be educated on the importance of quitting. Identifiable risk factors

such as hypertension, diabetes, obesity, and hyperlipidemia should start being addressed while the patient is hospitalized for the acute event. For most patients, a hospitalization for a cardiac event is a motivating experience to improve lifestyle habits.

Cardiac rehabilitation is also an important component of recovery from an ACS event and is underused.[157] Cardiac rehabilitation is designed to address physical and psychological effects of cardiac disease. It is a long-term program that emphasizes exercise, lifestyle changes, and risk factor modification, while also providing medical evaluation and education. Importantly, participation in cardiac rehabilitation after an MI has been associated with a 28% reduction in the risk of recurrent MI (RR, 0.72; 95% CI, 0.52 to 0.99; $P = 0.049$) and a 56% improvement in survival (RR, 0.44; 95% CI, 0.36 to 0.54; $P < 0.001$) in a cost-effective manner.[158,159]

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Full Reference Lists can be found online

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CHAPTER

91 Pathophysiology of Thromboembolic Stroke

EMER R. MCGRATH AND MARTIN J. O'DONNELL

Stroke is defined by the World Health Organization (WHO) as "rapidly developing clinical signs of focal (at times global) disturbance of cerebral function, lasting more than 24 hours or leading to death with no apparent cause other than that of vascular origin."[1] This definition is conventionally considered to include ischemic stroke, intracerebral hemorrhage (ICH), and subarachnoid hemorrhage (SAH). Clinical presentations lasting <24 hours are defined as transient ischemic attacks (TIAs). Recently, there has been a proposal to change the definition of stroke and TIA to a tissue-based definition, that is, one based on the presence or absence of acute ischemia or hemorrhage on neuroimaging.

EPIDEMIOLOGY OF STROKE

Incidence and Prevalence of Stroke

Stroke is the leading cause of acquired adult disability worldwide and the second most common cause of death after cardiovascular disease.[2,3] The WHO estimates that 15 million people suffer a stroke each year, and of these, 5 million people are left with permanent disability.[4] In the absence of further effective population-based interventions, a projected 18 million first-time strokes will occur in 2015, rising to 23 million first-time strokes in 2030.[3] Typical estimates for stroke prevalence (the number of individuals living with a previous history of stroke) are approximately 500 per 100,000 people in developed countries. The estimated prevalence of stroke survivors worldwide was 62 million in 2005, and this is projected to increase to 67 million in 2015 and to 77 million in 2030.[3]

Stroke-Related Mortality

In 2005, there were an estimated 5.7 million deaths due to stroke, accounting for approximately 10% of total deaths worldwide. It is projected that 6.5 million stroke deaths will occur in 2015 and 7.8 million deaths in 2030. A small decrease in age-specific stroke mortality rates has been projected from 2005 to 2030, which is largely due to a decline in high-income countries. However, due to an increasingly ageing population worldwide, crude stroke mortality rates are actually projected to increase, from 89 per 100,000 in 2005 to an estimated 98 per 100,000 in 2030. The increase in stroke mortality will be most marked in developing countries, where increases in stroke incidence are most prominent.[3]

Socioeconomic Consequences of Stroke

Disease burden may be measured using disability-adjusted life years (DALYs). One DALY is 1 year of healthy life lost, due to premature death or living with a disability. The global burden of stroke is currently projected to rise from an estimated 38 million DALYs in 1990 to 61 million DALYs in 2020.[4] Globally, stroke accounts for approximately 2% to 4% of total health care costs, while in high-income countries, stroke is responsible for more than 4% of direct health care budgets.[5] The estimated direct and indirect cost of stroke in the United States for 2010 was $73.7 billion. The estimated mean lifetime cost of ischemic stroke in the United States is currently $140,048, encompassing immediate inpatient care, rehabilitation services, and follow-up care costs (including long-term care).[6] A cost analysis study by Brown et al.[7] estimated that the total cost of stroke for the United States from 2005 to 2050 will be US $2.2 trillion, based on 2005-dollar prices. The decline in case fatality of stroke reported in many high-income countries is expected to impact on health care resources in these countries, given the increased requirement for rehabilitation services and long-term care facilities.

Global Variation in the Burden of Stroke

Worldwide, stroke demonstrates significant geographical variations, in terms of incidence (and temporal trends), prevalence, case fatality, and case mix (i.e., stroke subtypes). A systematic review of population-based studies by Feigin et al. found that from 1970 to 2008 there was a 42% decrease in the incidence of stroke in high-income countries, compared to a more than 100% increase in the incidence of stroke in middle- and low-income countries. In addition, from 2000 to 2008, overall stroke incidence rates in low- to middle-income countries exceeded rates in high-income countries for the first time, by over 20% (117 per 100,000 and 94 per 100,000, respectively).[8] In 2005, 87% of deaths due to stroke occurred in middle- and low-income countries. Moreover, this figure rose to 94% of global deaths due to stroke in those under the age of 70 years, where projected age-standardized death rates from stroke in people aged 30 to 69 years were higher for middle- and low-income countries than for high-income countries. For example, the age-standardized death rate for stroke in Nigeria was more than 120 per 100,000 people, compared to rates of approximately 20 per 100,000 people in the United Kingdom and <15 per 100,000 people in Canada. Finally, stroke mortality from 2005 to 2030 is predicted to increase by almost 33% in middle-income countries, from approximately 3 to 4 million deaths, while a small or negligible change is predicted for high-income countries.[3] Therefore, regional variations in the burden of stroke are projected to become more marked in the coming decades. The determinants underlying these changes may include variations in the stages of the epidemiologic transition, differing access to

risk factor modification therapies, and availability of evidence-based acute stroke interventions (e.g., stroke units).

ETIOLOGY OF ISCHEMIC STROKE

Heterogeneity of Stroke

Unlike acute coronary syndrome, which is mostly due to large-vessel atherosclerosis, the underlying mechanisms for ischemic stroke are more heterogeneous. For each ischemic stroke subtype, thrombogenesis is the usual underlying pathophysiological mechanism, although the local factors responsible for development of thrombosis differ by ischemic stroke etiology.[9] Ischemic stroke may be categorized into distinct etiological subtypes, including large vessel, small vessel, cardioembolism, mixed etiology, and cryptogenic.

Etiological Criteria for Ischemic Stroke

Stroke can be classified based on primary etiological mechanism into ischemic stroke and hemorrhagic stroke. In North America and Europe, approximately 85% to 90% of strokes are due to ischemia, with the remaining 13% occurring due to hemorrhage. In developing countries, a larger proportion of strokes are due to ICH.[8] Within ischemic stroke, there are five subcategories based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification system. The TOAST classification documents the pathophysiological mechanism of ischemic stroke based on clinical features and the results of investigations that include brain imaging (computed tomography [CT]/magnetic resonance imaging [MRI]), cardiac imaging (transthoracic and transesophageal echocardiography) and cardiac rhythm monitoring (e.g., Holter monitor), neurovascular imaging (duplex ultrasound, CT or MR angiography, and contrast angiography), and laboratory tests (Table 91.1). For secondary ischemic stroke prevention, the cornerstones of evidence-based therapy are lifestyle modification (e.g., smoking cessation), antiplatelet therapy, antihypertensive therapy, and statin therapy. Identification of certain ischemic stroke etiologies does inform additional evidence-based management approaches, most notably for atrial fibrillation (AF) (introduction of oral anticoagulant therapy) and significant carotid artery disease (carotid endarterectomy). The five categories in the TOAST classification system are (a) large-artery atherosclerosis, (b) cardioembolism, (c) small-artery occlusion or lacunar ischemic stroke, (d) stroke of other determined etiology, and (e) stroke of undetermined etiology ([i] two or more causes identified, [ii] negative evaluation, or [iii] incomplete evaluation).[10] The etiology of hemorrhagic stroke can be due to primary ICH or SAH, with ICH often topographically subclassified into lobar and deep hemorrhage.

Table 91.1

Etiology of thromboembolic ischemic stroke

Etiological Mechanism	Frequency (%)
Large-artery atherosclerosis	~20
Cardioembolism	~20–30
Lacunar	~20–30
Cryptogenic	15–40[a]

[a]Depending on the extent of etiological diagnostic workup.

Large-Artery Atherosclerosis

Large-artery stroke is usually a consequence of atherosclerosis in the extracranial (carotid or vertebral) and/or intracranial arteries (e.g., middle cerebral or basilar artery), where plaque rupture and thrombus formation can result in local occlusion or distal occlusion due to artery-to-artery thromboembolism. Vascular locations that have a predilection for development of atherosclerosis include the arch of the aorta, the origin and bifurcation of the common carotid artery, the distal end of the internal carotid artery, the terminal segments of the vertebral arteries, the basilar artery, and less commonly, the middle cerebral and posterior cerebral arteries.[11,12] In large arteries, thrombosis typically occurs at the site of an atherosclerotic plaque, termed atherothrombosis. Ischemic stroke may result from atherothrombosis due to artery-to-artery thromboembolism or, less commonly, by acute occlusion with resultant hypoperfusion (e.g., watershed infarction).[13,14] Large-vessel atherosclerosis accounts for about 20% of all ischemic stroke in high-income countries and is predominantly extracranial in origin (e.g., carotid artery).[15] Large-vessel disease is reported to be more common in parts of Asia, with an apparent predominance of intracranial artery stenosis (e.g., middle cerebral artery). For example, atherosclerosis of intracranial vessels has been reported to account for up to 33% to 50% of ischemic strokes in China and Thailand, based on some hospital-based registries.[16–18] The factors underlying this global variation in the proportion, and location, of large-vessel disease are largely unexplained, but may relate to differences in risk factors profiles (e.g., smoking and hypertension) or underlying genetic susceptibility. From a management perspective, carotid artery stenosis is the most important source of large-vessel stroke to identify, as timely carotid endarterectomy dramatically reduces the risk of recurrent ischemic stroke in patients with recent minor ischemic stroke or TIA, based on the results of large randomized controlled trials. For large-vessel intracranial disease, a recent clinical trial (SAMMPRIS trial) did not report a benefit of endovascular stenting (plus aggressive medical management) over aggressive medical management alone; the trial was stopped early due to excess rate of stroke and death, within 30 days, in the endovascular group.

Less common large-vessel mechanisms of ischemic stroke include arterial dissection,[19] fibromuscular dysplasia,[20] Moyamoya disease, and large-vessel arteritis (e.g., Takayasu arteritis and giant cell arteritis) (see Chapter 100).[11] Arterial dissection, usually of the extracranial vessels, is the third leading cause of ischemic stroke in young people and can lead to ischemic stroke by either local occlusion or distal thromboembolism.[19] Predisposing factors include direct trauma,[21,22] family history,[23] migraine,[24] and underlying arteriopathies, such as Moyamoya disease[25–27] or fibromuscular dysplasia.[28,29] Takayasu arteritis is a chronic granulomatous inflammatory condition of the aorta and its major branches. It occurs most commonly in Asia and is rare in Western Europe and North America. It predominantly affects the media and adventitia of the arterial wall, leading to stenosis or aneurysm formation.[30]

Approximately 10% to 20% of patients with Takayasu arteritis will have a TIA or ischemic stroke.[31]

Cardioembolism

Cardioembolism is the mechanism responsible for approximately 20% to 30% of all ischemic strokes.[15] There are a number of conditions that predispose to cardiac sources of emboli, which may originate from the venous system (paradoxical embolism), intracardiac (e.g., AF, left ventricular impairment) or postcardiac (aortic arch disease). Although the aortic arch is a large-vessel structure, it is conventionally included as a cardioembolic source, likely because it is usually identified on transesophageal echocardiography.

Paradoxical Embolism

Paradoxical emboli are proposed to occur when emboli that arise in the venous circulation, for example, from a deep vein thrombus, cross into the arterial circulation through a patent foramen ovale (PFO) or atrial septal defect, or through a pulmonary arteriovenous malformation (PAVM). Paradoxical embolism can be quite difficult to diagnose, and it is usually presumed, rather than confirmed, based on visualizing a right to left communication and/or shunt. PFO is common in the general population, present in about 20% of people. An association between PFO and ischemic stroke was first reported in younger patients (<55 years),[32] but the association has also been reported in older adults more recently.[33–37] However, while case–control studies report a positive association between PFO and cryptogenic ischemic stroke (odds ratio [OR] 3.12; 95% confidence interval [CI] 1.98 to 5.10),[35] two prospective cohort studies have failed to demonstrate an independent association between PFO and an increased risk of stroke, after a mean follow-up of 5 years[38] and 7 years.[39] In addition, studies have failed to show that the presence of an isolated PFO is a risk factor for recurrent ischemic stroke. Diagnostic investigations include transthoracic echocardiogram, transesophageal echocardiogram (TEE), and transcranial Doppler with contrast.[40] TEE is the gold standard investigation for diagnosis of PFO and estimation of PFO size, with a specificity and sensitivity of 100% for color Doppler TEE and 100% and 89%, respectively, for contrast TEE.[41] The optimal management strategy for patients with PFO and cryptogenic ischemic stroke remains controversial, due to difficulties in establishing a true causal relationship between PFO and ischemic stroke and a lack of randomized controlled trials delineating the best treatment options.[42] Although some studies have suggested an adjunct role for thrombophilic disorders,[42–45] the role for thrombophilic testing also remains uncertain. In healthy individuals with PFO, primary prevention with antithrombotic therapy is not currently recommended. The American Stroke Association/American Heart Association (ASA/AHA) currently recommends antiplatelet therapy for patients with ischemic stroke or TIA and a PFO to prevent a recurrent event (class IIa, level B evidence). There are no randomized controlled trials comparing oral anticoagulation to antiplatelet therapy, other than a subgroup analysis of the WARSS trial, which did not find a difference in recurrence stroke rates between treatment groups. An emerging management approach has been PFO closure, which may be achieved by transcatheter device closure, percutaneous device-less closure, and surgical closure by open or thoracoscopic access.[47] However, the recently presented CLOSURE trial did not report a benefit from PFO closure in approximately 900 participants after ischemic stroke, who were randomized to receive PFO closure or usual medical care, but did report an associated increased risk of new AF in the closure group on follow-up.[48] Other randomized controlled trials, evaluating other approaches to PFO closure following ischemic stroke, are ongoing. Management challenges are faced in patients with large PFO, PFO associated with atrial septal aneurysm[33,49] or in younger patients with no other risk factor for ischemic stroke, especially in those who experience a recurrent episode of ischemic stroke despite antiplatelet therapy.

An atrial septal aneurysm is a protrusion of part of the atrial septum through the fossa ovalis into the right or left atrium or both,[50,51] TEE is considered to be the investigation of choice for detection of atrial septal aneurysm.[52] An atrial septal aneurysm is associated with an increased risk of ischemic stroke, especially in association with a PFO. A meta-analysis of case–control studies comparing patients with ischemic stroke to controls reported ORs of 1.83 for PFO, 2.35 for atrial septal aneurysm, and 4.96 for both PFO and atrial septal aneurysm, indicating a greater risk of stroke from the combination compared to either condition alone.[53] Other potential explanations for the increased risk of stroke associated with atrial septal aneurysm include a predisposition toward atrial arrhythmias, thrombus formation, and left atrial dysfunction.[51,54–56]

PAVMs, which are commonly congenital in origin, are abnormal connections between pulmonary arteries and veins. They are also associated with an increased risk of ischemic stroke,[57] and paradoxical embolism is considered to be the most likely underlying mechanism.[58] The reported incidence of stroke in studies of patients with PAVM has ranged from 2.6% to 25%.[59–63] The gold standard for diagnosis of PAVM is pulmonary angiography, although less invasive techniques such as contrast and helical CT, magnetic resonance angiograms, and contrast echocardiography are also useful.[64] Treatment is recommended for patients with PAVMs who are symptomatic or who have feeding vessel diameters >3 mm. Embolotherapy is the current treatment of choice for PAVMs. Surgical resection is reserved for those who fail to respond to, or are unsuitable for, embolotherapy.[65]

Intracardiac Sources of Thromboembolism

Left-sided cardiac sources of emboli include left atrial thrombus secondary to AF or flutter,[66–68] left ventricle (LV) thrombus subsequent to a transmural myocardial infarction (MI),[69,70] an old MI with akinetic segments of myocardium and ejection fraction ≤28%,[71] cardiac tumors such as left atrial myxoma,[72] and abnormalities of the mitral valve, both native and artificial.[73]

AF is the most common cardiac arrhythmia and is a significant risk factor for stroke, associated with a fivefold increase in risk.[74] Irregular contractions of the atria lead to stasis of blood and an increased risk of thrombus formation, particularly in the left atrial appendage. This in turn predisposes to intracardiac thrombosis and, subsequently, ischemic stroke. In high-income countries, about 25% of all ischemic strokes and 36% of strokes in patients >80 years are due to AF.[75]

AF may be categorized as valvular (mostly associated with mitral valve disease) or nonvalvular.[76] Aspirin reduces the risk of ischemic stroke by about 22%, the addition of clopidogrel reduces the risk of recurrent stroke by a further 18% (ACTIVE-A)

while anticoagulant therapy (warfarin; international normalized ratio [INR] 2 to 3) reduces the risk of ischemic stroke by 66% compared to control, and warfarin (INR 2 to 3) is superior to combination aspirin and clopidogrel (ACTIVE-W). Recently, large randomized controlled trials have reported on the effectiveness of novel anticoagulants (apixaban, dabigatran, and rivaroxaban), which all appear very promising as replacements for warfarin in patients with AF, see Chapter 92.[77]

Other Cardiac Causes: Coronary heart disease is a known risk factor for ischemic stroke, mediated through a number of mechanisms. After an MI, mural thrombi, and subsequently cardioemboli, can arise due to the presence of left ventricular aneurysms, akinetic segments of left ventricular myocardium, or new-onset AF or flutter. A greater degree of myocardial injury and left ventricular dysfunction after anterior MI have been shown to increase the risk of stroke in the early postinfarct period.[78–80] The risk of mural thrombi, and therefore early stroke, is much greater after anterior wall MI rather than inferior wall MI.[81,82] The reported incidence of stroke in the first 2 weeks after MI ranges from 0.7% to 4.7%.[71]

Left ventricular hypertrophy (LVH) has also been associated with an increased risk of stroke both from electrocardiogram- and from echocardiogram-based studies, and this risk is independent of other risk factors. One proposed explanation for this association is that LVH can predispose to left atrial dilatation and in turn AF, largely medicated though the effect of hypertension.[83–85]

Valvular Heart Disease: Valvular heart disease may be native (e.g., rheumatic) or prosthetic. Of native valvular disease, mitral stenosis has the strongest association with ischemic stroke,[86] and, less commonly, mitral annular calcification and mitral valve prolapse.[87] Mitral stenosis is commonly associated with AF, which further increases the risk of ischemic stroke. Rheumatic heart disease remains a common cause of mitral valve disease, and an important risk factor for cardioembolic ischemic stroke, in many regions of the world (e.g., Africa and India). Emboli can also arise from valvular vegetations consisting of fibrin and platelets in nonbacterial thrombotic endocarditis[88,89] and from infected valvular vegetations in infective endocarditis.[90] Patients with mechanical mitral and aortic valves, particularly mitral valves, have a sufficiently high risk of ischemic stroke that justifies indefinite oral anticoagulant therapy. In contrast, bioprosthetic valves require a finite course of antithrombotic therapy, usually 3 months after surgery, unless there is an alternative indication for indefinite therapy (e.g., AF).

Congestive heart failure (CHF) is an independent risk factor for stroke, associated with a twofold to threefold increase in the relative risk (RR) of stroke.[75,91–93] Various mechanisms have been proposed to explain this association. Dysfunction of the LV in CHF can promote stasis of blood in the LV and left atrium (ejection fraction < 30%[94]), thereby predisposing to thrombus generation and risk of cardioembolic stroke, while transient arrhythmias such as AF are more common in this patient population.[95] In South and Central America, Chagas disease (tropical parasitic disease *Trypanosoma cruzi*) is an important cause of dilated cardiomyopathy and risk factor for stroke, especially in younger patients.

Postcardiac sources: The prevalence of severe atheroma of the aortic arch (>4 to 5 mm) is approximately 30%, and its presence is associated with a fourfold increase in the risk of ischemic stroke and peripheral embolism.[96] Emboli can also arise from atherosclerotic plaques in the aortic arch, proximal to the left subclavian artery, and enter the cerebral circulation leading to an ischemic stroke.[97]

Small-Vessel Disease

Approximately 20% to 30% of all ischemic strokes are due to lacunar infarcts.[15] Lacunar infarcts occur due to occlusion of small deep penetrating arteries, such as the lenticulostriate branches of the anterior cerebral artery and middle cerebral artery. The terminal pathophysiological mechanism underlying small-artery occlusion is believed to be local thrombosis, secondary to microatheroma and lipohyalinosis. Microatheroma is thought to be the most common mechanism underlying symptomatic lacunar infarcts,[98] while lipohyalinosis is proposed to predominantly account for many asymptomatic lacunar infarcts, causing occlusion of very small penetrating arteries <200 μm in diameter.[99] A microatheroma is characterized by lipid-laden macrophages, cholesterol deposits, and subintimal fibroblast proliferation.[100] Lipohyalinosis is believed to be an intermediate stage between fibrinoid necrosis and microatheroma, having characteristics of both arterial (atheromatous lipid deposits) and arteriolar (hyalinization) disease.[101] Fibrinoid necrosis typically occurs in the setting of malignant hypertension and affects cerebral, retinal, and renal arterioles and capillaries.[100] It is characterized histologically by intensely eosinophilic and finely granular deposits in the arteriolar wall.[102] Other causes for small-artery occlusion include microemboli,[103] cholesterol emboli from atherosclerotic plaques,[104] polycythemia,[105] antiphospholipid antibodies,[106] amyloid angiopathy,[107] cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL),[108] Sneddon syndrome,[109] and various types of vasculitis.[110]

CADASIL is an uncommon inherited disorder that predominantly affects the small arteries of the brain, leading to an increased risk of early-onset strokes, typically lacunar infarcts, in the periventricular and basal ganglia regions. A study in Scotland estimated the prevalence at almost 2 per 100,000 adults, which is believed to be an underestimate.[111] Other clinical consequences include subcortical dementia, migraine with aura, and psychiatric symptoms.[112] It is caused by a mutation in the *Notch3* gene on chromosome 19, which leads to the deposition of the ectodomain of the *Notch3* receptor protein and granular eosinophilic material on vascular smooth muscle cells of arteries, resulting in their gradual degeneration.[113] The degeneration of arterial vascular smooth muscle cells results in progressive fibrosis and luminal stenosis of small penetrating arteries in the brain, reduced blood flow, and subsequently, lacunar infarcts.[114,115] Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) has a similar pattern of early-onset cerebral ischemia to CADASIL and is due to a mutation in HTRA1.

Sneddon syndrome is a rare disorder characterized by cerebrovascular disease occurring in association with livedo reticularis, an ischemic dermatopathy of the skin, and the absence of an infection or collagen vascular disease.[109,116] The definitive etiology is unknown, although it is thought to be autoimmune medicated.[117] Hypertension is reported in approximately 60% of patients with Sneddon syndrome, while 35% are noted to have antiphospholipid antibodies.[118] Previous neuropathological reports have noted multiple small infarcts, together with

smooth muscle cell hyperplasia and fibrotic occlusion of small- to medium-sized cerebral arteries, as well as occasional arterial thrombi. These findings suggest that the underlying pathogenesis for ischemic events in Sneddon syndrome is a noninflammatory arteriopathy affecting the small- to medium-sized cerebral arteries.[109,119]

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is an inherited progressive disorder due to mitochondrial dysfunction. It is characterized by early onset of stroke, typically before the age of 40.[120] The underlying pathophysiology for cerebrovascular events in MELAS has not been fully determined. MRI diffusion-weighted images in these individuals have demonstrated multiple hyperintense cortical laminar lesions, which do not follow the distribution of any single large artery.[120,121] The mitochondrial angiopathy hypothesis suggests that the lesions are secondary to ischemia which is caused by mitochondrial and vascular dysfunction of cerebral small arteries.[120]

Stroke of Other Determined Etiology

Other uncommon and rare causes of ischemic stroke are considered in the category of "stroke of other determined etiology," according to the TOAST classification system. This category can include hematological disorders, hypercoagulable states, nonatherosclerotic vasculopathies, and arterial dissection.[10] *Cerebral venous sinus thrombosis* accounts for <1% of ischemic strokes and typically affects younger people.[46,122] The superior sagittal sinus, the transverse sinuses, and the cavernous sinuses are most commonly affected by thrombosis.[100] Venous thrombosis results in localized edema and venous infarction, which often becomes hemorrhagic, as well as raised intracranial pressure.[122] Reported risk factors for cerebral venous sinus thrombosis include inherited prothrombotic states such as factor V Leiden (FVL) mutation[123] and prothrombin gene mutation 20210A (PT G20210A)[124]; acquired prothrombotic states such as antiphospholipid antibodies,[125] pregnancy, and the puerperium[126]; infections such as otitis, sinusitis, and mastoiditis[127]; chronic inflammatory conditions such as Wegener granulomatosis and sarcoidosis[128]; and trauma such as a head injury,[129] dehydration, and injury to the jugular veins or sinuses from neurosurgical procedures.[122] MRI combined with MR venography is the most sensitive and specific diagnostic test.[130] A hyperintense signal from thrombosed sinuses on MRI together with corresponding absence of flow on MR venography confirms the diagnosis.[122] AHA/ASA guidelines indicate that anticoagulation is probably effective for patients with acute cerebral vein thrombosis (class IIa; level of evidence B), although definitive clinical trials are lacking. In the absence of trial data to define the optimal duration of anticoagulation for acute cerebral vein thrombosis, it is recommended to administer anticoagulation for at least 3 months, followed by antiplatelet therapy (class IIa; level of evidence C).[46]

Stroke of Undetermined Etiology

In some cases, the cause of stroke cannot be definitively determined and the stroke is classified as "stroke of undetermined etiology." A stroke may be classified in this category when one of the following two conditions are met: (a) an extensive evaluation is negative, which includes large-vessel imaging and complete cardiovascular assessment, or (b) the diagnostic evaluation is incomplete.[10] Clearly, the most important determinant of the proportion of patients labeled as cryptogenic is the extent of the diagnostic testing, which makes between-study comparisons of etiological case mix difficult.

Covert Stroke

Clinically overt stroke is considered to represent only a fraction of all episodes of cerebral infarction. The advent of contemporary MRI sequences has identified a large burden of subclinical cerebrovascular disease, which includes covert infarction, white matter hyperintensities, cerebral atrophy, and microbleeds. Moreover, the presence (pattern and burden) of covert stroke has been associated with an increased risk of cognitive decline, dementia, depression, vascular parkinsonism, and gait impairment.[131,132,133–136] Previous studies report a twofold to fourfold increase in the risk of clinically overt stroke in the presence of covert cerebral infarcts, independent of other vascular risk factors.[135,136] Given their chronic clinical consequence, the term "covert" rather than "silent" has been adopted. A systematic review of eight population-based studies reported a prevalence of silent brain infarcts in the general elderly population of 8% to 28%.[132] Previous studies indicate that the strongest risk factors associated with silent cerebral infarcts are age, hypertension, diabetes mellitus, and smoking.[132]

TRADITIONAL RISK FACTORS FOR ISCHEMIC STROKE

Both ischemic and hemorrhagic stroke are strongly associated with a number of conventional risk factors, namely hypertension, smoking, diet, sedentary lifestyle, obesity, diabetes mellitus, and exercise. Many of these risk factors exert their effect through a number of intermediate mechanisms. For example, hypertension increases the risk of ICH, small-vessel disease, atherosclerosis, and AF.

The INTERSTROKE study, which included 6,000 participants from 22 countries, reported that 10 key risk factors were associated with 90% of the population-attributable risk (PAR) of ischemic stroke, namely hypertension, smoking, waist-to-hip ratio (WHR), diet, physical activity (PA) level, diabetes mellitus, alcohol intake, psychosocial stress and depression, cardiac causes such as AF, and ratio of apolipoprotein B (ApoB) to apolipoprotein A1 (ApoA1) (after adjustment for age and sex)[137] (Table 91.2). Since many of these risk factors are common and modifiable, these findings suggest that population-based programs, which target key risk factors, may have an enormous impact on the incidence of stroke.

Age

Age is the strongest independent risk factor for stroke. The incidence of stroke increases with age, with up to 75% of strokes occurring in individuals over the age of 65 in high-income countries.[138] For every 10 years above the age of 55, the risk of stroke more than doubles.[4,139,140] A 5-year follow-up of a population-based cohort of over 4,000 people >65 years reported a hazard ratio (HR) of 1.74 per 10-year increase in age over 65.[138]

Table 91.2

Traditional risk factors for ischemic stroke

Risk Factor	OR (99% CI)
Hypertension (self-reported history)	2.37 (2.00–2.79)
Current smoking[a]	2.32 (1.91–2.81)
Diabetes mellitus	1.60 (1.29–1.99)
Ratio of ApoB to ApoA1	2.40 (1.86–3.11)
Obesity (waist-to-hip ratio)	1.69 (1.38–2.07)
Regular physical activity	0.68 (0.51–0.91)
Diet risk score	1.34 (1.09–1.65)
Alcohol consumption[a]	
1–30 drinks per month	0.79 (0.63–1.00)
>30 drinks per month	1.41 (1.09–1.82)
Psychosocial factors	
Psychosocial stress	1.30 (1.04–1.62)
Depression	1.47 (1.19–1.83)

Odds ratios for waist-to-hip ratio and diet risk score are for the highest tertile compared to the lowest tertile.
[a]Comparator for current smoker and alcohol intake is never or former.
OR, odds ratio; CI, confidence interval; Apo, apolipoprotein.
From O'Donnell MJ, Xavier D, Lisheng L, et al. Risk factors for ischaemic and intracerebral haemorrhagic stroke in 22 countries (the INTERSTROKE study): a case-control study. *Lancet* 2010;376:112–123.

Sex

Population-based studies have demonstrated sex differences in the risk of stroke, with men having a greater risk of stroke than women.[139,141,142] The incidence of stroke in men has been reported to be 25% higher than the incidence in women.[143] However, women tend to live longer than men and so have a higher incidence of stroke in the subgroup of individuals over 85 years.[144]

Hypertension

Hypertension is the most important modifiable risk factor for stroke. In the INTERSTROKE study, approximately 35% of all stroke cases could be attributed to a patient reported history of hypertension (OR 2.64). Using a less stringent definition of hypertension, patient-reported history of hypertension, or a blood pressure >160/90 mm Hg, the PAR of stroke increased to almost 52%[137] (see Table 91.2), a finding that is consistent with a previous review.[145] The relationship between blood pressure and cardiovascular risk is "continuous, consistent, and independent of other risk factors."[146] Hypertension is a stronger risk factor for intracerebral hemorrhagic stroke than for ischemic stroke, and the preferential reduction in ICH in high-income countries is believed to be primarily due to improved blood pressure control.[137] According to the National Health and Nutrition Examination Survey (NHANES) 2003 to 2006, an estimated 33.6% of United States adults have hypertension, defined as blood pressure ≥140/90 mm Hg, equating to over 74 million people.[6] The worldwide prevalence of hypertension in 2000 was reported to be 26%, an estimated 972 million people.[147]

Hypertension increases the risk of ischemic stroke by causing endothelial damage, altering blood–cell endothelial interaction, and predisposing to thrombus formation. It accelerates atherosclerosis, particularly at extracranial sites such as the carotid bifurcation, and predisposes to cardiac conditions like AF and MI that promote cardioembolic stroke.[148] Meta-analyses of randomized controlled trials have shown a 30% to 40% decrease in stroke risk with lowering of blood pressure interventions. A dose–response relationship with a greater decrease in stroke risk for larger reductions in blood pressure has also been demonstrated.[149,150] Excess salt intake is an important, modifiable determinant of blood pressure and a risk factor for stroke.

Smoking

Smoking is an independent risk factor for stroke, associated with a twofold increase in risk for ischemic stroke overall[151] and a twofold to fourfold increase in risk of hemorrhagic stroke.[152,153] A meta-analysis of 32 studies in the late 1980s found that compared to nonsmokers, smokers had almost double the risk of ischemic stroke, with an RR of ischemic stroke of 1.9.[154] The INTERSTROKE study reported an increased risk of stroke in smokers (OR 2.09) compared to nonsmokers, and smoking was a consistent risk factor for stroke across all regions and patient subgroups[137] (see Table 91.2). There is a dose-response relationship between smoking and risk of stroke, with an increasing stroke risk with increasing numbers of cigarettes smoked per day.[137,154] In the Framingham study, heavy smokers (>40 cigarettes per day) were reported to have twice the RR of a stroke compared to light smokers (<40 cigarettes per day). According to WHO estimates, the prevalence of tobacco smoking in the United States is 23.6%, and there are currently about 1.2 billion individuals smoking tobacco worldwide.[155] Smoking contributes to atherosclerosis by causing vascular endothelial dysfunction[156–158] and also promotes a procoagulant state by increasing fibrinogen concentration, decreasing fibrinolysis, and enhancing platelet aggregation.[159–161] Cessation of smoking is associated with a reduction in the risk of stroke and other cardiovascular events to a level approaching, but not reaching, that of individuals who have never smoked.[162–164] Recent evidence also supports that exposure to environmental tobacco smoke is an important risk factor for stroke.[165,166]

Diabetes Mellitus

Diabetes mellitus predisposes to an increased risk of atherosclerosis and subsequently ischemic stroke. Epidemiological studies have shown that the increased risk of stroke in patients with diabetes is independent of other risk factors. In the Honolulu Health program study, Japanese American men with asymptomatic hyperglycemia and overt diabetes mellitus had an increased risk of thromboembolic stroke compared to those with low glucose levels 22 years after follow-up (RR 3.45 and 1.43, respectively).[167] A population-based cohort study in California was carried out amongst almost 4,000 adults aged 50 to 79 years, with an average follow-up of 12 years. It was found that individuals with either a history of diabetes or a fasting blood glucose level >7.8 mmol/L had a greater age-adjusted risk of stroke morbidity and mortality compared to normoglycemic individuals (RR 1.8 for men and RR 2.2 for women). This risk was again found to be independent of other cardiovascular risk

factors.[168] The estimated prevalence of diabetes amongst the US adult population is 8%.[169] The estimated worldwide prevalence of diabetes across all age groups in 2,000 was 2.8%, representing approximately 171 million people.[170] Three large randomized controlled trials of intensive glycemic control in individuals with diabetes and additional cardiovascular risk factors or a history of cardiovascular disease or stroke have failed to demonstrate a benefit of intensive glycemic control in the reduction in risk of stroke.[171,172,173] The results of these trials indicated that there was no benefit in lowering HbA1c < 6.5% in such patients.

Lipids

Elevated levels of total cholesterol (TC) and reduced levels of high-density lipoprotein cholesterol (HDL-C) have been established as important modifiable risk factors for coronary heart disease.[174] However, epidemiological evidence to date has provided mixed results for the association between lipoproteins and ischemic stroke risk.[175–178] A population-based cohort of over 14,000 men and women with a mean follow-up of 10 years found weak and inconsistent associations between risk of ischemic stroke and five lipid factors—HDL-C, low-density lipoprotein cholesterol (LDL-C), ApoB, ApoA1, and triglycerides.[178] A recent prospective cohort study of almost 28,000 women ≥45 years with 11 years of follow-up found that when comparing the highest to the lowest quintiles, the HRs for ischemic stroke were 2.13 for TC, 1.68 for LDL-C, and 2.26 for non-HDL-C, after adjustment for other risk factors. After multivariable adjustment, no significant association was found between HDL-C and ischemic stroke risk.[179] In the INTERSTROKE study, while increased HDL-C concentration was associated with a decreased risk of ischemic stroke, no association was recorded with total or non-HDL cholesterol. Increased ApoA1 concentration was associated with a decreased risk of stroke, whereas increased ApoB was associated with an increased risk[137] (see Table 91.2). Apolipoproteins were more strongly associated with ischemic stroke than lipoproteins, a finding that was also noted for carotid artery disease in the NOMASS study.[180] The prevalence of hypercholesterolemia (total serum cholesterol levels > 240 mg/dL) amongst US adults over 20 years is 16.2%, an estimated 35 million people.[181] Results from trials of statin therapies have consistently reported a reduced risk of ischemic stroke with statin use.[182–187] In addition, a meta-analysis of over 90,000 patients included in trials of statin therapies reported a greater reduction in stroke risk with larger reductions in LDL-C.[188]

Obesity

A high body mass index (BMI) as a measure of general obesity and a high WHR as a measure of abdominal obesity are both associated with an increased risk of stroke, and this risk persists after adjusting for other established stroke risk factors.[189,190] In the INTERSTROKE study, individuals with a WHR in the higher tertile had an increased risk of stroke (OR 1.65) compared to those in the lowest tertile[137] (see Table 91.2). A prospective cohort study of over 20,000 male physicians found that during 12.5 years of follow-up, those with a BMI ≥ 30 kg/m^2 had twice the risk of total stroke (adjusted RR 2.0) compared to those with a BMI < 23 kg/m^2. In addition, for every unit increase in BMI, there was a 4% increase in the adjusted RR for all stroke.[189] A population-based case–control study in Northern Manhattan found that individuals with a WHR in the third and fourth quartiles had a higher risk of ischemic stroke (OR 2.4) compared to those in the first quartile, after adjustment for other risk factors including BMI.[190] A Swedish study which followed over 7,000 healthy males for 28 years also found an increased risk of stroke in those with a BMI > 30 kg/m^2 (adjusted HR 1.93) compared to those with a BMI of 20 to <23 kg/m^2.[191] Although adiposity and percentage body fat can increase as people grow older, a stable or decreasing BMI can also occur due to a loss of lean body mass and muscle.[192–194] This could explain the observation that WHR appears to be a better predictor of stroke than BMI.[190] There are a variety of proposed mechanisms for the association between obesity and stroke. To date, no clinical trials have reported a reduced risk of first or recurrent stroke with weight loss, although large long-term clinical trials are lacking.[46,151]

Physical Inactivity

Moderate and higher levels of PA have been associated with a decreased risk of stroke, even after adjusting for major cardiovascular risk factors.[195] A meta-analysis of 31 observational studies found that moderately intense levels of PA decreased the risk of all stroke subtypes, and this effect was seen with both occupational PA (RR 0.64) and leisure time PA (RR 0.57).[196] In the Physicians' Health Study, there was a 14% reduction in RR of stroke among those men who exercised vigorously ≥5 times per week, after adjustment for other risk factors.[197] In the Framingham study, medium-level PA was associated with a decreased risk of stroke (RR 0.41) with no additional benefit occurring with higher levels of PA.[198] A more recent observational study of over 47,000 Finnish people followed up for a mean of 19 years looked at the relationship between various types of PA and risk of stroke. The HR for total stroke in individuals with moderate (>4 hours per week of walking, cycling, or light gardening) and high (>3 hours per week of jogging, swimming, heavy gardening, or regular sports) levels of leisure time PA was significantly decreased, compared to individuals with low levels of PA (HR 0.86, 0.74, and 1.00, respectively). In addition, there was a decreased HR for total stroke in individuals with active commuting (>30 minutes daily) (HR 0.89).[195] The beneficial effects of exercise are believed to be mediated by its impact on other known risk factors for stroke. PA leads to decreased blood pressure, body weight, and LDL-C; increased HDL-C; and increased glucose tolerance and insulin sensitivity.[199,200] Other possible mechanisms for the decrease in stroke risk with PA include a decrease in platelet aggregation,[201] a decrease in fibrinogen,[202] and increased fibrinolysis.[203,204]

Diet

A diet with consumption of grains, fruit and vegetables, and fish has been associated with a decreased risk of stroke. In the INTERSTROKE study, increased consumption of fruit (OR 0.61) and fish (OR 0.78), but not vegetables was associated with a significantly decreased risk of stroke. Increased risk of stroke was associated with higher consumption of red meat, organ meats, or eggs (OR 1.35); higher consumption of fried foods, pizza, or salty snacks (OR 1.16); and cooking with lard (OR 1.66)[137] (see Table 91.2). A meta-analysis of seven prospective

cohort studies found an 11% decrease in the risk of stroke for each additional portion of fruit per day consumed, after adjustment for factors known to be more likely amongst consumers of fruit such as higher education, more regular exercise, and decreased likelihood of smoking. There was no significant decrease in stroke risk with vegetable intake.[205] Another study found that consumption of over six servings of fruit or vegetables per day provided little further reduction in the risk of ischemic stroke, compared to five or six servings.[206] In the Nurses' Health Study of over 75,000 women, compared to individuals in the lowest quintile for whole-grain intake, those in the highest quintile had a decreased RR of stroke (RR 0.69), after adjustment for known cardiovascular risk factors.[207] The Nurses' Health Study also investigated the association between fish and risk of stroke. Compared to women who ate fish less than once per month, those who ate fish more frequently had a decreased risk of stroke, after adjustment for other cardiovascular risk factors: one to three times per month (RR 0.93), once per week (RR 0.78), two to four times per week (RR 0.73), and ≥5 times per week (RR 0.48).[208]

Alcohol

The relationship between alcohol and stroke risk depends on the amount of alcohol consumed and the stroke subtype. For ischemic stroke, a J-shaped association has been reported.[137,209] Compared to moderate alcohol intake, both high levels of alcohol consumption and minimal or no alcohol consumption have been associated with an increased risk of ischemic stroke. The risk of hemorrhagic stroke appears to increase with the amount of alcohol consumed in a more linear fashion.[210] A meta-analysis of 35 observational studies found that compared to abstainers of alcohol, consumption of <12 g/d (one standard drink) was reported to be associated with a decreased risk of ischemic stroke (RR 0.80), as was consumption of 12 to 24 g/d (one to two standard drinks) (RR 0.72). However, consumption of >60 g/d (five standard drinks) was associated with an increased risk of ischemic stroke (RR 1.69).[209] A recent prospective cohort study of over 38,000 male physicians with a mean follow-up of 14 years found that compared with abstainers of alcohol, individuals who consumed one to two standard drinks per day on more than 4 days per week had a decreased risk of ischemic stroke (RR 0.68).[211]

There are various proposed mechanisms for the association between alcohol consumption and stroke risk. High alcohol intake can predispose to hypertension, AF, and cardiomyopathy, thereby increasing the risk of ischemic stroke.[212–215] One proposed explanation for the reduction in ischemic stroke risk with moderate alcohol intake is that alcohol tends to increase HDL-C levels,[216,217] reduce platelet aggregation,[218] enhance fibrinolysis,[219] and decrease the levels of fibrinogen.[220]

Psychosocial Factors

Chronic stress at home or in the workplace, social isolation, and anxiety are believed to be associated with an increased risk of stroke.[4] In the INTERSTROKE study, the presence of psychosocial stress, defined as a combined measure of general stress at home and in the workplace (permanent or several periods of stress versus no or some periods of stress in the past year) was reported to be associated with an increased risk of stroke (OR 1.30). Depression, defined as feeling sad, blue, or depressed for two or more consecutive weeks during the past 12 months, was also noted to be associated with an increased risk of stroke (OR 1.35, PAR 5.2%)[137] (see Table 91.2). A prospective cohort study of elderly Australians reported that, compared to individuals in the lowest tertile for depression score, those in the third tertile had a 41% greater risk of ischemic stroke (adjusted RR 1.4).[221] A second prospective study reported an increased risk of stroke/TIA in individuals with depressive symptoms >65 years (adjusted HR 3.43), but not in those >65 years.[222] Major life events have also been associated with an increased risk of stroke.[223]

EMERGING RISK FACTORS

Genetic Factors

A family history of stroke is considered a risk factor for stroke. Evidence for such an association comes from population-based studies,[224–226] family studies,[227,228] and twin studies.[229] In the Family Heart Study, a positive history of paternal or maternal stroke was associated with an increased risk of stroke, with adjusted ORs of 2.00 (95% CI 1.13 to 3.54) and 1.41 (0.80 to 2.50), respectively.[227] The increased risk of stroke in individuals with a positive parental history can be due to (a) genetic heritability of cerebrovascular risk factors such as hypertension and diabetes; (b) an inherited susceptibility to the effects of cerebrovascular risk factors; (c) familial sharing of the same environmental and lifestyle factors such as diet and levels of PA; and (d) the interaction between genetic factors and the environment.[227] Many stroke risk factors have both genetic and environmental elements, such as hypertension and diabetes.[230,231]

Twin studies also provide evidence of a genetic susceptibility to stroke, with markedly higher concordance rates for stroke in monozygotic twins (17.7%), compared to dizygotic twins (3.6%), and an almost fivefold increase in stroke prevalence in monozygotic, compared to dizygotic, twins.[229] Some cases of ischemic stroke can be caused by monogenic disorders with a Mendelian pattern of inheritance, such as CADASIL,[112] CARASIL, and sickle cell disease (SCD).[232] However, the majority of ischemic stroke cases are sporadic and of multifactorial origin. Genetic involvement in the pathogenesis of multifactorial ischemic stroke is likely to be polygenic, involving more than one gene. The genetic influence on stroke risk can be mediated in several ways; the presence of several genes may have an additive effect on risk (gene–dose effect), a synergistic effect with a multiplicative increase in risk, or genes may interact with acquired risk factors, such as smoking, and modulate their effects.[233]

The different phenotypes of ischemic stroke, such as cardioembolism and large-artery atherosclerosis, may reflect these differences in genetic influences, as for each subtype genetic factors may act differently, for example, predispose to typical stroke risk factors, modulate the effect of these risk factors, or independently increase the risk of stroke.[15,233]

Recent genome-wide association studies have found an association between various single-nucleotide polymorphisms (SNPs) and both stroke subtypes and risk factors, such as diabetes and AF. The CHARGE study identified two SNPs at a genetic locus on chromosome 12p13 associated with an increased risk of stroke.[234]

Monogenic disorders that have been associated with ischemic stroke include hematological disorders, such as FVL mutation[235] and protein C deficiency[236]; connective tissue disorders, such as Ehlers-Danlos syndrome type IV[237]; metabolic disorders such as MELAS[120] and hyperhomocysteinemia[238]; and inherited small-vessel disease, such as CADASIL.[112] Examples of polygenic disorders associated with ischemic stroke include Marfan syndrome,[239] familial Moyamoya disease,[240] and familial hemiplegic migraine.

HEMATOLOGICAL RISK FACTORS FOR STROKE

Thrombophilia and Ischemic Stroke

Inherited prothrombotic disorders include FVL mutation, protein C deficiency, protein S deficiency, antithrombin deficiency, and PT G20210A. Acquired disorders of coagulation that predispose to a hypercoagulable state include the antiphospholipid syndrome, hyperhomocysteinemia, malignancy, dysfibrinogenemia, elevated von Willebrand factor (vWF) levels, and elevated plasminogen activator inhibitor-1 levels.

The FVL mutation, the most common inherited disorder leading to a hypercoagulable state, leads to resistance of FV to degradation by activated protein C.[241] It is the most frequent genetic risk factor predisposing to venous thrombosis worldwide.[242] However, large prospective and nested case–control studies,[243,244] as well as two meta-analyses,[245,246] have failed to demonstrate a significant association between FVL mutation and risk of ischemic stroke in adults. Some small case–control studies have reported an increased risk of ischemic stroke in women using oral contraceptives who had either the FVL or PT G20210A mutation.[247–249] Other studies in children have suggested that FVL may be a risk factor for ischemic stroke in this group, although sample sizes were small.[250,251]

Protein C is a vitamin K–dependent factor that when activated, along with its cofactor protein S, induces proteolysis of factors V and VIII:C, thereby inhibiting the coagulation cascade and thrombus formation.[252] Deficiency of protein C and/or protein S leads to a hypercoagulable state and an increased risk of venous thrombotic events, most commonly deep venous thrombosis, pulmonary embolism, and superficial thrombophlebitis.[242,253,254] Studies investigating an association between protein C deficiency and ischemic stroke in adults have produced conflicting results. Some smaller case–control studies and case reports have reported a significant association between protein C deficiency and ischemic stroke,[236,255] but this has been contradicted in larger studies.[256,257] A prospective cohort studies and a meta-analysis have reported a significant association between protein C deficiency and ischemic stroke in children.[250,258] An association between protein S deficiency and ischemic stroke is also uncertain. Small studies and case reports have reported a possible association between protein S deficiency and risk of ischemic stroke[259,260]; however a larger case–control study failed to confirm an association.[256] A meta-analysis of case–control studies also failed to demonstrate a significant association in children.[250]

The PT G20210A mutation leads to an increase in plasma levels of prothrombin (factor II), a key enzyme in the coagulation cascade and the precursor of thrombin. Carriers of the mutation have an approximately threefold increased risk of venous thrombotic events.[261,262] Studies investigating the possible association between PT G20210A and risk of ischemic stroke have produced conflicting results. Two large prospective population studies, the Physicians' Health Study and the Cardiovascular Health Study, failed to demonstrate a significant association between PT G20210A and stroke risk[263,264]; however, a large meta-analysis of 19 case–control studies reported a modest, but significant, association.[265] Antithrombin is a serine protease inhibitor that inactivates thrombin and activated coagulation factors such as FX, FIX, FXI, and FXII, thereby inhibiting coagulation.[265] Deficiency of antithrombin results in a hypercoagulable state and an increased risk of venous thrombotic events. The deficiency can also be acquired, for example, due to severe liver disease, nephrotic syndrome, acute thrombosis, and disseminated intravascular coagulation or through the use of medications such as oral contraceptives or heparin.[265] There is no substantial evidence to date to demonstrate an association between antithrombin deficiency and risk of ischemic stroke in adults or children based on a prospective cohort study,[266] a large case–control study,[256] and a meta-analysis of eight case–control studies in children.[250]

The antiphospholipid syndrome (APL) syndrome is a systemic autoimmune disorder characterized by recurrent thrombosis or pregnancy morbidity, in association with increased levels of antiphospholipid antibodies.[242] The pathophysiological mechanism underlying the association between APL syndrome and an increased risk of recurrent venous and arterial thrombosis is still debated but is mainly believed to be caused by antiphospholipid antibody-mediated activation of endothelial cells and platelets.[267] Studies investigating the association between APL syndrome and the risk of ischemic stroke have produced contradictory results. Some case–control studies, such as the RATIO study, have reported a positive association between APL antibodies and an increased risk of ischemic stroke,[268,269] while others have failed to report such an association.[270,271] In the APASS case–control study of patients presenting with first ischemic stroke, anticardiolipin antibodies were detected in 9.7% of stroke patients, compared to 4.3% of controls. The OR for stroke risk independently associated with anticardiolipin antibody positivity was 2.31.[272] A large prospective cohort study demonstrated an association between elevated serum concentration of anticardiolipin antibodies and ischemic stroke risk in women, but not in men.[273] Several studies in young adults have demonstrated an association between APL antibodies and ischemic stroke.[274,275]

Homocysteine is an amino acid derived from methionine in the diet. Hyperhomocysteinemia can be inherited, due to inborn errors of amino acid metabolism such as cystathionine β-synthase deficiency, or more commonly it is acquired, for example, due to dietary deficiency of vitamin B_{12} or folic acid, increased age, or renal failure.[276] Homocysteine promotes atherogenesis and thrombosis[277]; however the pathophysiological mechanism underlying the association between hyperhomocysteinemia and risk of ischemic stroke is poorly understood.[242] Studies have consistently reported a strong and independent association between elevated plasma homocysteine levels and stroke risk, and a meta-analysis of eight prospective studies demonstrated a significant association between elevated homocysteine levels and risk of stroke.[238,278] Several large randomized trials, such as vitamin intervention for stroke prevention (VISP) and more recently vitamins to prevent stroke (VITATOPS), have failed to demonstrate an overall benefit from vitamin supplementation

(folic acid, vitamins B_6 and B_{12}) in reducing the recurrence of major vascular events such as stroke.[279,280]

Fibrinogen is a key factor in the coagulation cascade and in the promotion of thrombosis. High levels of fibrinogen are consistently associated with an increased risk of ischemic stroke.[281–283] One large meta-analysis of 31 population-based prospective studies reported a significant association between high plasma fibrinogen levels and ischemic stroke risk (HR 2.06, per 1 g/L increase in typical fibrinogen levels).[284]

vWF is a glycoprotein that is produced in the endothelial cells and platelets and plays a key role in hemostasis. It mediates platelet adhesion and aggregation at sites of endothelial injury and serves as a carrier protein for coagulation factor VIII.[285] Studies investigating the possible association between high vWF levels and ischemic stroke risk have produced inconsistent results. One case–control study reported a significantly increased risk of first ischemic stroke in patients with high vWF activity and antigen levels.[286] The Atherosclerosis Risk in Communities (ARIC) prospective study found a significant association between vWF levels and ischemic stroke risk[287]; however another large prospective study failed to demonstrate a similar association.[288]

Essential thrombocythemia (ET), one of the myeloproliferative disorders, is an uncommon cause of ischemic stroke, accounting for <1% of cases.[289] The hypercoagulable state in ET is believed to be due to increased platelet activation and turnover, an acquired protein C resistance and decrease in protein S levels, as well as endothelial activation.[289]

SCD is associated with an increased risk of stroke, particularly in those with the homozygous HbSS genotype.[46] The risk of stroke is up to 11% by 20 years of age, 15% by 30 years, and 24% by 45 years.[290] The most common mechanism underlying ischemic stroke in SCD is believed to be large-artery arteriopathy, secondary to hyperplasia of the intima from repeated endothelial injury.[291–293]

Thrombotic thrombocytopenic purpura (TTP) is a rare systemic disorder that increases the risk of ischemic stroke. It is characterized by thrombocytopenia, microangiopathic hemolytic anemia, fever, renal failure, and neurological abnormalities,[294] although the full pentad of clinical features rarely occurs. It can occur in familial or acquired forms. Familial TTP typically appears in childhood, although familial cases can also present later in life.[295] Acquired TTP occurs in older children and adults and is typically characterized by one episode, although recurrences do occur in 11% to 36% of individuals.[296–299] It is generally idiopathic, although it can also occur secondary to clopidogrel[300] or ticlopidine use,[301] malignancies, or occasionally during pregnancy or the peurperium.[302] Systemic aggregation of platelets in the microvasculature, mediated by large vWF monomers, leads to platelet-rich thrombi which can occlude the microvasculature and lead to ischemic events such as stroke.[295] The activity of ADAMTS 13, a vWF-cleaving metalloprotease, has been noted to be <5% of normal levels in the majority of patients with familial or acquired TTP.[295]

CONCLUSION

Ischemic stroke can be categorized into distinct etiological subtypes, which include large-vessel, small-vessel, cardioembolism, and cryptogenic subtypes. Unlike acute coronary syndrome, the mechanisms underlying the subtypes of ischemic stroke demonstrate greater heterogeneity. However, thrombogenesis is the underlying pathophysiological mechanism, common to all ischemic stroke subtypes.

Clinically overt stroke is considered to represent only a fraction of all strokes. The advent of contemporary MRI sequences has identified a large burden of subclinical cerebrovascular disease, which includes covert infarction, white matter hyperintensities, cerebral atrophy, and microbleeds.

Risk factors for stroke can be classified as traditional risk factors, such as hypertension, smoking, diabetes mellitus, and obesity; hematological risk factors, including FVL mutation, protein C deficiency, and antithrombin deficiency; and emerging risk factors namely, SNPs and monogenic and polygenic disorders. Many stroke risk factors have both genetic and environmental elements. The majority of ischemic stroke cases are sporadic and of multifactorial origin.

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CHAPTER

92

Prevention of Thromboembolic Stroke in Patients with Atrial Fibrillation, Valvular Heart Disease, or Cardiomyopathy

THOMAS L. KAWANO AND JONATHAN L. HALPERIN

Patients with various forms of cardiac disease are at risk of ischemic stroke and systemic embolism due to migration of thrombus that forms on endocardial or prosthetic valve surfaces or in the cardiac chambers. The risk varies with the nature of the underlying heart disease, but clinically these events are typically severe, resulting in substantial disability and mortality. Highly effective prophylactic therapy is available in the form of anticoagulant drugs, but treatment is associated with a risk of bleeding complications. Hence, an understanding of the pertinent pathophysiology, clinical factors responsible for variation in individual risk, and evolving management alternatives is crucial to optimum patient care.

ATRIAL FIBRILLATION

Atrial fibrillation (AF) is the most common sustained cardiac rhythm disturbance and an important independent risk factor for ischemic stroke. AF affects approximately 2.5 million individuals in the United States[1,2] and millions more worldwide. Prevalence is greater in men than in women[3–5] and increases with age, rising rapidly beyond the 6th decade to approximately 10% among those older than 80 years (see FIGURE 92.1).[2–6] The median age of patients with AF is approximately 72 years. As the population ages, the number of individuals with AF is likely to increase substantially in the coming decades.[2]

Patterns of AF

AF can be classified according to pattern of occurrence as paroxysmal, persistent, or permanent, based on the duration of the arrhythmia and reversibility.[7] *Paroxysmal* AF may occur once or multiple times, but involves self-terminating episodes. *Persistent* AF does not convert to normal sinus rhythm (NSR) spontaneously but NSR can be restored, at least temporarily, with pharmacologic or electrical cardioversion. *Permanent* AF is refractory and implies a clinical decision to allow AF to continue. Either paroxysmal or persistent AF may occur as a single or recurrent episode. Risk factors associated with the future development of AF have led to the concept that some patients may have "pre-AF," and the early success of catheter-based ablation therapy has created a cadre of patients with "post-AF," who merit special considerations with respect to antithrombotic therapy.

Risk of Stroke Associated with AF

As a result of disorganized and ineffective atrial systole, the fibrillating atria become passive conduits of blood from the systemic and pulmonary venous systems to the ventricles. Impaired atrial emptying leads to stasis and increases the risk of thrombus formation, particularly in the left-atrial appendage (LAA).[8] The factors that promote embolism of intracardiac thrombus and subsequent ischemic events (including stroke and peripheral arterial occlusion) are incompletely understood.[9,10] In addition to cardiogenic embolism, patients with AF commonly have other cardiovascular disease states, such as hypertension and atherosclerosis, which raise the risk of ischemic events.

Approximately 15% of all strokes are attributed to AF.[11] The rate of ischemic stroke among patients with AF not given antithrombotic therapy averages 4.5% per year,[12,13] approximately fivefold greater than in age- and gender-matched individuals with NSR. In the Framingham Heart Study, the risk of stroke attributable to AF rose from 1.5% in patients aged 50 to 59 years to 23.5% in those aged 80 to 89 years.[14] Because both the prevalence of AF and the risk of stroke increase with patient age, the problem of stroke prevention is amplified among the elderly; AF is the most common cause of stroke in women older than 75 years.[15] When compared to ischemic stroke due to other causes, ischemic stroke due to AF is typically more severe and carries a significantly higher mortality rate.[16] Several hypotheses have been advanced to explain the pathogenesis of stroke in patients with AF and the poor clinical outcomes associated with these events. Embolism of thrombus from the LAA may cause complete occlusion of relatively large cerebral arteries, consistent with the relatively frequent finding of large cortical infarcts on brain imaging and less frequent lacunar infarcts.[17] Chronically reduced cerebral blood flow, due either to the arrhythmia itself or to more extensive cerebrovascular disease associated with AF due to hypertension or atherosclerosis, may compromise collateral perfusion when acute infarction develops and contribute to greater stroke severity.

AF in the Setting of Valvular Heart Disease

Patients with AF and rheumatic mitral valve disease or mechanical or biologic prosthetic heart valves have been considered at high risk of stroke[10] and were excluded from most randomized

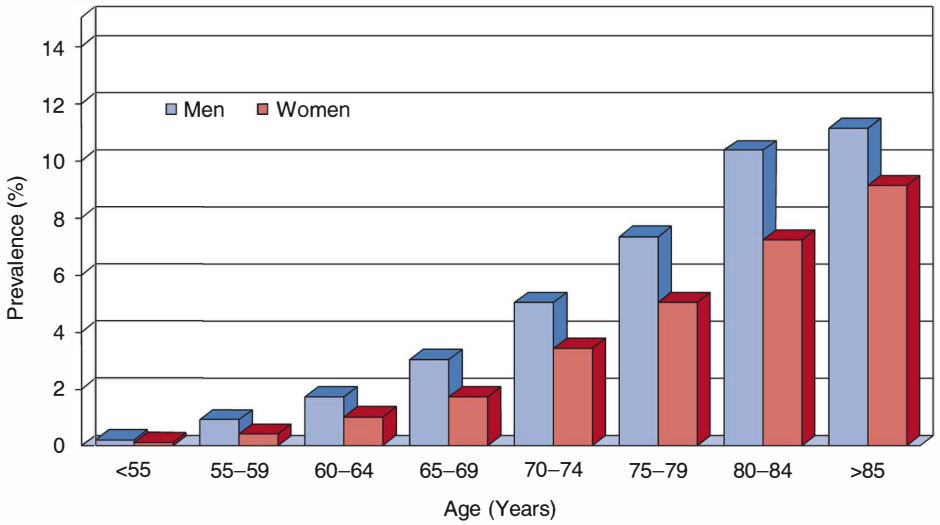

FIGURE 92.1 Prevalence of atrial fibrillation according to sex and age.

trials of antithrombotic therapy because of the perceived need for anticoagulation. Hence, available empirical data pertain mainly to patients with nonvalvular AF.

Atrial Flutter

Sustained atrial flutter is uncommon because the rhythm typically either degenerates to AF or reverts to NSR. Patients with persistent atrial flutter may have periods of AF and *vice versa*. There are scant data from longitudinal studies assessing the thromboembolic risk associated with isolated, sustained atrial flutter. Echo-Doppler examinations of patients with atrial flutter demonstrate more organized atrial mechanical function and greater LAA flow velocities than are typical in patients with AF.[18] Despite this functional distinction, intra-atrial thrombus and stroke have been documented in patients with atrial flutter.[6,19] In a transesophageal echocardiographic (TEE) study before cardioversion, 25% of patients with atrial flutter of slightly longer than 6 months mean duration had spontaneous echocardiographic contrast, a marker of stasis thought to represent a prethrombotic state, and 7% had LAA thrombus.[20] In patients with prior cerebral ischemic events, the prevalence of intra-atrial thrombus associated with atrial flutter was even higher.[21] A retrospective study of 100 patients with persistent atrial flutter found a higher than anticipated risk of stroke.[22] Although the role of anticoagulant therapy for patients with atrial flutter has not been evaluated in clinical trials, current treatment guidelines recommend that antithrombotic therapy follows the same risk-stratification approach applied to patients with AF.

Stroke Risk Stratification in Patients with AF

A combined analysis of untreated control groups in five primary prevention trials found a stroke rate of over 8% per year in patients with AF older than 75 years who had one additional clinical risk factor (hypertension, diabetes mellitus, heart failure (HF), or prior stroke or transient ischemic attack [TIA]). For patients of any age with a history of thromboembolism, the annual risk of stroke was 12%.[12,23] In a study of elderly nursing home patients who were not anticoagulated, the 3-year stroke rate exceeded 50%.[24]

Risk factors that increase the risk of stroke among patients with AF not treated with anticoagulants have been determined from randomized trials of antithrombotic therapy.[23–28] The most commonly cited risk-stratification strategies were generated from pooled analyses of five trials by the Atrial Fibrillation Investigators (AFI)[12] and from the Stroke Prevention in Atrial Fibrillation (SPAF) cohorts.[25,28] The AFI group[12] found the following independent risk factors: age (relative risk [RR] 1.4 per decade), prior stroke or TIA (RR 2.5), a history of hypertension (RR 1.6), and diabetes mellitus (RR 1.7). Analysis of 854 patients assigned to aspirin from the first two SPAF trials[29] identified three independent risk factors for stroke: women older than 75 years (RR 3.7), systolic hypertension >160 mm Hg (RR 2.2), and impaired left ventricular (LV) function defined as recent (within 3 months) HF, moderate-to-severe systolic dysfunction as assessed by two-dimensional echocardiography, or fractional shortening <0.25 by M-mode echocardiography (RR 1.8). Extending the analysis to 2,012 patients allocated to the aspirin or combination therapy arms of the SPAF-I to SPAF-III studies[30] identified five characteristics significantly associated with an increased risk of stroke: prior thromboembolism (RR 2.9), age (RR 1.8 per decade), female gender (RR 1.6), history of hypertension (RR 2.0), and systolic blood pressure >160 mm Hg (RR 2.3).

Although prior stroke or TIA, older age, and hypertension were identified as risk factors for stroke in patients with AF in both the AFI and SPAF risk-stratification schemes, there was a differential impact of age in these cohorts. The AFI scheme classified all patients with AF 65 years or older as at elevated risk, whereas the SPAF criteria classified women 75 years or younger and men of any age without other risk factors as at low risk. Uncertainty about risk in either gender aged 65 to 75 years and in men of any age without other risk factors applies to approximately 20% of the population with nonvalvular AF.[31]

Current guidelines recommend a risk-based approach to selection of patients with AF for oral anticoagulation to prevent stroke.[7] The main clinical risk factors are presented

Table 92.1

Principal clinical risk factors associated with stroke and systemic embolism in patients with nonvalvular atrial fibrillation[a]

High-Risk Factors
Mitral stenosis
Prosthetic heart valve
History of stroke or TIA
Moderate Risk Factors
Age > 75 y
Hypertension
Diabetes mellitus
HF or ↓ LVEF
Less Validated Risk Factors
Age 65–75 y
Coronary artery disease
Female gender

[a]Patients with any of the high-risk factors should be anticoagulated. Those with one moderate risk factor also benefit from anticoagulation, but the number of patients needed-to-treat with an anticoagulant as compared with aspirin is considerably larger, so some patients with one moderate risk factor may choose platelet inhibitor therapy. The moderate risk factors shown here and prior thromboembolism form the basis for the $CHADS_2$ risk score (see Table 92.2), while the less-validated risk factors have been incorporated into the CHA_2DS_2VASc score (see Table 92.3).
LVEF, left ventricular ejection fraction.
Adapted from Singer DE, Albers GW, Dalen JE, et al. Long-term antithrombotic therapy for chronic atrial fibrillation or atrial flutter: anticoagulants and antiplatelet agents. In Seventh ACCP Consensus Conference on Antithrombotic Therapy: Antithrombotic Therapy in Atrial Fibrillation. American College of Chest Physicians. *Chest* 2004;126(Suppl):429S–456S; Fang MC, Singer DE, Chang Y, et al. Gender differences in the risk of ischemic stroke and peripheral embolism in atrial fibrillation: the AnTicoagulation and Risk factors In Atrial fibrillation (ATRIA) study. *Circulation* 2005;112:1687–1691.

in Table 92.1. A modified scale, $CHADS_2$ (Cardiac failure, Hypertension history, Age >75 years, Diabetes, and Stroke or TIA [doubled]), integrates elements from the AFI and SPAF schemes in assessing the annual risk of stroke. This index allots two points for a history of stroke or TIA and one point each for age over 75 years, history of hypertension (not further defined), diabetes mellitus, recent clinical HF, or impaired LV systolic function (generally taken as an ejection fraction ≤35%) (Table 92.2). The scheme was initially evaluated in 1,733 Medicare beneficiaries with nonvalvular AF aged 65 to 95 years who were discharged from hospital off warfarin[32] and has been validated in several cohorts.

The $CHADS_2$ index has the advantage of simplicity, but classifies a relatively large proportion of patients with AF as at intermediate risk and has imperfect predictive capacity overall (c-statistic ~0.6). Additionally, many of those designated as low risk carry as high as a 1.3% annual risk of stroke. Additional risk factors, albeit less well validated than those that comprise the $CHADS_2$ index, should be considered in the stratification of stroke risk in patients with AF. Women with AF appear at higher risk than men with otherwise comparable risk profiles.[33–35] Vascular disease, including peripheral arterial disease, coronary disease (specifically prior myocardial infarction [MI]),

Table 92.2

$CHADS_2$ score[a]

	Score (Points)	Prevalence (%)
Congestive HF	1	32
Hypertension	1	65
Age > 75 y	1	28
Diabetes mellitus	1	18
Stroke or TIA	2	10
Moderate–high risk	≥2	50–80
Low risk	0–1	40–50

[a]For estimation of thromboembolic risk in patients with nonvalvular atrial fibrillation. One point each is assigned for a history of clinical HF or impaired left-ventricular systolic function (corresponding roughly to ejection fraction ≤35%), hypertension (not further defined), and diabetes mellitus and two points for prior ischemic stroke, TIA, or systemic arterial embolism. The approximate prevalence in the Euro Heart survey cohort is shown in the right-hand column, and proportion of patients falling into low-, intermediate-, and high-risk strata is given at the bottom.
Adapted from van Walraven C, Hart RG, Wells GA, et al. A clinical prediction rule to identify patients with atrial fibrillation and a low risk for stroke while taking aspirin. *Arch Intern Med* 2003;163:936–943; Nieuwlaat R, Capucci A, Lip GY, et al. Antithrombotic treatment in real-life atrial fibrillation patients: a report from the Euro Heart Survey on atrial fibrillation. *Eur Heart J* 2006;27:3018–3026.

and morphologically complex atheromatous plaque involving the thoracic aorta, is also associated with an increased risk of stroke, but its independent predictive value is controversial. An updated risk-stratification scheme, the CHA_2DS_2VASc score (Table 92.3), incorporates vascular disease (V) and female sex (S) into the risk-stratification schematic, assigning each of these factors one point, and distinguishes two age categories to

Table 92.3

CHA_2DS_2VASc risk-stratification scheme[a]

	Weight (Points)
Congestive HF or LVEF ≤ 35%	1
Hypertension	1
Age >75 y	2
Diabetes mellitus	1
Stroke/TIA/systemic embolism	2
Vascular disease/peripheral arterial disease/aortic plaque	1
Age 65–74 y	1
Sex: category (female)	1
High risk	≥2
Low risk	0–1

[a]This scheme has the advantage of assigning fewer patients than the $CHADS_2$ score to the intermediate-risk category. This score was adopted in the European clinical practice guidelines for management of patients with AF in 2010 but is not universally accepted as superior to the $CHADS_2$ score.
LVEF, left ventricular ejection fraction.
Adapted from Lip GYH, Halperin JL. Improving stroke risk stratification in atrial fibrillation. *Am J Med* 2010;123:484.

account for the continuous relationship of risk to age, allocating one point to patients with AF beyond age 65 and two points to those over 75 years.[36] The CHA_2DS_2VASc score was validated in the Euro Heart Survey cohort, anticoagulated clinical trial cohorts, and a national patient registry of patients with AF who were not anticoagulated.[37] When compared with the $CHADS_2$ risk score, CHA_2DS_2VASc had greater negative predictive value for stroke for patients classified in the low-risk category and classified fewer patients as intermediate risk, but the c-statistics still indicated imperfect capture of all attributable risks.[36]

The controlled clinical trials of warfarin included mainly patients with chronic, persistent, and permanent AF and smaller proportions of patients with paroxysmal AF.[7] For the purpose of determining stroke risk, the distinction between paroxysmal, persistent, and permanent AF proved immaterial. Patients with paroxysmal AF typically have a lower risk of stroke than those with persistent or permanent AF, as they are often younger and have fewer associated risk factors,[38,39] but multivariate analyses of prospectively followed cohorts found that the pattern of AF was not a significant predictor of stroke risk.[12,40] Current recommendations for anticoagulation, therefore, do not distinguish patients on the basis of the pattern of AF, though the available evidence does not address transient self-limited AF caused by acute medical illness or recent cardiac or noncardiac surgery.[7]

Postoperative Atrial Fibrillation

Patients who develop AF after undergoing cardiothoracic surgery are also at an increased risk of thromboembolic stroke. While there is a small but significant increased risk of in-hospital stroke in patients who develop AF after surgery involving cardiopulmonary bypass, the risk of stroke seems related to underlying comorbidities rather than to AF itself. This was demonstrated in a series of close to 4,000 patients who had cardiac surgery on cardiopulmonary bypass. The rate of AF after surgery was reduced with prophylactic therapy, but the incidence of stroke was not reduced.[41]

The decision to anticoagulate patients with AF in the postoperative period is confounded by the risk of bleeding and its associated sequelae (cardiac tamponade, hematoma, hemothorax, etc.). Additionally, a significant percentage of patients who develop AF postoperatively convert back to sinus rhythm. The duration of AF postoperatively, the associated risk of thromboembolic stroke, and the decision of when to anticoagulate are unclear. Practice guidelines differ on when to start anticoagulation for postoperative AF. At the time of this writing, the American College of Chest Physicians (ACCP) guidelines recommend initiating anticoagulation with vitamin K antagonist (VKA) 48 hours after a patient has developed AF. The American Heart Association/American College of Cardiology (AHA/ACC) guidelines recommend starting a VKA after 24 hours of developing AF. Both guidelines agree that anticoagulation should continue for 4 weeks following reversion to and maintenance of sinus rhythm, but the level of evidence supporting this duration of therapy is low.

Echocardiographic Markers of Stroke Risk

Although left-atrial size and LV systolic function can be assessed by transthoracic (precordial) echocardiography, TEE is necessary to consistently visualize abnormalities of the LAA and aortic arch linked with thromboembolism. This approach is used not only as an adjunct to elective cardioversion (see below) but sometimes in patients with persistent AF to assist in thromboembolic risk assessment.[42,43] Visible thrombus or dense spontaneous echo-contrast in the left atrium (LA), and particularly in the LAA, is associated with a two- to fourfold increased risk of stroke.[7,8] In the SPAF-III study, patients with atheromatous plaque in the thoracic segments of the aorta that had complex morphology (mobility, pedunculation, ulceration, or thickness >4 mm) had high stroke rates. Because many of these abnormalities were observed in the descending aorta beyond the origins of the cerebral vessels, the association with stroke may reflect either associated cerebrovascular disease or the pathogenic role of risk factors such as hypertension that are common both to atherosclerosis and to conditions of atrial stasis in patients with AF.[43]

Other Potential Stroke Risk Factors in AF

Other characteristics that may augment stroke risk include genetic polymorphisms, abnormalities of hemostatic and thrombotic function, increased platelet activation and aggregation, endothelial dysfunction,[44,45] and serum levels of troponin and B-type natriuretic protein, but none have yet proven sufficiently robust for routine clinical use. Thyrotoxicosis is a poorly understood risk factor for stroke among patients with AF, because patients with thyrotoxicosis were excluded from the randomized trials of antithrombotic therapy. AF develops in 10% to 15% of patients with hyperthyroidism, and thyrotoxicosis is evident in 2% to 5% of patients with AF.[46] A high frequency of stroke and systemic embolism has been reported in some[47–51] but not all studies.[52] The association, if real, may be mediated partly by concomitant HF.

Antithrombotic Drug Therapy for Prevention of Thromboembolism in Patients with AF

Oral Anticoagulant Therapy with a Vitamin K Antagonist

Several randomized trials demonstrated the efficacy of adjusted-dose warfarin for prevention of first or recurrent ischemic stroke in patients with AF with relative risk reductions (RRR) of 47% to 86% versus placebo or no therapy. Results from these trials are summarized in FIGURE 92.2. In a pooled analysis, adjusted-dose warfarin reduced the risk of stroke by about two-thirds.[27,46] The magnitude of benefit was fairly consistent among trials despite variations in design and in the intensity of anticoagulation. Compared with aspirin, adjusted-dose warfarin reduced the overall risk of stroke by 36% (95% CI 14% to 52%) and ischemic stroke by 46% (95% CI 27% to 60%).[30] The discrepancy is driven largely by the results of the SPAF-II trial (mean international normalized ratio [INR] 2.6), which involved an older patient cohort than enrolled in most other studies,[53] and the risk of intracranial bleeding among patients receiving adjusted-dose warfarin was substantially increased among patients more than 75 years old. Exclusion of SPAF-II data from the meta-analysis revealed a 49% reduction in the risk of all stroke for adjusted-dose warfarin compared with aspirin (95% CI 26% to 65%).[30] Anticoagulation lowered all-cause mortality by 33% (95% CI 9% to 51%) and the combined outcome of stroke, systemic

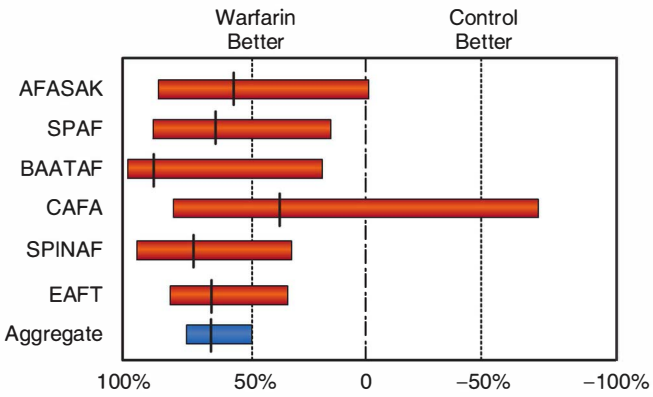

FIGURE 92.2 Stroke risk reductions with warfarin compared to control (placebo or no treatment) in patients with nonvalvular atrial fibrillation. The first five trials listed mainly involved primary prevention, whereas the last was confined to patients with prior nondisabling stroke or TIA. Values shown were obtained according to intention-to-treat analysis. AFASAK, Atrial Fibrillation, Aspirin and Anticoagulation trial; SPAF, the Stroke Prevention in Atrial Fibrillation trial; BAATAF, the Boston Area Anticoagulation Trial for Atrial Fibrillation; CAFA, the Canadian Atrial Fibrillation Anticoagulation trial; SPINAF, the Veterans Administration Stroke Prevention in Atrial Fibrillation trial; EAFT, the European Atrial Fibrillation Trial. (Adapted from Hart RG, Halperin JL. Atrial fibrillation and stroke: concepts and controversies. *Stroke* 2001;32:803.)

embolism, and death by 48% (95% CI 34% to 60%).[12] Due to concern about bleeding, particularly intracranial hemorrhage, when warfarin is given to elderly patients, the BAFTA trial compared open-label warfarin and aspirin in patients 75 years of age and older during treatment by primary doctors in the UK.[54] The annual risk of the primary outcome of (ischemic or hemorrhagic) stroke, other intracranial hemorrhage, or systemic embolism was significantly lower in the warfarin group than in patients randomized to aspirin (yearly risk 1.8% vs. 3.8%, RR 0.48, 95% CI 0.28 to 0.80, $P = 0.003$).[54] These data support the advantage of treating older patients who have AF with warfarin, unless there are other contraindications.

AF had been present for months or years in most of the patients enrolled in these studies, yet each was terminated earlier than planned because of the large benefit of antithrombotic therapy (most often warfarin) for prevention of ischemic stroke and systemic embolism. In aggregate, by intention-to-treat analysis, the annual stroke rate was reduced from 4.5% in the control arms to 1.4% with adjusted-dose warfarin. The effect was consistent across studies with an overall RRR of 68% (95% CI 50% to 79%).[12] The absolute risk reduction implies prevention of 31 ischemic strokes each year for every 1,000 patients treated (or 32 patients needed-to-treat [number needed to treat for 1 year] to prevent a single stroke).[12]

The efficacy of anticoagulation with warfarin was consistent across all patient subgroups and for preventing strokes of all degrees of severity. Most strokes occurring in the warfarin arms involved patients who had stopped warfarin or in whom the INR or prothrombin time ratio fell below the therapeutic range. In summary, evidence for the efficacy of anticoagulation with warfarin in patients with AF is strong and consistent, based on several adequately powered, randomized trials, though an important caveat is that these were mostly open-label studies. The one trial that involved double-blind treatment included no women.[55]

In these trials, anticoagulation proved relatively safe when the upper limit of the therapeutic range was less than INR 3.0, but whether comparable safety can be reliably achieved in less carefully selected patients outside the rubric of clinical research protocols is a persistent controversy. Pooled analysis of the first five primary prevention trials reported a 1.0% per year rate of major bleeding in control patients versus 1.3% in those treated with warfarin. Several schemes have been developed to predict the risk of bleeding in patients with AF during anticoagulant therapy, and though several have been validated in various cohorts none has been uniformly accepted (Table 92.4).

Intracranial hemorrhage is the only complication of anticoagulation that regularly produces deficits more severe than the ischemic strokes the therapy is intended to prevent, and in these trials the rate of intracranial hemorrhage was 0.3% per year in anticoagulated patients compared with 0.1% in the control groups.[12] Intracranial hemorrhage is more common in patients with prior ischemic stroke, hypertension, and advanced age.[56] One reason intracranial hemorrhages occurred at a low rate in these trials may have been the exclusion of patients considered at high risk of bleeding. Even when aggregated, these trials contribute less to understanding the determinants of intracranial hemorrhage than do large observational studies, which show a correlation with the intensity of warfarin anticoagulation (INR > 4.0),[56,57] although even with INR values in the

Table 92.4
HAS-BLED score[a]

	Weight (Points)
Hypertension (>160 mm Hg systolic)	1
Abnormal renal or hepatic function	1–2
Stroke	1
Bleeding history or anemia	1
Labile INR (TTR < 80%)	1
Elderly (age > 75 y)	1
Drugs (antiplatelet, NSAID) or alcohol	1–2
High risk (>4% per y)	≥4
Moderate risk (2%–4% per y)	2–3
Low risk (<2% per y)	0–1

[a]One of several risk scores for predicting bleeding in anticoagulated patients with atrial fibrillation. Although adopted in some clinical practice guidelines, no single scheme has yet been widely accepted to guide selection of patients for anticoagulant therapy for prevention of thromboembolism, and bleeding risk must be considered in the context of the patient's inherent risk of thromboembolism.
INR, International Normalized Ratio of prothrombin suppression; TTR, percent time in therapeutic range; NSAID, nonsteroidal antiinflammatory drug.
Adapted from Pisters R, Lane DA, Nieuwlatt R, et al. A novel user-friendly score (HAS-BLED) to assess one-year risk of major bleeding in atrial fibrillation patients: the Euro Heart Survey. *Chest* 2010;138(5):1093–1100; Lip GYH, Frison L, Halperin JL, et al. Comparative validation of a novel risk score [HAS-BLED] for predicting bleeding risk in anticoagulated patients with AF. *J Am Coll Cardiol* 2010;57:173–180.

therapeutic range of 2.0 to 3.0 warfarin doubles the risk.[58] Even more important is that intracerebral hemorrhage in anticoagulated patients is usually catastrophic and often fatal.

Efficacy of Aspirin

Evidence that aspirin is effective for prevention of thromboembolism in patients with nonvalvular AF is considerably weaker than that supporting warfarin. Meta-analysis of five controlled studies[23,59–64] found a 22% (95% CI 2% to 38%) reduction in stroke rates.[27] Four of these were placebo-controlled trials,[64] with aspirin doses ranging from 50 to 325 mg daily. A patient-level meta-analysis of data from the placebo-controlled AFASAK-1, SPAF-I, and EAFT trials estimated that the RRR was a marginally significant 21% (95% CI 0% to 38%).[67] The SPAF-I trial, in which the RRR was significant at 42%, had two randomized cohorts, one of patients eligible for randomization to warfarin and the other deemed ineligible on the basis of age, physician judgment, or patient refusal. In warfarin-eligible cases, the RRR afforded by aspirin was striking (94%), whereas that in the ineligible cohort was minimal (8%, not significant [NS]), similar to findings in the AFASAK-1 and EAFT studies. In other studies involving patients with AF, the efficacy of aspirin for prevention of thromboembolism was negligible or inconsistent.[61,62,64,66] Aspirin may be more efficacious for patients with AF and hypertension and diabetes[65] and for preventing noncardioembolic rather than cardioembolic ischemic strokes.[67] Cardioembolic strokes are, on average, more disabling than noncardioembolic strokes,[68] and aspirin appears to prevent nondisabling more than disabling strokes[27]; therefore, the greater the risk of disabling cardioembolic stroke in patients with AF, the less protection afforded by aspirin.[68]

In trials that compared the two therapies, oral VKA therapy was considerably more effective than aspirin.[20,23,49,53,54,59,62,63,68,69] Meta-analysis of six studies found an RRR of 46% (95% CI 27% to 60%) with oral anticoagulation compared with aspirin for ischemic stroke and 36% (95% CI 14% to 52%) for all (ischemic or hemorrhagic) strokes related to AF.[27] The hazard ratio (HR) for major bleeding associated with anticoagulation was 1.7 (95% CI 1.2 to 2.4).[70] Hence, treatment of 1,000 patients with AF with adjusted-dose oral anticoagulation instead of aspirin for 1 year would avoid 23 ischemic strokes but cause nine additional major bleeds.

Other Platelet-Inhibitor Agents and Lower-Intensity Anticoagulation

A randomized trial comparing adjusted-dose warfarin with the platelet inhibitor indobufen found no significant difference in rates of the combined endpoint of stroke, MI, pulmonary embolism, or vascular death (12% with indobufen vs. 10% with warfarin over an average follow-up of 12 months; $P = 0.47$).[71] None of the patients treated with indobufen developed major bleeding, which occurred in 0.9% of those treated with warfarin.

Trials of very low-intensity anticoagulation with or without aspirin have generally not identified a suitable alternative to adjusted-dose warfarin at INR 2.0 to 3.0 for prevention of thromboembolism in patients with AF. Meta-analysis of three studies[64,69,72] comparing fixed, low-dose warfarin (1.25 mg daily) with adjusted-dose warfarin (INR 2.0 to 3.0) found a mean RRR of 38% (95% CI 20% to 68%) favoring more intensive dosing.[27] Aspirin may augment protection against ischemic coronary events but raises the risk of hemorrhage in anticoagulated patients, and the incremental coronary benefit of adding aspirin to oral anticoagulation seems modest.[73] The SPAF-III trial compared a combination of warfarin (targeting INR initially to 1.2 to 1.5 with a maximum daily dose of 3 mg) plus aspirin (325 mg daily, enteric coated) with adjusted-dose warfarin (target INR 2.0 to 3.0). The trial was terminated early because of a higher rate of ischemic stroke and systemic embolism in patients taking the combination therapy (7.9% per year) than in those assigned to conventional warfarin (1.9% per year). There was no significant difference in rates of major hemorrhage between the two groups, and no significant advantage in terms of coronary outcomes because of the relatively low frequency of such events.

Two small-scale studies have addressed the use of lower-intensity warfarin. In one, targeting a lower INR value (1.9) was comparable to more conventional warfarin therapy (target INR 2.5) for secondary stroke prevention in patients with nonvalvular AF and was associated with a lower frequency of severe bleeding ($P = 0.0103$).[74] Patients in the conventional therapy group who developed bleeding complications were older (mean age 73.7 years), but the results must be interpreted cautiously because the study was terminated prematurely while the sample size was small ($n = 115$). The other trial compared low-intensity (target INR 1.8) and standard (target INR = 2.5) warfarin therapy for primary stroke prevention in patients over 75 years old.[75] There was no significant difference in the combined rate of thromboembolism or major bleeding with the two regimens (HR 0.7, 95% CI 0.4 to 1.1, $P = 0.1$). Despite a combined endpoint constellation, the event rate was lower than expected, and the results raise the possibility that less intensive anticoagulation may be adequate for primary stroke prevention in low-risk patients with AF.

Dual Antiplatelet Therapy

Although monotherapy with aspirin offers marginal benefit for thromboembolism prevention in patients with AF, the combination of aspirin plus clopidogrel, which blocks platelet aggregation through inhibition of adenosine diphosphate-dependent pathways,[76] reduces the incidence of ischemic events compared to aspirin alone. Early studies of clopidogrel showed a reduction of ischemic events with clopidogrel when added to aspirin therapy[77] or in place of aspirin[78] in patients with AF and atherosclerosis. The ACTIVE (Atrial fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events) studies compared dual antiplatelet therapy with aspirin and clopidogrel to adjusted-dose warfarin (ACTIVE-W) and to aspirin alone (ACTIVE-A) in patients with AF and a high risk of stroke ($CHADS_2$ score > 1).[79,80] The ACTIVE-W study was terminated early because of the overwhelming superiority of warfarin (RR with aspirin plus clopidogrel = 1.44, 95% CI 1.18 to 1.76, $P = 0.0003$) without any increase of the risk for major hemorrhage. ACTIVE-A was a randomized, double-blind study of 7,554 patients with AF who were not considered candidates for VKA therapy. Reasons for enrollment in ACTIVE-A included specific risk of bleeding (22.9% of patients), physician's judgment that the patient was a poor candidate for anticoagulation (49.7%), or the patients' preferences (26%). The primary endpoint included major vascular events (strokes, noncentral nervous system systemic embolic events, MIs, and deaths from vascular causes). The main secondary outcome was stroke. In ACTIVE-A, the combination of aspirin plus clopidogrel was

superior to aspirin alone for prevention of the primary outcome (RR 0.89, 95% CI 0.81 to 0.98, $P = 0.01$) and stroke alone (RR 0.72, 95% CI 0.62 to 0.83, $P < 0.001$). There was, however, a significantly higher risk for major bleeding with dual antiplatelet therapy (RR 1.57, 95% CI 1.29 to 1.92).

Novel Oral Anticoagulants

Despite their unequivocal efficacy against ischemic stroke in patients with AF, the narrow therapeutic margin of oral VKAs and interactions with numerous drugs and foods require frequent INR testing and dose adjustments. The quest for safer, more convenient alternatives has been particularly active and productive in recent years. Clinically available agents and others in advanced stages of development fall into two broad categories: direct inhibitors of thrombin (factor IIa) and inhibitors of activated factor X (factor Xa). The pharmacology of these agents is addressed elsewhere in this textbook.

Direct Thrombin Inhibitors

Ximelagatran was an orally administered prodrug converted after absorption to the active direct thrombin inhibitor melagatran. SPORTIF III and V were two long-term trials that compared ximelagatran to warfarin for prevention of stroke and systemic embolism in patients with AF.[81–83] While both studies found ximelagatran noninferior to warfarin (INR range 2.0 to 3.0), the compound was withdrawn from further development because of potential hepatotoxicity.

Dabigatran etexilate is another oral prodrug converted by a ubiquitous esterase to dabigatran, a potent direct inhibitor of thrombin. Its utility for prevention of thromboembolism in patients with nonvalvular AF was addressed in the RE-LY trial, a multicenter study of over 18,000 patients comparing dabigatran in twice-daily doses of either 150 or 110 mg to adjusted-dose warfarin (INR range 2.0 to 3.0).[84] This open-label study involving patients with a mean $CHADS_2$ risk score or 2.1 found dabigatran, 150 mg twice daily, superior to warfarin for prevention of stroke or systemic embolism (RR 0.66, 95% CI 0.53 to 0.82, $P < 0.001$). The rate of major bleeding with this dose of dabigatran was similar to that with warfarin. The 110 mg twice daily dose of dabigatran was noninferior to warfarin for prevention of stroke or systemic embolism and associated with a significantly lower risk of major bleeding (RR 0.80, 95% CI 0.69 to 0.93, $P = 0.003$) (FIGURE 92.3).[84] There was a higher risk of major gastrointestinal bleeding with dabigatran at 150 mg twice-daily dose than with warfarin (RR 0.91, 95% CI 0.85 to 0.97, $P = 0.005$),[84] but a lower risk of intracerebral hemorrhage (0.12 vs. 0.38% per year, $P < 0.001$). There was no indication of hepatic toxicity, but dyspepsia occurred more often in the dabigatran groups, sometimes limiting treatment. The FDA approved dabigatran in the 150 mg twice-daily dose for use in the United States; a dose of 75 mg twice daily was also approved for patients with impaired renal function (creatinine clearance 15 to 30 mL/min) based on pharmacokinetic modeling, but clinical experience with this low dose is limited. Regulators in Canada approved both the 110 and 150 mg twice-daily doses; and the European regulatory agency (EMA) also approved the drug.

Factory Xa Inhibitors

Several inhibitors of factor Xa have been investigated as therapeutic agents for prevention of thromboembolism

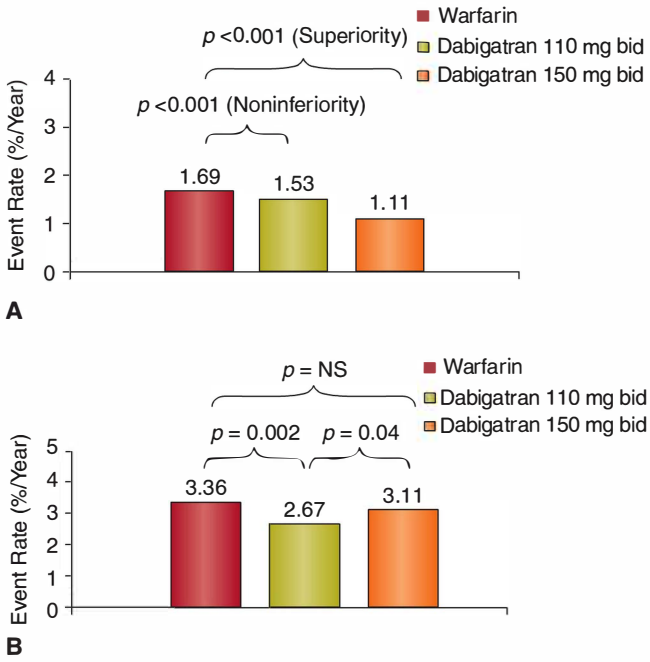

FIGURE 92.3 Main results of the RE-LY trial of the fixed-dose, oral, direct thrombin inhibitor, dabigatran etexilate, versus adjusted-dose warfarin (target INR 2.0 to 3.0) in patients with nonvalvular atrial fibrillation. **A:** Rates of the primary events (all strokes [ischemic and hemorrhagic] and systemic embolic events). **B:** Rates of major bleeding (bleeding that was fatal, was permanently disabling, involved a critical anatomic site, resulted in a ≥2 g/dL decline in hemoglobin content, or required transfusion of ≥2 units of whole blood or packed red blood cells). (Adapted from Connolly SJ, et al. Dabigatran versus warfarin in patients with atrial fibrillation. *N Engl J Med* 2009;361:1139.)

in patients with AF. Rivaroxaban is an orally active, direct factor Xa inhibitor effective for prevention and treatment of venous thromboembolism. It was evaluated in the multicenter, double-blind ROCKET-AF trial of 14,264 patients comparing rivaroxaban, 20 mg once daily (15 mg daily for patients with creatinine clearance 30 to 49 mL/min), to adjusted-dose warfarin (INR range 2.0 to 3.0) for prevention of stroke or systemic embolism in patients with AF at increased risk of stroke (mean $CHADS_2$ score = 3.5).[85] Rivaroxaban proved noninferior to warfarin (HR 0.79, 95% CI 0.66 to 0.96, $P < 0.001$) without increasing the risk of major bleeding. By intention-to-treat analysis, rivaroxaban was not superior to warfarin (2.20% vs. 2.40% per year, HR 0.91, 95% CI 0.78 to 1.07, $P = 0.26$). Based on the data from ROCKET-AF, the 20 mg daily dose of Rivaroxaban has been approved by the FDA for the prevention of stroke and systemic embolism in patients with nonvalvular AF.

Another direct factor Xa inhibitor, apixaban, has been investigated as a potential alternative to vitamin K antagonism. The AVERROES trial compared apixaban, 5 mg twice daily, to aspirin (81 to 324 mg daily) in patients with AF with at least one additional stroke risk factor who were not considered candidates for therapy with warfarin.[86] The study was stopped on

the advice of the Data Safety Monitoring Committee because of the superior efficacy of apixaban for prevention of stroke or systemic embolism in this patient population (RR = 0.46, $P < 0.001$). There was no significant difference in major bleeding between the two therapies, but there was an excess of minor bleeding in the group given apixaban, a substantial proportion of which was taking aspirin concurrently, and early termination of the study may have prevented detection of a difference in rates of major bleeding. The Apixaban for the Prevention of Stroke in Subjects with Atrial Fibrillation trial[87] compared apixaban, 5 mg twice daily, versus warfarin (target INR 2.5) in 18,201 moderate- to high-risk patients ($CHADS_2 \geq 1$) with nonvalvular AF. The primary outcomes were stroke or systemic embolism, and the key secondary efficacy outcome was death from any cause. The primary safety outcome was major bleeding defined as clinically overt bleeding accompanied by a decrease in the hemoglobin level ≥2 g/dL or transfusion of ≥2 units of packed red cells. Over a median follow-up of 1.8 years, apixaban was associated with a 21% reduction in primary events (HR 0.79, 95% CI 0.66 to 0.95, $P = 0.01$), a 31% reduction in the risk of bleeding (HR 0.69, CI 0.60 to 0.80, $P < 0.001$), a significant reduction in intracerebral bleeding, and an 11% reduction in all-cause mortality (HR 0.89, CI 0.80 to 0.99, $P = 0.047$) but no significant reduction in rates of ischemic stroke or cardiovascular mortality compared to warfarin. As with rivaroxaban, there was a trend toward lower rates of MI with apixaban compared to warfarin, but this was not statistically significant (0.53% vs. 0.61%, HR 0.88 [0.66 to 1.17], $P = 0.37$). Unlike dabigatran or rivaroxaban, apixaban therapy was associated with a lower rate of gastrointestinal bleeding than warfarin.

A third oral factor Xa inhibitor, edoxaban, is currently in phase 3 investigation in the ENGAGE-AF TIMI 48 trial of patients with AF.[88] Several other agents in this class (including betrixaban and darexaban) have completed phase 2 studies, but adequately powered phase 3 trials have yet to begin.

Other molecules under development for this indication include antagonists of the initial phase of tissue factor activation of factor VII and agents that stimulate fibrinolysis. Evaluation of each will require large trials because available active comparators (e.g., warfarin) are highly effective, resulting in low event rates.

Until recently, warfarin was the sole anticoagulant proven effective for patients with AF at risk of stroke; but its narrow therapeutic margin and variable dose response compromised clinical utility. The emergence of new anticoagulants that offer equal or superior efficacy, the convenience of fixed oral dosing, and greater safety, particularly with respect to reduction in the rate of intracerebral hemorrhage—the most feared complication of antithrombotic therapy and in many respects the sole complication that often leads to worse outcomes than the ischemic strokes treatment is intended to prevent—may make warfarin the less preferred option for many patients. Clinical practice guidelines undergoing revision to address these alternatives may support a multiplicity of options for anticoagulation therapy in patients with AF, but the limitations of crosstrial comparisons and lack of prospective comparative effectiveness studies addressing the newer agents will challenge clinicians to select therapy based on economics, availability, tolerability, convenience, and other factors.

Nonpharmacologic Therapy for Prevention of Thromboembolism

Cardioversion

Systemic embolism is the most serious complication of direct-current or pharmacologic cardioversion of AF to NSR. Observational evidence favoring anticoagulation has impeded trials comparing anticoagulation to no therapy.[89–93] In the prospective cohort study of Bjerkelund and Orning,[94] cardioversion without anticoagulation was associated with a 5.3% incidence of clinical thromboembolism versus 0.8% in patients selected for treatment with a VKA. Most ischemic events are presumed to result from embolism of either preexisting thrombus or new thrombus forming in the LA or LAA when atrial mechanical dysfunction worsens.[95–99] Most published guidelines recommend anticoagulation with adjusted-dose warfarin (INR 2.0 to 3.0) for 3 to 4 weeks before and after elective cardioversion of patients with AF.[100,101] Serial TEE studies have demonstrated resolution of thrombus after 1 month of warfarin in most patients with AF of recent onset,[8,18,102] but thrombus may persist in a minority of cases.[104] In addition, a month of anticoagulation may enhance thrombus organization and adherence to the endocardial surface, reducing the risk of embolism.

In the prospective Assessment of Cardioversion Using Transesophageal Echocardiography (ACUTE) trial,[104] the incidence of clinical thromboembolism in 603 patients after treatment with warfarin (INR 2.0 to 3.0) for 3 weeks before cardioversion was 0.5%. During the conventional treatment phase of the Ludwigshafen Observational Cardioversion study,[103] 357 patients undergoing successful cardioversion had a 0.8% incidence of neurologic events.

Most information on cardioversion-related thromboembolism is based on electrical cardioversion, but anticoagulation is also recommended for patients undergoing pharmacologic conversion. Following restoration of normal atrial electrical activity on the surface ECG after cardioversion, normal mechanical contraction of the LA may be delayed for more than a week[105–107] and appears related to the duration of AF before cardioversion.[108,109] Anticoagulation should be continued for 1 month following apparently successful cardioversion and for longer in patients with additional stroke risk factors, as AF may recur without symptoms.

Transesophageal Echocardiography-Guided Cardioversion

The accuracy of TEE for detection of thrombus in the LA and LAA and the association of this finding with the risk of thromboembolism following cardioversion[8,110] offers an opportunity to perform cardioversion earlier in patients without thrombus. In several studies,[8,18,99,102,104,107] rates of thromboembolism were similar to those associated with standard therapy for 3 to 4 weeks of anticoagulation before cardioversion, with the advantages of an earlier recovery of atrial mechanical function, ease of anticoagulation management, elimination of readmission for elective cardioversion, and potential cost-effectiveness.[111] Limitations of the TEE approach include patient discomfort, rare procedural complications, and limited availability at some centers. Despite the absence of LAA thrombus on precardioversion TEE, stroke has been described among patients who did not receive anticoagulation during the pericardioversion period and for at least 3 weeks after cardioversion.[112–115]

The ACUTE trial enrolled 1,222 patients with AF for whom elective electrical cardioversion was planned to compare the conventional and TEE-guided approaches.[7] In the TEE-guided arm, five ischemic neurologic events occurred among 619 subjects compared with three events among 603 patients managed conventionally (P = NS). Most adverse events in the TEE-guided group were associated with recurrent AF and subtherapeutic anticoagulation intensity (INR < 2.0). Many of the patients in the conventional management arm converted to NSR spontaneously during the month of anticoagulation before scheduled cardioversion. In general, cardioversion occurred earlier in the TEE-guided group, but there was no difference in the prevalence of NSR by 8 weeks following randomization. In smaller, nonrandomized studies in which the duration of AF was <3 weeks, however, the rate of recurrent AF was lower and the likelihood of NSR at 1 year greater among subjects undergoing TEE-guided cardioversion.[116]

When AF is known to have been present for <48 hours, cardioversion is commonly performed without either prolonged antecedent anticoagulation or TEE examination. In a study of 357 patients with symptomatic AF for <48 hours,[117] 250 converted spontaneously and 107 underwent pharmacologic or electrical cardioversion without prolonged anticoagulation or TEE before cardioversion, and the risk of clinical thromboembolism was <1%. In another retrospective study of 258 patients undergoing cardioversion for AF of <2 days duration, one ischemic event occurred among 198 patients not anticoagulated before or following cardioversion (0.5%) compared with none among 60 patients treated with anticoagulation.[118] Given the limitations of these studies, heparin is generally recommended immediately before and during cardioversion and warfarin for 4 weeks following cardioversion of patients with AF of known recent onset (<48 hours duration). In an analysis of patients in the RE-LY trial undergoing cardioversion, the frequencies of stroke and major bleeding within 30 days of cardioversion on dabigatran etexilate were low and comparable to those on warfarin with or without TEE guidance,[119] making dabigatran an alternative to warfarin in patients requiring cardioversion. For high-risk patients, it seems prudent either to perform TEE or to delay cardioversion for a month to allow prior anticoagulation. When emergency cardioversion is necessary to terminate AF associated with a rapid ventricular response causing angina, HF, hypotension, or syncope, heparin is generally recommended at the time of cardioversion followed by oral anticoagulant therapy for at least 3 to 4 weeks.

Alternatives to Antithrombotic Therapy

Antiarrhythmic Therapy

Until recently, most physicians favored cardioversion and a strategy of rhythm control over rate control for patients with AF of recent onset, presuming that restoration and maintenance of NSR would avoid the adverse hemodynamic consequences of AF and reduce the risk of thromboembolism. Several randomized studies, most notably the Atrial Fibrillation Follow-up Investigation of Rhythm Management (AFFIRM)[120] and the comparison of Rate Control and Rhythm Control in Patients with Recurrent Persistent atrial fibrillation (RACE)[121] trials, demonstrated that the frequency of ischemic events was equal regardless of whether a rate control or rhythm control strategy was followed. Most of these events occurred after warfarin was stopped or when the INR fell below the therapeutic range. The largest trial, AFFIRM,[120] enrolled 4,060 patients with recurrent AF older than 65 years or with other risk factors for stroke or death who had no contraindications to anticoagulation. All were initially anticoagulated, but warfarin was sometimes withdrawn from those in the rhythm control arm who appeared to maintain NSR. After a mean follow-up of 3.5 years, 35% of patients in the rate control group were in NSR, compared to 63% of those in the rhythm control group. More than 85% of patients in the rate control arm were treated with warfarin, compared to 70% in the rhythm control arm. All-cause mortality was not reduced by rhythm control (26.7% vs. 25.9%, P = 0.08), and there was no significant difference in ischemic stroke between the rhythm control and the rate control strategy (7.1% vs. 5.5% respectively, P = 0.79).

The RACE trial enrolled 522 patients with recurrent AF or atrial flutter of <1 year duration undergoing cardioversion on one or two occasions over a period of 2 years, randomized to rate control or to rhythm control strategies.[121] The primary outcome was a composite of death from cardiovascular causes, HF, thromboembolism, bleeding, pacemaker implantation, and severe adverse drug effects. After 2.3 years follow-up, there was a trend toward a lower incidence of the primary endpoint with rate control than with rhythm control (17.2% vs. 22.6%, HR 0.73, 90% CI 0.53 to 1.01). There was no difference in cardiovascular mortality (6.8% vs. 7%) and a trend toward more nonfatal endpoints among patients in the rhythm control arm. The results were marred, however, by imbalance between arms in the prevalence of hypertension.

The ATHENA trial compared the antiarrhythmic drug dronedarone against placebo in patients with paroxysmal AF.[122] Patients randomized to dronedarone had a lower incidence of the primary outcome of first hospitalization due to cardiovascular events or death.[122] A *post hoc* analysis of ATHENA demonstrated that dronedarone was also associated with a lower rate of stroke and TIA (HR = 0.66, 95% CI 0.46 to 0.96, P = 0.027).[123] It is not clear, however, whether this benefit accrued as a consequence of successful rhythm control or some other effect of dronedarone.

Although both rate and rhythm control approaches are reasonable clinical options, the foregoing findings support continuation of anticoagulation in high-risk patients even after NSR is restored following spontaneous, pharmacologic, or direct-current cardioversion. One reason for the failure of rhythm control to reduce thromboembolism is that AF recurs in 40% to 60% of patients by 1 year following initially successful cardioversion, even when antiarrhythmic drug therapy is employed,[124,125] and many of these episodes of recurrent AF are asymptomatic.[126] Another potential explanation for the frequency of ischemic events following apparently successful cardioversion is that patients with AF typically have associated cardiovascular diseases such as atherosclerosis and hypertension that are independently implicated as causes of stroke and responsive to antithrombotic therapy.

The Maze Operation and Catheter-Based Pulmonary Vein Isolation

A cardiac surgical operation, the Maze procedure, involves creation of scars on the endocardial surface of the atria to interrupt reentrant circuits and maintain NSR; concurrently, the LAA is either amputated or closed.[127] For carefully selected patients in

the hands of experienced operators, the long-term success of the Maze procedure in preventing recurrent AF exceeds 90%,[128] and this combination of rhythm control and obliteration of the LAA may reduce the risk of thromboembolism, although no controlled studies have been conducted. In a series of 306 patients followed for 11.5 years, there were two perioperative strokes and one later stroke, corresponding to an annual risk well below 1%, comparable to that reported by other investigators.[31,129,130] Although the original procedure was scalpel based, modifications using intraoperative delivery of radiofrequency, microwave, or cryoablation energy have been described.[131] Because patients and physicians are reluctant to embrace major surgery as a strategy for management of AF, the Maze procedure has been largely restricted to adjunctive use during other cardiac operations, such as coronary bypass or valve repair or replacement operations, although less invasive options are under investigation.[132]

An alternative treatment involves catheter-based radiofrequency ablation of intra-atrial pathways that participate in the initiation or perpetuation of AF. On the basis of the observation that regions in the LA proximate to the insertions of the four pulmonary veins are a common site of earliest activation of atrial ectopic beats that engender AF, radiofrequency encirclement, and electrical isolation of the pulmonary veins has become an accepted form of catheter-based treatment.[108,133] A nonrandomized, multicenter, prospective study of 79 patients documented freedom from recurrent AF in 84% over follow-up of approximately 1 year,[109,134] but larger studies are needed to address the safety and efficacy of this procedure.

No adequate studies have yet been published that specifically assess the risk of thromboembolism in patients who have undergone radiofrequency pulmonary vein isolation. Although some patients undergoing ablation or the Maze operation may be poor candidates for long-term anticoagulation, there is presently a dearth of evidence to support withdrawal of anticoagulation following either procedure. In the absence of trial-based conclusions, consensus recommendations advocate treating such patients longer than those undergoing apparently successful cardioversion of AF, basing the duration of anticoagulation more on the patient's intrinsic risk of thromboembolism than on the likelihood of arrhythmia recurrence.[135]

Left-Atrial Appendage Closure

Because the LAA has been implicated as an important source of thrombus formation in patients with nonvalvular AF,[136] closure or obliteration of the appendage offers a potential alternative to anticoagulation for prevention of embolism. Percutaneous, catheter-based devices for occlusion of the LAA have been developed. Limited observational data in North America and Europe suggested that the Percutaneous Left-Atrial Appendage Transcatheter Occlusion (PLAATO) device delivered by the transseptal approach and deployed in the LAA[137] was effective and relatively safe in patients with AF who are not candidates for warfarin therapy, but the device is no longer manufactured and no comparative studies are available. The WATCHMAN LAA closure device is a self-expanding device with a polytetrafluoroethylene membrane and nitinol frame that secures it to the ostium of the LA. The device is deployed into the LAA using a transseptal approach under fluoroscopic and TEE guidance. The device eventually becomes endothelialized, but because the membrane is permeable to blood, patients treated to date have been given anticoagulation therapy for at least 45 days after implantation; TEE is used to confirm proper device position and absence of flow into and out of the LAA before stopping anticoagulation therapy. PROTECT-AF, a randomized, multicenter study, compared the WATCHMAN device to standard long-term warfarin therapy in patients with nonvalvular AF and risk factors for thromboembolism.[138] In this study, the device compared favorably for prevention of stroke, systemic embolism, and cardiovascular or unexplained death (RR 0.62, 95% CI 0.35 to 1.25), but those treated with the device faced procedural complications including bleeding (RR 1.69, 95% CI 1.01 to 3.19). Other devices are under development, including not only catheter-based methods for LAA occlusion but investigational methods for obliteration of the LAA under thoracoscopic guidance.[139] These approaches are currently confined mainly to patients with relative contraindications to chronic anticoagulation with warfarin, but their use might eventually be extended to a wider array of patients once safety and efficacy are established.[140] Amputation, ligation, or plication of the LAA has become conventional during cardiac surgical operations involving the mitral valve, though definitive evidence that this is associated with a lower rate of postoperative thromboembolism is lacking.

CARDIOMYOPATHY

Though death in patients with chronic HF is usually caused by progressive hemodynamic failure, thrombotic coronary occlusion, or ventricular arrhythmias, patients with systolic HF are also at risk of thromboembolic stroke.[141] One or more components of Virchow's triad (stasis, endothelial injury, or a hypercoagulable state) predispose patients with systolic dysfunction to formation of mural thrombus in the LV. Wall motion abnormalities, particularly when they involve the LV apex, create zones of stasis, as occurs when a ventricular aneurysm develops following MI. Stagnant blood can occasionally be distinguished by spontaneous echo contrast on echocardiography as a result of interaction between red blood cells and plasma proteins, particularly fibrinogen. Spontaneous echo contrast is a precursor of thrombus development and a proven risk factor for stroke.[142]

Impaired release of nitrous oxide (NO) from endothelial cells has been implicated as a cause of vasoconstriction in patients with HF, and reduced NO release promotes monocyte and platelet adhesion. This mechanism and elevated levels of von Willebrand factor, a marker of endothelial damage, predispose to thrombus formation.[143,144] Circulating platelet aggregates are increased in patients with HF.[145] Compared to patients with coronary artery disease, patients with HF display higher levels of D-dimer and thrombin activity but varying degrees of platelet activation.[146,147] Elevated levels of fibrinopeptide A and thrombin–antithrombin complex have been correlated with increased LV end-diastolic volume and diminished fractional shortening, while levels of platelet factor 4, β-thromboglobulin, and plasmin–α_2-plasmin inhibitor complex are comparable in patients with HF and controls. Plasma viscosity in patients with HF is negatively correlated with LV ejection fraction,[148] and hemorheologic indices improve after treatment with angiotensin-converting enzyme inhibitors but not with β-blockers.[149]

The reported incidence of thromboembolism in the setting of HF varies substantially from as high as 50% in historical autopsy series to as low as 1% per year for those in sinus rhythm

in more recent clinical reports.[150–159] Fuster et al. reported an 18% prevalence of arterial thromboembolism (3.5 per 100 patient-years) in a review of 104 patients with idiopathic dilated cardiomyopathy,[160] but in that series the strongest independent predictor of thromboembolic events was AF. A later study of 264 patients with mean left-ventricle ejection fraction (LVEF) of 27% reported a cerebral ischemic event rate of 1.7% per year and higher thromboembolism and mortality rates when LV mural thrombus was detected.[161] These findings were substantiated in a series of 224 patients awaiting transplantation, in whom the risk of systemic embolism during follow-up was 3.2% per year.[162]

PREVENTION OF THROMBOEMBOLISM

The Survival and Ventricular Enlargement (SAVE) trial reported an inverse relationship between stroke risk and EF, with an 18% increase in risk for every 5% reduction in EF, relating thromboembolism to HF and severity of LV dysfunction.[154] The annual risk of thromboembolism in stable patients with chronic HF is relatively low (1% to 3% per year), even when the EF is severely reduced or LV thrombus is evident by echocardiography. This low annual risk limits the ability to detect a benefit of anticoagulation if it exists.[154]

Observational data from early studies have yielded conflicting results regarding the advantage of anticoagulation for prevention of thromboembolism. In the SAVE trial, treatment with warfarin was associated with an 81% lower risk of stroke.[154] A retrospective analysis of 6,767 patients with EF < 35% enrolled in the Studies of Left Ventricular Dysfunction (SOLVD) trials found an overall risk reduction of 32% in cardiovascular and sudden death with warfarin.[163] While the cohort included a high proportion of patients with ischemic heart disease, a benefit of warfarin was also seen in those without coronary disease. The SOLVD trials failed to demonstrate a reduction with warfarin in the combined rate of fatal and nonfatal thromboembolic events. Observational analysis of the Vasodilator Heart Failure Studies (V-HeFT) provided additional, albeit contradictory, data on the effect of anticoagulation. In V-HeFT I, there was no reduction in thromboembolic events during treatment with warfarin (2.9% vs. 2.7% per year in those not receiving chronic oral anticoagulation). In the V-HeFT II study, in contrast, the incidence of thromboembolism was higher in those receiving warfarin ($P < 0.01$), but antithrombotic therapy in these studies was not randomized.[164]

A Cochrane systematic review concluded that the limited evidence from observational studies regarding a reduction in mortality and cardiovascular events and the lack of controlled trials did not support routine use of anticoagulation in patients with HF in sinus rhythm.[165] The authors also concluded that there was no evidence that the efficacy of anticoagulation was superior to aspirin in this disease state.[166] Large, randomized trials of anticoagulation in patients with HF conducted over 50 years ago included patients with AF and rheumatic heart disease, and few trials were designed to address the issue in patients with HF in sinus rhythm. The available evidence does not show a clear advantage to anticoagulation therapy for prevention of thromboembolism in this situation. The Warfarin and Antiplatelet Therapy in Chronic Heart Failure (WATCH) trial was a randomized, double-blind trial intended to determine the optimum antithrombotic regimen for patients with HF ($n = 1,587$) and reduced LVEF in sinus rhythm based on the hypothesis that warfarin would be superior to antiplatelet therapy with aspirin and that clopidogrel would be superior to aspirin in preventing major cardiovascular events.[167] The primary endpoint was the constellation of all-cause mortality, nonfatal MI, and nonfatal stroke; secondary endpoints were the three components of the primary endpoint considered individually. The study was terminated early because of slow enrollment, however, and the statistical analysis was adjusted for reduced sample size. In conflict with the primary hypothesis, the number of primary endpoints was similar across treatment groups. While the overall incidence of nonfatal stroke was low in all three treatment groups, there was a statistically significant lower rate of nonfatal strokes in the warfarin arm than in the antiplatelet arms of the study ($P < 0.01$). This difference was eliminated, however, when fatal stroke and intracranial bleeding were included.

Because 9% of patients with a stroke have a left-ventricular EF ≤30%, finding the optimum treatment to reduce the probability of recurrent stroke is of clinical significance, yet there are no large randomized studies to resolve this conundrum. The Warfarin versus Aspirin in Reduced Cardiac Ejection Fraction (WARCEF) study is comparing warfarin (INR 2.5 to 3.0) versus aspirin (325 mg daily) in the 3 to 5-year event-free survival for the composite endpoint of death or stroke (ischemic or hemorrhagic) among 2,860 patients with an LVEF ≤35% who do not have AF or mechanical heart valves.[168] The results may help guide clinicians in selecting optimum antithrombotic therapy for patients with reduced LVEF with or without clinical HF.

While the benefit of anticoagulation in patients with HF in NSR is not yet clear, clinical practice guidelines generally support the use of anticoagulation in patients with HF and AF. Given the lack of compelling evidence for patients with NSR, the use of warfarin is not well defined and is recommended when there is another clear indication for anticoagulation or a disorder associated with increased thromboembolic risk (e.g., amyloidosis or noncompaction of the ventricular myocardium—a condition in which the LV myocardium consists of a highly trabeculated layer with deep recesses in addition to the normally "compacted" myocardium) and in those with familial dilated cardiomyopathy and a history of thromboembolism in first-degree relatives, but these recommendations are based more on consensus than empirical evidence.[169]

The Risk of Embolic Events Associated with Intracardiac Thrombus

Whereas the presence of an LV thrombus in a patient who has suffered a recent, large MI likely increases the risk of thromboembolism, the significance of mural thrombi in the setting of chronic HF is less clear. The incidence of mural thrombus after acute anterior MI ranges from 21% to 41%.[36,170] Several longitudinal studies failed to demonstrate consistent resolution of thrombus by echocardiography, though these studies suggested protection against stroke for patients managed with chronic anticoagulation.[37–44,171–178] Because the sensitivity of echocardiography may be insufficient to reliably predict systemic embolism in patients with dilated cardiomyopathy and intracavitary thrombus, treatment is largely empirical.[41,42,175,176] Guidelines recommend long-term anticoagulation for those with LV thrombus complicating acute MI based on meta-analysis of limited studies.[45,46,179,180] Consistent data support the

notion that mobile or protuberant mural thrombus conveys a high risk of thromboembolism, and patients with this characteristic should generally be anticoagulated.[47–49,181–183]

VALVULAR HEART DISEASE

Valvular heart disease spans a diverse range of endocardial pathology. While data support antithrombotic therapy with either aspirin or warfarin for prevention of thromboembolism in relation to specific valve lesions (e.g., mitral stenosis [MS]), there is relatively scant evidence with regard to other forms of valvular heart disease. This section addresses specific valvular pathology where an association with thromboembolic disease has been established and where antithrombotic therapy may be appropriate.

Diseases of Native Heart Valves Associated with Thromboembolism

Rheumatic Mitral Stenosis

MS is usually caused by rheumatic fever; congenital MS and MS due to other etiologies (e.g., annular calcification, carcinoid syndrome) are relatively rare. MS carries a 10% to 20% lifetime risk of clinically apparent systemic embolism (incidence 1.5% per year),[10,184,185] though the risk increases nearly sevenfold in the presence of AF. Following an initial thromboembolic event, the risk of recurrence over the patient's lifetime is up to 65%, and two-thirds of these recur within the first year after the initial event. Most clinical trials and meta-analyses involving prevention of cardiogenic embolism associated with AF have been restricted to patients with nonvalvular (nonrheumatic) AF. Patients with MS at highest risk are those with prior events or AF, who benefit substantially from anticoagulation. Patients with rheumatic MS who remain in NSR also carry a risk of thromboembolism. It remains unclear, however, whether patients at higher risk of embolism without AF or prior embolism uniformly benefit from anticoagulation.

While systemic embolism in patients with MS is thought to originate from left-atrial thrombus, the presence or absence of thrombus does not correlate closely with clinical ischemic events.[186] In a prospective analysis of 534 patients with MS, risk factors for embolism in patients with NSR included increasing age, diminishing mitral orifice area, atrial thrombus, and aortic regurgitation.[187] In this analysis, early balloon mitral valvuloplasty (BMV) was a negative predictor for the development of systemic embolism. The frequency of embolism was not directly related to the transmitral gradient, cardiac output, HF symptoms, or diameter of the LA.

The need for yearly echocardiograms for patients with MS is controversial and not currently recommended unless change in symptoms or functional status occurs. Asymptomatic patients with MS should be followed annually by clinical evaluation that includes an ECG. For patients who complain of palpitations, Holter monitoring or cardiac event monitoring is reasonable to detect AF, the finding of which warrants anticoagulation.

Current practice guidelines recommend anticoagulation for patients with MS who have AF, a history of systemic embolism (even when sinus rhythm is present), or atrial thrombus detected by TEE, though the evidence supporting these recommendations is limited to observational reports.[186] Anticoagulation may be considered for patients with hemodynamically severe MS, atrial enlargement >55 mm diameter, or in those with spontaneous echo contrast (class IIb).

Balloon Mitral Valvuloplasty

BMV is contraindicated when atrial thrombus is detected, owing to the potential for dislodgement during catheter manipulation. To avoid this complication, TEE should be performed prior to the procedure to exclude thrombus, and if thrombus is detected, BMV should be deferred until resolution of the clot is proven by repeat TEE after at least 3 weeks of anticoagulation with a VKA (INR 2.5 to 3.5) or heparin (target-activated partial thromboplastin time twice the control value).[188] Predictors of clot resolution include NYHA functional class II or better, thrombus diameter ≤1.6 cm, less dense spontaneous echo contrast, and INR ≥ 2.5.[189] Following BMV, recommendations for anticoagulation therapy are the same as those prior to the procedure.

Mitral Valve Prolapse

Mitral valve prolapse (MVP) refers to billowing of redundant tissue of one or both mitral leaflets during systole. The condition is often congenital but may result from breakdown of one or more components of the mitral valve apparatus (papillary muscles, chordate tendineae, leaflets, or annulus). It is commonly accompanied by mitral regurgitation (MR) and is the etiology of up to 50% of cases of pure MR.

Whether MVP is associated with an increased risk of TIA or stroke has been debated since Barlow and Bosman[190] in 1966 described a 28-year-old woman with MVP who had transient left-arm weakness. Subsequently, a case-controlled study of 60 patients <45 years old who experienced ischemic stroke or TIA found MVP in 40%, compared with prevalences of 6.8% in 60 age-matched controls, and 5.7% in 42 stroke victims >45 years old.[191] In contrast, survival in asymptomatic or minimally symptomatic patients with MVP was similar in an age-matched cohort over a follow-up of >6 years.[192] Only patients with redundant mitral valve leaflets had significant complications, including infective endocarditis or cerebral embolism.

Variation in the echocardiographic criteria for diagnosis of MVP confounds assessment of the association of this valvular lesion with cerebral embolism and casts doubt on the conclusions of earlier studies. Current diagnostic criteria require superior displacement of the mitral valve >2 mm beyond plane of the annulus in the parasternal long-axis view. In a prospectively followed cohort of 777 patients in Olmsted County, MN, with MVP based on these criteria, there was an excess lifetime risk of ischemic neurologic events compared to the expected risk in the community (RR 2.2, 95% CI 1.5 to 3.2, $P < 0.001$).[193] Patient age, AF, MV thickening, and, to a lesser degree, LA enlargement were independent predictors of ischemic neurologic events by multivariate analysis.

The exact mechanism of TIA or stroke in relation to MVP is not clear. Possible explanations include endocardial disruption and fibrin deposition on denuded surfaces of the valve, mural thrombus at the junction of the prolapsed leaflet with the atrial wall, fibrinous endocarditis, and intrinsic abnormalities of platelet function linked to β-receptors.[194,195] No randomized trials have tested the efficacy of antithrombotic therapy or the superiority of one therapy over another in patients with MVP for prevention of thromboembolism. Given the uncertain

association of MVP with cerebral ischemic events, guidelines issued by various organizations differ. Some recommend aspirin (75 to 325 mg daily) for patients with MVP who have experienced stroke or TIA and those <65 years old with MVP and AF without MS, hypertension, or HF.[186] Warfarin is recommended for patients >65 years old with MVP and AF or a history of hypertension, HF, MS, or murmur of MR. Additionally, for patients with MVP and a history of stroke, anticoagulation should be employed if there is a history of MS, MR, AF, or LA thrombus. Anticoagulation is recommended as well for patients with a history of stroke and thickened valve leaflets (>5 mm) and for those with recurrent TIA despite aspirin therapy.

Thromboembolism Associated with Prosthetic Heart Valves

Available heart valve prostheses can be broadly categorized into biologic and mechanical types. Mechanical prostheses carry the advantage of greater long-term durability, but the potential for thromboembolic complications exceeds that of bioprosthetic valves. The structural integrity of bioprosthetic valves diminishes over time, resulting in valve failure at a certain point. In early studies, approximately 50% of bioprosthetic valves implanted in patients younger than 40 years failed within 10 years of implantation. Mechanical valves have a low rate of structural deterioration, but require long-term anticoagulation therapy with warfarin to prevent thromboembolism and therefore impose on the patient an inherently greater risk of bleeding. With improvement in the durability of newer generation bioprosthetic valves lowering the risk of a second or third operation and the potential for percutaneous approaches to valve replacement that might reduce the need for reoperation in the future, there has been a trend in the United States to prefer bioprosthetic valve replacement even in younger patients.

Mechanical Valves

All patients with mechanical prosthetic heart valves require anticoagulation, but the target intensity varies from INR range 2.0 to 3.0 to as high as 3.0 to 4.5 depending on the type of prosthesis, valve position, and presence or absence of additional risk factors. These risk factors, which are largely anecdotal and not derived from high-quality randomized trial databases, include AF, previous thromboembolism, hypercoagulable states, and LV dysfunction. A minimum INR range of 2.5 to 3.5 is recommended for patients with any of these risk factors regardless of the valve replacement or type of mechanical prosthesis, though higher intensities of anticoagulation are necessary for patients with older models that are more prone to thromboembolic complications. Additionally, an INR range of 2.5 to 3.5 is recommended for patients with tilting-disc or Starr-Edwards valves in the aortic position and for all mechanical prostheses in the mitral position. Bileaflet prostheses associated with high orifice-to-annulus ratios, manufactured from modern thromboresistant materials like pyrolytic carbon such as the St. Jude Medical or Medtronic-Hall prostheses placed in the aortic position, are associated with relatively low rates of thromboembolism and require less intensive anticoagulation (target INR 2.0 to 3.0) in the absence of other risk factors. In addition to warfarin, it has been recommended that patients with mechanical valve prostheses be treated with low-dose aspirin (50 to 100 mg daily) in addition to warfarin unless there is a contraindication due to gastrointestinal intolerance or bleeding.[196] A systematic review (*Cochrane Database Syst Rev* 2003;(4):CD003464.), however, showed that the reduced risk for thromboembolic events (OR 0.39) was counterbalanced by an increased risk for major bleeding (OR 1.66). Even with anticoagulation, the annual risk of thromboembolism is approximately 1% to 2%.[197] The risk is greater with mitral valve prostheses, which carry a risk roughly twice that of aortic valves,[197] and greater for caged-ball devices and multiple prostheses.[198]

While there are scant data to support the use of unfractionated heparin (UFH) as a bridging therapy, it is often initiated early after valve replacement surgery once anticoagulation therapy is safe from the standpoint of postoperative bleeding, usually 24 to 48 hours after the operation. Heparin can be stopped after 3 to 5 days of warfarin therapy, once the INR has reached the therapeutic range.

Data supporting the use low molecular weight heparin (LMWH) is also meager. A "black box" warning on the package insert of LMWH preparations cautioning that use in patients with mechanical heart valves was not adequately studied has been removed. The ACCP guidelines for antithrombotic therapy endorse the use of LMWH as a modality for bridging therapy following valve replacement surgery or when VKA therapy must be interrupted.[199] The AHA/ACC guidelines list a class IIb recommendation regarding bridging therapy with LMWH when anticoagulation must be stopped for noncardiac surgery or other invasive medical or dental procedures in a patient with mechanical cardiac valve prosthesis.

None of the novel oral anticoagulants (dabigatran, rivaroxaban, apixaban, edoxaban, or others) has been studied to determine their safety or efficacy for bridging or long-term therapy in patients with mechanical prosthetic heart valves and thus should not be used as a substitute for warfarin, UFH, or LMWH in this situation.

Bioprosthetic Valves

Over the long term following implantation, the frequency of thromboembolism in patients with biologic prosthetic heart valves without anticoagulation is similar to that in patients with mechanical valves receiving therapeutic anticoagulation.[198] Bioprosthetic heart valves, however, are associated with an elevated risk of thromboembolism in the first 90 days after implantation, especially when the valve has been placed in the mitral position.[200] Aside from the early postoperative state, factors associated with thromboembolism are older patient age, AF, absence of anticoagulation, and previous thromboembolism. Anticoagulation with warfarin for 90 days after valve implantation should be considered in patients with bioprosthetic valves, though this is not routine at all centers.[186] After the initial 90-day period, anticoagulation therapy can be withdrawn unless the patient has AF, a previous thromboembolic event, hereditary thrombophilia, or other hypercoagulable condition, in which case long-term anticoagulation is usually required, though the evidence supporting this approach is relatively weak.

Interruption of Anticoagulation in Patients with Prosthetic Heart Valves

When a patient requires noncardiac surgery or another type of invasive medical or dental procedure associated with an escalated risk of bleeding, the risks of thromboembolism as a result

of interrupting anticoagulation must carefully be weighed against the risks of procedure-related bleeding. If periprocedural bleeding is unlikely or inconsequential, as is usually the case with prophylactic dental hygienics or ocular surgery, anticoagulation should be continued near the lower end of the range of therapeutic intensity. When the risk of serious bleeding is greater, anticoagulation therapy should be adjusted or interrupted accordingly. Bridging with UFH or LMWH should be initiated when the INR falls below 2.0 to 2.5 in patients who are at high risk of thromboembolism, including patients with mechanical heart valve prostheses in the mitral position, patients with bileaflet mechanical valves in the aortic position who have additional risk factors for thromboembolism, and patients with bioprosthetic valves who have AF. The parenteral anticoagulant can be interrupted 6 to 12 hours prior to the invasive procedure, restarted as early after the procedure as considered safe to do so while warfarin is resumed, and continued until the INR has returned to the therapeutic range. Patients who are not at high risk, including those with bileaflet mechanical aortic valve prostheses without other thromboembolism risk factors, do not generally require parenteral bridging therapy. These individuals can interrupt warfarin approximately 3 days prior to the procedure and resume the VKA 24 hours following the intervention. For patients taking antiplatelet therapy, aspirin therapy should be stopped 1 week prior to the procedure and clopidogrel 5 days prior. For reversal of anticoagulation in patients requiring urgent or emergency procedures, administration of fresh-frozen plasma or four-factor prothrombin complex concentrate is preferred over low-dose (<5 mg) intravenous or oral vitamin K, which takes 12 to 24 hours to reach full effect; high-dose vitamin K (10 mg or more) is contraindicated because resistance to warfarin may continue for several weeks.

Thromboembolism Associated with Abnormalities of Atrial Septation

A remnant of the fetal circulation, patent foramen ovale (PFO) develops when there is failure of the septum primum and septum secundum to fuse after birth, allowing for left-to-right shunting at atrial level. It is a common finding with a prevalence of 15% to 25%[201] that is sometimes associated with an atrial septal aneurysm (ASA), in which the redundancy of atrial septal tissue results in >10 mm excursion during respiration. The prevalence of ASA is much lower (1% to 2%) than that of PFO. Though usually benign, PFO has been associated with cryptogenic ischemic stroke in otherwise apparently healthy, younger individuals. A meta-analysis in 2000 found an OR of 3.1 (95% CI 2.29 to 4.21) for the association of PFO with cryptogenic stroke in patients younger than 55 years old.[202] The data from patients 55 years and older were less impressive, though there was still a trend toward increased risk (OR 1.27, 95% CI 0.8 to 2.01). While a PFO provides an anatomic substrate for paradoxical embolism of thrombus from the peripheral venous system and thrombus in transit has occasionally been documented, deep-vein thrombosis is found in only a small proportion of patients with cryptogenic stroke and PFO. Other possible mechanisms of stroke in PFO include embolism of thrombus formed on the rim or left-atrial side of the PFO itself, asymptomatic paroxysmal AF, or passage through the shunt of nonthrombotic, vasoactive humoral substances that escape degradation of the pulmonary bed.

The treatment for prevention of initial or recurrent stroke in patients with a PFO remains controversial. Most studies have focused on secondary prevention of cryptogenic stroke in patients with PFO. The French PFO-ASA study followed 216 patients younger than 55 years who had a cryptogenic stroke up to 4 years[203]; all were treated with aspirin. Patients with a PFO alone had the same risk of recurrent stroke or TIA at 4 years as those with no identified atrial abnormality (HR 1.34, 95% CI 0.62 to 2.90, $P = 0.45$). The risk of stroke was significantly higher in those who had both PFO and ASA (HR = 3.91, 95% CI 1.59 to 9.59, $P = 0.003$), raising the possibility that patients with both lesions might benefit from more aggressive therapy (anticoagulation or anatomical closure). Similarly, the PFO in Cryptogenic Stroke Study (PICSS) found no significant difference in time to recurrent stroke or death between patients with and without PFO treated with aspirin over 2 years (event rates 14.8% vs. 15.4%, HR 0.96, 95% CI 0.62 to 1.48, $P = 0.84$).[204] Comparing aspirin to warfarin in patients with PFO, the PICSS investigators found no significant advantage of warfarin for prevention of subsequent stroke, though the study was underpowered to exclude a treatment benefit. Current AHA/ASA and ACCP guidelines recommend antiplatelet medication as the initial therapy for patients with PFO and cryptogenic stroke,[199] based on "insufficient data to establish whether anticoagulation is equivalent or superior to aspirin for secondary stroke prevention in patients with PFO."

Should recurrent stroke occur in a patient with PFO (with or without ASA) and no other apparent cause, it has been common practice to close the PFO using direct surgical patch or ligation or percutaneous catheter-directed techniques. No devices designed specifically for the closure of PFO are currently approved for use by the U.S. Food and Drug Administration. No randomized studies comparing medical therapy with antiplatelet agents or anticoagulants, percutaneous therapies, or surgical correction have provided clear evidence of the superiority of one approach over another for secondary prevention of stroke in patients with PFO. The CLOSURE I trial was an multicenter, open labeled trial that randomized 909 patients aged 18 to 60 with a PFO and a history of TIA or stroke to the STARFlex closure device plus antiplatelet therapy or medical therapy alone. After 2 year follow up there was no significant difference between the two groups in the primary endpoint of recurrent stroke or TIA.[206]

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CHAPTER

93

Ischemic Stroke: Acute Thrombolytic Therapy, Antithrombotic Approaches, and General Management

GREGORY J. DEL ZOPPO AND JEFFREY L. SAVER

PREVENTION AND TREATMENT OF ACUTE STROKE

Stroke is a syndrome of fixed or transient neurologic deficits that result from atherothrombotic events, thromboembolism, subarachnoid hemorrhage, intracerebral hemorrhage, lacunae, and other cerebrovascular causes (Table 93.1).[1,2] Atherothrombotic stroke refers to cerebral arterial occlusions from either *in situ* arterial thrombosis or artery-to-artery emboli originating in intracranial arteries or the internal carotid artery (ICA), the aortic arch, and the vertebral-basilar (VB) artery system.

Platelet-fibrin thrombi arise on arteriosclerotic lesions in the extracranial portion of the ICA (i.e., at the flow divider) or in the aortic arch and can embolize downstream, predominantly into the middle cerebral artery (MCA) and anterior cerebral arterial territories. A similar process can send emboli from atheromata at the subclavian-vertebral artery junctions into the basilar artery. Emboli from the heart originate from left ventricular mural thrombi formed during myocardial ischemia (infarction) (MI) or other injury; from atrial thrombi formed in association with (nonvalvular) atrial fibrillation (AF); from valvular injury; from prosthetic valves; or, in association with patent foramen ovale and atrial septal aneurysm. Less common than atherothrombotic stroke, thrombosis of small, penetrating cerebral arteries leads to lipohyalinosis and lacunae formation.[3] Studies employing angiography within 6 hours of onset of carotid artery territory ischemic stroke have shown a high frequency of atherothrombotic or thromboembolic arterial occlusions in the symptomatic territory (FIGURE 93.1).[4,5,6,7,8,9,10]

NATURAL HISTORY OF CEREBRAL ATHEROTHROMBOTIC AND THROMBOEMBOLIC DISEASE

Transient ischemic attacks (TIAs) are often accompanied by subsequent stroke or other cardiovascular disease. Completed stroke has been reported in 40% to 75% of individuals with one or more TIAs,[11,12] with a prevalence of approximately 30% per year.[11,12] Among individuals with a premonitory TIA, approximately 50% may have a stroke within the 1st year.[13] The early risk of stroke is high, occurring in 3% to 5% of patients within 48 hours after TIA and in 8% to 14% within 3 months.[14] Stroke-related death and cardiac death occur in 28% and 37% of TIA individuals, respectively, indicating that cardiovascular mortality is significant in the population of patients with stroke.[13] More recent evaluations, which have included data from placebo groups in large trials of antithrombotic efficacy, suggest a lower mortality. For example, the combined outcome events of stroke, MI, and death occurred in 14% of individuals treated with placebo for more than 2 years in the United Kingdom transient ischemic attack (UK-TIA) Study Group Trial.[15] Approximately 64% of individuals with TIAs showed evidence of cerebral infarction in their initial computed tomography (CT) scan.[16]

Patients presenting with an ischemic stroke are at risk for recurrence, often within the same vascular territory. The 5-year cumulative incidence of secondary stroke was 42% among men in one prospective follow-up study. A separate study noted a 32% 7-year cumulative incidence for recurrent stroke.[17] Again, the highest recurrence of stroke is within the 1st year following the initial event.

Stroke-related mortality during the first 7 days after ictus was examined prospectively by Silver et al.[18] Cerebral edema from large, hemispheric ischemic lesions led to transtentorial herniation and death in 78% of 46 patients who died in that interval. This is consistent with the number of fatal events (82%) in one retrospective pathology study.[19] Generally, however, mortality is not considered to be the primary outcome in current acute stroke intervention trials because of its low incidence and multifactorial basis.

Improvements in neurologic outcome are commonly observed among patients who survive a stroke. Both neurologic presentation and outcome depend on stroke subtype.[20] Patients with lacunar strokes fare better than those patients with thromboembolic events. The conditions under which stroke occurs can alter outcome. It has been recently appreciated, both clinically and experimentally, that female patients have a better 1-year survival and lower infarct volume than male patients, although the reasons are unclear.[21–23] Experience from two statin intervention trials for prevention of MI demonstrated a significant reduction in stroke incidence in patients treated with the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors.[24–28] Although, epidemiologic studies have not established cholesterol levels as a risk factor for ischemic stroke,[29] in both situations, the mechanisms of protection are unknown.

STROKE AS A SYSTEMIC DISORDER

Stroke is a systemic disorder, and as such, in the acute and early postictal phase, a number of conditions can influence stroke outcome. These require acute and intensive medical attention. Systemic *oxygen desaturation* exacerbates brain injury that originates from focal cerebral ischemia. One-third of ischemic stroke patients suffer aspiration acutely,[30] while pneumonia

Table 93.1

Etiology of focal cerebral ischemia

Source	Frequency (%)
Ischemic Stroke	
Atherothrombotic events	40–57
Thromboembolism	16–23
Lacunae	14
Hemorrhagic Stroke	
Intracerebral hemorrhage	4–18
Subarachnoid hemorrhage	10–19

complicates acute stroke in 5% to 12% of patients.[31] All stroke patients should be monitored for hypoxia, with a goal of maintaining O_2 saturation levels of >95%.

Focal ischemia impairs autoregulation in the injured territory so that regional cerebral blood flow (rCBF) fluctuates with mean arterial pressure.[32–34] Management of postischemic *hypertension* is problematic, and the approaches have been open to debate.[35] In select patients carefully managed in intensive care, induced hypertension can improve cerebral perfusion and may favorably influence the clinical course.[36] It is recommended that antihypertensive treatment be withheld in the first few hours after ischemic stroke onset, unless the systolic blood pressure is above 220 mm Hg or the mean arterial pressure >130 mm Hg on two consecutive measurements at least 5 minutes apart.[37,38] This approach is recommended unless the patient has a concurrent condition that independently requires moderation of blood pressure (e.g., acute MI, aortic dissection, or early hemorrhagic transformation of cerebral infarct). Notable, to minimize the risk of cerebral hemorrhagic transformation, is the requirement that in the first 24 hours after thrombolytic treatment the systolic blood pressure should be maintained at <180 mm Hg and diastolic blood pressure <105 mm Hg.

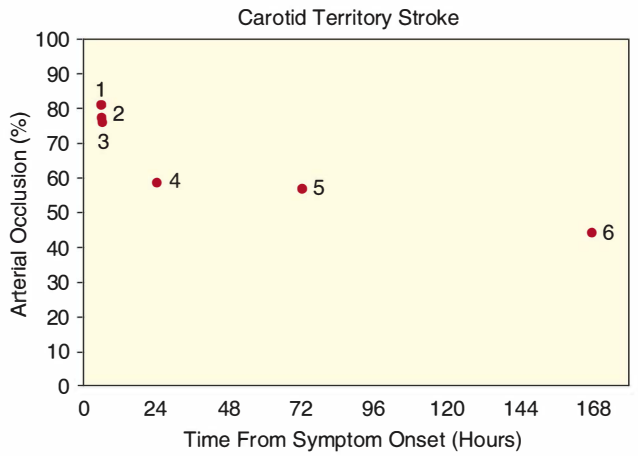

FIGURE 93.1 Series of angiography-based studies from separate trials demonstrating the frequency of carotid artery territory obstruction accompanying symptomatic ischemic stroke. Data points 1 to 6 are derived from citations[4,5,7,8,9,10] in order.

Careful *fluid management* is mandatory in patients with acute cerebral ischemia. This is usually accomplished with intravenous administration of normal saline with infusion rates of 75 to 125 mL/h to maintain intravascular volume.[37]

Hyperglycemia is the most common metabolic derangement in acute ischemic stroke, and elevated blood glucose at admission in acute ischemic stroke correlates with poor outcome.[39] However, it has not been demonstrated whether this association reflects a direct adverse effect of hyperglycemia upon outcome, or an epiphenomenon of severe initial brain injury producing a secondary rise in blood sugar. In patients receiving intravenous thrombolysis, acute hyperglycemia is a risk factor for hemorrhagic transformation.[40] It is suggested that severe hyperglycemia should be treated promptly in acute stroke, aiming to maintain a glucose level of <200 mg/dL.[41,42]

Hyperthermia on admission is an independent risk factor for poor outcome from acute ischemic stroke, and increased core temperature markedly worsens ischemic and cerebral injury in animal models.[43] It is sometimes uncertain whether hyperthermia is a consequence of severe stroke, rather than a promoter of increased injury. Induced hypothermia is beneficial as a neuroprotective treatment in human global brain ischemia after cardiac arrest and has been a promising strategy in focal ischemic stroke. Interventional trials are underway to determine whether systemic or selective cerebral hypothermic treatment can improve stroke outcome. Fever should be investigated for a source of infection and treated, and the elevated temperature reduced promptly with antipyretics and cooling blankets if necessary.

HEMOSTASIS AND CEREBROVASCULAR ISCHEMIA

Thrombosis and thromboembolism play a central role in focal cerebral ischemia, as suggested by clinical, angiographic, and laboratory observations. The findings of migrating thromboemboli in the retinal artery and refractile bodies in patients with focal cerebral ischemia[44–46] and of thrombi in cortical arteries during cerebral ischemia and on affected carotid arteries support a pathogenic role for these thrombotic events.[47,48]

Select acute angiographic studies have documented cerebrovascular occlusions in individuals with focal ischemia. In three prospective studies, symptomatic occlusion of a brain-supplying artery within the carotid territory was documented in 81% of patients within 8 hours of symptom onset.[9] Separate angiographic studies have shown arterial occlusions in 59% of patients at 24 hours and in 41% at 1 week after symptom onset in patients with focal cerebral ischemia.[4,5,7,8,9] Furthermore, large-artery events in the carotid territory are primarily thrombotic or embolic in origin. VB ischemia results from *in situ* thrombosis on atheromata in the basilar or vertebral arteries or from emboli from more proximal sources.[49] Indeed, atherosclerosis of brain-supplying arteries is a thrombophilic state (FIGURE 93.2).

There are differences among races with regard to thrombosis predisposition. For instance, among white populations, cardiogenic thromboemboli or extracranial arterial sources are common, whereas *in situ* intracranial atherosclerosis with supervening thrombosis is more frequent in the Japanese population.[50] Thrombi derived from the ICA or brain-supplying arteries have mixed architectural features of erythrocytes and fibrin, rather than one or the other, with fibrin/platelet deposits interspersed with linear collections of monocytes and

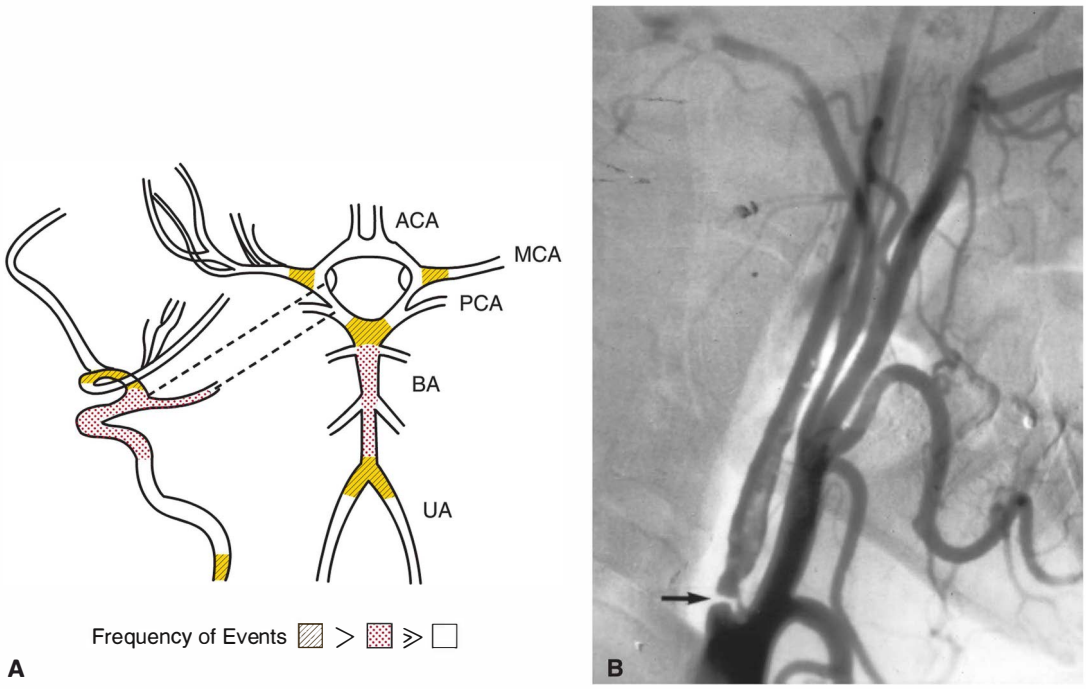

FIGURE 93.2 Atherosclerosis as a source of thrombotic and embolic obstruction of arterial circuits in the central nervous system (CNS). **A**: Predilection sites for atheromata in brain-supplying arteries. **B**: Example of severe (99%) stenosis of the ICA in patient with TIAs (*arrow*). ACA, anterior cerebral artery; MCA, middle cerebral artery, PCA, posterior cerebral artery; BA, basilar artery; UA, uncal artery.

polymorphonuclear leukocytes and confined erythrocyte-rich regions.[51] It has been suggested that red cell and mixed thrombi frequently produce hyperdense middle cerebral artery (HMCA) signs sometimes observed on CT and hypointense artery signs on magnetic resonance imaging (MRI).[52]

Indirect evidence for the involvement of thrombi in cerebrovascular ischemia derives from observations of platelet, coagulation, and fibrinolytic system activation in patients with thrombotic stroke or TIAs.[53–57] Platelet activation, spontaneous platelet aggregation, and circulating platelet aggregates have been reported in patients with recent atherothrombotic and thromboembolic cerebral ischemia.[53,56–63] Experimental studies confirm the deposition of fibrin and activated platelets in microvessels of the ischemic regions shortly after MCA occlusion.[64–69] These observations implicate the local activation of hemostasis in the ischemic microvascular bed.

HEMORRHAGIC TRANSFORMATION IN CEREBRAL ISCHEMIA

Hemorrhage accounts for approximately 10% to 15% of all strokes.[1,6,7,9,70–75] Normal platelet function appears necessary to maintain the integrity of the cerebrovascular beds and to prevent clinically detectable hemorrhage. Antithrombotic agents increase hemorrhagic risk in the ischemic territory.[76] Increases in the incidence of symptomatic intracerebral hemorrhage were observed with aspirin (acetylsalicylic acid [ASA]) in the UK-TIA Study, the International Stroke Trial (IST), and the European Stroke Prevention Study-2 (ESPS-2) trial.[72,73,77] An increased risk of intracerebral hemorrhage is associated with advanced age (>75 years), the intensity of anticoagulation, and the concomitant use of antithrombotics.[76]

Hemorrhage within the ischemic lesion occurs normally during thromboembolic stroke and is classified as hemorrhagic infarction (HI), parenchymal hematoma (PH), or both (FIGURE 93.3). HI refers to petechial or confluent petechial hemorrhage in the area of ischemic injury, typically involving cortical or basal ganglia gray matter.[78–81] HI occurs in 50% to 70% of individuals in postmortem studies,[78–80] in 10% to 43% of nonanticoagulated individuals with acute cerebral infarction in CT scan-based studies,[82,83] in 37.5% of patients with cardiogenic cerebral embolism, but in only 1.9% of patients with carotid territory thrombosis.[84] Petechial hemorrhage can result from ischemia-related degradation of the microvessel basal lamina matrix components.[85,86]

PH is a homogeneous mass of blood (coagulum) that can displace brain tissue. It is most often the cause of symptomatic hemorrhage. Many reports of PH in patients with cerebral embolism are associated with anticoagulant treatment.[87,88] In addition, PH can result from the rupture of small penetrating arteries (FIGURE 93.4).

THE PENUMBRA

Astrup and colleagues depicted the development of the core of ischemic injury destined for tissue destruction (infarction), as a circumferential region, or "penumbra," of metabolically metastable tissue that has the potential for full recovery, following occlusion of a brain-supplying artery.[89,90–93] This depiction has greatly influenced the concept of ischemic stroke following occlusion of brain-supplying arteries and the attempts to enhance tissue recovery clinically in the acute phase. This

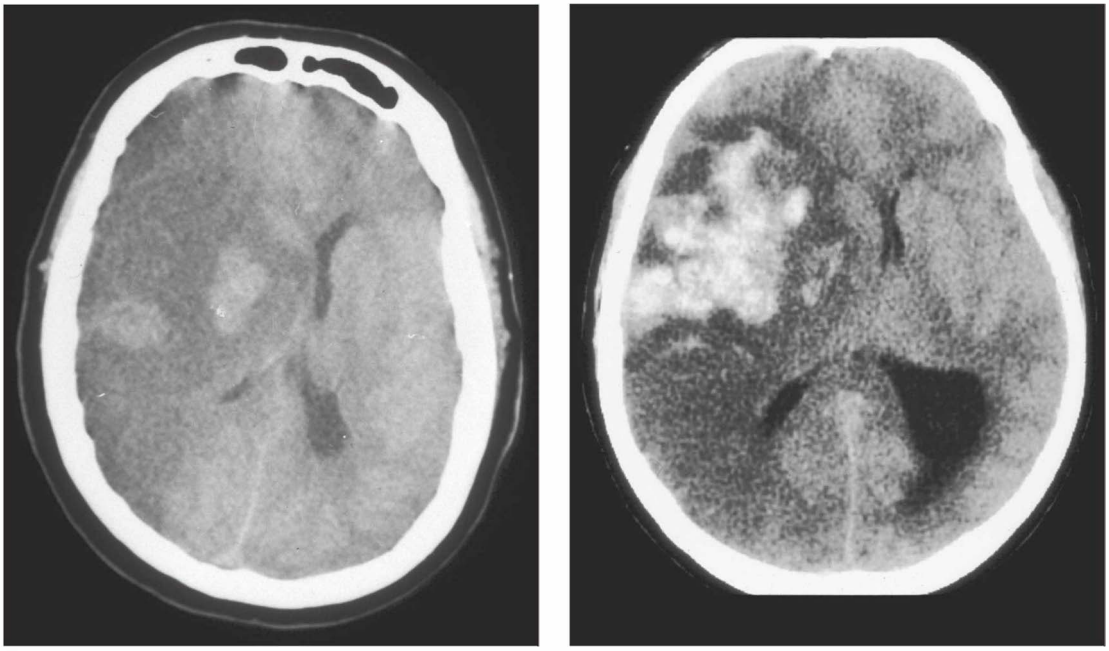

FIGURE 93.3 Hemorrhagic transformation. **A**: HI typified by regions of confluent petechiae 24 hours after MCA occlusion. Note midline shift due to tissue swelling. **B**: Parenchymal hemorrhage marked by coagulum displacing midline structures and compressing ventricle. (Image in panel **A**: From von Kummer R, Bozzao L, Manelf C. *Early CT diagnosis of hemispheric infarction*. Berlin, Germany: Springer Verlag, 1995:1, with permission.)

concept encompasses characteristic electrophysiologic changes, biochemical and molecular alterations, microvessel responses, metabolic changes, and regional differences in tissue perfusion and H_2O diffusion as displayed by imaging studies (e.g., the DEFUSE study).[93–95] By positron emission tomography, the infarction corresponds to rCBF below 12 mL/100 g/min and a cerebral metabolic rate for oxygen ($CMRO_2$) below 65 μm/100 g/min.[96] The penumbra has been defined as rCBF decreased to 12 to 22 mL/100 g/min, $CMRO_2$ above 65 μm/100 g/min, and an oxygen extraction fraction of 50% to 90%.[96] Experimental data from vascular and molecular modeling studies as well as recent imaging work indicate that the penumbra is dynamic and

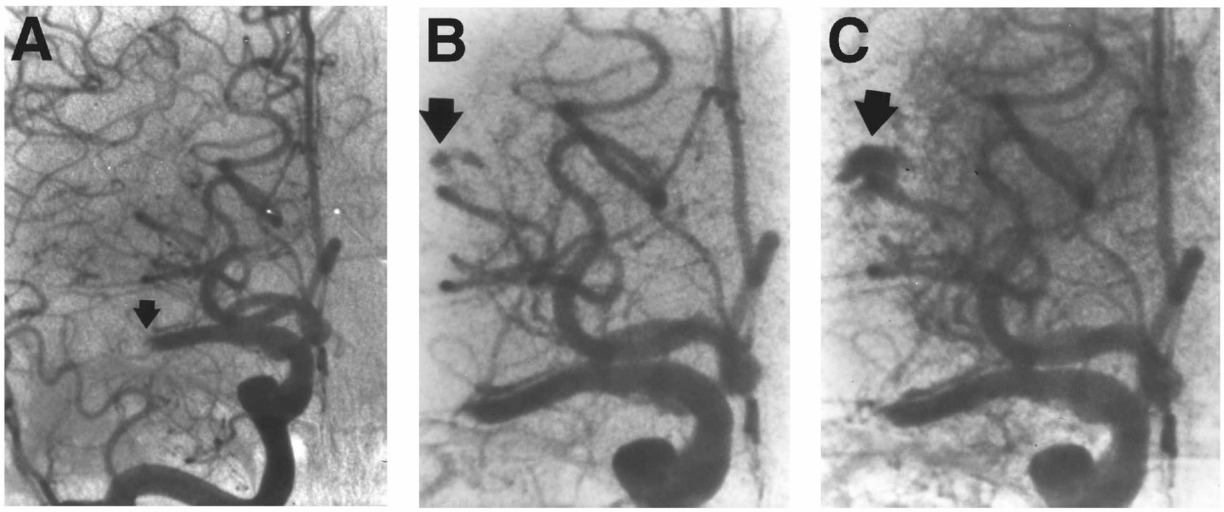

FIGURE 93.4 Evolution of parenchymal hemorrhage following intravenous infusion of recombinant tissue-type plasminogen activator (rtPA). *Arrows* indicate evolving hemorrhage. **A**: Obstruction of proximal middle cerebral artery (MCA) (M1 segment). **B,C**: During systemic rtPA infusion, following partial recanalization of the MCA, progressive extravasation from a distal lenticulostriatal artery was noted. A large PH with ventricular extension resulted (see FIGURE 93.3). (From the collection of M. Pessin, with permission.)

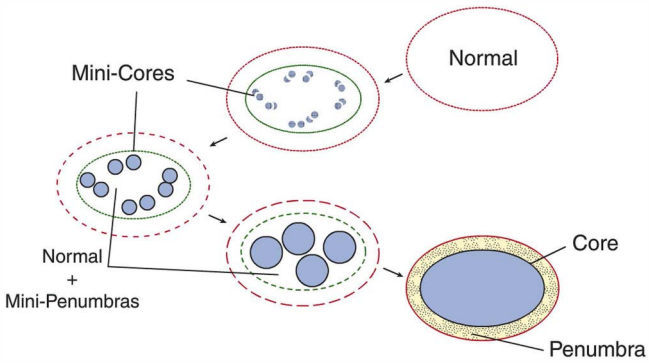

FIGURE 93.5 A proposed construct of the "penumbra" that takes into account the heterogeneous evolution of injury within the 1st hours of ischemia onset after the occlusion of a brain-supplying artery. The lesions and their reversibility depend upon the time and location of the reduction in regional cerebral blood flow (rCBF) in the territory-at-risk. If the injury stimulus cannot to be curtailed, the mini-cores coalesce with the duration of rCBF reduction devouring micro-penumbras. The evolution from normal function to the final lesion may depend upon a number of factors, including the territory of vascular supply, depth of reduction of rCBF, inflammatory state at baseline and/or degree of inflammatory response, and other factors.[93]

demonstrates that in the early minutes and hours after ischemia onset the core contains pockets of injury surrounded by "mini-penumbras."[93] It has been hypothesized that failure to resolve these mini-penumbras into viable tissue leads ultimately to homogenous injured tissue (FIGURE 93.5). The cell and microvessel events in large part relate to local microvascular flow and may underlie recoverability of the ischemic territory-at-risk when flow is reestablished. Normal collateral arterial circuits protect cerebral cortical tissue and may contribute to the reversibility of the ischemic penumbra. Importantly, acute reperfusion of the occluded artery (arteries) is taken to decrease the penumbra and recover normally functioning tissue.

IMAGING OF CEREBRAL TISSUES/VASCULATURE

To define the regions of evolving injury, imaging approaches provide information about tissue status, vessel patency and morphology, and cerebral perfusion. With acquisition times of only 10 to 20 minutes, both CT scans and MR imaging can distinguish infarction from hemorrhage, identify the site of vascular occlusions, and delineate already irreversibly infarcted tissue from potentially reversible penumbra.

Brain Parenchymal Imaging

Tissue injury. Noncontrast CT excludes hemorrhagic transformation and other nonischemic causes of acute neurologic deficits (e.g., tumor, infection). It has the advantages of being rapid and inexpensive with widespread availability. CT imaging can identify signs of early injury (e.g., loss of gray-white differentiation, hypodensity, and sulcal effacement) and of cerebral artery occlusion infarct signs (HMCA sign). These signs occur in up to 80% of patients within 6 hours from symptom onset.[97] Pathophysiologic studies suggest that early infarct signs represent edema. The presence of early infarct signs can be associated with a poorer outcome.[98,99]

MR imaging is sensitive to tissue injury. Within the first few hours of ischemia onset, standard MR protocols (T1-weighted, T2-weighted, and proton density–weighted sequences) are relatively insensitive to ischemia, showing abnormalities in <50% of cases.[100] The earliest changes, seen as increased signal on T2-weighted and fluid attenuated inversion recovery sequences, are due to a net increase in tissue water content. Although the most ischemic lesions are evident on both CT and conventional MR by 24 hours, standard MR is superior to CT in identifying posterior fossa and subcortical lesions.

Extravasated blood can be reliably detected with gradient recalled echo or echo-planar susceptibility-weighted MR imaging.[101] MR is at least equally accurate to CT in distinguishing acute intracerebral hemorrhage from acute ischemic stroke.[102,103]

Diffusion-weighted images (DWI) allow visualization of regions of cerebral ischemia within minutes of symptom onset, indicating lesion size, lesion age, neuroanatomic site, and the vascular territory involved.[104–107] Decreased flux of free H_2O from the extracellular to intracellular space, during the early moments of ischemia, can be quantified on the apparent diffusion coefficient (ADC) maps. While the increased DWI signal may persist for several weeks or longer partially due to a T2 effect, the ADC returns to normal or increased levels within 7 to 10 days from ischemia onset.[108] DWI has a high sensitivity (95% to 100%) and specificity (95% to 100%) for acute ischemia.[109–111] The initial diffusion lesion volume correlates well with final infarct volume, as well as neurologic and functional outcomes in stroke patients.[105,112,113] Early reperfusion can normalize DWI images.[114,115] Therefore, DWI can distinguish acute from chronic ischemia[104,116,117] and provide frequent*** visualization of multiple acute lesions in different vascular territories in patients who have only one clinically symptomatic acute insult, providing evidence of an embolic stroke mechanism.[118]

Perfusion Imaging

Perfusion CT assesses rCBF, identifying regions of irreversible tissue injury as areas of reduced cerebral blood volume (CBV).[119] Perfusion CT has the advantage of rapid data acquisition and rapid postprocessing and can be performed in conjunction with the baseline head CT and CT angiography scans. *MR perfusion-weighted imaging* follows rapid injection of an intravenous paramagnetic contrast agent and relies upon generation of concentration–time curves throughout the tissue under study. Perfusion measures derived from this technique include mean transit time, relative CBV, and rCBF. Baseline MR perfusion lesion volumes correlate well with final infarct volume as well as neurologic and functional outcome.[113] Perfusion lesion volume likely identifies all tissue-at-risk of infarction if vessel recanalization does not occur, while diffusion imaging identifies only tissue that has already sustained advanced metabolic failure.

Vascular Imaging

Digital subtraction angiography (DSA) is the standard imaging modality to assess vessel anatomy and pathology, capable of visualizing medium and small vessels, collateral circulation,

and specific vascular lesions (e.g., arterial dissection, small caliber vessels such as vasculitis). Imaging of the cervicocephalic vessels to demonstrate evidence and site of stenosis and occlusions is achieved by contrast-enhanced *helical CT angiography.*[120] Image postprocessing permits the data to be visualized using multiplanar reformatting, surface or three-dimensional volume rendering, and maximum-intensity projection techniques.[121] *MR angiography* has sensitivity and specificity ranges of 70% to 100% when compared to DSA for the detection of cervical and intracranial stenoses.[122] In the intracranial vasculature, MRA is useful in identifying acute proximal large-vessel occlusions, but cannot reliably identify distal or branch occlusions. Combined with data from DWI, MRA substantially improves acute diagnosis of stroke mechanism.[106] Contrast-enhanced MRA techniques that employ gadolinium contrast to outline vascular structures provide more reliable images of the thoracocervical vasculature. Flow artifacts can occur, including in-plane flow saturation, susceptibility to turbulent or complex flow, and flow-like effects from adjacent short T1 substances, such as thrombus and fat, which distort the degree of stenosis.

Carotid and transcranial Doppler (TCD) ultrasound techniques offer another noninvasive method to assess the neurovasculature acutely. Ultrasound studies of the carotid bifurcation are a well-established noninvasive test in the diagnosis of stroke. A full carotid ultrasonography battery includes continuous-wave Doppler measurement of blood velocity, B-mode imaging of vessel anatomy, and color-flow imaging of flow direction and lumen caliber. Carotid ultrasound visualizes only the proximal cervical carotid arteries and very limited segments of the cervical vertebral arteries. TCD ultrasound employs a low-frequency probe to penetrate the skull and interrogate the major basal intracranial arteries.[123] TCD offers a practical, noninvasive assessment of intracranial vessel status that provides early diagnostic and prognostic information in patients with acute stroke.[124] In addition, TCD can be used to monitor cerebral hemodynamic status and response to recanalization therapy.[125]

ANTITHROMBOTIC APPROACHES TO CEREBRAL ISCHEMIA

The direct and indirect evidence that platelet activation, acceleration of coagulation, and thrombosis underlie most ischemic strokes supports the use of antithrombotic agents in the management of cerebrovascular ischemia. Plasminogen activators (PAs) have been used to limit the consequences of recent transient or fixed neurologic deficits in the acute phase. Recombinant tissue plasminogen activator (rtPA) is now used in patients within 3 (to 4.5) hours of symptom onset and in the absence of detectable cerebral hemorrhage to reduce injury or to improve clinical outcome. Antiplatelet agents and anticoagulants have also been used as prophylaxis against recurrence of ischemia (i.e., secondary prevention). Antiplatelet agents play a role in the reduction of TIAs and ischemic strokes associated with thromboemboli of atherosclerotic origin. Anticoagulants are employed for prevention of cardiogenic emboli, arising in the setting of nonvalvular AF, ventricular dysfunction, or valvular injury or prostheses. Both approaches have been combined, but neither has been used in acute treatment of thrombotic stroke.

TRANSIENT CEREBRAL ISCHEMIA (TRANSIENT ISCHEMIC ATTACKS)

Because TIAs and ischemic stroke (e.g., transient monocular blindness [amaurosis fugax])[126–128] can often be manifestations of ongoing platelet and coagulation activation and of vascular injury in brain-supplying arteries, antiplatelet agents have been applied with benefit.[4,5,6,7,8,44,46,57,63,86,129]

Antiplatelet Agents

Aspirin (Acetylsalicylic Acid). In ischemic cerebrovascular disease, ASA can reduce the incidence of TIAs and subsequent ischemic strokes. Despite the large number of prospective evaluations of ASA, a small number of well-conducted level I studies "drive" the efficacy of ASA in the carotid artery territory (Table 93.2).

The Aspirin in Transient Ischemic Attacks (AITIA) study—which randomized patients with a 3-month history of TIAs, who were not considered candidates for carotid endarterectomy, to ASA (1,300 mg/day) or to placebo—demonstrated a reduction in stroke and vascular death at 6 months with ASA exposure.[130] There was a considerable decrease in the combined outcomes of recurrent TIAs, cerebral/retinal infarction, and death among those receiving ASA, but no reduction by life-table analysis at the 24-month follow-up and no reduction in stroke incidence. In the Canadian Cooperative Study Group trial, patients with a history of TIAs were randomized to ASA (1,300 mg/day), sulfinpyrazone (800 mg/day), and the combination ASA/sulfinpyrazone, or placebo.[131] Patients who received ASA had a significant decrease in the incidence of stroke and death compared to sulfinpyrazone or placebo.[131] The risk reduction for stroke and death with ASA was most significant for men. Among patients with a history of TIAs in the double-blind "Accidents, Ischemiques Cerebraux Lies a l' Atherosclerose (AICLA)" trial who were randomized to ASA (990 mg/day), the combination of ASA (990 mg/day) with dipyridamole (225 mg/day), or placebo, those who received ASA or the combination demonstrated a reduced incidence of stroke, MI, and death compared to patients who received placebo.[132] Efficacy in the combined outcome seemed to be associated with an ASA dose range of 990 to 1,300 mg/day.

However, the ASA doses used were associated with considerable side effects. This was confirmed by the UK-TIA aspirin trial, which randomized 2,435 individuals with TIAs or minor ischemic stroke to "high dose" ASA (1,200 mg/day), "low-dose" ASA (300 mg/day), or placebo.[15] The 4-year incidence of nonfatal MI, nonfatal major stroke, and death was significantly reduced by 18% in a dose-dependent manner in patients who received ASA. But, gastrointestinal side effects were more common with the high-dose regimen, and excess mortality was attributed to intracranial hemorrhage in the ASA-treated groups. In a separate study, a decrease in major hemorrhagic events, including fatal intracerebral hemorrhage, was associated with lower ASA doses (30 vs. 283 mg/day).[133]

The Swedish Aspirin Low-Dose Trial (SALT) Collaborative Group reported an 18% risk reduction of stroke and death among TIA patients who started ASA (75 mg/day) within 1 to 4 months of their initial symptoms compared to patients who received placebo.[134] A 16% to 20% reduction in the risk of stroke, frequent TIAs, and MI was also observed.

Table 93.2

TIAs (± stroke): antiplatelet agents

Study	Agent	Dose (per Day)	Patients (n)	Stroke (n)	Mortality (n)
AITIA Study[130]	ASA	1,300 mg	88	10	3
	Placebo	—	90	12	7
Canadian Cooperative Study Group[131]	ASA/placebo 1	1,300 mg/—	144	22	4
	Sulfinpyrazone/placebo 2	800 mg/—	156	29	9
	ASA/sulfinpyrazone	1,300 mg/800 mg	146	14	6
	Placebo 1/placebo 2	—/—	139	20	10
Danish Cooperative Study[374]	ASA	1,000 mg	101	18	7
	Placebo	—	102	11	7
AICLA[132]	ASA/dipyridamole	990 mg/225 mg	202	18	8
	ASA	990 mg	198	17	10
	Placebo	—	204	31	7
UK-TIA Study Group[15]	ASA	1,200 mg	815	66	111
	ASA	300 mg	806	68	106
	Placebo	—	814	88	122
Dutch TIA Trial Study Group[133]	ASA	283 mg	1,576	109	151
	ASA	30 mg	1,555	90	160
SALT[134]	ASA	75 mg	676	93	61
	Placebo	—	684	112	69
European Stroke Prevention Study Group[139]	ASA/dipyridamole	975 mg/225 mg	1,250	114	108
	Placebo	—	1,250	184	156
American-Canadian Cooperative Study Group[140]	ASA/dipyridamole	1,300 mg/300 mg	448	53	46
	ASA	1,300 mg	442	60	38
Matias-Guiu et al.[375]	ASA/dipyridamole	50 mg/300 mg	115	3	2
	Dipyridamole	400 mg	71	3	3
European Stroke Prevention Study-2[77]	ASA/dipyridamole[a]	400 mg/50 mg	1,650	157	285
	Dipyridamole[a]	400 mg	1,654	211	288
	ASA	50 mg	1,649	206	282
	Placebo	—	1,649	250	202
Hass et al.[148]	Ticlopidine	500 mg	1,529	172	175
	ASA	1,300 mg	1,540	212	196
ESPRIT Study Group[142–144]	ASA/dipyridamole	30–325 mg/400 mg	1,363	96	93
	ASA	30–325 mg	1,376	116	107

[a]Extended-release dipyridamole.
ASA, aspirin (acetylsalicylic acid); UK-TIA, United Kingdom Transient Ischemic Attack; AITIA, Aspirin in Transient Ischemic Attacks; TIA, transient ischemic attacks; SALT, Swedish Aspirin Low-Dose Trial.

The results of those trials, and meta-analyses of all clinical series, suggest that even low doses of ASA benefit early stroke and vascular mortality in patients with a history of TIAs or minor ischemic events.

Combination ASA/Dipyridamole. Clinical use of the combination of ASA with dipyridamole has been based upon positive interactions between the agents in both preclinical experiments and in clinical platelet survival studies.[135–137] The efficacy of this combination, however, has been challenged.[136] Dipyridamole (400 to 800 mg/day) alone did not affect stroke incidence or related mortality compared with placebo in one limited double-blind, level I randomized trial.[138]

The European Stroke Prevention Study (ESPS) Group demonstrated a 33% reduction in risk of stroke and death with ASA (975 mg/day) and dipyridamole (225 mg/day) compared to placebo.[139] The three-arm "AICLA" study also demonstrated the benefits from the combination of ASA with dipyridamole over placebo (but no difference with ASA alone) for patients with TIAs when the outcome events of stroke, MI, and mortality were combined.[132] However, there was no difference in stroke or mortality between the combination of ASA (1,300 mg/day) and dipyridamole (300 mg/day) and ASA alone in the American-Canadian Cooperative Study Group.[140]

On the basis of experimental evidence suggesting that sustained release extended-release dipyridamole (ER-DP) could produce elevated plasma adenosine levels, the relative efficacy of the fixed combination of low-dose ASA (50 mg/day)/ER-DP (400 mg/day) was tested in the ESPS-2 against ER-DP, ASA, or placebo. At 2-year follow-up, low-dose ASA alone and dipyridamole alone (relative risk reduction [RRR] for stroke, 15.8% and 17.7%, respectively) and the combination (RRR, 36.7%) were found to be superior to placebo in limiting stroke, death, or both for individuals with a history of TIAs or recent stroke.[77,141]

The European/Australian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT) Group examined the impact of combination ASA (30 to 375 mg/day)/dipyridamole (400 mg/day) or ASA alone on vascular death, nonfatal stroke, nonfatal MI, or major hemorrhage in patients presenting with TIA or minor ischemic stroke.[142–144] With a mean follow-up of 3.5 years, the combination of ASA/dipyridamole was superior to ASA alone (HR = 0.80, 95% CI = 0.66 to 0.98) in reduction of the primary outcome events. While headache occurred in the combination, overall ASA/dipyridamole improved outcome in this trial and produced a decrease in ischemic events.[143] The greater efficacy of the combination supports its use in the clinical setting.

In a separate study, clopidogrel was prospectively compared to the combination of aspirin/ER-DP with or without the angiotensin receptor blocker telmisartan in the Prevention Regimen for Effectively avoiding Second Strokes (PRoFESS) study of first recurrent stroke in a 2 × 2 factorial design.[145] In total, 20,332 patients were randomized within 120 days of the initial event and followed for a mean of 2.5 years. There was no difference in the incidence of recurrent strokes between the two groups, although there were more hemorrhagic events in the aspirin/ER-DP arms. This trial did not meet the predefined criteria for noninferiority.

Sulfinpyrazone. The Canadian Cooperative Study Group compared ASA, sulfinpyrazone (800 mg/day), a uricosuric agent with antiplatelet effect, and placebo but found no significant difference in the incidence of stroke and mortality between sulfinpyrazone and placebo over 2.2 years.[131] Sulfinpyrazone (800 mg/day) and placebo were also tested in a double-blind, crossover trial.[146] A nonsignificant reduction in TIAs and in stroke and mortality at 4-month follow-up was recorded in patients receiving sulfinpyrazone. Another level I study comparing sulfinpyrazone (800 mg/day) and ASA (1,000 mg/day) in individuals with TIAs concluded that there was a higher incidence of stroke, MI, and vascular death in the sulfinpyrazone cohort at 11-month follow-up.[147]

Adenosine Diphosphate Receptor Antagonists. Thienopyridines irreversibly block the adenosine diphosphate platelet receptor $P2Y_{12}$ and inhibit other downstream events, thereby blocking platelet activation. Ticlopidine found benefit in patients with TIA/minor stroke[148] and in prevention of secondary stroke (see below). Ticlopidine (500 mg/day) was associated with a statistically significant 12% reduction in the risk of stroke and death from any cause over ASA (1,300 mg/day) and a 21% reduction in the risk of secondary outcomes of stroke and stroke-related death following TIAs. Reversible leukopenia occurred in 0.8% of patients taking ticlopidine, whereas gastrointestinal symptoms were more common in patients receiving ASA. Intracerebral hemorrhage occurred equally in both groups. Because of concerns about the frequency of thrombotic thrombocytopenic purpura, ticlopidine has been supplanted by the related thienopyridine clopidogrel.

In summary, ASA reduces the risk of stroke, MI, and mortality in individuals with a history of TIAs or minor stroke. This risk reduction is obviously not dose dependent. The fixed combination of low-dose ASA/ER-DP also produces a substantial risk reduction. Ticlopidine was more effective than ASA in preventing stroke and mortality after TIAs in one study but is seldom used now because of unwanted side effects. When used alone, dipyridamole and sulfinpyrazone have little demonstrated benefit.[149,150]

Anticoagulation

Changes in plasma thrombin concentration and FDP levels occur following ischemic stroke that suggest that interruption of coagulation may be beneficial. In the 1960s and 1970s, clinical experience with anticoagulation in patients with TIAs or minor stroke was limited.[151–154] Uncontrolled reports suggested that heparin could decrease the incidence of basilar artery TIAs.[155,156] Fisher observed a transient reduction in the incidence of TIAs in 97% (of 29) patients treated with anticoagulants, with TIAs returning in 60% of those in whom anticoagulation was discontinued.[157] Two studies reported that long-term anticoagulation decreased the incidence of strokes and/or vascular death among patients with recent TIAs[158,159]; a third study showed that despite a decrease in TIA frequency, there was no decrease in the incidence of stroke or vascular death.[160] Two other studies failed to show superiority of anticoagulation over its comparator for the incidence of stroke or death.[161,162] In a small Medical Research Council (MRC)-sponsored trial of phenindione, patients with TIA receiving fixed "low-dose" anticoagulation had a lower incidence of vascular events, stroke, and death than those patients receiving an adjusted "high-dose" regimen.[163] Only the Cerebral Embolism Study Group trial indicated a benefit from immediate anticoagulation for cardiogenic embolism.[164]

Subsequently, there have been few controlled prospective randomized level I studies. The Warfarin-Aspirin Recurrent Stroke Study (WARSS) found no difference in outcome between TIA and stroke patients treated with oral anticoagulation under tight control compared to patients treated with ASA.[165] There was no increase in intracerebral hemorrhage in the anticoagulated group, most probably because of the rigid control of the international normalized ratio (INR).[165]

The risk of intracerebral hemorrhage increases with age in patients treated with oral anticoagulants which inhibit vitamin K activity.[166,167] With careful monitoring and dose adjustment, this risk can be reduced.[167] The Stroke Prevention in Reversible Ischemic Trial[168] was terminated because of an unacceptably high incidence of intracranial hemorrhages in the group at a high target INR of 3.0 to 4.5.

In summary, excluding individuals with AF or cardiac valvular prostheses, the results of various small studies do not convincingly support a role for anticoagulation in patients with TIA or minor stroke, considering the risk of hemorrhage. The risk of intracerebral hemorrhage is increased for patients receiving oral anticoagulants at INRs in excess of 3.0 to 4.5.

Direct Thrombin Inhibitors

A single small safety study of the parenteral thrombin inhibitor argatroban in patients presenting with TIAs or minor ischemic stroke suggesting no detriment has appeared.[169] No

phase III experience exists; however acute use has been under limited study.[170]

Plasminogen Activators

The use of fibrinolytic agents in patients with cerebral ischemia is confined to acute intervention in ischemic stroke; there is no indication for the use of these agents in TIAs.

STROKE-IN-PROGRESSION

Progressive deterioration following ischemic stroke has been ascribed to (a) anterograde extension of *in situ* thrombus to occlude critical arterial branches or (b) recurrent embolism. The role of anticoagulation in patients with stroke-in-progression, however, remains unsettled, in large part, because of the difficulty in defining what constitutes "progression" at entry and uncertainties about the pathogenesis of "evolving stroke." Early studies also reveal methodologic weaknesses and conflicting outcomes of this diagnosis.[171–173] Furthermore, deterioration following ischemic stroke may be due to edema, hemorrhage, or a combination of the events that lead to tissue swelling and injury.[174]

The Trial of Org 10172 in Acute Stroke Treatment (TOAST), a level I prospective randomized placebo-controlled study of the heparinoid danaparoid for stroke-in-evolution, demonstrated no benefit to the anticoagulant regarding disability indices, neurologic status, or mortality.[175–177] Entry was opened to all patients with hemispheric stroke symptoms for up to 24 hours.[175] A short-lived reduction in stroke incidence was seen. Serious intracerebral hemorrhage was significantly more frequent in patients who received danaparoid (14 of 646 patients) compared to placebo (4 of 635 patients). Danaparoid decreased the incidence of deep venous thrombosis (DVT). Stroke-in-progression is no longer identified as a separate entity for which a specific treatment is required.

COMPLETED STROKE

Prospective CT scan and MRI[178–180] have confirmed the experimental observation that the ischemic lesion stabilizes, as an infarct, by 24 hours after occlusion of the supply artery.[181,182] These congruent findings suggest that antithrombotic interventions beyond 24 to 72 hours after symptom onset are unlikely to have a beneficial impact on the initial ischemic stroke. Following a first-time ischemic stroke, the rate of subsequent ischemic stroke, MI, death, or vascular-related death is 10% to 12% per year.[183–185] Hence, the initial ischemic stroke offers a target for antithrombotic agents to reduce the incidence of second ischemic events (secondary prevention).

Antiplatelet Agents

Meta-analyses suggest benefit from antiplatelet agents in patients with TIAs or first stroke.[186,187] When considered with TIAs, ASA or ticlopidine can significantly decrease the risk of second cerebral ischemic events (Table 93.3). The combination of low dose ASA and ER-DP considerably reduces the incidence of second stroke and/or death in patients presenting with a history of TIAs or stroke compared to ASA or dipyridamole alone.[141]

The IST randomized 19,435 patients with presumed ischemic stroke within 48 hours of symptom onset to placebo, ASA (300 mg/day) alone, subcutaneous heparin (low dose, 10,000 IU/day; or medium dose, 25,000 IU/day), or both ASA and heparin for 14 days in a 3 × 2 factorial design.[72] ASA was associated with an overall statistically significant reduction in total recurrent ischemic strokes within 14 days ($P < 0.001$) and also a significant 0.1% increase in symptomatic intracranial hemorrhage. At 6 months, a modest decrease in the risk of death or dependency was seen. The Chinese Acute Stroke Trial (CAST) demonstrated similar outcomes.[188,189] Together, the IST and CAST demonstrated that ASA is associated with a small reduction in the incidence of recurrent stroke and mortality in the first weeks after signal stroke. This did not persist.

Ticlopidine was found to be superior to placebo in reducing the risk of second stroke, MI, and vascular mortality when patients were treated within 1 to 16 weeks following the signal thromboembolic stroke.[183] Side effects related to ticlopidine, including reversible leukopenia, diarrhea, or rash, occurred in 8% of the treated patients. Concerns about the side effects of ticlopidine prompted a study of the efficacy of the related thienopyridine derivative clopidogrel.

The Clopidogrel versus Aspirin in Individuals at Risk of Ischaemic Events (CAPRIE) study, a prospective, randomized, double-blind level I comparison of clopidogrel (75 mg/day) against ASA (325 mg/day) in patients presenting with ischemic or lacunar stroke, MI < 35 days old, or symptomatic atherosclerotic peripheral arterial disease (PAD), demonstrated a statistically significant 8.7% reduction in the combined risk of ischemic stroke, MI, or vascular-related death by clopidogrel compared to ASA.[190] The overall benefit associated with clopidogrel was driven by the PAD outcome; there was no independent significant difference in outcome in the stroke cohort. Because of the near equivalent frequency of intracranial hemorrhage (clopidogrel = 0.33% vs. ASA = 0.47%), clopidogrel is now used clinically. The large (7,599 patients) Management of Atherothrombosis with Clopidogrel in High-risk patients trial compared the efficacy of ASA (75 mg)/clopidogrel (75 mg) to clopidogrel (75 mg) alone for ischemic events including stroke. No difference was seen in outcome between the two groups, although the ASA/clopidogrel combination was associated with significantly higher incidence of life-threatening hemorrhage (including intracranial hemorrhage).[191] The Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance trial, a prospective double-blind placebo-controlled study of combination ASA (75 mg)/clopidogrel (75 mg) *versus* ASA (75 mg)/placebo for patients with multiple atherothrombotic risk factors, demonstrated no significant difference for the primary efficacy endpoint (the composite of MI, stroke, or cardiovascular mortality).[192] Long-term clopidogrel use was associated with an increased risk of hemorrhage, particularly in the first year.[193] Overall, there was no clear benefit in stroke efficacy outcome (a *post hoc* analysis of secondary prevention) with the combination over ASA alone. That study has provided much for discussion. The PRoFESS study and its outcomes have been mentioned above.

A recently undertaken trial, the Platelet-Oriented Inhibition in New TIA and minor ischemic stroke trial (http://clinicaltrials.gov/ct2/show/NCT00991029), is a randomized double-blind multicenter study to investigate whether clopidogrel (75 mg/day) alone is superior to aspirin (50 to 325 mg/day) in improving survival free from major ischemic vascular events (ischemic stroke, MI, and ischemic vascular death). The projected sample size is 4,150 patients.

Table 93.3

Completed stroke: antiplatelet agents

Study	Agent	Dose (per Day)	Patients (n)	Stroke (n)	Mortality (n)
Swedish Cooperative Study[184]	ASA	1,500 mg	253	32	34
	Placebo	—	252	32	37
Gent et al.[185]	Suloctidil	600 mg	218	29[a]	13[a]
	Placebo	—	220	28[a]	25[a]
Blakeley[194]	Sulfinpyrazone	800 mg	145	—	25
	Placebo	—	145	—	28
Canadian-American Ticlopidine Study[183]	Ticlopidine	500 mg	525	54	30[a]
	Placebo	—	528	89	38[a]
CAPRIE[190b]	Clopidogrel	75 mg	3,233	315	—
	ASA	325 mg	3,198	338	—
IST[72]	ASA[c]	300 mg	9,720	362[d]	872
	No ASA[c]	—	9,715	452[d]	909
CAST[188]	ASA	160 mg	10,554	335	343
	No ASA	—	10,552	351	398
ESPS-2[77]	ERDP	400 mg/50 mg	1,650	157	285
	Dipyridamole	400 mg	1,654	211	288
	ASA	50 mg	1,649	206	282
	Placebo	—	1,649	250	202
Diener et al.[191]	ASA/clopidogrel	75 mg/75 mg	3,420	299	201
	Placebo/clopidogrel	—/75 mg	3,454	309	201
Bhatt et al.[192,193]	ASA/clopidogrel	75–162 mg/75 mg	7,802	132	371
	Placebo/clopidogrel	—/75 mg	7,801	163	374
Sacco et al.[145]	ASA/ERDP	50 mg/400 mg	10,181	916	739
	Clopidogrel	75 mg	10,151	898	756

[a]Eligible events only, excluding events >28 days after study drug was permanently discontinued.
[b]Stroke subgroup.
[c]Factorial design (include heparin ± ASA).
[d]14-day outcomes.
ASA, aspirin; CAPRIE, Clopidogrel versus Aspirin in Individuals at Risk of Ischaemic Events; IST, International Stroke Trial; CAST, Chinese Acute Stroke Trial.

The novel antiplatelet agents sulfinpyrazone[194] and suloctidil[185] failed to demonstrate benefit over placebo in separate trials. Neither agent is now used in stroke management.

The value of the glycoprotein IIb/IIIa (integrin $\alpha_{IIb}\beta_3$) antagonist abciximab given within 24 hours of the onset of ischemic stroke was examined in a phase II study.[195] No significant improvement in outcome efficacy or increased risk of intracerebral hemorrhage was seen in 74 patients at the doses tested.[195] The follow-on phase III randomized, placebo-controlled double-blind study stratified for time (<3 to 6 hours from symptom onset) and severity was terminated at 808 of the projected 1,800 patients due to excessive symptomatic or fatal intracranial hemorrhage (~5.5% abciximab vs. 0.5% placebo-treated patients).[196] Hence, this strategy has been abandoned. This observation contrasts with experimental studies with polypeptide or organic molecule integrin $\alpha_{IIb}\beta_3$ inhibitors in experimental models of focal ischemia that demonstrate reduction in injury volume.[65,69]

Those clinical studies confirm that in the setting of ischemic stroke, some antiplatelet agents given alone are beneficial in outcomes of recurrent stroke but when used in combination can potentially increase the risk of significant hemorrhage.

To test a related notion (i.e., the acute use of an antiplatelet agent in combination), a prospective randomized trial of acute intervention rtPA with or without the GPIIb-IIIa antagonist eptifibatide (the Combined Approach to Lysis Utilizing Eptifibatide *a*nd rtPA in Acute Ischemic Stroke) defined a dose of eptifibatide that was safe.[197] Recently, the Combined Approach to Lysis Utilizing Eptifibatide and rtPA in Acute Ischemic Stroke—Enhanced Regimen has begun comparing rtPA (0.6 mg/kg total) and eptifibatide (bolus 135 μg/kg and 2-hour infusion at 0.75 μg/kg/min) compared to standard-dose rtPA (0.9 mg/kg) in patients with ischemic stroke that can have the rtPA initiated within 3 hours of symptom onset.

Because of the effects on stroke outcome in patients with TIAs, ASA is recommended for secondary prevention. ASA/ER-DP has been shown to be superior to ASA in secondary prevention. On the basis of its similar mode of action to ticlopidine, clopidogrel has been substituted in practice. Current American College of Chest Physicians recommendations for

secondary prevention in noncardioembolic ischemic stroke are management with ASA (50 to 100 mg/day), ASA/ER-DP, or clopidogrel (75 mg/day).[149,150]

Anticoagulants

The evidence that heparin or long-term oral anticoagulation benefits patients with presumed atherothrombotic "completed" stroke is sparse. Early randomized, controlled trials and uncontrolled studies of oral anticoagulation have given the impression that anticoagulant therapy does not prevent second stroke events in patients with an initial TIA or atherothrombotic or thrombotic stroke.[161,198–202]

IST and CAST were conducted on the premise that proper use of subcutaneous unfractionated heparin (UFH) should not be associated with an increase in intracerebral hemorrhage and may therefore be safe in the treatment of presumed primary atherothrombotic stroke.[72,73,189] However, the significant increase in symptomatic intracerebral hemorrhage nullified the benefits of heparin on recurrent stroke. It is uncertain why hemorrhagic complications were so prominent in that study. Moreover, UFH used in this way is not common practice.

Low molecular weight heparins (LMWHs) have been tested with the intent to alter the outcome of first-ever stroke, usually not before 24 hours after symptom onset (Table 93.4). In the Fraxiparin in Stroke Study (FISS), patients were randomized to subcutaneous "high-dose" nadroparin (8,200 IU of antifactor Xa per day), "low-dose" nadroparin (4,100 IU/day), or placebo for 10 days beginning within 48 hours after onset of stroke.[203] A significant dose-dependent reduction in 6-month mortality and dependence was observed favoring the nadroparin arm. FISS-bis, a follow-on trial of nadroparin in stroke, compared similar doses, but demonstrated no difference in combined outcome or mortality (unpublished). In the Heparin in Acute Embolic Stroke Trial (HAEST), dalteparin produced no significant improvement in favorable outcome compared to placebo when both were delivered early following the onset of stroke.[204] No difference in detectable intracerebral hemorrhage was observed. The effect of certoparin on ischemic stroke outcome was examined in a level II dose-finding trial. No significant difference in outcome was detected among dose steps ranging from 3,000 to 16,000 U/day.[205] A modest increase in the risk of symptomatic hemorrhage was seen at the high dose. Certoparin failed to demonstrate a benefit in patients treated within 12 hours of symptom onset.[205] The Tinzaparin in Acute Ischaemic Stroke trial, a randomized double-blind trial, comparing high-dose tinzaparin, low-dose tinzaparin, and ASA patients treated within 48 hours of stroke onset displayed no difference in modified Rankin scale (mRS) at 6 months follow-up.[206] In short, no LMWH to date has been shown to demonstrate benefit in patients treated late after stroke onset. However, in animal models, acute intervention with enoxaparin has been shown to decrease injury volume by several measures.[207]

An unresolved problem has been whether anticoagulation or antiplatelet treatment is appropriate (superior) in ischemic stroke of unknown origin for recurrence. To determine this, the WARSS trial was undertaken by the National Institute of Neurological Disorders and Stroke (NINDS).[208] The study found no differential benefit of warfarin or ASA on the frequency of secondary cerebral ischemic events in patients presenting within 30 days of the signal stroke.[208] Warfarin was not associated with an increase in intracerebral hemorrhage, probably because of the tight monitoring of the INR.

At present, there is no evidence of an advantage to the use of anticoagulants for completed stroke.[209,210] This conclusion is reflected in a Cochrane analysis of some 24 trials, in which the majority of patients were treated late after stroke onset.[210] However, none of those studies instituted anticoagulants in the acute phase.

Table 93.4

Completed stroke: anticoagulation

Study	Agent	Dose (per Day)	Patients (n)	Stroke (n)	Mortality (n)
FISS[203]	Nadroparin[a]	8,200 aFXa	100	—	45[a]
	Nadroparin[a]	4,100 aFXa	101	—	53[a]
	Placebo	—	105	—	68[a]
FISS-bis	Nadroparin[a]	172 aFXa/kg	245	—	145[a]
	Nadroparin[a]	86 aFXa/kg	272	—	156[a]
	Placebo	—	250	—	142[a]
HAEST[204]	Dalteparin	200 IU/kg	224	19[b]	148[a]
	ASA	160 mg	225	17[b]	146[a]
TOPAS[205]	Certoparin	16,000 aFXa	97	13[c]	12
	Certoparin	10,000 aFXa	103	10[c]	7
	Certoparin	6,000 aFXa	102	6[c]	7
	Certoparin	3,000 aFXa	98	11[c]	4

[a]Death or dependency at latest follow-up.
[b]At 14 days.
[c]Including TIAs.
aFXa, antifactor Xa activity; FISS, Fraxiparin in Stroke Study; HAEST, Heparin in Acute Embolic Stroke Trial; TOPAS, Therapy of Patients with Acute Stroke.

Plasminogen Activators

Early experience with PAs in patients with completed stroke failed to demonstrate efficacy when symptomatic improvement and death were the primary outcomes. Level I clinical trials evaluating intravenous infusion of thrombolytic agents in completed stroke were inconclusive. Fletcher et al.[211] observed no benefit in 31 patients treated with urokinase plasminogen activator (uPA) within 10 to 12 hours of symptom onset, but observed a very troublesome incidence of symptomatic intracranial hemorrhage and related mortality. Consequently, the use of PAs in individuals with stroke was generally contraindicated.[212] The long interval to treatment after the stroke onset and the possibility that the stroke was caused by undiagnosed hemorrhage (scanning technologies were not available) were common concerns with those early studies.[213] A group of prospective, randomized, controlled low-dose studies of intravenous uPA or tPA in patients with stable focal neurologic deficits of <5 days duration demonstrated no difference in clinical outcome or in the incidence of symptomatic intracerebral hemorrhage.[214–217]

ACUTE INTERVENTIONS IN ISCHEMIC STROKE

A major change in the approach to ischemic stroke followed the proposal that PAs should be applied acutely (within 6 to 8 hours) after symptom onset.[213] Conceptually, it was reasoned that rapid and adequate return of flow to the metastable, potentially reversible, zones of neuron and tissue injury in the ischemic vascular territory, the "penumbra," should provide benefit.[89,218] The contributions of local vascular anatomy and collateral vascular protection to tissue perfusion, as well as the predominantly thrombotic basis for focal cerebral ischemia, underlie attempts to achieve early recanalization with PAs.

Acute intervention with PAs in patients selected by strict CT scan and clinical criteria is associated with evidence of benefit.[6,74] In contrast, although experimental studies suggest that acute interventions with antiplatelet agents and anticoagulants could increase vascular patency and decrease injury, no clinical studies of acute intervention in ischemic stroke patients have been pursued.

Systemic Infusion of Plasminogen Activators

Recanalization of ICA occlusions by plasminogen activities is infrequent (0% to 25%),[6,9,214,215,219,220] but partial or complete arterial recanalization has been reported in 34% to 59% of patients with distal carotid artery territory occlusions who were treated within 6 to 8 hours of symptom onset.[6,219,220,221] Mori et al.[219] demonstrated, in a randomized controlled trial, that patients treated with 20 or 30 MIU of recombinant tPA (rtPA) (duteplase) had improved recanalization and considerable clinical improvement at 30 days compared to patients treated with placebo. Patients demonstrating early recanalization had better neurologic outcome than those not exhibiting recanalization. In those studies, detectable hemorrhagic transformation occurred in 29% to 53% of treated individuals,[6,219,220,221] which was similar to natural history.[79] Recanalization of vertebral or basilar artery occlusions with rtPA has been reported.[220,221]

Three symptom-based, randomized, controlled trials of the acute intravenous delivery the streptokinase (SK) in patients with ischemic stroke were terminated because of excessive early mortality and symptomatic intracranial hemorrhage in the SK treatment groups.[222–224] No preparatory dose-finding studies were performed in any of those trials. In the Multicentre Acute Stroke Trial-Italy (MAST-I),[224] the combination of ASA with SK produced excessive early case fatality. The outcome of that trial has not been explained, and the potential utility of SK in ischemic stroke has not been pursued further.

rtPA (alteplase) is approved for intravenous use within 3 hours of symptom onset under strict patient-selection criteria (Table 93.5). This is based upon the results of a two-part, level I, placebo-controlled outcome study.[6] In part 1, there was no difference in neurologic status at 24 hours between the rtPA (0.9 mg/kg) and placebo-treated groups according to the National Institutes of Health Stroke Scale (NIHSS) score. In part 2, at 3 months posttreatment, rtPA recipients displayed a statistically significant 11% to 13% absolute improvement over placebo in Barthel index, mRS score, Glasgow outcome scale score, and NIHSS for evidence of no or minimal disability/deficit. Symptomatic hemorrhage was significantly more frequent among patients treated with rtPA (6.4%) than placebo (0.6%). Mortality was unchanged, but intracerebral hemorrhage contributed to death. In follow-up, the outcome benefits in that population were sustained at 12 months and were most apparent in moderately severe strokes.[225] Independent postlicensing use of rtPA in general practice has confirmed the original experience.[226] Benefit was associated with age <75 years and absence of "early signs" of ischemic injury.[226]

Three other phase III prospective randomized safety and efficacy studies of intravenous rtPA (alteplase) broaden the impression from that study and emphasize limitations to the acute use of rtPA. The European Cooperative Acute Stroke Study (ECASS) compared parenteral infusion rtPA (1.1 mg/kg to a maximum of 100 mg) to placebo in patients within 6 hours of symptom onset. No significant difference was observed between the two groups for 90-day median mRS.[74] A subgroup of patients with evidence of ischemia on the entry CT scan, who should have been excluded ("protocol violators"), displayed increased mortality and hemorrhage when treated with rtPA. A *post hoc* analysis of the "target population" suggested an 11% to 12% absolute improvement over placebo in mRS 0 and 1 (no or minimal disability) in the rtPA-treated group, when the "protocol violator" data were excluded. However, a significantly higher proportion of rtPA patients (6.1%) had intracerebral hemorrhage causing neurologic deterioration or death (PH2) than placebo patients (2.6%). ECASS-2, the follow-on randomized double-blind nonangiographic study, required a careful review of the baseline CT scans for "early signs of ischemia" for exclusion of those patients.[98,227] There, 40.3% of patients who received rtPA within 6 hours of symptom onset had a favorable outcome (mRS = 0 or 1), an insignificant 3.2% improvement over placebo. Severe symptomatic parenchymal hemorrhage (PH2) was significantly more frequent in the rtPA group (11.7%) than in the placebo group (3.1%).

Viewed together, the results of the ECASS and NINDS studies indicate the enormous importance of individual patient selection in benefit and in reducing the hemorrhagic risk accompanying the use of PAs in acute stroke.[6,74,75] Heterogeneity among clinical studies of acute intervention with rtPA is well-recognized.[228] Today, rtPA is available for treatment of ischemic stroke in appropriately selected patients within 3 hours

Table 93.5

Acute stroke: plasminogen activators

Study[a]	Agent	Patients (*n*)	Δ(T – 0)[b] (h)	Clinical Improvement (%)	Hemorrhage			
					Nil	HI	PH	%
NINDS (part 1)[6]	rtPA	144	≤1.5, ≤3.0	1.2[c]	—	—	13	5.6
	C	147			—	—	3	0.0
NINDS (part 2)[6]	rtPA	168	≤1.5, ≤3.0	50[d] 31[e]	—	—	21	7.1
	C	165		38[d] 20[e]	—	—	8	2.1
ECASS[74]	rtPA	313	<6.0	35.9	179	72	62	19.8
	C	307		29.3	184	93	30	6.5
ECASS-2[227]	rtPA	409	<6.0	40.3[d]	217	142	48	11.8
	C	391		36.6[d]	233	141	12	3.1
ECASS-3[229]	rtPA	418	3.0–4.5	52.4[d]	305[f]		22[f]	5.3
	C	403		45.2[d]	332[f]		9[f]	2.2

[a]Randomized studies without vascular diagnosis.
[b]Time from symptom onset to treatment.
[c]Relative risk reduction.
[d]mRS score 0 and 1.
[e]National Institutes of Health Stroke Scale (NIHSS) score.
[f]According to the ECASS II definition any ICH.
C, control; HI, hemorrhagic infarction; PH, parenchymal hematoma; rtPA, recombinant tissue-type plasminogen activator; SK, streptokinase; NINDS, National Institute of Neurological Disorders and Stroke; ECASS, European Cooperative Acute Stroke Study; ICH, intracerebral hemorrhage.

of the onset of symptoms in North America and certain other countries.

In order to secure the approval for the acute use of rtPA stroke patients in Europe, two registries and a phase III trial were undertaken. One prospective registry of patient treatment experience with rtPA treatment within 3.0 hours of stroke onset (SITS-MOST) demonstrated that the incidence of symptomatic intracerebral hemorrhage was not different than a pool of randomized controlled trials. Those safety impressions and the experience with patients treated after 3.0 hours (SITS-ISTR registry) were the basis for ECASS-3. ECASS-3 was a multicenter, prospective, randomized, placebo-controlled trial comparing patients best medical treatment together with either rtPA ($n = 418$) or placebo ($n = 403$) between 3.0 and 4.5 hours from symptom onset.[229] The primary efficacy outcome in ECASS-3 (mRS = 0 to 1 at 90 days) was significantly greater with rtPA (52.4%) than placebo (45.2%) (risk ratio = 1.16, 95% CI 1.01 to 1.34, $P = 0.04$). Implications of these findings have been reviewed recently.[230]

The studies of intravenous rtPA underscore the major contributors to the risk of intracerebral hemorrhage in ischemic stroke. These include excessive time from symptom onset to treatment, low body mass, diastolic hypertension, older age, and the use of rtPA.[9,231,232] The appearance of "early signs of ischemia," marked by low attenuation and/or sulcal effacement on the initial CT scan, is associated with an increased risk of death and hemorrhage.[74,232–235]

Direct Local Infusion of Plasminogen Activators

Angiography-controlled intraarterial infusion allows delivery of higher local concentrations of PAs to the thrombus, where local flow is stagnant, and definition of both vascular anatomy and recanalization outcome. Refinements of flow-directed and guidewire-directed catheter techniques pioneered by Zeumer et al. and catheter systems for the acute delivery of thrombolytic agents in the cerebral circulation have broadened the experience with local delivery,[236] although the approach remains experimental (FIGURE 93.6). Among larger series, recanalization of symptomatic carotid artery territory occlusions has approached 46% to 90% of symptomatic patients treated with intraarterial infusion of SK or uPA within 8 hours.[237–239] Detectable hemorrhagic transformation in the ischemia territory occurred in 18% to 33% of treated patients (Table 93.6).[237–239]

Vertebrobasilar artery territory ischemia can produce considerable disability.[240,241] Nenci et al.[242] first demonstrated benefits after local intraarterial treatment of symptomatic basilar thrombosis. One retrospective comparison of the clinical outcome in 43 patients who received intraarterial uPA or SK with 22 patients who received conventional therapy (i.e., heparin) suggested considerable survival benefit in those demonstrating recanalization (who received the fibrinolytic agent).[243] Subsequent reports have supported recanalization efficacy with these agents.[236,239,244] However, significant hemorrhagic transformation also occurs in this setting.

The Prourokinase in Acute Cerebral Thromboembolism (PROACT) study was the first randomized double-blinded placebo-controlled examination of PAs by intraarterial infusion to demonstrate benefit.[7] In that phase II study, direct arterial infusion of recombinant single chain urokinase (rscuPA, recombinant prourokinase [rproUK]; dose of 6 mg) was compared with placebo for recanalization of M1 and M2 MCA occlusions and safety outcomes.[7] Overall, rscuPA produced a significant increase in MCA recanalization and in hemorrhagic transformation, which were heparin dependent. The follow-on open study, PROACT-2, randomized patients with symptomatic proximal MCA occlusions to recombinant scuPA (9 mg) or to placebo for recanalization and for 3-month disability outcome.[245] Both groups also received heparin. Recanalization was significantly

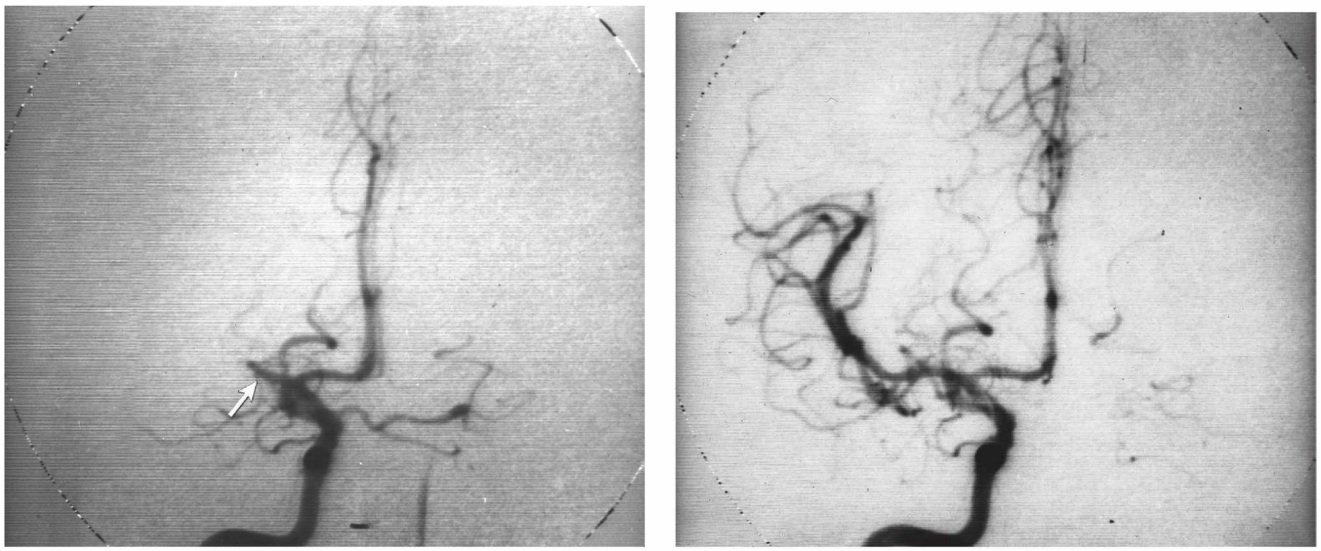

FIGURE 93.6 Recanalization of proximal middle cerebral artery (MCA) occlusion. **A**: Obstruction of the proximal MCA (M1 segment). *Arrow* indicates the site of thrombotic occlusion of the M1 segment. **B**: Relief of flow obstruction by direct intraarterial urokinase infusion, with return of downstream arterial patency (Angiograms courtesy of H. Zeumer).

greater with recombinant scuPA (65.7%) than no intervention (18.0%), as was the frequency of symptomatic intracerebral hemorrhage. Whereas the proportion of patients with no or minimal disability (mRS = 0 to 1) was not significantly different between the two groups, rscuPA was associated with an improvement in outcome measured as mRS = 0 to 2. No further exploration of rscuPA has ensued, and it is not available for clinical use.

Table 93.6

Acute stroke: plasminogen activators

Study[a]	Agent	Patients (n)	Δ(T – 0)[b] (h)	Recanalization (%)	Hemorrhage (%)
Carotid Territory: Intraarterial Delivery					
del Zoppo et al.[238]	SK/uPA	20	1–24	90.0	20.0
Mori et al.[237]	uPA	22	0.82–7	45.5	18.2
Matsumoto and Satoh[239]	uPA	40	1–24	60.0	32.5
PROACT[7]	scuPA/h	26	<6.0	57.7	42.3
	C/h	14	<6.0	14.3	7.1
PROACT-2[245]	scuPA/h	121	<6.0	65.7	10.2
	—/h (iv)	59	<6.0	18.0	1.9
Carotid Territory: Intravenous Delivery					
Yamaguchi[220]	rtPA	52	<6.0	38.5	28.6
von Kummer et al.[221]	rtPA	22	<6.0	59.1	36.4
del Zoppo et al.[9]	rtPA	93 (104)[c]	<8.0	34.4[c]	30.8
Mori et al.[219]	rtPA	19	<6.0	47.4	52.6
	C	12		16.7	41.7
Yamaguchi[376]	rtPA	47 (51)[c]	<6.0	21.3	47.1
	C	46 (47)[c]		4.4	46.8

[a]Studies employing angiography.
[b]Time from symptom onset to treatment.
[c]( ) refers to the total number of patients treated in intention-to-treat.
C, control; h, heparin; rtPA, recombinant tissue-type plasminogen activator; scuPA, single-chain urokinase plasminogen activator; SK, streptokinase; PROACT, Prourokinase in Acute Cerebral Thromboembolism.

Defibrinogenating Agents

Fibrinogen contributes to thrombus formation and to plasma viscosity. With reduction in plasma fibrinogen comes decreased viscosity, increased flow, and, potentially, limitation of thrombus formation. Reduction of fibrinogen to 100 mg/dL by the defibrinogenating agent ancrod has been considered to be safe in patients with ischemic stroke.[246,247] According to prespecified covariate-adjusted analyses (taking into account baseline stroke severity), favorable outcome was more frequent in the ancrod-treated group (42.2%) than in the placebo-treated group (34.4%) in the Stroke Treatment with Ancrod Trial (STAT).[247,248] Intracranial hemorrhage was moderately more frequent in the ancrod group. In the European Stroke Treatment with Ancrod Trial, no advantage to ancrod was observed.[249] However, differences in study design and patient recruitment with STAT were observed.[249] The potential benefit of ancrod for the acute treatment of ischemic stroke was resolved by the test of the hypothesis that rapid defibrinogenation in patients within 6 hours of stroke onset could improve efficacy and safety. No difference in the efficacy outcome (positive responder status) was observed in the combination of two studies of 500 patients randomized to multiple-day dosing with ancrod or placebo.[250] Treatment levels intended for fibrinogen were reached in >90% of the ancrod patients, however. No further work with this agent has been undertaken.

Mechanical Interventional Approaches

Coincident with the evolution of directed delivery of the PAs SK, uPA, rscuPA, and rtPA, mechanical devices designed to capture and retrieve thrombi or reestablish flow in the cerebral arterial circuit have been devised. Apparent advantages of some of these devices are the rapid achievement of recanalization and application to refractory proximal thrombi, such as carotid "T" occlusions.[251] No studies to date have demonstrated superiority or equivalence of such devices to current acute applications of the antithrombotic agents in use.

Augmented Fibrinolysis. Strategies that disrupt thrombus integrity can increase arterial perfusion. For instance, passage of the guidewire through the occlusive thrombus increases the rapidity of thrombus lysis. PROACT prohibited penetration of the thrombus with guidewire or catheter.[7] More recently, both *endovascular ultrasound* techniques, based upon limited bench studies, and the direct application of 2 MHz diagnostic TCD, developed in the clinical setting, have been shown to enhance thrombus lysis initiated by PAs.[252,253]

Angioplasty and Stenting. Angioplasty and stenting have been undertaken in select patients presenting with occlusions caused by atherosclerotic plaques. While reestablishment of flow is possible with angioplasty to 84% of patients,[254] significant generation of thromboemboli with downstream ischemia occurs. Stent placement both in the carotid artery and in downstream self-expanding stents have been promoted as less traumatic with relatively low associated symptomatic hemorrhage frequencies (~5%).[255,256] However, recently in a prospective study, placement of self-expanding nitinol stents in the carotid artery was no more efficacious than carotid endarterectomy in patients presenting with TIAs and was associated with an increased incidence of stroke.[257]

Suction Thrombectomy. Devices employing vacuum aspiration can generate significant recanalization. In a multicenter, single-arm cohort trial, the Penumbra System yielded minimal or better recanalization (Thrombolysis in Myocardial Infarction [TIMI] 2 or 3) in 81.6% of 125 target vessels.[258,259] Symptomatic intracranial hemorrhage occurred in 11.2% of patients. Functional independence was not significantly more frequent among patients in whom recanalization was achieved. Overall, similar devices can decrease the risk of uncontrolled thrombus fragmentation and downstream embolization.

Endovascular Retrieval Devices. Thrombus retrieval devices have evolved out of devices for removing coils and include the Merci Retrievers, helical nitinol coils that ensnare the thrombus. Moderate or complete recanalization by use of the device alone was achieved in 48% in one study.[260] Successful recanalization was associated with improved clinical outcome (90-day mRS 0 to 2 in 53% recanalization vs. 6% nonrecanalization, $P < 0.0001$). Symptomatic hemorrhage occurred in 5% of patients treated with the device alone and 24% treated with the device and an additional intervention (e.g., local fibrinolysis) due to incomplete recanalization response to the device. In a subsequent trial employing a later generation device, among 164 patients enrolled, moderate or complete recanalization was achieved in 57% by use of the device alone and in 70% with adjunctive approaches (intraarterial thrombolysis or angioplasty).[261] Technical advantages of using retrievable stents have been recently described.[262,263] Several multicenter trials comparing stent retrievers with earlier generation devices are underway.

For practical reasons, at some centers, patients may receive intravenous rtPA and subsequently be treated with an endovascular procedure to remove residual obstructing material. There is no apparent advantage in safety.[264,265] Such approaches must be considered experimental. All devices should be subject to controlled trials.

CAROTID ARTERY ATHEROTHROMBOTIC DISEASE

Atherosclerosis is the most prevalent arterial thrombophilic state. The carotid artery bifurcation and other large-artery branch points are predilection sites for atheroma formation and are sources of cholesterol- and thrombus-containing emboli to the cerebral hemispheres (see FIGURE 93.2). Endarterectomy of the extracranial portion of the carotid artery can resolve the local vascular flow abnormalities, but cerebral ischemic symptoms can recur.

Medical versus Surgical Intervention. TIAs and minor stroke are *symptomatic* products of carotid artery atherosclerosis. Although a major source of thromboemboli, 20% to 45% of strokes are unrelated to carotid artery stenosis (e.g., lacunes).[266] The North American Symptomatic Carotid Endarterectomy Trial and the MRC-sponsored European Carotid Surgery Trial demonstrated significant survival benefit and symptomatic relief from endarterectomy over medical (i.e., antiplatelet agent) therapy for carotid stenoses of 70% to 99%.[267,268] The benefits in reduction of ipsilateral stroke also accrue for endarterectomy of symptomatic stenosis of intermediate extent (50% to 69%), but depend on the balance of surgical risk.[269]

The surgical management of *asymptomatic* carotid artery stenosis has been controversial. The multicenter comparison of carotid endarterectomy with optimal medical management (including antiplatelet agents [ASA]) in 444 men with asymptomatic carotid artery stenosis by the Veterans Affairs Cooperative Study Group demonstrated a statistically significant

reduction in combined ipsilateral ischemic neurologic events in the surgical group (8.0% vs. 20.6%, $P < 0.001$).[270] The frequency of ipsilateral stroke was 4.7% in the surgical group and 9.4% in the medical group; however, there was no difference in stroke and stroke-related mortality at 30 days. Asymptomatic Carotid Atherosclerosis Study (ACAS) prompted examination of local single-center experiences with surgical treatment of asymptomatic carotid lesions and a meta-analyses.[271–273] Although benefit could be reproduced, postoperative mortality was a major variable.[273] Overall, experience has indicated that benefit from carotid endarterectomy for asymptomatic lesions is modest at best, with no difference between medical management or surgery for perioperative mortality or stroke, although selected patients may benefit.[273] Hence, carotid endarterectomy would be expected to benefit a select subset of asymptomatic patients and would depend upon the frequency of surgical complications.

Antiplatelet agents are adjuncts to carotid endarterectomy that lower the incidence of embolic stroke and death or decrease the incidence of carotid restenosis after carotid endarterectomy. In one prospective double-blind, placebo-controlled trial, ASA (1,300 mg/day), given within 5 days of carotid endarterectomy, reduced the incidence of stroke or death at 6 months.[274] In a separate trial, ASA (50 to 100 mg/day) started 1 to 12 weeks after carotid endarterectomy had no appreciable effect on strokes, MI, or vascular death.[275] Following a retrospective analysis of experience with ASA (1,500 mg/day),[276] a prospective comparison of presurgical ASA (1,000 mg/day) or no treatment was undertaken.[277] Survival was extended in the ASA-treated group, although cerebral events occurred equally in both. Subsequently, a prospective comparison of four doses of ASA after endarterectomy (81, 325, 650, or 1,300 mg/day) concluded that the lower dose tiers (81 and 325 mg/day) were superior in averting stroke, MI, and death within the next 3 months.[278]

Current practice is to continue patients undergoing endarterectomy on ASA (81 to 325 mg/day) after the procedure. There is little information regarding a role for anticoagulants after endarterectomy, and fibrinolytic agents are contraindicated.

Carotid Stenting/Endarterectomy. Carotid stenting has emerged as a viable alternative revascularization procedure to carotid endarterectomy on the basis of controlled trials.[257,279] The appropriate antithrombotic regimen after stent placement in the carotid system has not been defined by those trials.

CEREBRAL EMBOLISM FROM A CARDIAC SOURCE

Emboli from cardiac injury sites can cause focal cerebral ischemia. These sources include mural thrombi associated with left ventricular dyskinesia (e.g., after MI), prosthetic valves, rheumatic valvular vegetations, and thromboemboli arising during AF. Because of the thrombotic nature of most cardioembolic events, a role for anticoagulation has been suggested in both primary and secondary prevention.

Cardioembolic Stroke Following Myocardial Infarction

The incidence of systemic thromboembolism, including stroke, after MI varies from 1% to 3% per year, but may be as high as 3.7% during the first month after an MI (i.e., the acute phase).[280–285] Antiplatelet agents could be effective in decreasing the incidence of post-MI cerebral events. However, evidence continues to be scant. The CAPRIE study suggested that clopidogrel could reduce the combined events of second MI, stroke, or peripheral artery disease following an initial MI, although the benefit for MI was not significant.[190]

Anticoagulation during the acute in-hospital and in the chronic post-hospitalization phases reduces the number of cerebrovascular events.[280–286] Secondary prevention trials designed to determine the incidence of recurrent MI as the main outcome event have also examined the effect of long-term anticoagulation on stroke incidence. Level II trials suggested a trend in favor of decreased post-MI stroke incidence. In the Veterans Administration Cooperative Study, however, there was no reduction in stroke incidence 1 to 5 years after MI for patients randomized to long-term anticoagulation within 21 days of their MI.[280] The trend favoring anticoagulation for stroke was mirrored by an overall reduction in the combined outcomes of stroke, second MI, and death. A similar trend was seen in two subsequent trials and appeared independent of the type of oral anticoagulant.[282,283] The Sixty Plus Reinfarction Study randomized MI patients more than 60 years to oral anticoagulation or placebo.[284] At 2-year follow-up, anticoagulation was associated with a trend toward a lower stroke incidence.

Those studies also demonstrated an increased incidence of serious hemorrhages, which contributed to morbidity or death in the anticoagulated group.[280,282–284] In German-Austrian Aspirin Trial (GAAT) and the Sixty Plus Reinfarction studies, the number of serious anticoagulation-associated hemorrhages, including intracerebral events, was considered excessive.[283,284] In the latter, seven intracranial hemorrhages occurred in the anticoagulated group, compared to one in the placebo group, although fatal intracranial events were equal. Approximately 72% of the anticoagulated patients had an INR of 2.7 to 4.5.[284]

Two level I placebo-controlled trials demonstrated a significant reduction in stroke incidence with anticoagulation.[281,285] The Warfarin Reinfarction Study (WARIS) also reported a significant decrease in the incidence of cerebrovascular events in patients who received warfarin.[285] In both trials, hemorrhage was more common in the active treatment group. In WARIS, the target INR was 2.8 to 4.8, considered high by current measures. The follow-on randomized three-armed open trial (WARIS-II) of warfarin (INR = 2.8 to 4.2), warfarin and ASA (75 mg/day), or ASA (160 mg/day) alone in patients with MI demonstrated that the combination warfarin/ASA was superior to the other regimens for the combined outcomes of death, nonfatal reinfarction, and cerebral stroke.[287,288] While the incidence of hemorrhage was greatest in the warfarin/ASA cohort at study close, the incidence of occult hemorrhage at 3 months and 4 years was not different from normal in all three cohorts, suggesting overall benefit with INR = 2.0 to 3.0 in patients with a low or intermediate risk of hemorrhage.[288,289]

Three trials of anticoagulation during the acute in-hospital phase of MI demonstrated a decreased incidence of stroke.[290–292] In a single-blind test which randomized patients to heparin/warfarin or placebo post-MI, anticoagulation was associated with a significant decrease in strokes.[291] A second study demonstrated a trend in favor of heparin and oral anticoagulation.[290] Both studies, however, demonstrated no change in the combined outcomes of stroke, second MI, and death. A separate level I MRC-sponsored trial randomized patients to heparin/adjusted high-dose phenindione or heparin/fixed low-dose phenindione for 28 days within

Table 93.7

AF (nonvalvular): antiplatelet agents/anticoagulants

Study	Agent	Dose (per Day)	Patients (n)	Stroke (n)	Mortality (n)
AFASAK[296]	Warfarin	INR = 2.8–4.2	335	5	—
	ASA	75 mg	336	17(3)[a]	—
	Placebo	—	336	19(2)[a]	—
SPAF[297]	1. Warfarin/ASA	INR = 2.0–3.5/325 mg	393	7	14
	Placebo	—	195	17(1)[a]	8
	2. ASA	325 mg	517	18(1)[a]	31
	Placebo	—	528	34(4)[a]	39
BAATAF[298]	Warfarin	INR = 1.5–2.7	212	2	11
	Placebo	—	208	—	26
CAFA[299]	Warfarin	INR = 2.0–3.0	187	4(1)[a]	7
	Placebo	—	191	9(2)[a]	6
SPAF-II[300]	Warfarin[b]	INR = 2.0–4.5	358	13(1)[a]	36
	ASA	325 mg	357	19(2)[a]	41
	Warfarin[c]	INR = 2.0–4.5	197	13(1)[a]	26
	ASA	325 mg	188	18(0)[a]	24
SPAF-III[301]	Warfarin	INR = 2.0–3.0	523	11(0)[a]	35
	Warfarin	INR = 1.2–1.5	521	43(1)[a]	42
RE-LY[314]	Dabigatran (150 mg)		6,076	111	438
	Warfarin	TTR = 64.0%	6,022	142	487
ROCKET-AF[315]	Rivaroxaban		7,081	149	208
	Warfarin	TTR = 55.0%	7,090	161	250
ARISTOTLE[318]	Apixaban		9,120	199	603
	Warfarin	TTR = 62.2%	9,080	250	669

ASA, aspirin; INR, international normalized ratio; AFASAK, Atrial Fibrillation, Aspirin, Anticoagulation; SPAF, Stroke Prevention in Atrial Fibrillation; BAATAF, Boston Area Anticoagulation Trial for Atrial Fibrillation; CAFA, Canadian Atrial Fibrillation Anticoagulation.
[a]Numbers in parentheses indicate systemic embolic events.
[b]Patients ≤ 75 y.
[c]Patients >75 y.
[d]TTR = time in therapeutic range (INR = 2.0–3.0).

14 days of acute MI. A significant decrease in the incidence of systemic embolism with the high-dose group was seen, although there was no difference in recurrent MI or death.[292] Both the incidence and severity of hemorrhagic complications were quite low. This experience suggests that cerebrovascular ischemic events during the early post-MI period can be reduced with anticoagulation.

In summary, long-term anticoagulation can decrease the number of cerebrovascular events after recovery from MI. In practice, however, concerns about the higher incidence of serious hemorrhagic events, including intracerebral hemorrhage, have limited routine use of anticoagulants.

Cardioembolic Stroke in Atrial Fibrillation

The pathogenesis of stroke and the role of antithrombotic regimens in individuals with nonrheumatic AF have been reviewed.[293] In a retrospective study, Sage and Van Uitert[294] indicated that the risk of cerebral infarction after an episode of nonvalvular AF can be 20% per year, with a mortality rate from the initial infarct of 38% per year. This is in accordance with the experience of Hart et al.,[295] who reported a 34.8% incidence of symptomatic embolism (13% cerebral) in individuals not undergoing immediate anticoagulation. Furthermore, the population incidence of nonvalvular AF is increasing with the age. These, and other data, have suggested the need for prospective clinical trials (Table 93.7).

Primary Prevention

Vitamin K Antagonists (VKAs). The AFASAK (Atrial Fibrillation, Aspirin, Anticoagulation) study, an open randomized trial of warfarin, ASA (75 mg/day), or placebo in nonrheumatic, nonvalvular AF, demonstrated a significant decrease in cerebral embolic events in the warfarin group at the 2-year follow-up.[296] But, hemorrhagic complications were significantly more common in the warfarin group.[297,298] The Stroke Prevention in Atrial Fibrillation (SPAF) study randomized patients with AF to warfarin, ASA, or placebo (group 1) if they were eligible for warfarin, or to ASA or placebo (group 2, double-blind) if they were not warfarin eligible.[297] SPAF-I was terminated when the warfarin and ASA arms of group 1 demonstrated a statistically significant combined 81% risk reduction in ischemic stroke and

systemic embolism. The relative benefit of warfarin over ASA was not reported, but 10.9% of patients randomly assigned to warfarin were withdrawn because of drug intolerance. The Boston Area Anticoagulation Trial for Atrial Fibrillation (BAATAF), an open randomized study of long-term, low-dose warfarin or no treatment, demonstrated a significant 86% risk reduction for stroke and death in favor of the warfarin group.[298] No differences in fatal hemorrhages were seen between the warfarin and no-therapy groups. The Canadian Atrial Fibrillation Anticoagulation (CAFA) Study was terminated early based on the results of those two studies, with a 44.8% risk reduction in ischemic stroke and systemic thromboembolism favoring warfarin.[299]

Concerned that the risk of intracranial hemorrhage could be associated with warfarin-based approaches using ASA[300] or low-dose oral anticoagulation,[301] the SPAF-II trial examined the relative efficacies of warfarin and ASA in individuals with nonvalvular AF.[300] That study prospectively stratified patients into two age cohorts: ≥75 years and those <75 years. A modest, but not statistically significant, reduction in ischemic stroke events was associated with warfarin over ASA. There was a significantly greater frequency of major hemorrhagic events with warfarin in the ≥75-year cohort than in the <75-year cohort, however. The low overall annual thromboembolic event rate was a concern. Nonsignificant differences between the ASA treatment and control arms of the AFASAK study, the European Atrial Fibrillation Trial (EAFT), and ESPS-2 were observed in patients with nonvalvular AF.[175,296,302] ASA was associated with a significant 21% RRR in annual stroke events in an individual patient combined analysis of AFASAK, EAFT, and SPAF-I,[303] a conclusion supported by a broader meta-analysis.[304] Heterogeneity in the results could not be excluded.[305]

Age >80 years, intensity of anticoagulation, and prolonged prothrombin time from the therapeutic range are all contributors to an increased risk of hemorrhage.[306] The SPAF-III trial addressed the intensity of anticoagulation.[301] Patients were randomized either to adjusted-dose warfarin to maintain the INR between 2.0 and 3.0, or to low-intensity warfarin with ASA (325 mg/day) to maintain the INR between 1.2 and 1.5. That study was terminated when the annual disabling stroke rate of the combination therapy exceeded that of adjusted-dose warfarin (5.6% vs. 1.7%, respectively). When cumulative event rates were calculated, a nearly significant difference ($P = 0.07$) favoring warfarin seemed to support the results of previous studies (see Table 93.7). The results of SPAF-III led to the early termination of the AFASAK-2 study of patients with moderate risk.[307,308] A review of published data to 2001 indicated that anticoagulation is superior to antiplatelet agents for the reduction of nonfatal stroke risk in patients with AF.[309] Evaluation of the quality of anticoagulation for nonvalvular AF demonstrates that the ischemic stroke difference was significantly less among patients with INR ≥ 2.0 compared to INR < 2.0.[310] Overall, adjusted-dose warfarin to a trough INR of 2.5 is superior to antiplatelet agents or no treatment and is the current accepted management approach for nonvalvular AF.

To emphasize this observation, and despite the accepted superiority of warfarin anticoagulation over ASA, the Atrial Fibrillation Clopidogrel Trial with Irbesartan for prevention of Vascular Events (ACTIVE) was undertaken to compare the combination clopidogrel/ASA with warfarin (ACTIVE-W) and with ASA alone (ACTIVE-A) for the prevention of vascular events in stroke patients with a high risk for AF. The ACTIVE-W portion of the study complex was terminated before the intended completion of the study when it was shown that oral anticoagulation (INR = 2.0 to 3.0) was superior to ASA (75 to 100 mg/day)/clopidogrel (75 mg/day) displaying annual combined risks of stroke, embolism, MI, or vascular death of 3.93% and 5.60%, respectively (RR 1.44 [1.18% to 1.76%], $P = 0.0003$).[311]

Nonvitamin K Antagonists. Oral alternatives to warfarin have been attractive, conceivably intended to be given without monitoring. Concern about the ability to sustain target levels of anticoagulation with warfarin in a number of settings and the infrastructure needed to monitor this has stimulated the search for agents that affect coagulation system differently. One direct thrombin inhibitor that could be administered orally, ximelagatran, was found to be not inferior (±2.5%) to warfarin in preventing systemic embolic events in patients with nonvalvular AF.[312,313] In the Stroke Prevention Using Oral Thrombi Inhibitor in Atrial Fibrillation (SPORTIF) III trial, warfarin generated a nonsignificant decrease in stroke incidence compared to ximelagatran.[312,313] Because of hepatic toxicity, the development of ximelagatran was stopped and the agent is not available. The parenteral factor Xa inhibitors argatroban and bivalirudin are not used for nonvalvular AF.

However, potential alternatives to warfarin in primary prevention of thromboembolic stroke in patients with nonvalvular AF have been tested recently. The Randomized Evaluation of Long-term Anticoagulation Therapy (RE-LY) trial demonstrated that dabigatran, an orally delivered direct thrombin inhibitor, was not inferior to warfarin, and dabigatran at 150 mg twice per day is indeed superior for the combined primary outcomes of stroke or systemic embolism.[314] It is now indicated for nonvalvular AF. The results of the Rivaroxaban Once Daily Oral Direct Factor Xa Inhibition Compared with Vitamin K Antagonism for Prevention of Stroke and Embolism Trial in Atrial Fibrillation (ROCKET-AF) trial have been reported.[315] The ROCKET-AF trial compared rivaroxaban, an oral direct factor Xa inhibitor, with warfarin in a double-dummy placebo-controlled format, demonstrating that rivaroxaban was not inferior to warfarin in the composite efficacy outcome of stroke (ischemic and hemorrhagic) and systemic embolism.[315,316] The novel antifactor Xa inhibitor apixaban has been shown to be superior to ASA (81 to 324 mg/day) in patients with nonvalvular AF in a prospective randomized study (AVERROES).[317] In a recently completed prospective randomized trial, Apixaban for Reduction of Stroke and Other Thromboembolic Events in Atrial Fibrillation (ARISTOTLE), the antifactor Xa inhibitor was found superior to warfarin in the reduction of stroke and systemic embolism.[318]

Interestingly, while the frequency of major hemorrhagic events in RE-LY, ROCKET-AF, and ARISTOTLE was not different between the test agent and warfarin, these studies demonstrated a decrease in the frequency of intracranial hemorrhage (hemorrhagic stroke). Although the reasons for the observed reduction in intracranial hemorrhage by these antithrombotic agents are not clear, it has been suggested that neither rivaroxaban nor dabigatran affects the tissue factor:factor VIIa complex, whereas warfarin decreases factor VIIa.[319] Rosenberg and Aird[320] suggested that the cerebrovasculature has unique hemostatic features unlike other vascular beds, although these features have not been described. Fundamental studies are required to further delineate the nature of the hemostatic responses in the central nervous system that the use of these new antithrombotic agents

has uncovered. This is a potentially exciting area of research. At this writing, dabigatran and rivaroxaban are licensed for primary prevention of stroke in nonvalvular AF patients.

Secondary Prevention

Information from secondary prevention trials involving patients with AF who present with a signal stroke is limited. In an open trial of anticoagulation in patients with stroke and nonvalvular AF, Lodder et al.[321] found no difference in the incidence of second stroke or death at a mean follow-up of 2.25 years. Death from serious hemorrhage occurred in 6 of the 70 anticoagulated patients (18.6%). The Cerebral Embolism Study Group performed a randomized, open trial of immediate versus delayed anticoagulation patients with AF and cardioembolic strokes for the principal outcome event of recurrent embolism.[164] Anticoagulation was initiated immediately after a signal stroke or 10 days after the first stroke. In that small series, two patients in the delayed treatment group had second embolic events. This limited experience suggests caution in the use of anticoagulants in secondary prevention of recurrent cerebral embolism in AF. More recently, data from ROCKET-AF indicate that rivaroxaban can produce modest, but significant reduction in embolic events in secondary prevention, suggesting that other oral non-VKA agents may have this activity.[315]

In summary, the results of randomized, controlled trials of individuals with nonvalvular AF support the use of oral anticoagulation to reduce the incidence of embolic stroke.[322] For patients with a high risk to AF (with a prior TIA/stroke or systemic embolus, history of hypertension, poor left ventricular function, age >75 years, rheumatic mitral valve disease, or a prosthetic valve), the current recommended therapy is adjusted-dose warfarin anticoagulation at a target INR of 2.5 (range 2.0 to 3.0) rather than ASA.[323] New oral non-VKA agents have been found to be noninferior to warfarin, and, in the case of dabigatran and apixaban, are superior for efficacy. All such agents appear to have a lower incidence of intracerebral hemorrhage.

Stroke in Low Cardiac Ejection Disease

Patients with partly compensated left ventricular function and low cardiac output are at increased risk of ischemic stroke. Congestive heart failure is accompanied by increased prevalence of AF, which ranges from 0% to 42%.[324] Antithrombotic treatment options are uncertain. The Warfarin-Aspirin in reduced Cardiac Ejection Fraction study is a prospective double-blind double-dummy superiority trial comparing ASA with warfarin to decrease the incidence of ischemic stroke in the setting of CHF.[324] This trial is nearing completion.

VALVULAR CARDIOVASCULAR DISEASE

Rheumatic valvular disease, mechanical or xenograft prosthetic cardiac valves, and calcified mitral annuli generate thromboemboli. Clinically detectable systemic embolism from untreated rheumatic mitral stenosis and mitral insufficiency occurs with a 3.7% and 1.9% yearly incidence, respectively. Recurrent embolism occurs frequently within 6 to 12 months after the signal embolism.[325–327] The risk of cerebral embolism is greatly increased by concurrent AF.[326,328,329] And, the incidence of systemic embolism is greater for mechanical devices than for xenograft prostheses. Adjunctive antithrombotic strategies offer protection against thromboembolic complications of valve prostheses.[328,329] But, the use of xenograft prostheses has significantly altered the antithrombotic approach to prophylaxis.

Mechanical Prosthetic Cardiac Valves

The incidence of cardiac source thromboembolic events depends upon the type of mechanical valves, their location, and the age of the patient. The rate of systemic embolic events (per year) appears greatest with caged disk or ball valves (2.5%), in excess of tilting disk (0.7%) or bileaflet (0.5%) valves.[330] Generally, thromboembolic risk is higher for mechanical valves in the mitral position than in the aortic position,[331] perhaps in part because of the association of AF.[332,333] Finally, the incidence of systemic thromboembolism is greatest in the elderly (≥70 years).[330]

The use of oral anticoagulants has generally reduced the incidence of embolic events. Although individual series varies, and few prospective controlled studies have been undertaken, the higher incidence of emboli with caged devices suggests the need for increases of anticoagulation intensity. In one study of complications with bileaflet aortic valves, a greater reduction in thromboembolic rate was seen with an INR of 2.5 to 3.5.[334] In one multiseries review, this range of anticoagulation appeared efficacious.[335] For valves in the aortic position (bileaflet and tilting variety), anticoagulation to a target INR of 2.5 (range 2.0 to 3.0) is recommended, and for valves in the mitral position the target INR of 3.0 (range 2.5 to 3.5) is recommended.[331]

The addition of antiplatelet agents can increase the protection afforded by oral anticoagulation, but may increase the risk of hemorrhagic complications. Two level I studies demonstrated reduced thromboembolic risk in patients receiving combination anticoagulation with ASA over anticoagulation alone.[336,337] The rate of systemic thromboembolic or vascular death was significantly reduced by addition of ASA (100 mg/day) to oral anticoagulation at INR 3.0 to 4.5.[338,339] ASA doses of 500 to 1,000 mg/day reduced the number of cerebral embolic and systemic embolic events considerably, although the hemorrhagic risk was dose dependent.[337] In view of the gastrointestinal side effects of ASA-containing regimens, addition of dipyridamole to oral anticoagulation has been successfully tested in patients with mechanical prosthetic cardiac valves.[329] Whereas those studies suggested no increase in hemorrhage, the general experience supported an increase in major hemorrhage with this regimen.

Dipyridamole (400 mg/day) with warfarin considerably reduced thromboembolism or death compared to warfarin and placebo.[340,341] The combination of dipyridamole with oral anticoagulation (INR 2.0 to 2.5) was also associated with a low thromboembolic rate in a separate study (without a control arm).[342] In an open study of patients with mechanical prostheses, dipyridamole (400 mg/day) and warfarin were superior to warfarin and ASA (500 mg/day),[343] and major hemorrhagic complications were considerably fewer with the addition of dipyridamole.

The possibility that low-dose ASA might also further reduce embolic events in patients with heart valve replacement already managed with warfarin was tested by Turpie et al.[344] In an open prospective trial, patients randomized to 100 mg/day ASA or 650 mg/day ASA (both receiving warfarin adjusted to a target INR of 2.0 to 3.0) showed no statistically

significant difference with respect to systemic embolism, vascular death, or total death. However, the rate of hemorrhagic events in the high-dose ASA group (13.4 vs. 7.9 events per 100 patient-years) significantly exceeded that of the low-dose group. On this basis, low-dose ASA is often added to warfarin. However, meta-analysis of trials combining dipyridamole with oral anticoagulation indicated a decrease in fatal and nonfatal thromboemboli.[345,346]

A vexing issue is how to anticoagulate women with mechanical valves during pregnancy. One recent limited series indicated success with enoxaparin as treatment for varying periods during pregnancy when antifactor Xa levels were maintained at 1.0 to 1 U/mL.[347]

Xenograft Cardiac Valves

Systemic thromboembolic events occurred in 4% of patients with bioprostheses at 3 years who are in sinus rhythm and in 16% of patients with AF.[348,349] The greatest risk of thromboembolism occurred during the first 3 months after placement of the bioprosthesis.[350,351] A lower frequency of major embolic events was seen in patients with porcine biosynthetic valves receiving anticoagulation for 6 to 12 weeks in one study.[349] There was no difference in incidence of systemic emboli in patients with xenograft prostheses randomized to oral anticoagulation at an INR of 2.0 to 2.25 or at an INR of 2.5 to 4.0 (standard anticoagulation) for the first 3 postoperative months, although, clinically significant hemorrhagic events were more frequent in the group receiving standard anticoagulation.[352]

The benefit of antiplatelet agents has been evaluated in several level III studies.[353–355] Those studies implied that warfarin or ASA (1,000 or 500 mg/day) produced similar risk reduction in patients in sinus rhythm. Patients with mitral valve bioprostheses who received 500 mg ASA every 2 days were less likely to have embolic events than those who received 1,000 mg/day.[355] However, this conclusion has not been rigorously tested. Accepted clinical practice requires anticoagulation for the initial 3 months following placement of the bioprosthesis.

THROMBOTIC EVENTS ASSOCIATED WITH CENTRAL NERVOUS SYSTEM INJURY

Deep Venous Thrombosis

DVT can complicate the early course of recovery from stroke.[356] This is attributed to immobility of the affected limb or prolonged recumbency, although, contributions of the activated coagulation state is also likely. Heparinoids and LMWHs have been tested as alternatives to prophylactic UFH. Early experience with prophylactic use of two LMWH preparations, however, was mixed.[357–359] Twice-daily subcutaneous treatment with one preparation was associated with considerably reduced frequency of DVT.[359] Turpie et al.[360] demonstrated an advantage of twice-daily subcutaneous administration of a low molecular weight heparinoid over subcutaneous heparin in decreasing the incidence of DVT. In the TOAST trial, danaparoid produced a considerable reduction in the frequency of poststroke DVT, compared to placebo.[176,177,361–363] A recent review of published data and guidelines points out the concerns of conflicting information.[364] Prophylactic use of anticoagulants to decrease venous thromboembolism is reasonable in patients having suffered recent stroke, but the use must be tempered with an increased risk of hemorrhage. Patients suffering an intracerebral hemorrhage appear at particular risk of DVT.[365] Here, too, the recommendation for the use of heparin or LMWHs is tempered with caution and should not be instituted before the second day after ictus.[149,150]

Sagittal Sinus Thrombosis

Cortical vein thrombosis can accompany local inflammation, thrombophilic states (including anticardiolipin or antiphospholipid antibodies), and pregnancy, or it can be idiopathic. Although thrombolytic agents have been employed unusually, anticoagulation is the treatment of choice. Recent epidemiologic report underscores the relative lower morbidity of this condition compared to ischemic stroke.[366] Petechial hemorrhage is a common finding with sagittal sinus thrombosis.

Retinal Vascular Thrombosis

Retinal arterial occlusions are commonly related to carotid artery stenosis.[367] In one series, approximately 40% of patients with retinal vein thrombosis had evidence of circulating anticardiolipin antibodies.[368] Fibrinolytic agents have been used successfully in patients with retinal artery and retinal vein occlusion.[369,370] Partial recovery of form vision was possible in some patients with acute retinal artery occlusion.[371,372] Antiplatelet agents or anticoagulants have been used in patients with cerebral retinal thrombosis before neovascularization.[373] To date, these approaches have not been tested prospectively.

CONCLUSIONS

Atherothrombosis and thromboembolism that lead to acute cerebrovascular disease are accompanied by activation of the coagulation system and of platelets. Antithrombotic agents can preserve or reconstitute vascular patency and thereby minimize permanent injury from focal cerebral ischemia. Both patient selection and the subtype of stroke are relevant to benefit from treatment.

PAs can increase the probability of minimal disability or no abnormality in selected patients with acute cerebral ischemia. Intravenous infusion of rtPA has displayed this activity. Recanalization of symptomatic, documented carotid territory occlusions and vertebrobasilar arterial occlusions has been achieved by local intraarterial PA delivery. Recent studies have underscored the need for careful individual selection, because fatal hemorrhage is a consistent risk.

Antiplatelet agents can reduce the risk of the combined outcomes of subsequent stroke, MI, and mortality in individuals with a history of recent TIAs. For secondary prevention of recurrent stroke in patients presenting with a stable, recent focal cerebral deficit, ASA/ER-DP produces additional benefits over ASA. Thienopyridines have been associated with risk reduction in second ischemic events. When TIAs are associated with carotid artery stenosis of 70% to 99%, carotid endarterectomy and ASA are superior to ASA alone in preventing subsequent stroke and stroke-related mortality.

Patients with AF without a valvular abnormality benefit from long-term anticoagulation with VKAs with or without an antiplatelet agent. Anticoagulation can reduce the risk of systemic and cerebral embolism in individuals with a signal MI,

nonvalvular (i.e., nonrheumatic) AF, mechanical valve prosthesis, and xenograft bioprosthesis (for the initial 3 months after placement). Recent data with non-VKA oral agents indicate that they are not inferior to, and could be superior to, warfarin for primary prophylaxis of patients with nonvalvular AF. Early use of heparin in individuals with sinus venous thrombosis may produce clinical improvement.

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Full Reference Lists can be found online

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CHAPTER

94 Medical and Surgical Treatment of Peripheral Arterial Disease

PHILIP JOSEPH, SAURABH KALRA, AND SONIA ANAND

Peripheral arterial disease (PAD) affects approximately 27 million individuals in North America and Europe, and its prevalence is increasing as life expectancy increases.[1] Atherosclerosis is the most common cause of PAD, with symptoms primarily attributable to arterial stenosis and reduced blood flow. Nonatherosclerotic PAD is far less common, resulting from vasculitis, arterial vasospasm, or thromboembolism. Moreover, the development of PAD represents one manifestation of an atherosclerotic process, which in the majority of individuals affects the entire vascular tree. Consequently, patients with PAD are at an increased risk for coronary artery disease and cerebrovascular disease.

The diagnosis and management of PAD has evolved considerably over the past few decades. Advances have stemmed from improved diagnostic modalities as well as from new medical and surgical treatments, with a greater focus on overall cardiovascular risk reduction. Despite these improvements, the successful treatment of individuals with PAD remains a challenge and encompasses a multifaceted strategy involving lifestyle modification, pharmacologic therapy, and peripheral arterial interventions. This chapter provides an overview of the pathogenesis, epidemiology, and management of PAD related to the lower extremities.

PATHOGENESIS OF PAD AND LIMB ISCHEMIA

Atherosclerotic Plaque Development (see Chapter 89)

Briefly, atherosclerosis results from adverse interactions between vessel hemodynamics, blood elements, and the vascular intima.[2] Covering the intimal surface of the vessels, the vascular endothelium regulates several important aspects of vascular homeostasis including vascular tone, leukocyte adhesion and migration, platelet function, and coagulation. Vascular homeostasis is achieved through several autocrine and paracrine factors mediated by endothelial cells. Nitric oxide is released by the endothelium, exerting vasodilatory, antiproliferative, and antithrombotic effects.[3] Endothelial dysfunction develops in the presence of cardiovascular risk factors (such as hypertension, diabetes, smoking, and hyperlipidemic disorders) and results in the decreased availability of nitric oxide or an imbalance in vascular homeostasis favoring deleterious circulating factors. The development of endothelial dysfunction promotes atherosclerotic plaque development, hypercoagulability, and thrombosis. The nascent step in atherogenesis involves the endothelial mediated migration of low-density lipoprotein (LDL) from the blood plasma into the vessel intima.[4,5] Subsequent oxidative stress and intimal inflammation lead to the increased expression of leukocyte adhesion molecules along the vascular surface. Once monocyte and T-lymphocyte adherence occurs, chemokines facilitate leukocyte migration into the vessel intima.[6–11]

As monocytes differentiate into macrophages within the vessel intima, they internalize oxidized LDL via scavenger receptors and develop into foam cells. Macrophage colony stimulating factor has been shown to be present in human atherosclerotic lesions and hypothesized to promote macrophage replication within the intima.[7] Foam cells coalesce to form early atherosclerotic plaques, commonly described as "fatty streaks" (FIGURE 94.1). Activated macrophages and T-lymphocytes cause further plaque progression through the release of inflammatory cytokines and the promotion of smooth muscle cell (SMC) migration and proliferation within the vessel intima. As foam cells, SMCs, and extracellular lipids accumulate, the atherosclerotic plaque continues to organize and grow within the intimal layer. Simultaneously, macrophage and SMC apoptosis within the plaque result in the development of a relatively acellular, collagen-based fibrous cap, which provides stability to the lipid core (FIGURE 94.2). With the continuation of these inflammatory, replicating, and apoptotic processes, the atherosclerotic plaque becomes more complex, creating an abundant extracellular matrix (comprised of interstitial collagen, proteoglycans, and elastin fibers) within the vessel wall, developing calcification, and undergoing neovascularization.[7]

Vascular Ischemia and Thrombosis

As the atherosclerotic plaque enlarges, an outward, "positive" remodeling phase initially predominates preventing luminal obstruction. However, luminal stenosis eventually develops as the enlarging plaque overcomes the vessel's capacity to positively remodel. Moreover, proteolytic enzymes exuded from macrophages degrade and weaken the fibrous cap, creating a "vulnerable plaque" susceptible to rupture.[12] With plaque rupture, the thrombogenic inner vessel becomes exposed to circulating platelets and coagulation factors, resulting in thrombosis. If thrombosis results in occlusion of the vessel, acute ischemia develops.[7] However, repetitive, subclinical thrombotic events also occur; with subsequent healing, and fibrous tissue and collagen fiber formation, resulting in progressive plaque expansion and luminal stenosis.[12] Thus, atherosclerosis progression may span many years without the development of symptoms. However, progressive obstruction of the arterial lumen eventually impedes blood flow through the affected vessel.

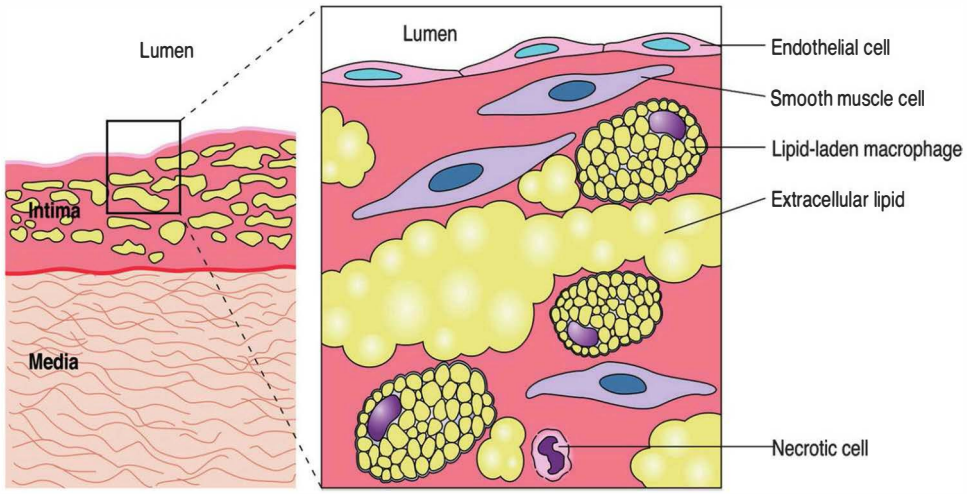


FIGURE 94.1 Fatty streak of atherosclerosis. The fatty streak, composed largely of foamy macrophages, is presumed to be an early stage in the formation of atherosclerotic lesions. Note the intimal thickening in the left panel and the infiltrating cells in the enlargement on the right. (From Rubin R, Strayer DS. *Rubin's pathology: clinicopathologic foundations of medicine*, 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2008.)

EPIDEMIOLOGY OF PAD AND VASCULAR RISK FACTORS

Prevalence and Clinical Course of PAD

Both symptomatic and asymptomatic patients with PAD are at increased risk for adverse cardiovascular events. The prevalence of asymptomatic PAD varies with the age of the population studied, from 3% in 15,106 asymptomatic individuals between the ages of 45 and 60 years[13] and 4.3% among a population >40 years of age[14] to 29% of higher risk individuals over age 70 with cardiovascular risk factors.[15] The prevalence of symptomatic PAD is 3% to 6% in the general population, also increasing with age (FIGURE 94.3).[16]

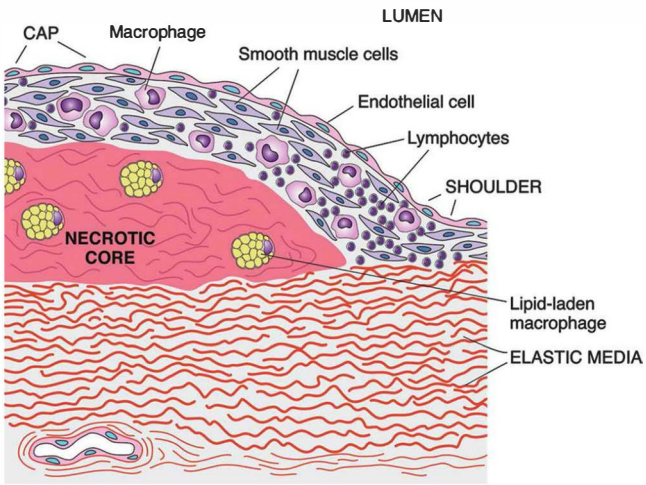


FIGURE 94.2 Fibrofatty plaque of atherosclerosis. In this fully developed fibrous plaque, the core contains lipid-filled macrophages and necrotic SMC debris. The "fibrous" cap is composed largely of SMCs, which produce collagen, small amounts of elastin, and glycosaminoglycans. Also shown are infiltrating macrophages and lymphocytes. (From Rubin R, Strayer DS. *Rubin's pathology: clinicopathologic foundations of medicine*, 5th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2008.)

The natural history of patients with PAD varies widely depending on disease severity and progression of atherosclerosis, with symptoms ranging from intermittent claudication to critical limb ischemia (CLI) and limb loss (FIGURE 94.4). The majority of individuals with PAD have stable leg symptoms, with worsening claudication occurring in approximately 25% of patients,[17] in association with diabetes, smoking, a low initial ankle–brachial index (ABI), and multiple stenotic lesions.[18] CLI is the presenting symptom in 1% to 3% of PAD patients,[17] and 5% to 10% of patients with previously stable PAD develop CLI over a 5-year period; risk factors being diabetes, advanced age, dyslipidemia, smoking, and an ABI of <0.5.[16] In symptomatic individuals, only 1% to 3% require limb amputation over a 5-year period,[17] but individuals with diabetes or those who present with CLI are at increased risk for limb loss.

Between 40% and 65% of patients with PAD are at increased risk for major adverse cardiovascular events (vascular-related death, myocardial infarction, or stroke),[16,19] the annual event rate being 5% to 7% and mortality related to cardiovascular disease being the most common cause of death. Coronary artery disease accounts for 40% to 60% of patient mortality, cerebrovascular disease for 10% to 20%, and other vascular events for 10% of deaths associated with PAD.[16]

RISK FACTORS FOR THE DEVELOPMENT OF PAD

The risk factors for PAD are the same as those for other atherosclerotic disease processes, modifiable ones including smoking, dyslipidemia, diabetes, and hypertension. In the National Health and Nutrition Examination Survey (NHANES), 95% of patients had at least one modifiable cardiovascular risk factor and 75% of patients had at least two cardiovascular risk factors.[14] Advanced age represents a nonmodifiable risk factor for PAD development.

Smoking causes several deleterious alterations in lipid metabolism, including reduced high-density lipoprotein (HDL) and increased LDL, triglyceride, and total cholesterol levels.[20] In

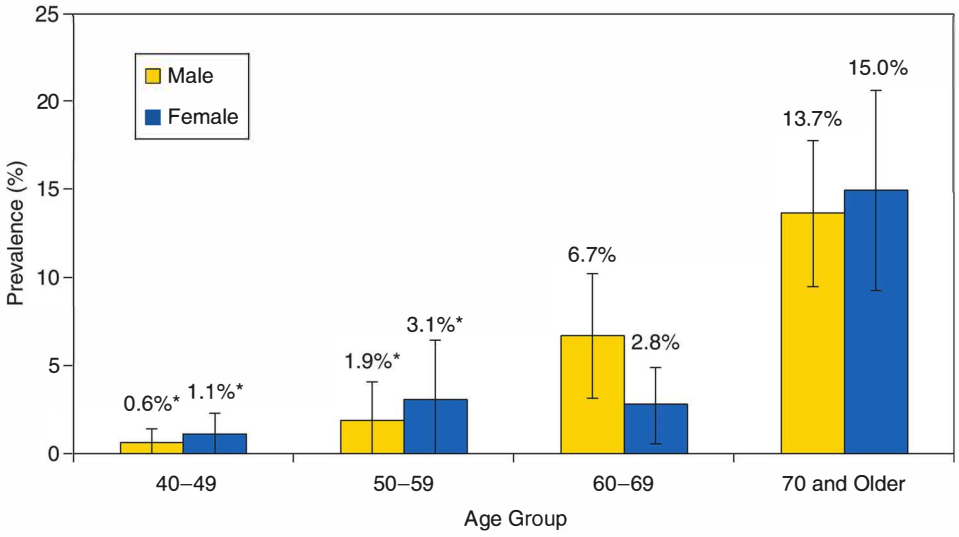


FIGURE 94.3 Prevalance of PAD in adults over 40 years by age and gender. (Reprinted from Selvin E, Erlinger TP. Prevalence of and risk factors for peripheral arterial disease in the United States: results from the National Health and Nutrition Examination Survey, 1999–2000. *Circulation* 2004;110:738–743, with Permission.)

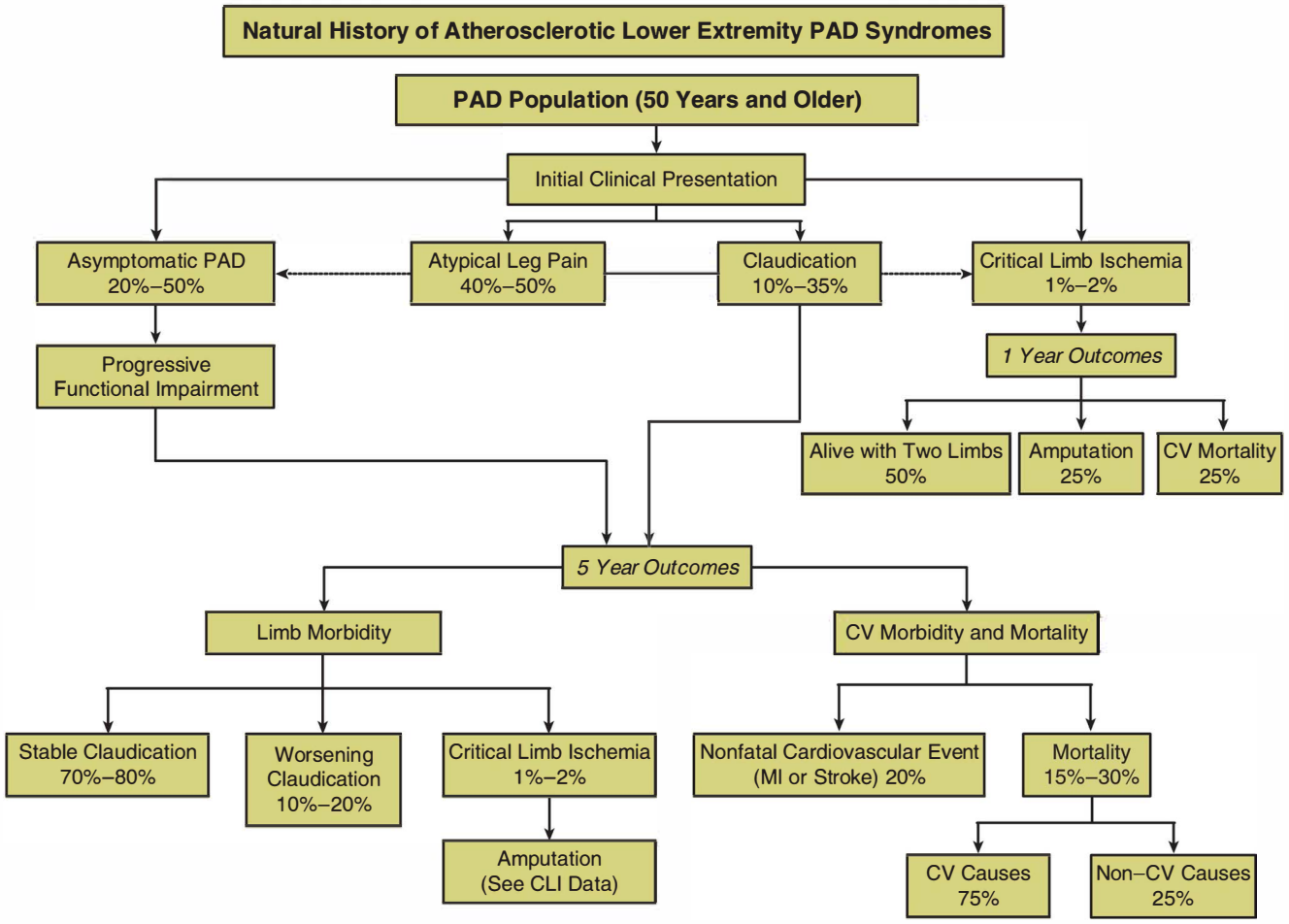


FIGURE 94.4 Natural history of lower extremity PAD. Individuals may be asymptomatic or present with symptoms of leg pain or CLI. All individuals with PAD face a risk of progressive limb ischemia symptoms, increased cardiovascular ischemic event rate, and increased mortality. CV, cardiovascular; MI, myocardial infarction. (Reprintedfrom Hirsch AT, Haskal JZ, Hertzer NR, et al. ACC/AHA 2005 practice guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic). *Circulation* 2006;113:e462–e654, with permission. ©2006 American Heart Association, Inc.)

addition, smoking directly influences atherosclerosis by causing endothelial dysfunction, inflammation, and coagulation within the vascular bed.[21] Individuals who smoke are at risk for cardiovascular events, such as sudden cardiac death, nonfatal myocardial infarction, and ischemic stroke. Active smokers are at a 4.6-fold increased risk of developing PAD compared to nonsmokers,[14] and smoking is associated with a threefold increased risk of CLI, with both PAD severity and progression directly correlated to the number of cigarettes consumed.

Diabetes is a strong modifiable risk factor for PAD, associated with a 2.71-fold increased risk.[14,22] In the Framingham registry, men were at a twofold increased risk for stroke in the presence of PAD and diabetes compared to either disease alone, and women with diabetes and claudication symptoms were at a three- to fourfold increased risk for coronary artery disease, stroke, and heart failure.[22,23] The presence of diabetes signifies a more aggressive course of PAD, with diabetic patients at a 5- to 10-fold increased risk for major limb amputation compared to those without diabetes.[16]

Several mechanisms have been proposed to explain the aggressive nature of vascular disease observed in diabetics. Clustering of cardiovascular risk factors, such as hypertension and dyslipidemia, partly account for the increased cardiovascular event rate. Patients with diabetes frequently develop dyslipidemia secondary to insulin resistance, resulting in decreased HDL, with increased LDL and triglycerides.[24] Significant deleterious effects on vascular biology also occur in the setting of insulin resistance and hyperglycemia.[25] Increased oxidative stress and platelet activation occur in the setting of hyperglycemia, placing patients with diabetes at increased risk for vascular thrombotic events. Negative effects of insulin resistance on SMC proliferation and cellular apoptosis promote further atherosclerosis progression. The development of endothelial dysfunction can impair cellular glucose transport, potentially leading to further insulin resistance and worsening diabetes.[26] Clinical studies support the causal relationship of hyperglycemia, insulin resistance, and accelerated atherosclerosis, as reflected by a direct correlation of cardiovascular disease with glycosylated hemoglobin levels in diabetic populations.[27]

Dyslipidemia: Patients with PAD demonstrate significant abnormalities in lipid metabolism; however, the associated risk appears to be lower compared to other vascular risk factors such as diabetes or smoking. In the NHANES registry, hypercholesterolemia (defined as a total cholesterol level 240 mg/dL [6.2 mmol/L]) was associated with a 1.68-fold increase in the prevalence of PAD.[14] Abnormalities in total cholesterol, LDL, very low-density lipoprotein, and lipoprotein (a) levels are associated with PAD, while serum triglyceride levels are only modestly associated with PAD.[28,29]

Hypertension results in a 1.5- to 2-fold relative risk (RR) increase for PAD.[14] Hypertension significantly alters vascular through increased endothelial dysfunction, loss of precapillary networks, and increased microvascular vasoconstriction in hypertensive animal models; all of which beget further increases in blood pressure, promoting both atherosclerosis and thrombosis.

Advanced age represents a risk factor for cardiovascular events and worse clinical outcomes in patients with established PAD. In the SMART registry of patients with symptomatic PAD, each 10-year increase in age was associated with a 1.85-fold risk increase in fatal and nonfatal vascular events.[30]

Genetic risk: The genetic contribution to PAD has not been clarified to the same extent as in other cardiovascular diseases. Several family studies suggest that PAD is in part related to genetic risk, estimating that genetic variation accounts for 21% to 48% of the interindividual variability in the ABI.[31] Genome-wide association studies have enabled further insight into the role of common genetic variants in cardiovascular disease. The genetic epidemiology network of arteriopathy study demonstrated that PAD (identified as a reduction in the ABI) was modestly heritable in African American and white hypertensive populations. Single-nucleotide polymorphisms (SNPs) associated with PAD were observed in several loci, but no single SNP reached genome-wide significance ($P \leq 5 \times 10^{-8}$).[32] A common variation of the chromosome 9p21 allele has been associated with several forms of vascular disease, particularly myocardial infarction and stroke.[33–36] In elderly individuals, Cluett et al.[37] demonstrated a modest association between the 9p21 allele and PAD detected by ABI (odds ratio [OR] 1.29, 95% CI 1.06 to 1.56). Further studies are necessary to identify common genetic variants associated with PAD development and to clarify the role of genetic testing in risk stratification of PAD patients.

DIAGNOSIS OF PAD

Symptoms of PAD

Approximately, 20% to 50% of individuals with PAD do not report any disease-related symptoms.[17] The classic symptom of PAD is intermittent leg claudication, occurring as the initial presenting complaint in 10% to 35% of patients. Caused by flow-limiting vascular stenosis, intermittent claudication is characterized by pain due to reversible muscle ischemia during activity, improving with rest.[38] Typically, claudication symptoms occur in the calves, although patients may also present with upper leg, hip, or buttocks pain. Patients frequently experience reduced exercise performance, and symptoms may potentially become debilitating.

With worsening PAD severity, CLI may develop. CLI reflects a state of persistent, reduced perfusion at rest, and is characterized by the presence of resting claudication occurring for >2 weeks, or the development of ischemia-related lesions, such as arterial ulcers or gangrene (FIGURE 94.5). Both the Fontaine

FIGURE 94.5 Ischemic ulcers and gangrene in a patient with CLI. (From Topol EJ, Califf RM, et al. *Textbook of cardiovascular medicine*, 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2006.)

Table 94.1

Classification of PAD: Fontaine stages and Rutherford categories

Fontaine		Rutherford		
Stage	**Clinical**	**Grade**	**Category**	**Clinical**
I	Asymptomatic	0	0	Asymptomatic
IIa	Mild claudication	I	1	Mild claudication
IIb	Moderate-to-severe claudication	I	2	Moderate claudication
		I	3	Severe claudication
III	Ischemic rest pain	II	4	Ischemic rest pain
IV	Ulceration or gangrene	III	5	Minor tissue loss
		III	6	Major tissue loss

Reprinted from Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg* 2007;45(1):S5–S67. © 2007, with permission from Elsevier.

and Rutherford classification systems may be used to organize PAD severity systematically based on the degree of leg ischemia (Table 94.1). Individuals who present with symptoms suggestive of intermittent claudication or CLI should undergo objective testing to confirm the presence of PAD. Acute limb ischemia (ALI) occurs due to a sudden interruption of the blood flow to an extremity, with patients presenting with a combination of limb pain, pallor, pulselessness, paresthesia, or paralysis signifying threatened limb viability.[16]

Ankle–Brachial Index

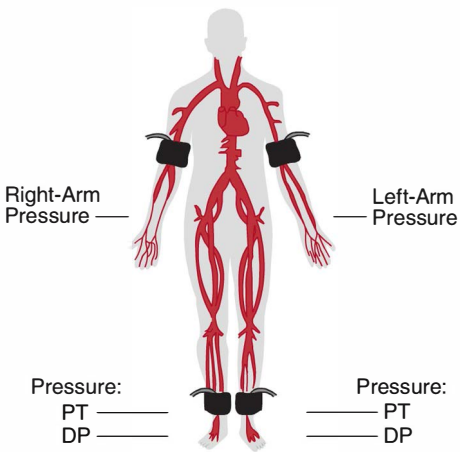
The ABI is the primary test used for the diagnosis of PAD. The ABI represents the ratio of the systolic blood pressure recorded in the brachial artery of either upper extremity to the systolic pressure recorded in either lower extremity (using the posterior tibial or dorsalis pedis artery), measured noninvasively (FIGURE 94.6). The ABI is typically measured by Doppler ultrasonography; however, studies show comparable results with auscultation techniques.[39] The ABI is an accurate measure of PAD, with value of ≤0.90 demonstrating a 95% sensitivity and >95% specificity for detecting angiographically significant arterial stenoses.[40,41] The ABI can also be used to further classify individuals into those with mild (ABI = 0.9 to 0.71), moderate (ABI = 0.70 to 0.41), or severe (ABI ≤ 0.4) disease.[25,42]

An abnormal ABI is associated with an increased mortality and a higher incidence of major adverse cardiovascular events, with lower ABI indexes predictive of a worse clinical outcome (FIGURE 94.7).[43–47] In a meta-analysis of 16 studies, the use of the ABI in asymptomatic patients altered the cardiovascular risk profile for 1-in-5 men and 1-in-3 women, when added to the Framingham risk score.[48] An ABI index >1.3 may represent a calcified and noncompliant artery and is also associated with increased vascular events, which should prompt further investigations (such as pulse volume recording, toe–brachial index measurement, or duplex ultrasound) to determine if PAD is

FIGURE 94.6 Measurement of the ABI. DP, dorsal pedis artery; PAD, peripheral arterial disease; PT, posterior tibial artery. (Adapted from Topol EJ, Califf RM, et al. *Textbook of cardiovascular medicine*, 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2006.)

ABI	Severity of Disease
0.91–1.3	Normal
0.71–0.90	Mild Disease
0.41–0.70	Moderate Disease
≤0.40	Severe Disease

ABI is 95% Sensitive and 99% Specific for PAD



$$\text{ABI} = \frac{\text{Lower Extremity Systolic Pressure}}{\text{Brachial Artery Pressure}}$$

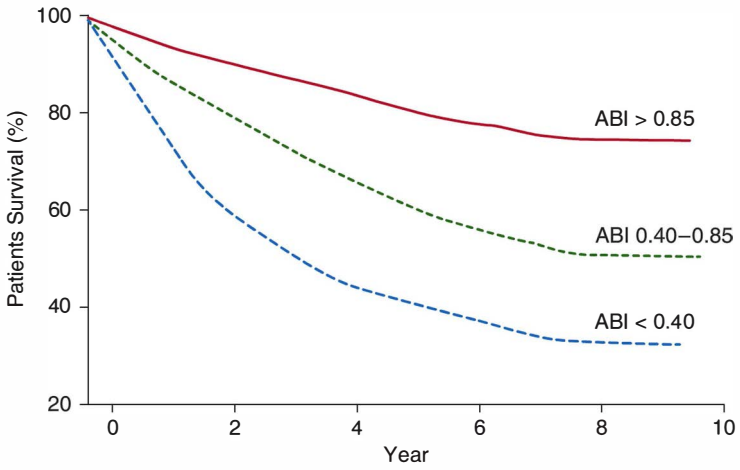


FIGURE 94.7 Survival with PAD severity based on the ABI. (From Topol EJ, Califf RM, et al. *Textbook of cardiovascular medicine*, 3rd ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2006.)

present.[42] Due to the accuracy of the ABI in predicting PAD, and the increased cardiovascular risk associated with an abnormal ABI measurement, patients who are asymptomatic with an ABI ≤0.9 should undergo lifestyle and pharmacologic risk factor modification in accordance with a diagnosis of vascular disease.

Additional Functional Modalities

In patients with suspected PAD but normal ABI measurements, additional measures may be performed to confirm the diagnosis, including ABI measurements after exercise, toe–brachial index, and pulse volume recording.[49] The toe–brachial index may be performed in symptomatic patients with calcified, noncompressible proximal arteries causing either a "false-negative" or significantly elevated ABI. A small occlusive cuff is placed on the great toe, with the systolic blood pressure recorded at the point when pulsatile flow is first detected by plethysmography. A toe–brachial index of <0.7 is considered specific for PAD.[17]

Pulse volume recording involves the measurement of the pulse amplitude and contour in the proximal and distal lower extremities using noninvasive thigh and ankle cuffs, with abnormalities in the lower extremity pulse amplitude or contour signifying reduced arterial blood flow.[50] The brachial artery pulse volume is recorded simultaneously as a measure of normal flow. Segmental evaluation of the lower extremities by continuous-wave Doppler ultrasound can further assist in PAD diagnosis. Abnormalities in Doppler morphology and the Doppler waveform pulsatility index suggest impaired arterial flow due to arterial stenosis.[17] Both pulse waveform and continuous Doppler can be used to establish a diagnosis of PAD in patients with equivocal ABI results, to follow PAD progression, and to potentially assess the effectiveness of revascularization.

Additional Imaging Modalities

Invasive angiography is the gold standard for the diagnosis of PAD and for the localization of arterial stenoses. However, with the introduction of accurate and readily available noninvasive imaging modalities, invasive angiography is becoming more commonly used in conjunction with revascularization, rather than to establish a diagnosis or guide treatment decisions.

Duplex ultrasonography utilizes both functional and anatomic imaging to evaluate for PAD. Markers of PAD by duplex ultrasonography include the presence of turbulent flow, alterations in Doppler waveform, and a peak-to-peak velocity ratio (measured within the obstruction and distally) >2.[17] Although Duplex ultrasonography has >90% sensitivity and specificity for detecting a significant stenosis above the popliteal artery, its accuracy is reduced in the presence of poorly visualized vessels, or with multiple stenoses.[17]

Computed tomographic angiography (CTA) is being utilized with increasing frequency to diagnose PAD, with sensitivities ranging from 89% to 99% and specificities from 83% to 100%.[51] Compared to conventional angiography, CTA allows for fast acquisition of accurate images in a noninvasive manner, although the risks related to radiation exposure and contrast-induced nephrotoxicity remain.[52] Magnetic resonance angiography (MRA) provides accurate localization of arterial stenoses noninvasively, without exposure to radiation, and may be used to diagnose PAD or to select potential candidates for revascularization (FIGURE 94.8). Disadvantages of MRA include long acquisition times, reduced diagnostic accuracy with infrapopliteal arterial segments, and the risks of renal toxicity and nephrogenic systemic fibrosis with gadolinium in patients with preexisting renal insufficiency.[51] A meta-analysis of 23 studies demonstrated that the accuracy of MRA to detect obstructive PAD has improved with advancing technology; for the detection of a >50% stenosis on invasive angiography, two-dimensional time-of-flight MRA had a sensitivity of 64% to 100% and a specificity of 68% to 96%; with three-dimensional gadolinium-enhanced MRA, the sensitivity and specificity improved to 92% to 100% and 91% to 99%, respectively.[53] While the ABI remains the primary test to diagnose PAD, several noninvasive functional and imaging modalities are available to establish a diagnosis with equivocal ABI results, and to identify potential candidates for revascularization.

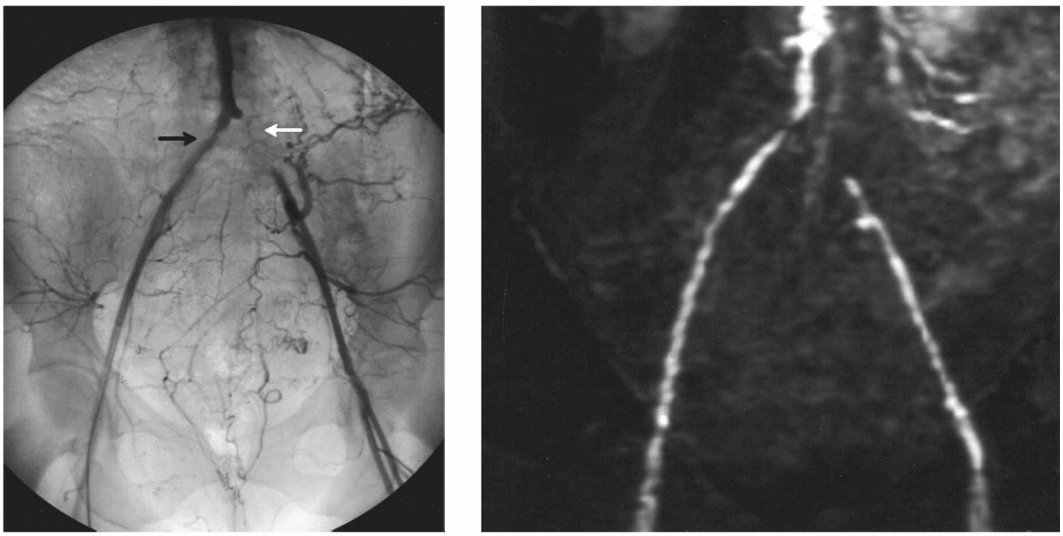


FIGURE 94.8 **A:** Pelvic arteriogram of a 75-year-old female with bilateral hip claudication. The study demonstrates diffuse infrarenal aortic atherosclerosis, right common iliac artery stenosis (*black arrow*), and left common iliac artery occlusion (*white arrow*) with external iliac artery reconstitution via collaterals. **B:** Corresponding MRA with 2-D gadolinium-enhanced technique. (From Baim DS. *Grossman's cardiac catheterization, angiography, and intervention*, 7th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2006.)

TREATMENT OF PATIENTS WITH PAD

The primary goals for PAD management are to reduce cardiovascular events and to improve PAD-related symptoms. Secondary prevention of cardiovascular disease is achieved through lifestyle modification and medical therapy (Table 94.2). This section focuses on (a) medical management to reduce cardiovascular risk, (b) the medical and surgical management of symptomatic PAD with respect to intermittent claudication, and (c) specific management issues surrounding CLI and ALI. A summary of the management of asymptomatic and symptomatic patients with PAD is shown in FIGURE 94.9.

CARDIOVASCULAR RISK MODIFICATION

Antiplatelet Therapy

Platelet activation is a crucial component of vascular atherothrombosis and mediated by several compounds including thromboxane A_2, adenosine diphosphate (ADP), serotonin, and norepinephrine.[54] The antiplatelet therapies most extensively studied in PAD are acetylsalicylic acid (aspirin) and thienopyridines (clopidogrel and ticlopidine). Aspirin reduces platelet activation through the inhibition of cyclooxygenase, a crucial enzyme for the synthesis of thromboxane A_2. Thienopyridines impair binding of ADP to the $P2Y_{12}$ receptor, consequently inhibiting platelet activation through the ADP pathway.

Antiplatelet therapy is primarily utilized to reduce the risk of cardiovascular events in patients with PAD. A large meta-analysis by the Antiplatelet Trialists collaboration involving 287 studies examined the effects of antiplatelet agents on cardiovascular outcomes in patients with a history of vascular disease. In a subgroup analysis of 42 studies involving 9,214 patients with PAD, antiplatelet therapy (primarily aspirin, alone or in combination with dipyridamole) was associated with a 23% odds reduction in subsequent vascular events.[55] In a recent meta-analysis of 18 randomized studies involving 5,269 patients with PAD, Berger et al.[56] reported a significant reduction in nonfatal cerebrovascular events with aspirin therapy compared to placebo (RR 0.66, 95% CI 0.47 to 0.94). However, no significant differences were demonstrated for all-cause mortality, cardiovascular mortality, nonfatal myocardial infarction, or bleeding. Although numerous studies have reported significant improvements in various cardiovascular endpoints with aspirin therapy, recent evidence suggests that the overall cardiovascular benefits demonstrated with aspirin may not be applicable to very low-risk patients who have asymptomatic PAD without concomitant cardiovascular disease. A large, randomized control trial of 28,980 patients with asymptomatic PAD (diagnosed by an ABI of <0.95) failed to demonstrate a reduction in cardiovascular events in participants treated with aspirin 100 mg daily compared to placebo.[57] However, the low overall mortality rate (1.9%) and mean ABI score (0.86) in the trial suggests a lower risk population compared to those evaluated in previous studies. In PAD patients undergoing revascularization, aspirin improves vessel patency after lower extremity bypass or percutaneous angioplasty. Overall, aspirin is recommended as first-line antiplatelet therapy for the majority of patients with a diagnosis of PAD.

Thienopyridines also benefit patients with PAD. The CAPRIE study showed an 8.7% RR reduction in vascular events with clopidogrel compared to aspirin therapy in patients with atherosclerotic vascular disease, including PAD.[58] The greatest benefit was demonstrated within the PAD subgroup, achieving a 23.8% RR reduction in vascular events. Thienopyridines also improve symptoms related to PAD. In a randomized study of 151 PAD patients with intermittent claudication, Balsano et al.[59] reported an improved ABI index, pain-free walking distance, and maximal walking distance with ticlopidine compared to placebo. Current guidelines support the use of clopidogrel as

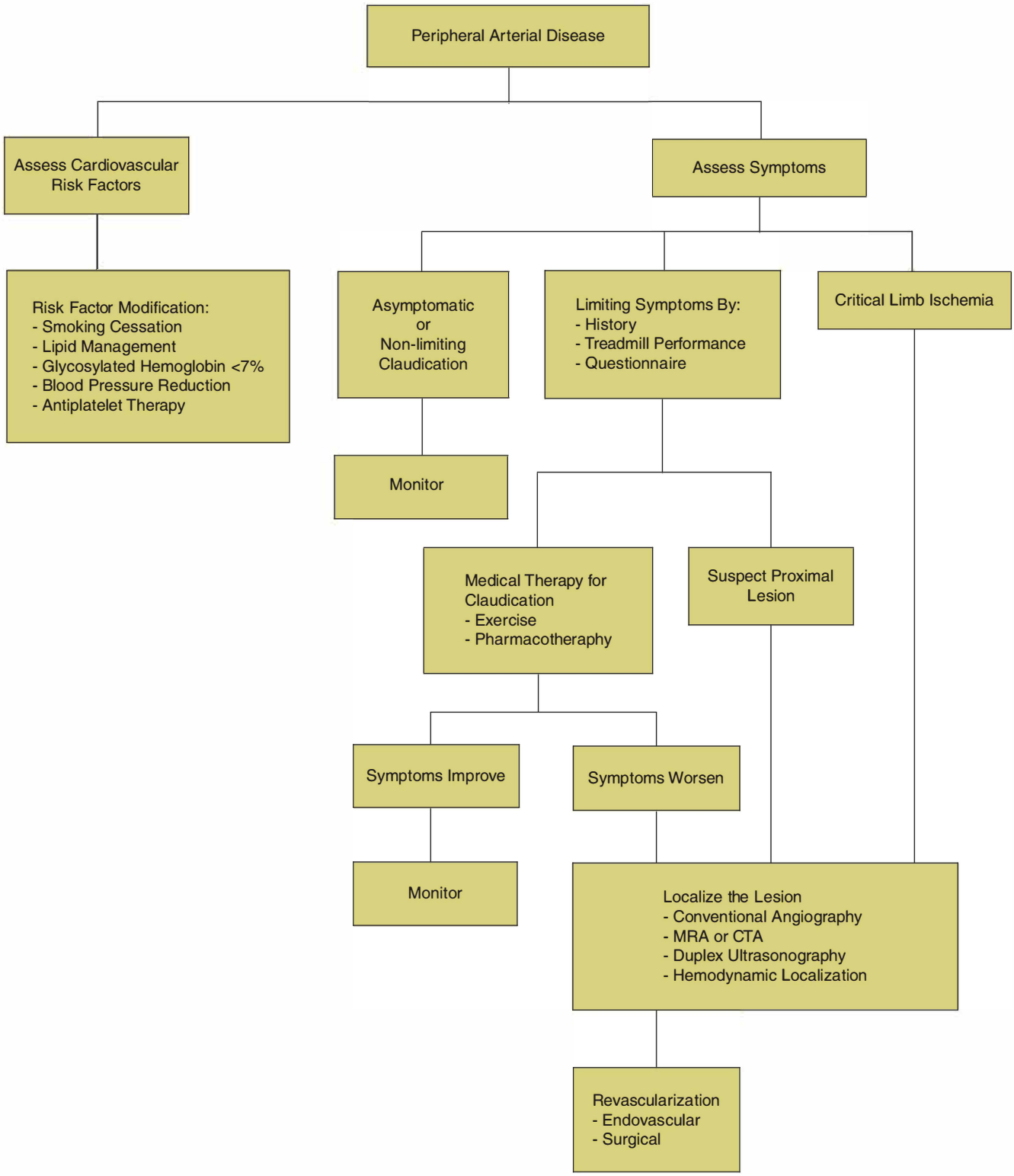


FIGURE 94.9 Overview of the management of patients with asymptomatic PAD, intermittent claudication, or CLI. (Adapted from Hiatt WR. Medical treatment of PAD and claudication. *N Engl J Med* 2001;344(21):1608–1621, with permission. © 2001, Massachusetts Medical Society. All rights reserved.)

Table 94.2

Treatment guidelines for cardiovascular risk reduction in patients with PAD

Treatment	Goals of Management
Smoking cessation	• Always address smoking cessation in PAD • Both nonpharmacologic and pharmacologic methods may be utilized
Antiplatelet therapy	• Antiplatelet therapy should be considered in all PAD patients • Aspirin (81mg daily) is the preferred antiplatelet agent • Clopidogrel (75 mg daily) may be considered as an alternative
Lipid reduction	• LDL reduction to <2.59 mmol/L (100 mg/dL) in all PAD patients • Consider further LDL reduction to <1.8–2 mmol/L (70–78 mg/dL) • HMG coenzyme A reductase inhibitors (statins) are first-line therapy for treatment of dyslipidemia in PAD
Diabetes control	• Target HbA1C level of <7.0%
Blood pressure reduction	• Target blood pressure <140/90 in PAD • Consider further reduction of blood pressure to <130/80 in patients with coexisting renal failure or diabetes • Consider ACE inhibitor for blood pressure reduction and cardiovascular protection

PAD, peripheral arterial disease; LDL, low-density lipoprotein; HbA1C, hemoglobin A1C; ACE, angiotensin-converting enzyme.

an alternative antiplatelet agent to aspirin for cardiovascular risk reduction in patients with PAD. The development of severe neutropenia, a potentially fatal side effect, has been reported in 2.1% of patients prescribed ticlopidine, which has significantly limited its use in clinical practice.[60]

The use of dual antiplatelet therapy to reduce secondary events in patients with established cardiovascular disease remains controversial. The CHARISMA study is the largest randomized trial which evaluated dual antiplatelet therapy with aspirin and clopidogrel in patients with known vascular disease or multiple vascular risk factors.[61] Dual antiplatelet therapy did not reduce cardiovascular events compared to aspirin alone and was associated with a significant increase in bleeding events. Given the results of CHARISMA, there is currently insufficient evidence to support the routine use of dual antiplatelet therapy in patients with PAD without secondary indications (i.e., post-myocardial infarction or vascular stent implantation).

Anticoagulant Therapy

While a theoretical rationale exists for improved antithrombotic effects with the addition of an oral anticoagulation agent to antiplatelet therapy, studies examining the combination have failed to demonstrate a benefit and have shown increased bleeding. The WAVE study compared the combination of warfarin and antiplatelet therapy to antiplatelet therapy alone in patients with a history of PAD.[62] Combination therapy did not reduce cardiovascular events and was associated with increased life-threatening bleeding. Combination therapy has also been studied for the prevention of graft occlusion in patients following peripheral bypass surgery with either venous or arterial grafts. In the Dutch BOA study, there were no significant differences in cardiovascular events or limb salvage with combination therapy compared to antiplatelet therapy alone in patients undergoing infrainguinal bypass.[63] Although the Dutch BOA study reported fewer ischemic events with combination therapy in patients with venous grafts, other studies have not demonstrated similar results.[64] Currently, the routine use of anticoagulants in addition to antiplatelet therapy is not recommended for routine use in patients with PAD to reduce cardiovascular risk or prevent graft occlusion.

Lipid-Lowering Therapy

As patients with PAD are a high-risk population for major cardiovascular events, aggressive lipid management is recommended. HMG coenzyme A reductase inhibitors (statins) are the principal agents for risk reduction in patients with vascular disease, with each reduction of 1 mmol/L of LDL with statin therapy associated with a 20% reduction in cardiovascular events.[65] Large randomized control trials demonstrate profound benefits with statin therapy in patients with cardiovascular disease or vascular risk factors. The Heart Protection Study compared simvastatin 40 mg to placebo in 20,536 individuals with cardiovascular disease, including 6,748 patients with PAD.[66] Simvastatin was associated with significant reduction in all-cause mortality (12.9% vs. 14.7%, $P = 0.0003$), primarily driven by a decrease in coronary-related death (5.7% vs. 6.9%, $P = 0.0005$). Moreover, aggressive lipid control provides further benefit compared to moderate lipid control in patients with cardiovascular disease. The Treat to New Targets study reported significant improvements in cardiovascular events with a reduction in LDL to <1.8 mmol/L (<70 mg/dL), compared to moderate LDL control in patients with coronary artery disease (RR 0.78, 95% CI 0.69 to 0.89).[67] Current PAD guidelines recommend aggressive LDL reduction in PAD patients to <2.59 mmol/L (100 mg/dL).[16] A number of lipid guidelines advocate for even lower LDL targets to <1.8 to 2.0 mmol/L (70 to 77 mg/dL) in patients with established vascular disease.[65]

Clinical studies suggest that statins may improve cardiovascular outcomes by reducing vascular inflammation, an important mediator of atherosclerosis progression. The PROVE-IT study reported significant reductions in C-reactive protein (CRP) levels with high-dose compared to low-dose statin therapy amongst 3,745 patients presenting with acute coronary syndromes who were followed over a mean period of 24 months. Patients with a reduced CRP had significantly fewer cardiovascular events compared to those with an increased CRP, regardless of LDL concentration.[68] Complementary results were reported in the REVERSAL study, which examined the effects of high-dose statin therapy on atherosclerosis progression in CAD patients by coronary intravascular ultrasound.[69] Participants who received high-dose statin therapy had reduced coronary atherosclerosis progression compared to those who received moderate-dose statin therapy, atheroma progression being directly related to changes in both LDL and CRP. Both PROVE-IT and REVERSAL provide support for the additional vascular benefits statins provide as potent mediators of vascular inflammation. Statin therapy also exerts positive effects on endothelial activity, likely mediated through increased endothelial nitric oxide synthase expression.[70,71] Tsunekawa et al.[72] demonstrated increased brachial artery flow-mediated vasodilation and plasma nitrate levels in prediabetic patients treated with statin therapy compared to placebo. Additional proposed vascular effects of statins include decreased oxidative stress through the downregulation of angiotensin II and NAD(P)H oxidase; and the stimulation of circulating endothelial progenitor cells which assist in ischemic tissue neorevascularization and repair.[73] In addition to cardiovascular risk reduction, several studies have reported improvements in claudication symptoms and walking performance with statin therapy in patients with PAD.[74–76]

Individuals with PAD frequently have further disorders of lipid metabolism, such as reduced HDL and elevated triglycerides. Although epidemiologic studies have demonstrated that both HDL and triglyceride disorders are associated with increased cardiovascular events, there is limited evidence supporting targeted therapy to improve HDL or triglycerides in the current era of aggressive LDL lowering using statin therapy. Lifestyle modifications, including exercise and weight loss, have beneficial effects on both HDL and triglycerides. Niacin therapy can increase HDL up to 15% to 25%, and reduce LDL by an additional 20% in patients, and may be considered as a second agent in patients with persisting lipid abnormalities despite adequate statin therapy.[65] Niacin improves surrogate markers of cardiovascular disease such as carotid artery intimal thickness, although whether this translates to a reduction in cardiovascular events in patients already receiving statin therapy remains uncertain.[77] Fibric acid derivatives (fibrates) may be considered in patients with severe hypertriglyceridemia or those with persistently increased triglycerides despite statin therapy and lifestyle modification.

Blood Pressure Control

Aggressive blood pressure is recommended in all patients with vascular disease. The intersociety consensus for the management of PAD (TASC II) guidelines suggest that patients with PAD should have blood pressure controlled to <140/90, or <130/80, in the presence of diabetes or renal impairment.[16] Weight reduction, limitation of alcohol consumption, and dietary changes are important lifestyle modifications which reduce blood pressure. In separate populations of overweight individuals, a weight reduction of 10 lbs resulted in a significant reduction in blood pressure ranging from 5 to 20 mm Hg.[78–80] Maintenance of a normal body weight (BMI 18.5 to 24.9 kg/m^2) should be targeted in all patients to improve blood pressure and reduce overall cardiovascular risk.[80,81] Alcohol consumption should be limited to no more than 2 drinks per day in men, and no more than 1 drink per day in women.[80] Dietary salt intake should be limited to 65 to 100 mmol per day in hypertensive individuals. Consumption of a diet rich in fruits, vegetables, and low-fat dairy products with limited saturated fat has been shown to lower blood pressure.[80,82,83]

Several classes of medications may be used alone or in combination for hypertension treatment in patients with PAD. Angiotensin-converting enzyme (ACE) inhibitors and thiazide diuretics are considered first-line therapies for hypertension management in PAD patients. Ostergren et al.[84] evaluated the effects of ramipril in a subset of 3,099 patients with PAD enrolled in the HOPE study. Ramipril was associated with a 25% RR reduction in vascular events in individuals with clinical PAD, with similar benefits also demonstrated in those without clinical PAD but a reduced ABI. Long-acting calcium channel blockers and angiotensin receptor blockers (ARBs) can also be used as initial monotherapy for hypertension treatment. Despite previous concerns of worsening claudication symptoms with β-adrenergic blocking drugs, multiple studies have demonstrated their safety and effectiveness in patients with PAD.[16] However, the antihypertensive effects of β-adrenergic blocking drugs are diminished in patients >60 years of age. ACE inhibitors and ARBs should not be routinely used in combination due to the increased risks of renal dysfunction and hyperkalemia.

Diabetes Management

Several studies have demonstrated significant vascular benefits from aggressive glucose control in patients with diabetes. The Diabetes Control and Complications trial reported decreased microvascular complications (i.e., retinopathy and nephropathy) and a trend toward reduced macrovascular complications (i.e., stroke, myocardial infarction, claudication, amputation, and arterial events requiring peripheral bypass) with intensive glucose control in type I diabetic patients. The UK Prospective Diabetes Study demonstrated similar microvascular benefits with intensive glucose control in patients with type II diabetes. Although single studies have not demonstrated significant macrovascular benefits with intensive glucose control, a meta-analysis of five studies involving 32,629 individuals did report significant reductions in incident cardiovascular events (OR 0.89, 95% CI 0.83 to 0.95) and myocardial infarctions (OR 0.86, 95% CI 0.78 to 0.93) with intensive therapy.[85] Current guidelines suggest a target hemoglobin A1C (HbA1C) of <7% in patients with PAD and a history of diabetes.[16,86]

Two recent studies have evaluated the effects intensive glucose control to "near euglycemic" levels on cardiovascular outcomes in diabetic patients. Both the ADVANCE and ACCORD studies demonstrated no significant cardiovascular benefits with lowering the HbA1C to ≤6.5%, with intensive treatment associated with a higher overall mortality in ACCORD.[87,88] Given the marginal benefit and potential harm observed with aggressive antiglycemic treatment to a nearly euglycemic HbA1C level, there is insufficient evidence to support further treatment of

HbA1C levels when they are below 7%, as recommended in current guidelines. The presence of small vessel disease, associated peripheral sensory neuropathy, and severity of atherosclerosis in diabetic patients place them at an increased risk of arterial and foot ulcer development. Careful foot examinations and foot care education should be advocated for all individuals with diabetes, particularly for those with documented PAD.

Smoking Cessation

Observational studies have demonstrated significant beneficial effects with smoking cessation in patients with PAD including reduced stoke, myocardial infarction, symptoms of claudication and risk of amputation, and improved graft patency after peripheral bypass surgery.[89,90] Unfortunately, long-term smoking cessation is achieved in only 2.5% of individuals who do not utilize behavioral or pharmacologic aids.[91] All patients with PAD should be advised to quit smoking. Behavior therapy includes stress management, group therapy, identification of social cues that trigger smoking, and the development of coping strategies to aid quitting. Smoking cessation rates are significantly increased with the involvement of an expert, such as a smoking councilor or physician.[92,93]

Pharmacotherapy includes nicotine replacement therapy (NRT) and non-nicotinic treatment regimens. NRT is considered safe to use in patients with stable cardiovascular disease. NRT is currently available in the form of the patch, gum, nasal spray, and inhaler. A Cochrane review of 132 studies examining NRT on smoking abstinence reported significant improvements in smoking cessation rates compared to no treatment (RR 1.58, 95% CI 1.50 to 1.66). These benefits occurred across all forms of NRT.[94] Hajek et al. compared the efficacy and compliance rates of different forms of NRT over 12 weeks of therapy. The highest compliance was reported with the nicotine patch (82%), followed by the nicotine gum (38%), nicotine spray (15%), and nicotine inhaler (12%), although there were no significant differences in overall smoking cessation rates with any form of NRT.[95]

Non-nicotinic therapies approved for smoking cessation are bupropion SR (Wellbutrin SR, Zyban) and varenicline (Champix). Studies have reported improved quitting rates in patients prescribed bupropion combined with behavior therapy. Moreover, the combination of bupropion and NRT is safe and may result in improved smoking cessation compared to each product individually.[95] Varenicline is a high-affinity $\alpha_4\beta_2$ partial agonist that promotes dopamine release and reduces nicotine craving and has been compared for up to 52 weeks of therapy with bupropion, or placebo in active smokers attempting to quit.[96] Varenicline was associated with improved abstinence from smoking compared to bupropion and placebo during the 12-week active therapy and at 24-week follow-up, but at 52 weeks, there was no significant differences in smoking cessation rates for patients who had previously received varenicline or bupropion. With the addictive properties and behavioral dependencies associated with smoking, long-term compliance remains a challenge.

MANAGEMENT OF PAD SYMPTOMS

Initial medical strategies for symptomatic PAD management involve pharmacologic therapies and lifestyle modification to improve claudication symptoms and exercise tolerance. Percutaneous or surgical revascularization procedures are reserved for patients who do not respond adequately to medical therapy, or in the management of proximal lower extremity stenosis (i.e., iliac or femoral involvement). This section discusses medical and revascularization options for patients with PAD with respect to symptoms of intermittent claudication. The treatment of CLI and ALI differ significantly and will be discussed in subsequent sections.

MEDICAL MANAGEMENT OF INTERMITTENT CLAUDICATION

Supervised Exercise Rehabilitation

Exercise can alleviate claudication symptoms through several potential mechanisms, including improved endothelial function, improved oxidative metabolism and oxygen extraction, increased ischemia threshold, reduced inflammation, and further optimization of atherosclerotic risk factors (e.g., hypertension, obesity, and dyslipidemia).[97] Several studies have reported significant benefits associated with exercise on claudication relief and functional improvement in patients with PAD. Supervised programs have shown more benefit than unsupervised regimens. A meta-analysis of 21 studies examining exercise in patients with claudication demonstrated improved distance to onset of claudication pain and distance to maximal claudication pain with supervised exercise rehabilitation. Exercise duration >30 minutes, frequency >3 times per week, program duration >6 months, walking as the primary mode of exercise, utilizing increased pain thresholds for cessation of activity, and supervised lower extremity resistance training were associated with the greatest improvements in PAD symptoms. A recent randomized control study demonstrated significant improvements in maximal walking distance and overall physical functioning with treadmill exercise and resistance training, compared to the control group, in 156 patients with asymptomatic or symptomatic PAD.[98]

A typical supervised exercise program consists of walking with a treadmill for three sessions per week, with each session ranging from 30 minutes to 1 hour in duration.[16] Treadmill intensity is modified to elicit claudication symptoms between 3 and 5 minutes of exercise. Patients should engage in a cycle of walking until moderate claudication occurs, at which point they should stop until the pain has resolved, then resume exercising until symptoms recur. Once an exercise can be performed for 10 minutes without signs of claudication, the intensity can be increased. Patients with PAD may have several barriers to participating in supervised exercise rehabilitation, including severe symptoms of claudication, coexisting angina, musculoskeletal impairments, facility distance, and financial constraints. One small study has shown the clinical utility of arm ergometry in patients with PAD, which may be used as an alternative to treadmill exercise in patients with limited mobility.[99]

Cilostazol

Cilostazol promotes arterial vasodilation through the inhibition of phosphodiesterase III. Secondary effects include inhibition of platelet aggregation, *in vitro* inhibition of vascular SMCs, and marginal benefits on lipid metabolism. A 2002

meta-analysis involving 1,751 patients concluded that cilostazol was associated with a significant increase in maximal treadmill performance from baseline (100 ± 143 vs. 50 ± 127 m, $P < 0.001$) compared to placebo.[100] Similar results were obtained in a Cochrane meta-analysis involving seven studies, which reported improved initial distance to the onset of claudication symptoms with cilostazol compared to placebo.[101] The most frequently studied dose of cilostazol (100 mg twice daily) resulted in an improvement of the initial claudication distance of 31 m (95% CI 21.3 to 40.9 m). A randomized study conducted by Dawson et al.[102] in 698 patients with symptomatic PAD showed that cilostazol was associated with a significant improvement in the mean distance to the onset of claudication, while no improvement was noted with pentoxifylline (218 m for cilostazol vs. 202 m for pentoxifylline, $P = 0.0001$). Fewer patients experienced deterioration or had no change in walking distance with cilostazol (23% vs. 34%). Studies examining cilostazol have not demonstrated any differences in cardiovascular events or overall mortality compared to placebo. Cilostazol is contraindicated in PAD patients with coexisting congestive heart failure. For patients who have limiting claudication despite antiplatelet therapy, risk factor optimization, and lifestyle modification, PAD guidelines suggest a 3- to 6-month course of cilostazol for symptom relief.[16]

Naftidrofuryl

Naftidrofuryl is a 5-hydroxytryptamine type II antagonist that inhibits the effects of serotonin.[103,104] Although the precise mechanism of its vascular effects remains elusive, increased aerobic tissue metabolism, vasodilation, and decreased platelet aggregation through the secondary expression of type II nitric oxide synthase have been observed Kieffer el al.[105] demonstrated a 92% improvement in pain-free walking distance and an 83% improvement in maximum walking distance in patients treated with naftidrofuryl. Similar benefits were reported in a Cochrane meta-analysis of 1,083 patients with symptomatic PAD treated with naftidrofuryl compared to placebo.[104] Naftidrofuryl was associated with a significant improvement in pain-free walking distance, with a number need to treat of 4.48 patients to achieve a benefit in claudication symptoms. Side effects of naftidrofuryl include hepatitis, dizziness, and headache.[106] Naftidrofuryl is not recommended in patients with hyperoxaluria or kidney stones. PAD guidelines suggest that naftidrofuryl can be used as an alternative to cilostazol in patients with claudication.[16]

Pentoxifylline

Pentoxifylline is a methylxanthine derivative, which improves blood viscosity and vascular blood flow by decreasing red blood cell rigidity. The use of pentoxifylline for the relief of claudication remains controversial. Several small studies have shown modest improvements in maximal walking distance with pentoxifylline compared to placebo, but the previously described study by Dawson et al.[102] examining pentoxifylline, cilostazol, and placebo in patients with claudication demonstrated no improvement in maximal walking distance with pentoxifylline. Moreover, current studies provide limited knowledge regarding the safety profile and frequency of side effects with pentoxifylline. Therefore, further studies examining the benefits and risks of pentoxifylline are required to determine its clinical utility in patients with symptoms of intermittent claudication.

PERCUTANEOUS REVASCULARIZATION FOR SYMPTOMATIC PAD

Percutaneous Endovascular Interventions

Percutaneous endovascular interventions are recommended for patients with limiting symptoms of claudication who have had an inadequate response to exercise rehabilitation and pharmacologic therapy.[17] These procedures include percutaneous transluminal angioplasty with ballooning, endovascular stenting, and atheroma debulking procedures.[16] Endovascular procedures displace or remove atheromatous plaque obstructing the vessel lumen, resulting in improved vascular blood flow that can relieve claudication symptoms. However, vascular injury and remodeling also occur, which may result in postprocedural vessel thrombosis or recurrent stenosis (FIGURE 94.10). Mechanical forces created during the procedure cause endothelial disruption with subsequent platelet activation and activation of coagulation. Studies have demonstrated elevations in several markers of thrombosis after intervention, including the thrombin–antithrombin complex, D-dimer, fibrinopeptide A, thrombomodulin, and von Willebrand factor.[107–110] With the activation of platelets and procoagulants, the risk of vessel thrombosis is increased in patients during the acute period (<30 days) following intervention.[111] Vessel restenosis occurs in 30% to 50% of patients after balloon angioplasty and is the consequence of three primary mechanisms: elastic arterial recoil, negative remodeling, and excessive SMC proliferation within the vessel intima.[112,113] While endovascular procedures generally achieve high periprocedural success rates, maintaining vessel patency by preventing vessel thrombosis or restenosis remains a challenge.

Patient Selection for Endovascular Intervention

Procedural success is heavily dependent on appropriate patient selection. Short- and long-term outcomes after the intervention are dependent on lesion type, lesion location, and clinical factors. TASC II guideline categorizes aortoiliac and femoral–popliteal lesions separately into four anatomic types based on length and complexity (Tables 94.3a and 3b).[16] Endovascular intervention is primarily utilized for type A and B lesions, although type C lesions are treated with increasing frequency. Long-term vessel patency is highest with revascularization of iliac stenosis compared to infrainguinal disease (i.e., common femoral, superficial femoral, and infrapopliteal arteries). A systematic review by Kandarpa et al.[114] examined initial and long-term vessel patency rates in patients undergoing percutaneous transluminal balloon angioplasty for PAD. The initial success of angioplasty in iliac disease was 91%, with a 4-year vessel patency rate of 58%. Angioplasty of infrainguinal disease had slightly inferior outcomes, with initial and 4-year vessel patency rates of 89% and 49%, respectively. Several factors unique to infrainguinal disease act to decrease vessel patency after endovascular procedures. These include a higher frequency of multiple arterial occlusion levels, biomechanical forces within the arterial tree influencing vessel patency, and poor distal run-off to the site of stenosis.[115] Reduced vessel patency is observed at a higher

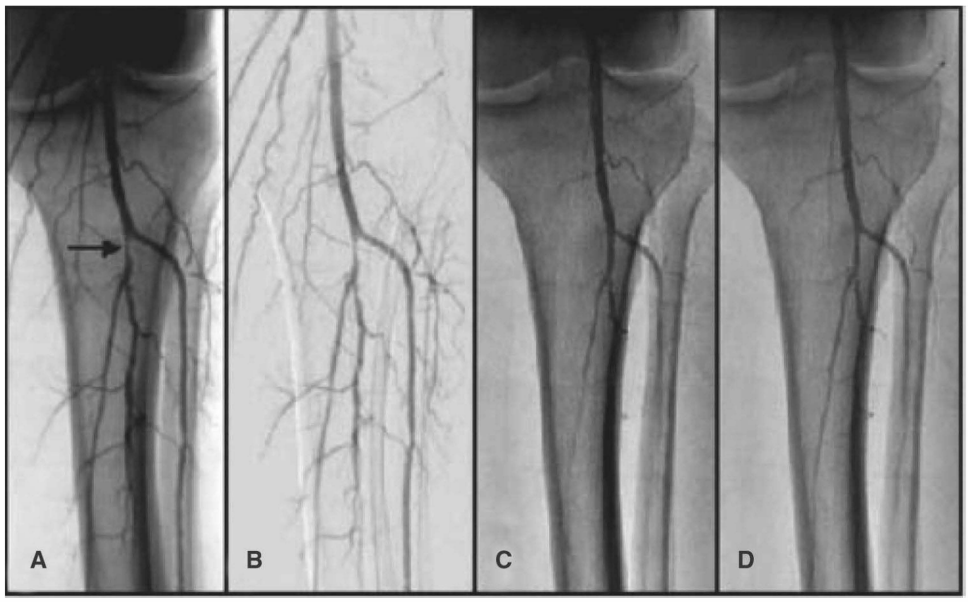


FIGURE 94.10 Infrapopliteal stenosis (**A**, *arrow*) causing lifestyle-limiting claudication. The lesion was treated successfully with balloon angioplasty, resulting in a 20% residual stenosis; however, recurrence of symptoms resulted in repeat angiography, which demonstrated significant restenosis at the site of balloon angioplasty **(B)**, which was treated with a Taxus drug-eluting stent **(C)**. Repeat angiography at 6 months demonstrated a widely patent stent but progression of other disease **(D)**. (From Baim DS. *Grossman's cardiac catheterization, angiography, and intervention*, 7th ed. Philadelphia, PA: Lippincott Williams & Wilkins, 2006.)

Table 94.3a

TASC classification of aortoiliac obstructive lesions

Type A	• Unilateral or bilateral stenoses of CIA • Unilateral or bilateral single short (≤3 cm) stenosis of EIA
Type B	• Short (≤3 cm) stenosis of infrarenal aorta • Unilateral CIA occlusion • Single or multiple stenosis totaling 3–10 cm involving the EIA not extending into the CFA • Unilateral EIA occlusion not involving the origins of internal iliac or CFA
Type C	• Bilateral CIA occlusions • Bilateral EIA stenoses 3–10 cm long not extending into the CFA • Unilateral EIA stenosis extending into the CFA • Unilateral EIA occlusion that involves the origins of internal iliac and/or CFA • Heavily calcified unilateral EIA occlusion with or without involvement of origins of internal iliac and/or CFA
Type D	• Infrarenal aortoiliac occlusion • Diffuse disease involving the aorta and both iliac arteries requiring treatment • Diffuse multiple stenoses involving the unilateral CIA, EIA, and CFA • Unilateral occlusions of both CIA and EIA • Bilateral occlusions of EIA • Iliac stenoses in patients with AAA requiring treatment and not amenable to endograft placement or other lesions requiring open aortic or iliac surgery

CIA, common iliac artery; EIA, external iliac artery; CFA, common femoral artery; AAA, abdominal aortic aneurysm. Reprinted from Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg* 2007;45(1):S5–S67. © 2007, with permission from Elsevier.

Table 94.3b

TASC classification of femoral-popliteal obstructive lesions

Type A	• Single stenosis <10 cm in length • Single occlusion <5 cm in length
Type B	• Multiple lesions (stenoses or occlusions), each <5 cm • Single stenosis or occlusion <15 cm not involving the infrageniculate popliteal artery • Single or multiple lesions in the absence of continuous tibial vessels to improve inflow for a distal bypass • Heavily calcified occlusion <5 cm in length • Single popliteal stenosis
Type C	• Multiple stenoses or occlusions totaling >15 cm with or without heavy calcification • Recurrent stenoses or occlusions that need treatment after two endovascular interventions
Type D	• Chronic total occlusions of CFA or SFA (>20 cm, involving the popliteal artery) • Chronic total occlusion of popliteal artery and proximal trifurcation vessels

CFA, common femoral artery; SFA, superficial femoral artery. Reprinted from Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg* 2007;45(1):S5–S67. © 2007, with permission from Elsevier.

frequency in patients with diabetes, renal failure, smoking, and severe claudication symptoms, and in those presenting with CLI.[17] Thus, maintaining vessel patency after the procedure is heavily dependent on a variety of vascular and clinical factors, and careful patient selection is crucial to optimizing success.

Endovascular Stenting and Atheroma Debulking Procedures

Endovascular stent placement after balloon angioplasty provides additional mechanical support to the vessel lumen, diminishing the effects of elastic recoil and negative vessel remodeling. However, vessel restenosis may still occur through excessive neointimal SMC proliferation. Angioplasty and stent deployment results in mechanical trauma and denudation of the vessel endothelium. Mechanical injury stimulates biologically active factors, promoting the migration and proliferation of vascular SMCs to the vessel intima, and forming a neointimal layer within the stent.[113] Clinical studies support the utilization of endovascular stenting as primary therapy for internal and external iliac artery lesions, and as salvage therapy for more distal lesions with supoptimal balloon angioplasty results. In iliac disease, primary stenting appears to reduce vessel restenosis. A meta-analysis of six studies involving 2,116 patients demonstrated improved long-term patency and similar complication rates with primary stenting for iliac disease compared to percutaneous transluminal angioplasty.[116] Primary stenting of infrainguinal arterial lesions is more challenging as vessel anatomy, external force applied to vessels from adjacent muscles, and axial vessel compression occurring with movement can lead to stent deformation, fracture, and reduced patency.[115] Several studies have failed to show a significant benefit with bare metal stenting compared to percutaneous transluminal angioplasty in the treatment of femoral–popliteal stenosis.[117–120] However, Schillinger et al.[121] demonstrated a benefit with routine nickel–titanium (nitinol) stenting compared to balloon angioplasty with salvage stenting in 104 patients with superficial femoral artery stenosis. Patients treated with routine nitinol stenting had reduced angiographic restenosis at 6 months (24% vs. 43%, $P = 0.05$) and improved maximal walking distance at both 6 (363 m vs. 270 m; $P = 0.04$) and 12 months (387 m vs. 267 m; $P = 0.04$).

A promising method to reduce vessel restenosis currently under evaluation is the use of antineoplastic agents to locally inhibit vascular SMC proliferation. The THUNDER study evaluated the use of paclitaxel-coated angioplasty balloons with nitinol stenting as needed in 154 patients undergoing revascularization for superficial femoral or popliteal disease.[122] Patients undergoing paclitaxel-coated angioplasty had reduced angiographical vessel stenosis at 6 months (late lumen loss 0.4 ± 1.2 mm vs. 1.7 ± 1.8 mm, $P < 0.001$) and target-lesion revascularization at 24 months (15% vs. 52%, $P < 0.001$) compared to the control group. Conversely, the SIROCCO II study failed to show a benefit of sirolimus-coated compared to bare metal stents in 57 patients with superficial femoral artery stenosis.[123] Initial studies examining the routine use of adjunct procedures such as laser or cutting balloon atherectomy have failed to show significant clinical benefits over either percutaneous transluminal angioplasty or stenting; thus, their routine use cannot be recommended in the treatment of PAD until further data are available.

Antiplatelet Therapy during and After Endovascular Intervention

The majority of patients with PAD undergoing endovascular intervention for claudication are already on antiplatelet therapy for cardiovascular risk reduction. However, studies also report benefits with antiplatelet therapy specific to revascularization. The Antithrombotic Trialists' collaboration meta-analysis of 46 studies evaluated the effects of antiplatelet therapy in patients who underwent various vascular procedures. Antiplatelet therapy was associated with a 43% risk reduction in vascular occlusive events in the subset of 3,000 patients who underwent a peripheral vascular

intervention.[124] Either aspirin or clopidogrel are considered appropriate antiplatelet agents after endovascular intervention.

Currently, there are no randomized studies examining the clinical utility of dual antiplatelet therapy after peripheral revascularization. The benefit of dual antiplatelet therapy after peripheral stenting has largely been inferred from surrogate endpoints of platelet studies and clinical trials involving coronary stenting.[125] There are limited data examining clinical outcomes with dual antiplatelet therapy in PAD patients undergoing endovascular intervention. Cassar et al.[126] compared the antiplatelet benefits of clopidogrel with aspirin to aspirin alone in patients undergoing lower limb angioplasty. A significant reduction in platelet fibrinogen binding and p-selectin expression by flow cytometry was demonstrated with dual antiplatelet therapy, indicating improved platelet inhibition. Wilson et al.[112] showed similar antiplatelet effects with dual therapy. However, no significant differences were demonstrated regarding smooth vessel proliferation, assessed *ex vivo* by extracellular signal–regulated kinases 1 and 2 activation, suggesting a mechanism of benefit primary related to reducing thrombotic events rather than vessel restenosis. Despite a lack of evaluation in randomized clinical studies, dual antiplatelet therapy is widely utilized after endovascular intervention, primarily after stent implantation, and many physicians have adopted the use of short-term clopidogrel in addition to indefinite aspirin therapy.

SURGICAL MANAGEMENT OF INTERMITTENT CLAUDICATION

Studies comparing the long-term efficacy of surgical and percutaneous endovascular revascularization have shown comparable outcomes. The BASIL study demonstrated similar 6-month amputation-free survival rates in 452 patients with severe ischemia undergoing surgical bypass or percutaneous endovascular revascularization.[127] Similarly, a randomized study of 263 patients comparing bypass surgery to endovascular revascularization showed no benefit of one therapy over the other with respect to overall survival or graft patency at 4 years.[128] However, both studies selected patients with obstructive lesions that were amenable to either intervention, while many patients who are evaluated in clinical practice have anatomic lesions that favor one revascularization strategy over another. In accordance with the TASC II anatomic classification system, focal and discreet stenoses are generally treated by endovascular therapy, while more complicated lesions and diffuse PAD should be managed surgically.[16]

Indications for surgical revascularization in patients with PAD and claudication are similar to those for endovascular treatment. Surgery is usually considered for appropriate anatomic stenosis and persistent, limiting claudication symptoms despite aggressive medical management.[16] Surgery is reserved for complicated lesions and diffuse disease, which would generally result in worse outcomes with endovascular intervention. As such, TASC II guidelines recommend surgical revascularization for type D anatomic lesions involving the iliac and femoral–popliteal arteries.[16] Although surgery is considered the preferred therapy for type C stenosis, endovascular outcomes for these lesions have improved considerably over time. Moreover, surgery portends a higher morbidity and mortality in the intraoperative and recovery period compared to endovascular procedures. Thus, assessing the patient's comorbidities in addition to anatomic disease severity is crucial for balancing the overall risks and benefits of surgery prior to proceeding with revascularization.

Surgical Management of Aortic–Iliac Disease

Surgical bypass is the preferred treatment for extensive or complicated lesions involving the aortic and iliac arteries. The most common surgical approach involves the implantation of a prosthetic conduit from the infrarenal aorta to the femoral arteries through an intra-abdominal approach. Despite the burden of cardiovascular disease and complexity of patients undergoing surgical bypass, procedural mortality and morbidity are relatively low. A meta-analysis of 23 studies, examining aortoiliac bypass outcomes since 1975, demonstrated an overall operative mortality of 3.3% and morbidity rate of 8.3% with aortobifemoral bypass surgery.[129] Moreover, favorable long-term outcomes have been demonstrated with aortofemoral bypass. For the treatment of claudication, limb patency rates at 5 and 10 years are 91% and 86%, respectively.[129] Smoking remains an important cause of graft failure after surgical revascularization. In a meta-analysis of 29 studies of both suprainguinal and infrainguinal bypass surgery, Willigendael et al.[130] demonstrated a 2.35-fold increase in graft failure in smokers compared to nonsmokers. Alternative bypass techniques are required for patients with unsuitable abdominal anatomy or significant comorbidities, which place them at increased risk with an intra-abdominal approach. In such cases, extra-anatomic bypass techniques, such as axillofemoral bypass using a retroperitoneal approach or crossover femoral bypass, may be considered. The long-term results of extra-anatomic bypass procedures are less favorable compared to aortofemoral bypass. The 5-year patency rates for axillounifemoral, axillobifemoral, and femoral–femoral bypass are 51%, 71%, and 75%, respectively.[16] Consequently, extra-anatomic bypass is reserved for situations where the perioperative risks of an intra-abdominal surgery outweigh the benefits of preserving long-term limb patency.

Surgical arterial endarterectomy is an alternative to bypass in patients with aortoiliac disease, but the procedure is less durable than surgical bypass, with 5-year vessel patency rates of 60% to 94%.[16]

Surgical Management of Infrainguinal Disease

The most important determinant of success following surgical bypass for infrainguinal disease is uncompromised flow to the inflow artery supplying the conduit. Targeting a specific arterial level for anastomosis (i.e., common femoral, superficial femoral, or popliteal) is less important than ensuring whichever inflow artery used is patent. When the inflow artery has obstructive disease, endovascular intervention or endarterectomy may be performed to improve vessel patency prior to distal bypass. The distal anastomosis site should also be based on the quality of the vessel rather on than a specific arterial level. Typically, the tibial or peroneal artery is used for the distal anastomosis, although grafting of the plantar artery has also been performed with success. Kram et al.[131] evaluated the long-term patency of infrainguinal grafting in 217 patients who underwent femoropopliteal surgery with either reverse saphenous vein or prosthetic conduits. The observed 5-year primary and secondary patency rates after femoropopliteal surgery were 61% and 78%, respectively.

Unlike aortoiliac disease, long-term graft patency in infrainguinal disease is dependent on conduit composition. Venous conduits generally have better long-term patency rates compared to prosthetic conduits. Various studies report a 5-year patency rate for above-knee femoropopliteal bypass surgery with venous grafts of 74% to 76%, compared with lower rates using polytetrafluoroethylene grafts of 39% to 52%.[16] The long saphenous vein is the preferred conduit for femoral to below-knee popliteal or distal bypass. The postoperative risk of graft failure remains higher in smokers compared to nonsmokers.[130]

Preliminary studies have reported positive results with the combination of percutaneous and surgical interventions in patients with multilevel PAD. In a study of 110 patients with PAD, combined superficial femoral artery balloon angioplasty with distal popliteal bypass grafting had comparable results to bypass grafting alone in patients with infrainguinal PAD,[132] the overall 5-year primary and secondary patency rates being 63% and 78% respectively, with 81% of patients avoiding major limb amputation. The combination revascularization strategy may become an appealing option for selected patients with multilevel vascular disease not easily amenable to either intervention alone.

Antithrombotic Therapy with Surgical Interventions

Both arterial and venous grafts are subject to thrombosis related to impairments in conduit blood flow, luminal reduction secondary to neointimal hyperplasia, and surface thrombogenicity (with prosthetic conduits).[133] Therefore, a rationale exists for the use of antithrombotic therapy to promote graft patency. In a meta-analysis of five studies involving 423 patients, aspirin therapy reduced graft occlusion following inguinal bypass surgery (RR 0.78, 95% CI 0.64 to 0.95) compared to placebo.[134] Given the beneficial effects of aspirin for maintaining graft patency, and for cardiovascular risk reduction in the PAD population as a whole, it should be considered for all patients undergoing peripheral bypass surgery.

There is limited data supporting the use of oral anticoagulation therapy routinely in patients after peripheral bypass surgery, particularly with the routine use of aspirin. Studies report improved graft patency following peripheral bypass surgery with vitamin K antagonists compared to placebo,[134,135] but these benefits were not reproduced in the large randomized Dutch BOA study of 2,690 patients receiving either aspirin or oral anticoagulation following infrainguinal bypass, in which there were similar graft occlusion rates with either therapy.[63] Subgroup analysis demonstrated that patients receiving venous grafts derived a benefit with oral anticoagulation (RR 0·69, 95% CI 0·54 to 0·88), while aspirin was beneficial for nonvenous grafts (RR 1·26, 95% CI 1·03 to 1·55). Vascular-related death and total mortality did not differ with either therapy, while major bleeding was increased in the oral anticoagulation group. A Veterans Affair study of 831 patients undergoing surgical bypass treated with aspirin with or without a vitamin K inhibitor reported no significant differences in graft patency or vascular-related mortality, with increased bleeding in the dual anticoagulation group.[136] Given the marginal benefits for preventing graft occlusion, and the increased bleeding risk compared to antiplatelet therapy, oral anticoagulation is not recommended for routine use in PAD patients following bypass surgery.[133]

MANAGEMENT OF CHRONIC CRITICAL LIMB ISCHEMIA

CLI is a chronic condition caused by severe flow limitation to the affected limb. Presentations of CLI include resting claudication and ischemia-related leg ulcers or gangrene (FIGURE 94.7). New-onset ischemic neuropathy can exacerbate leg pain, while the presence of long-standing peripheral neuropathy (which occurs in associated conditions such as diabetes) may paradoxically limit claudication symptoms. Thus, the development of arterial ulcers may follow resting claudication symptoms, or may be an initial presentation in patients with long-standing peripheral neuropathies. Considered an "end-stage" manifestation of PAD, CLI is associated with a greater severity of vascular disease, more multisegmental and distal disease, increased risk of leg amputation, and a worse overall prognosis compared to the general PAD population.[137] In a large prospective cohort following 1,586 patients with CLI, the 6-month amputation rate and 1-year mortality rate were 12% and 19.1% respectively.[138]

Once CLI is suspected clinically, the diagnosis should be confirmed by documenting reduced arterial blood flow by ABI, toe systolic pressure, or transcutaneous partial pressure of oxygen ($TcPO_2$) of the extremity. CLI complications typically develop at an ankle pressure of 50 to 70 mm Hg, a toe pressure of 30 to 50 mm Hg, and a $TcPO_2 < 30$ mm Hg.[16] Peripheral angiography may be considered to document severe PAD in indeterminate cases or if revascularization is considered (FIGURE 94.9).

MEDICAL THERAPY FOR CRITICAL LIMB ISCHEMIA

The goal of medical therapy is to reduce claudication pain, heal ischemic lesions, and avoid limb amputation. Unfortunately, patients who undergo medical therapy alone for CLI have a high incidence of adverse outcomes. Marston et al.[139] examined clinical outcomes in 142 patients treated medically for CLI and found a 1-year amputation rate of 38% and continued ischemic ulcers despite medical therapy in 50%; an ABI < 0.5 was the best predictor of a poor clinical outcome.

Secondary Prevention of Cardiovascular Events

All patients with CLI should undergo aggressive secondary prevention treatment, including antiplatelet therapy, blood pressure reduction, management of dyslipidemia, diabetes control, and smoking cessation independent of revascularization status (Table 94.2). There is some evidence supporting improved cardiovascular outcomes associated with aggressive secondary prevention in CLI. In the PREVENT III cohort, statin therapy was associated with an improved 1-year survival in patients who underwent revascularization surgery (hazard ratio 0.71, 95% CI 0.52 to 0.98).[140] The same analysis showed no improvement in survival with β-blockers or antiplatelet therapy, but given the significant benefits of antiplatelet therapy regarding cardiovascular risk reduction and following vascular intervention in the PAD population as a whole, it is recommended that patients with CLI also be treated with an antiplatelet agent.

Prostanoids

Prostanoids demonstrate several vascular actions that may potentially benefit patients with CLI, including antiplatelet and profibrinolytic effects, vasodilation, and improved endothelial function.[141] Despite these theoretical benefits, studies evaluating the role of prostanoids in patients with PAD have demonstrated inconsistent results. A randomized trial of 1,560 patients with CLI demonstrated a significant reduction in the combined endpoint of death, major limb amputation, and cardiovascular events at hospital discharge (RR 0.87, 95% CI 0.81 to 0.93) with prostaglandin E_1 compared with placebo therapy.[142] However, at 6 months, these benefits were no longer statistically significant. Similarly, a meta-analysis of seven randomized studies involving 643 patient with CLI reported a reduction in the outcome of death or amputation-free survival in patients treated with iloprost compared to placebo (22.6% vs. 36.2%, $P = 0.015$).[143] A recent Cochrane systematic review involving 20 studies evaluated the effects of various prostanoids in the management of patients with CLI. Overall, prostanoid therapy was associated with improved ulcer healing (RR 1.54, 95% CI 1.22 to 1.96) and relief of rest pain (RR 1.32, 95% CI 1.10 to 1.57) compared to standard therapy.[141] However, there were no significant differences in overall mortality (RR 1.07, 95% CI 0.65 to 1.75) or leg amputations (RR 0.89, 95% CI 0.76 to 1.04) and increased adverse effects observed with prostanoids (RR 2.35, 95% CI 1.99 to 2.78). In the subgroup of three trials comparing intravenous ilioprost to placebo, significant benefits were observed for relief of rest pain (RR 1.54, 95%CI 1.19 to 1.99), ulcer healing (RR 1.80, 95% CI 1.29 to 2.50), and major amputations (RR 0.69, 95% CI 0.52 to 0.93). No significant differences were observed for total amputations (RR 0.79, 95% CI 0.60 to 1.03), with increased adverse events noted in the ilioprost group (RR 2.05, 95% CI 1.68 to 2.49). Overall, prostanoids appear to provide improved short-term symptom control and ulcer healing in patients with CLI. However, consistent benefits have not been observed in more substantial clinical endpoints, arguing against the routine use of prostanoid therapy in patients with CLI.

Hyperbaric Oxygen Therapy

A Cochrane systemic review evaluated the effects of hyperbaric oxygen therapy (HBOT) in five studies of patients with PAD-related ulcers. Although sample size and methodologic issues limited the analysis, HBOT was associated with a significant improvement in diabetes-related major limb amputation (RR 0.31, 95% CI 0.13 to 0.71).[144] HBOT may be considered in selected CLI patients with limited therapeutic options, such as those who are not candidates for or who have responded poorly to revascularization.

Spinal Cord Stimulation

Spinal cord stimulation (SCS) has been utilized in patients with severe PAD who have failed other medical therapies and are not appropriate candidates for surgery. Although initially used to improve pain symptoms, animal models demonstrated increased vasodilation of the lower extremities, suggesting potential vascular benefits in the healing of ischemia-related ulcers.[145,146] A Cochrane review of six studies evaluated the effects of SCS compared to standard care in patients with inoperable CLI. SCS was associated with improved pain relief and limb salvage at 12 months (RR 0.71, 95% CI 0.56 to 0.90),[147] but no significant improvement in ulcer healing was observed. Limited evidence supports the use of SCS in selected patients with refractory disease and restricted therapeutic options.

REVASCULARIZATION FOR CRITICAL LIMB ISCHEMIA

Revascularization either by surgical bypass or by endovascular intervention is the optimal limb salvaging treatment for patients with CLI. Unfortunately, many patients with CLI are excluded from either or both modes of revascularization due to significant comorbidities, excessive surgical risk, or unsuitable anatomy. For surgical revascularization, risk assessment tools have been developed to help identify patients who will most benefit. The PREVENT CLI III risk score derived from five clinical variables (presence of dialysis, CAD, tissue loss, age >75, and hematocrit ≤30%) accurately predicts adverse cardiovascular events, death, and amputation-free survival following surgical revascularization for CLI.[148] Clinical risk assessment tools may be useful to assist in the appropriate selection of patients for surgical revascularization.[149]

Clinical studies have not established the superiority of surgical or endovascular revascularization techniques for CLI. The BASIL study did not show any significant difference in 5-year time to death or above-ankle limb amputation between revascularization strategies in 452 patients with CLI and infrainguinal vascular disease,[127] but surgery was associated with increased procedure-related morbidity and a longer hospital stay. Conversely, *post hoc* analysis showed that patients who survived without amputation for 2 years after surgical revascularization had a significant long-term reduction in mortality (OR 0·34, 95% CI 0·17 to 0·71) and limb loss (OR 0·37, 95% CI 0·17 to 0·77). Although not definitive, the results of BASIL suggest higher short-term complications but more durable long-term results with surgical revascularization. Following initial revascularization, partial foot amputation may be required in patients with gangrene or nonhealing ulceration after revascularization in order to preserve the rest of the limb.[16] Overall, revascularization in patients with CLI provides the best opportunity for limb preservation, but patients remain at an increased risk for limb loss, morbidity, and mortality.

Major Limb Amputation

Clinical factors associated with major limb amputation (above ankle) include diabetes, chronic renal failure, major tissue loss, and poor ambulatory status.[150] The main indications for primary limb amputation (no prior revascularization attempt) are life-threatening infection of the affected limb, extensive necrosis of the foot, or uncontrolled pain in patients who are not candidates for revascularization,[16] but it is also considered in patients with irreversible flexion contractures, terminal illness, and reduced life expectancy due to significant comorbidities.[137] Secondary amputation occurs in the context of a persistent infection or limb deterioration despite attempted revascularization.

The primary goal of major lower limb amputation is to preserve the limb at the most distal level possible in order to retain functional ability. However, as distal areas of the limb are prone to reduced blood flow, an amputation level must be chosen, which has adequate circulation to foster wound healing and prevent infection. Below-knee amputation is successful in 80% of cases, while above-knee amputation has a 90% success rate.[16] Successful lower amputation levels are generally associated with improved function and survival. Feinglass et al. examined short- and long-term mortality in 4,103 patients undergoing above- and below-knee amputation in Veterans Affairs hospitals from 1991 to 1995. Thirty-day mortality was 6.3% with below-knee amputations compared to 13.3% with above-knee amputations.[151] Three-year survival rates with the below- and above-knee amputation groups were 57% and 39% respectively.

ACUTE LIMB ISCHEMIA

ALI occurs due to a sudden interruption of the blood flow to an extremity, which often threatens limb viability.[152,153] Clinically, patients present with the "five P's" related to ischemia: pain, pallor, pulselessness, paresthesia, and paralysis, but they vary depending on the severity and duration of ischemia.[154–156] Mortality in ALI is high, from 15% to 20%,[16] and patients are at risk for permanent tissue loss and neuromuscular complications. Thus, early detection of ALI is essential, as expeditious management can improve limb salvage, while delayed recognition increases the risk for limb complications and overall mortality.

Etiology of Acute Limb Ischemia

Peripheral embolization and native thrombotic vascular occlusion are the two most common causes of ALI, additional causes being traumatic injury, postendovascular procedure thrombosis, and vascular graft-related thrombosis. Approximately 80% to 90% of peripheral embolic events are cardiac in origin, arising from thrombi secondary to postmyocardial infarction ventricular aneurysms, atrial fibrillation, native valvular disease, or prosthetic valves;[156] emboli also occur with proximal peripheral aneurysms, dislodgement of ulcerated arterial plaques, paradoxical embolization, and following endovascular procedures or vascular surgery.[133]

Unlike ALI secondary to an embolic event, thrombotic vascular occlusion is commonly associated with significant atherosclerosis. Due to the relatively protracted process of atheromatous plaque formation, progressive luminal obstruction, and collateral vessel formation, the presentation of ALI secondary to thrombosis may be delayed and less symptomatic. However, a thrombotic event occurring prior to adequate collateral formation will usually present with severe symptoms, similar to an embolic event.[156]

Initial Evaluation and Risk Stratification

The initial assessment of a patient with suspected ALI is focused on ensuring clinical stability, assessment for an etiology, and evaluating the severity of ischemia. In clinical practice, the degree of ischemia supersedes the duration of ischemia and is dependent on the anatomic level of obstruction, underlying cause, collateral circulation, and thrombus propagation. Although 6 hours has been accepted as the maximum time period of ischemia beyond which irreversible necrosis occurs, tolerance of skeletal muscle to ischemia is variable, as most patients do not have complete cessation of blood flow in the axial and collateral vessels. Not surprisingly, acceptable revascularization results have been achieved after relatively long periods of ischemia.[157,158]

The Rutherford classification is frequently utilized for initial risk stratification in patients presenting with ALI. Based on symptoms and physical examination, the affected limb is classified into one of three categories: viable (class I), threatened viability (class IIA and IIB), and irreversible ischemia (class III)[152,159] (Table 94.4 and FIGURE 94.11). When ALI is clinically suspected, impairment of vascular flow should be confirmed by Doppler ultrasonography of the peripheral pulses. Angiography by computed tomography, magnetic resonance imaging, or invasive methods may also be performed to visualize the site of obstruction, determine the extent of the disease, and guide revascularization decisions.[152]

Table 94.4

Classification of acute limb ischemia

		Findings		Doppler Examination	
Category	**Description/Prognosis**	**Sensory Loss**	**Muscle Weakness**	**Arterial**	**Venous**
I. Viable	Not immediately threatened	None	None	Audible	Audible
II. Threatened					
a. Marginally	Salvageable if promptly treated	None or minimal (toes)	None	Inaudible	Audible
b. Immediately	Salvageable with immediate revascularization	More than toes, associated with rest pain	Mild, moderate	Inaudible	Audible
III. Irreversible	Major tissue loss, permanent nerve damage inevitable	Profound, anesthetic	Profound, paralysis (rigor)	Inaudible	Inaudible

Reprinted from Rutherford RB, Baker JD, Ernst C, et al. Recommended standards for reports dealing with lower extremity ischemia: revised version. *J Vasc Surg* 1997;26(3):517–538. © 1997, with permission from Elsevier.

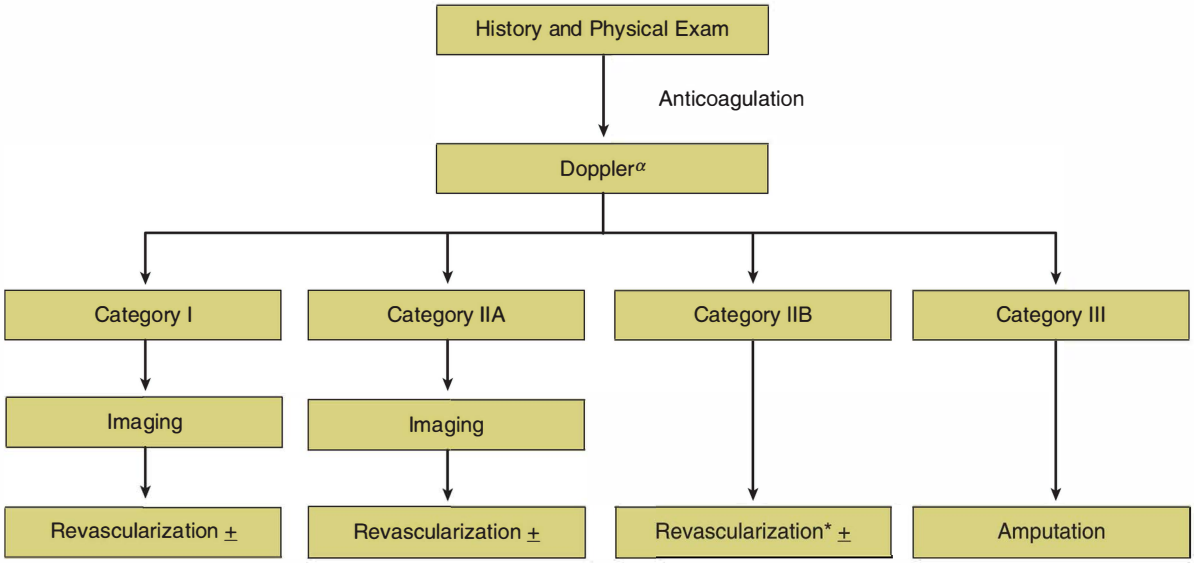


FIGURE 94.11 Management of patients presenting with ALI. Category I—viable, category IIa—marginally threatened, category IIB—immediately threatened; α confirming either absent or severely diminished ankle pressure/signals; *In some centers imaging would be performed. (Reprinted from Norgren L, Hiatt WR, Dormandy JA, et al. Inter-society consensus for the management of peripheral arterial disease (TASC II). *J Vasc Surg* 2007;45(1):S5–S67. © 2007, with permission from Elsevier.)

Anticoagulation in Acute Limb Ischemia

The goal of ALI therapy is to prevent further thrombosis or embolism, and ultimately restore adequate reperfusion to the affected limb.[16] Therefore, immediate anticoagulation therapy should be considered for all patients presenting with ALI, preferably with unfractionated heparin,[160] although randomized studies demonstrating a benefit of anticoagulation therapy in ALI are lacking. Several case series have supported this practice, reporting higher limb salvage and lower mortality rates in patients treated with anticoagulantion.[161,162]

Revascularization Strategies for Acute Limb Ischemia

Restoration of blood flow to the acutely ischemic limb can be accomplished either by surgical revascularization or endovascular procedures. Surgical thrombectomy involves open dissection and the direct removal of thrombus from the site of occlusion. A focal atherosclerotic lesion at the prior site of thrombosis can be treated with surgical revision and patch angioplasty, while more diffuse atherosclerotic disease warrants surgical bypass.[159] The primary percutaneous endovascular procedure for thrombus removal in ALI is catheter-directed thrombolysis; additional procedures include percutaneous aspiration thrombectomy and percutaneous mechanical thrombectomy (utilizing hydrodynamic recirculation to dissolve and evacuate thrombus).[16] Percutaneous angioplasty may be performed to provide long-term revascularization if a residual obstructive lesion is present at the site of occlusion. Five clinical trials have compared surgery and catheter-directed thrombolysis for the initial management of ALI.[163–165,166,167] The largest trial, TOPAS, evaluated reperfusion with catheter-directed urokinase or surgery in 548 patients with ALI of <14 days in duration involving either a native artery or bypass graft.[166] The primary endpoint of amputation-free survival was similar in the thrombolysis and surgical groups at 6 months (71.8% vs. 74.8%, $P = 0.43$) and 1 year (65.0% vs. 69.9%, $P = 0.23$). The thrombolysis group underwent significantly less open surgical procedures, although major hemorrhagic complications (including intracranial hemorrhage) occurred more frequently compared to surgery. Similar results were obtained in a meta-analysis of these five trials (including TOPAS) involving 1,283 patients with ALI. Overall, there was no significant difference between the two groups regarding limb salvage at 30 days (OR 1.21, 95% CI 0.74 to 1.99), at 6 months (OR 0.86, 95% CI 0.59 to 1.26), and at 1 year (OR 0.88, 95% CI 0.62 to 1.23). Moreover, overall mortality was similar at 30 days (OR 0.58, 95% CI 0.30 to 1.33), at 6 months (OR 1.35, 95% CI 0.83 to 2.18), and at 1 year (OR 0.87, 95% CI 0.61 to 1.25).[168] However, a higher incidence of stroke (OR 6.41, 95% CI 1.57 to 26.22), major hemorrhage (OR 2.80, 95% CI 1.7 to 4.6), and distal embolization (OR 8.35, 95% CI 4.47 to 15.58) was observed with catheter-directed thrombolysis. Studies comparing the two strategies have demonstrated no clear advantage of one therapy over the other, emphasizing the need to weigh the risks of hemorrhagic complications due to thrombolysis against those related to surgery.

Percutaneous Catheter-Directed Thrombolysis

Catheter-directed thrombolysis is an excellent option for the initial treatment of patients with mild-to-moderate limb ischemia, those with significant medical comorbidities in whom surgery presents too high a risk, and in those with multiple previous surgical interventions.[152,159] Potential advantages of catheter-directed thrombolysis include low pressure reperfusion, which may reduce reperfusion injury, and additional fibrinolysis in branch vessels that are too small to be treated with other

revascularization methods.[16] Although streptokinase was initially utilized during catheter-directed thrombolysis, newer thrombolytic agents such as urokinase and recombinant tissue plasminogen activator (rt-PA) have demonstrated a more rapid response, improved safety, and greater efficacy.[169] Additional thrombolytic agents such as reteplase, prourokinase, tenecteplase, recombinant staphylokinase, and alfimeprase have undergone limited evaluation in patients with PAD.[160,169]

Several randomized trials have compared the efficacy of various thrombolytic strategies in patients with critical ischemia. Berridge et al.[170] compared treatment with intravenous rt-PA, intra-arterial rt-PA, or intra-arterial streptokinase in 60 patients with symptoms of CLI for <30 days. Intra-arterial rt-TPA was associated with an improved mean ABI and a lower rate of limb amputation at 30 days compared to the other two treatment strategies.[169,170] Conversely, intravenous thrombolysis was associated with inferior reperfusion outcomes and increased bleeding complications compared to catheter-based thrombolysis.[170]

Three randomized trials have compared intra-arterial urokinase with intra-arterial rt-PA for PAD. Schweizer et al. demonstrated improved vessel patency with rt-PA immediately after thrombolysis and at 6 months.[169,171] Meyerovitz et al.[172] reported more rapid thrombolysis at 8 hours with rt-PA compared to urokinase in patients presenting with native arterial or graft occlusions. Regarding clinical outcomes, there was no difference in mortality or bleeding complications with either rt-PA or urokinase among the three studies. Mahler et al. did note a significantly higher amputation rate in the rt-PA group (OR 0.29, 95% CI 0.08 to 1.06), although this difference was not demonstrated in the other studies.[169,173] Ouriel et al.[174] compared three dosing regimens of intra-arterial prourokinase with intra-arterial urokinase in patients with peripheral arterial occlusions and reported no statistical differences in short-term vessel patency or clinical outcomes. The heterogeneity of these studies and small number of subjects make for difficult conclusions, but the overall results suggest that intra-arterial rt-PA may be more effective intra-arterial streptokinase, while there are no significant differences regarding clinical outcomes between intra-arterial rt-PA and intra-arterial urokinase.[169] The use of systemic intravenous thrombolysis is associated with reduced reperfusion and increased bleeding compared to intra-arterial strategies[170] and is not recommended in patients presenting with ALI.

Selection of the Appropriate Revascularization Strategy

Selecting the appropriate intervention is often done on an individual basis and is dependent on several considerations such as the location and cause of the occlusion, severity of ischemia, patient comorbidities, the presence of contraindications, and local practice patterns.[16] Using anatomic references, suprainguinal occlusions are generally treated surgically, while endovascular methods are favored for the management of infrainguinal disease. Patients presenting with significant ischemic symptoms and threatened viability (Rutherford class IIB) should undergo immediate revascularization.[159] Traditionally, surgery has been the preferred method of revascularization for these patients; however, advances in endovascular techniques have led to the increased use of percutaneous procedures in patients with severe ALI. Due to continuous advancements in endovascular management, time to revascularization has become a less important factor in deciding the appropriate mode of revascularization. Moreover, although endovascular techniques such as catheter-directed thrombolysis may require a longer time period to restore complete patency, a significant improvement in perfusion is seen well before complete thrombus dissolution.[152,159] Patients with Rutherford class I or IIa symptoms generally tolerate the longer reperfusion time required for endovascular catheter thrombolysis and may benefit from the less invasive procedure if significant comorbidities are present. Intravenous anticoagulation alone is also an option for patients presenting with Rutherford class I symptoms who have significant comorbidities or those at a high risk for complications with thrombolytic therapy.

Amputation may be necessary in patients with Rutherford class III signs and symptoms due to irreversible major tissue loss. Major limb amputation occurs in up to 25% of patients presenting with ALI; as initial therapy in 10% of patients presenting with unsalvageable limb necrosis, and in an additional 10% to 15% of patients following failed revascularization.[16]

PAD THERAPY: FUTURE DIRECTIONS

Despite advances in surgical and percutaneous revascularization techniques, a significant amount of patients with PAD require limb amputation, particularly when presenting with CLI. Consequently, novel methods of limb revascularization are currently being examined to improve limb salvage, including stem cell therapy and gene therapy.

Stem Cell and Progenitor Cell Therapy

Bone marrow-derived mononuclear cells (BMMNCs) are important mediators of angiogenesis, stimulating the enlargement of preformed arterioles parallel to a vascular obstruction, and restoring blood flow distally through the development of collateral networks.[175] Impairment of this regenerative process can result in poor collateralization with subsequent limb ischemia. Circulating endothelial progenitor cells are synonymous with BMMNCs and are reduced in conditions associated with poor collateralization in vascular disease, such as advanced age, diabetes, and smoking.[176] A limited number of studies have evaluated BMMNCs in PAD and have reported promising effects. Tateishi-Yuyama et al. conducted an unblinded randomized control study involving 22 patients with bilateral CLI undergoing BMMNC or peripheral blood mononuclear cell injection in each ischemic limb. Four weeks after injection of BMMNC, significant improvements were observed in ABI (0.37 to 0.46 95% CI 0·06 to 0·11; $P < 0·0001$), $TcPO_2$ (28.8 mm O_2 to 46.3 mm O_2, $P < 0.0001$), and ischemic symptoms.[177] Smaller benefits were reported following peripheral mononuclear cell injection. Pooled effects from small studies suggest an increase in ABI between 0.1 and 0.2, 10 to 20 mm O_2 increase in $TcPO_2$, and improved walking distance by 100 to 200 m.[176] However, clinical outcomes require replication in larger randomized studies, such as the BONMOT-CLI study, in order to establish the efficacy of this therapy in PAD.[178]

Gene Therapy

Gene therapy has undergone limited evaluation as an alternate method of therapeutic angiogenesis in patients with PAD. Gene therapy strategies have focused on the transfer of nucleic acids, which promote growth factors, into ischemic tissue to promote

angiogenesis.[179] Lederman et al. reported promising effects with intra-arterial infusion of recombinant fibroblast growth factor-2 (rFGF-2) in 190 patients with intermittent claudication, demonstrating a greater change in peak walking time at 90 days with rFGF-2 compared to placebo (increase of 0.60 minutes with placebo, 1.77 minutes with single-dose therapy, and 1.54 minutes with double-dose therapy, $P = 0.034$).[180] However, subsequent studies of encoded angiogenesis factors have failed to demonstrate improvements in exercise parameters or quality of life compared to placebo.[181,182] Therefore, further studies are needed to adequately characterize the benefits and side effects of gene therapy prior to its clinical use as a revascularization strategy in patients with PAD.

CONCLUSIONS

Many effective therapies are currently available for the management of patients with PAD. Given that mortality in PAD is primarily due to a vascular cause, strategies to reduce cardiovascular risk should be provided to all patients regardless of symptoms. Individuals with intermittent claudication have several lifestyle and medication options available to improve symptoms. Patients with persistent symptoms may benefit from additional surgical or percutaneous revascularization strategies. Those presenting with chronic limb ischemia or ALI are at an increased risk for limb-related complications, amputation, and mortality compared to the general PAD population. In these patients, aggressive revascularization should be sought in order to improve limb-related morbidity and overall survival. However, because many PAD patients have significant comorbidities, which may increase the risks associated with these procedures, careful patient selection for the appropriate revascularization strategy is essential to optimize clinical success. Ongoing advances in medical therapy and revascularization techniques hold the promise for even further improvements in the clinical outcomes of patients with PAD.

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CHAPTER

95 Unusual Sites of Arterial Occlusion

PHILIP A. KALRA, JON G. MOSS, AND PETER WIEDEMANN

UNUSUAL SITES OF ARTERIAL OCCLUSION

In this chapter, we consider occlusive arterial diseases as they affect three separate vascular sites, the renal, gut, and retinal vessels. In the renal vasculature, most efforts have been directed at diagnosing and treating significant renal artery narrowing, as opposed to complete occlusion, whereas the latter is more relevant in the clinical presentation of intestinal or retinal vascular diseases.

RENAL ARTERY STENOSIS AND OCCLUSION

Introduction

Renal artery narrowing, which is usually focal but can be both multiple and more diffuse, is termed renal artery stenosis (RAS), and it is of clinical importance because of its blood flow-limiting effects upon the renal circulation and consequent intrarenal ischemia. This in turn can perturb renal function, blood pressure control, and renal neuroendocrine activity, with clinical manifestations including fluid and salt retention. Interest in RAS accelerated during the 1980s and 1990s with the advent of interventional procedures that could dilate stenotic lesions, providing the possibility that renal revascularization might improve patient outcomes. However, recent trials have shown that such intervention for atherosclerotic lesions, by far the commonest cause of RAS, may not be associated with patient benefit in the majority and that there are still gaps in our knowledge regarding management of the condition.

Etiology

Atheromatous change within the main renal arteries is very commonly seen in ageing populations, but also in younger patients with risk factors or a predisposition for early development of atheroma, and hence atherosclerosis is the cause of over 90% of RAS in Western populations; the remainder is due to fibromuscular disease (FMD). The epidemiology is different in the Indian subcontinent and the Far East, with vasculitis, including Takayasu arteritis, said to be responsible for up to 60% of RAS cases.[1] The latter is discussed in Chapter 100.

As the epidemiology, clinical presentations, and outcomes after treatment of FMD and atherosclerotic RAS differ markedly, they are considered separately, with greatest attention being directed to atherosclerotic renovascular disease (ARVD), which is the preeminent condition.

Fibromuscular Disease

Epidemiology and Pathology

FMD accounts for 5% to 10% of RAS cases. However, autopsy studies and analysis of renal vascular anatomy in healthy renal donors suggest that its prevalence maybe as high as 2% in Western general populations.[2] FMD involves the renal arteries in 60% to 75% of cases, but it can involve other major arteries such as the carotids (in 30% to 60%), vertebral, mesenteric, celiac axis, and coronary vessels.[3] The pathogenesis of FMD is unknown, but there is suspicion that female hormones could be important, given the fact that up to nine times more females than males are diagnosed with the condition. Several different pathologic forms are recognized, the most common of which is medial fibroplasia, typically found in women who present in their third or fourth decades. Here the noninflammatory pathology involves areas of thinning of the intima and media of the vessel wall with loss of the elastic lamina, changes that lead to aneurysm formation. These areas alternate with localized regions of fibrotic narrowing, and so the result is the classical "string of beads" appearance at angiography (FIGURE 95.1). Stenoses tend to occur more distally in the renal circulation than atherosclerotic lesions, and they can be several centimeters in length and also present in multiple vessels; bilateral renal FMD is seen in 30% to 50% renal cases.

Clinical Picture

The classical presentation of FMD is with hypertension and usually well-preserved renal function in young adults, although there is a growing consensus that many cases present in later life. The diagnosis should certainly be considered in young patients (e.g., <35 years old) who present with severe or accelerated-phase hypertension, or in other patients where there is new-onset hypertension, deterioration in previously stable hypertension, or renal functional deterioration in patients treated with renin angiotensin blocking (RAB) agents. An abdominal bruit may be detected, but awareness of the possibility that neurologic features, mesenteric angina, or claudication may be manifestations of FMD at nonrenal arterial sites is important. Progressive narrowing of the renal arteries may be seen in a third of patients over time, but progression to complete occlusion is rare.

Management

The management of patients with fibromuscular RAS is centered upon control of hypertension. Medical therapy, most appropriately utilizing RAB agents, may be suitable in the majority. Renal revascularization with percutaneous renal angioplasty (PTRA)

FIGURE 95.1 FMD of the right renal artery. Typical "string of beads" appearance associated with medial fibroplasia.

is indicated for patients in whom blood pressure cannot be controlled to target via medical therapy, in those with accelerated hypertension, and in those intolerant of RAB. Unlike the situation for atherosclerotic RAS, success rates for hypertension control after PTRA are good in FMD, with cure (i.e., no further need for antihypertensive drugs) observed in 30% to 60% and an improvement in blood pressure control in the majority of the remaining cases.[4] The 10% of patients unresponsive to PTRA are more likely to include those with multiple FMD lesions or with intrarenal disease.

Atherosclerotic Renovascular Disease

Atherosclerotic narrowing of the renal arteries is by far the commonest cause of RAS.

The renovascular disease usually develops as part of a systemic inflammatory atheromatous syndrome and is accompanied by macrovascular disease in several other important vascular beds, notably the coronary, aortoiliac, and iliofemoral circulations. The term ARVD is preferentially used to describe all patients with atherosclerotic renal disease as around 40% have evidence of renal artery occlusion (RAO) at first clinical presentation. About 90% of RAS lesions are "ostial," that is occurring within 1 cm of the origin of the renal artery, and around 35% of patients have bilateral disease.[5]

Epidemiology and Risk Factors

ARVD is a disease of ageing. In our own local renovascular database, which comprises almost 1,000 patients with ARVD referred from a 1.5 million population over 20 years, the median age of the population is 70 years with 46% of patients in their seventh decade of life and 87.1% of patients being aged >60 years at study entry.[6] As would be expected, prevalence is increased in smokers and in patients with type 2 diabetes, and epidemiologic studies demonstrate that about 30% of typical ARVD cohorts are diabetic.[7] Although there is no clear association of ARVD with hyperlipidemia, the lipoprotein profile is similar to that found in other vascular diseases, with a very low apolipoprotein A1 concentration but only slight elevations of total and LDL cholesterol.[8]

The epidemiology of ARVD differs according to the studied population. Many patients have asymptomatic ARVD that is only identified during the investigation of other arterial conditions, and so an accurate prevalence figure for the general population is unknown. However, in North Carolina, 6.8% of 834 community-based people aged >65 years were shown to have incidental RAS of >60% by Doppler imaging.[9] Approximately, 1 in 200 (0.54%) US citizens aged over 65 years and receiving Medicare had a diagnosis of ARVD in 1998 to 1999,[7] but the prevalence is far greater in groups enriched with vascular disease, such as those with coronary artery disease (CAD) or peripheral vascular disease (PVD). Medicare data derive from the 5% annual random sample of patients aged >65 years available for analysis. New cases of ARVD were diagnosed with an incidence of 3.7 per 1,000 patient years (0.37% per year) in 2000 to 2001.[7] A study of 12 years of Medicare data (1992 to 2004) has shown a 3.35 times increase in ARVD diagnosis over the period, presumably reflecting a change in investigational and screening strategies rather than any change in frequency of disease development.[10]

Association of ARVD with Other Vascular Diseases

ARVD is often identified as an incidental or asymptomatic pathology in patients with other vascular diseases, and so it is of particular interest to the disciplines of cardiology, vascular surgery, and hypertension. Many studies have been undertaken in selected groups of patients who are likely to be enriched with ARVD. The most extensive interest in the association of ARVD with CAD has been developed by the group in Duke University Medical Centre in patients presenting predominantly with angina or myocardial infarction.[11] Up to 11% of these patients had significant unilateral RAS and 4% bilateral RAS >50% when investigated with abdominal aortography concomitant with coronary angiography. Patients with greater extent of CAD have greatest severity of RAS. As will be discussed later, the presence of RAS in this population indicates an adverse prognosis, which in part resulted in cardiologic enthusiasm to detect ("shoot the renals") and perform endovascular intervention ("drive by angioplasty") of RAS lesions during coronary investigation. In view of the frequent association of ARVD with CAD and hypertension, it is little surprise that structural heart disease can be detected in many patients with RAS and that syndromes of cardiac dysfunction are highly prevalent. Interest in the relationship between congestive heart failure (CHF) and RAS has been increasing, and estimates suggest that 30% to 50% of older CHF patients may have ARVD.[12,13] Clinical presentations with sudden-onset or "flash" pulmonary edema can be life-threatening and may be the first indication of ARVD in up to 10% of patients.[14] Its true incidence is most probably underestimated due to the fact that renovascular investigation is undertaken in a minority of patients with abrupt-onset left-ventricular failure. Patients with bilateral significant RAS or a solitary functioning kidney are those at most risk of this condition, which is one of the accepted indications for renal revascularization. As would be anticipated due to anatomical relationships, aortoiliac disease is

frequently associated with ARVD, with RAS often being detected incidentally during vascular surgical investigation. The prevalence of ARVD in patients undergoing aortography for intermittent claudication has been found to be 30% to 45%, and the presence of ≥60% RAS in patients with PVD has been associated with a 2.5-fold increased risk for major cardiovascular events and death during follow-up.[15] Carotid disease also frequently coexists with ARVD; in a Japanese study involving 729 high-risk cardiovascular patients, 22% of those with carotid artery stenosis had RAS. Conversely, carotid disease has been demonstrated in 46% of patients with RAS.[16] The above macrovascular disease prevalence figures are further emphasized in studies of the US Medicare population, and a study of ARVD patients in 20 hospitals in Spain found PVD present in 67.5%, CAD 44.8%, heart failure 32.6%, and stroke in 27.3%.[17]

ARVD Prevalence in Other Populations

In selected hypertensive populations, up to 2% of patients can be shown to have RAS, but it is quite likely that the lesion is not causal in most and that "renovascular hypertension" exists only in the minority. This assertion is based upon the fact that improvements in blood pressure are the exception rather than the rule after revascularization in the majority of patients with RAS, whereas a rigorous definition of renovascular hypertension would be cure or substantial improvement in hypertension after such procedures. Similarly, although impaired renal function is found in most patients with ARVD, its etiology is usually related to other factors causing renal injury, such as hypertension and an atherogenic milieu that predate RAS development, and the hemodynamic effects of a RAS may not necessarily be pathogenetically important. Up to 15% of patients with end-stage kidney disease (ESKD) on dialysis programs have ARVD, but in many cases the disease is a comorbid association rather than a cause of the ESKD.[18] Despite this, awareness of the association is important as some cases of ARVD will present subacutely with ESKD, and revascularization of critical RAS can occasionally lead to independence from a future of dialysis. Patients with ESKD have the poorest prognosis of all ARVD patients, presumably because of their extensive vascular comorbidities, and this will be discussed later.

Clinical Presentations of ARVD

A large proportion of patients diagnosed with ARVD are asymptomatic from the condition, and their disease is identified during investigation of other diseases (e.g., during coronary or peripheral angiography). Others are identified after referral to specific clinics (e.g., nephrology, hypertension, or heart failure clinics), whereas a minority are diagnosed during an acute hospital admission (e.g., acute kidney injury [AKI], pulmonary edema). Hypertension is usually present in patients with anatomically significant RAS; data from the angioplasty and stenting for renal artery lesions (ASTRAL) trial show that 98% of ARVD patients had this diagnosis at enrolment.[19] The typical pattern is of systolic hypertension with low diastolic pressure and widened pulse pressure, often resistant to medical therapy. Perhaps 2% of ARVD presentations are with AKI, the most likely scenarios being bilateral critical RAS (e.g., >95% RAS), acute RAO, in association with accelerated-phase hypertension or with cholesterol atheroembolization in patients with severe aortic atheroma, radiocontrast injury as a result of angiographic procedures or, most commonly, following the use of RAB therapy.

The most frequent nephrologic presentation is with asymptomatic chronic kidney disease (CKD), usually associated with hypertension, in patients referred to the nephrology clinic. The link between ARVD and sudden-onset heart failure, or CHF, has already been described, but many additional patients with primarily diastolic heart failure and hypertension have underlying contributory RAS. With all the above clinical presentations, a key pointer to the diagnosis of RAS is the presence of arterial bruits (epigastric and femoral).

Diagnosis of ARVD

ARVD should be suspected in patients with hypertension and/or CKD and any of the following:

- Audible vascular bruits
- >1.5 cm disparity in bipolar renal length on renal imaging
- Unexplained pulmonary edema
- Unexplained CKD in those with evidence of other vascular disease
- Significant deterioration of renal function in association with RAB therapy

There are several imaging techniques that can be used to diagnose RAS (FIGURE 95.2). Magnetic resonance angiography (MRA) has a sensitivity/specificity for detection of RAS exceeding 90%, but it can overestimate the degree of RAS. Contrast MRA should be avoided where possible in patients with stage 4 or worse CKD (glomerular filtration rate [GFR] of <30 mL/min) because of the risk of nephrogenic systemic fibrosis, a syndrome that can

FIGURE 95.2 Intra-arterial digital subtraction angiogram showing severe bilateral atherosclerotic renal artery stenosis (*arrowheads*).

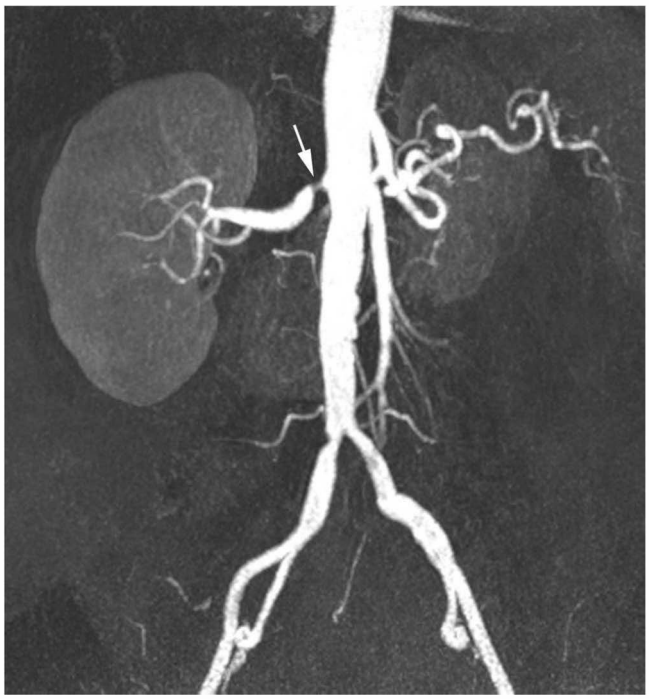

FIGURE 95.3 CT renal angiogram showing significant right atherosclerotic RAS (*arrowhead*).

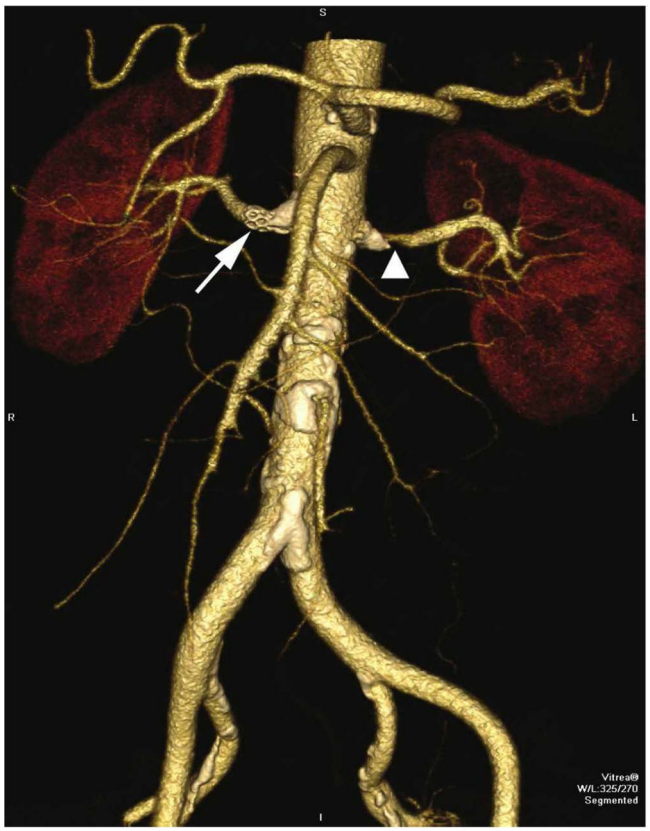

FIGURE 95.4 Spiral CT scan showing the quality of three-dimensional postprocess imaging. There is a left RAS (*arrowhead*) and a right renal artery stent (*arrow*).

be induced by certain preparations of gadolinium.[20] Computed tomography angiography (CTA) or multislice CT has similar sensitivity/specificity to MRA for RAS detection and evaluation (FIGURES 95.3 and 95.4). Its main limitation is risk of contrast nephropathy in patients with advanced CKD.[21] The likelihood of this complication occurring can be minimized by prophylactic protocols involving intravenous fluid infusion. Duplex ultrasonography is highly sensitive for the detection of RAS, and the Doppler waveforms provide information on intrarenal vascular resistance, which provides some indication of the extent of intrarenal parenchymal disease.[22] Limitations of the technique are patient body habitus and its operator dependency, but it is completely risk free and is the best screening tool for RAS in patients with advanced CKD. Conventional renal angiography (usually intra-arterial angiography) is utilized only to confirm the presence of RAS at the time of revascularization procedures, or in diagnosis of more complicated or uncertain cases. It only provides two-dimensional images, it is invasive, and use of contrast carries a risk of contrast nephropathy.

Treatment of ARVD

Medical Therapy

ARVD occurs as part of a diffuse inflammatory vascular disease process, and extrarenal vascular pathology (e.g., CAD, heart failure, cerebrovascular accident) is a major determinant of the poor outcome of the patients. Hence, although there is no evidence base to guide optimal vascular protective therapy, a sensible approach is to advise smoking cessation, and treatment with antiplatelet therapy and statins. The latter can stabilize atherosclerotic plaques, and there is also some evidence that they can ameliorate progression or even induce regression of atherosclerotic RAS.[23] The target blood pressure of <130/80 should be sought, but this may only be attained in a minority of patients, even when multiple antihypertensive agents are used in combination. Use of RAB therapy, although seemingly counterintuitive, is actually the most sensible approach for the majority of ARVD patients, especially for those with coexisting CAD and cardiac dysfunction, or proteinuria (indicative of chronic renal parenchymal injury). Careful monitoring of renal function is obviously required, particularly in patients with significant bilateral RAS, or RAS affecting a solitary kidney.

Renal Revascularization

Revascularization procedures have been undertaken to restore renal artery patency in patients with RAS for almost three decades, the rationale for treatment usually being one of the following:

- Control of severe hypertension that is unresponsive to medical therapy (e.g., blood pressure >160/90 despite four antihypertensive drugs)
- Improvement in serious associated cardiac disease, such as flash pulmonary edema
- Presentation with AKI
- Preservation of functioning renal mass by preventing RAO and renal atrophy in patients with severe RAS
- Ameliorating progressive deterioration of function in CKD

Although surgical reconstruction is still performed for complicated anatomical cases, including some cases of RAS in association with abdominal aneurysm, it has largely been replaced by percutaneous interventional techniques (angioplasty and/or stenting), which now account for >98% of all revascularization procedures.[10] Careful individualized patient evaluation is necessary as complications can occur in 5% to 10% of procedures. These are most frequently of minor seriousness (e.g., groin hematoma) or they resolve rapidly (e.g., contrast nephropathy); more serious complications such as arterial rupture, embolization or thrombosis, or significant cholesterol embolization may affect up to 2%.[24]

Despite the ready availability of interventional procedures for RAS, highlighted by the fact that 16% of Medicare patients diagnosed with ARVD during the last decade underwent a procedure within 6 months of diagnosis,[7,10] there is only limited evidence to suggest that patients benefit from revascularization. There have been five published randomized clinical trials (RCTs) investigating outcomes after percutaneous revascularization in patients with ARVD.[19,25,26–28] A meta-analysis of the first three small RCTs showed that angioplasty was associated with slightly improved systolic blood pressure control, but with no benefit to renal function.[29] The Dutch-led STAR study included 140 ARVD patients randomized equally to medical therapy or to stenting.[25] No benefit was seen with revascularization for the main outcome measure of reduced GFR, but the study was underpowered, especially as >25% of the patients randomized to revascularization did not receive the procedure; the intra- and periprocedural mortality rate was 5% in those receiving a stent. To date, the most robust evidence derives from the UK-led ASTRAL trial, in which 806 patients with significant RAS were randomized to medical therapy or medical therapy plus percutaneous revascularization.[19] The intervention led to no overall worthwhile clinical improvements in renal function (the primary outcome measure), blood pressure control, renal or cardiovascular events (10% per year in both arms), or mortality (8% per year) during a median follow-up period of 34 months. However, a serious adverse event rate of 6.8% was observed in revascularized patients. Despite the unequivocal results of ASTRAL, interpretation of the trial results must be considered in light of the investigated population. A large proportion of the ASTRAL patients had relatively asymptomatic or stable disease, presenting with CKD and hypertension, and very few patients presenting with conditions such as AKI or flash pulmonary edema were randomized as most of these would have received revascularization outside of the trial. Also, only a minority of patients (12%) had "rapidly deteriorating" renal function prior to study entry.

Notwithstanding the value and methodologic reliability of the RCT, there is a realization that overall grouped RCT results may disguise benefits in minority groups of patients. Hence, the results from some of the many retrospective, observational, or nonrandomized studies that have been reported in the literature are of value, although the latter are often flawed by selection and reporting bias. Some studies suggest that hypertension control can be improved in 50% to 85%[30] of patients, but these experiences are not uniform. There has been even more variability when considering renal function as the main outcome, but a summary of published studies suggests that overall 25% of patients show renal functional improvement, 25% deteriorate, and 50% remain unchanged following revascularization.[31–33] It is possible that intrarenal atheroembolism may be contributory for some of the patients who deteriorate, and studies using distal embolic protection in high-risk patients have shown a high yield of embolic material in the filter baskets.[34] A few investigators have shown that distal protection embolic devices benefit renal functional outcome,[35] but this has not been translated into widespread usage within the renal stenting community.

The following summarizes the current viewpoint regarding indication for revascularization therapy for ARVD[36]:

- Revascularization should not be recommended for patients with incidental or asymptomatic atherosclerotic RAS
- There is no evidence that revascularization benefits patients with stable CKD or hypertension
- Revascularization is indicated in those patients with ARVD and AKI or flash pulmonary edema (consensus view)
- Some clinicians would also recommend it being utilized in patients with RAS and very resistant hypertension, in those with rapidly deteriorating renal function, or in those intolerant of RAB, although no trial evidence is available to support this

Prognosis of ARVD

Before the introduction of statins, small studies showed that RAS lesions were likely to progress to more severe RAS or RAO. For example, an arteriographic study from the 1980s showed that progression of RAS occurred in 44% of patients, with progression to RAO in around 10% per year.[37] Hemodynamically significant RAS lesions also lead to renal atrophy; in a prospective duplex ultrasound study, the 2-year cumulative incidence of renal atrophy (defined as >1cm shrinkage in bipolar length) was 20% in patients with RAS > 60%, but interestingly atrophy was still seen in 5.5% of kidneys with normal renal arteries, the latter most prominent in patients with elevated systolic blood pressure.[38] Atrophy was associated with an increase in serum creatinine, which emphasizes the role of renal shrinkage in progression of renal impairment. Although few studies have examined the influence of statin therapy upon RAS progression, current rates of progression are undoubtedly less than that observed in the earlier studies.[23]

The severity of RAS often bears no relation to the extent of intrarenal injury and the resulting renal dysfunction.[39,40] Some patients with a severe RAS do develop a drop in renal perfusion and consequent impaired renal function simply via a hydraulic effect, but this does not explain why patients with unilateral RAS also develop renal failure or why isotope studies of individual kidney function can find similar or even worse function in the contralateral kidney supplied by a normal renal artery compared to the severe RAS kidney.[41] These observations explain why renal function in patients with severe RAS may only improve in the minority after revascularization and emphasize the importance of preexisting intrarenal parenchymal damage. Data from ASTRAL show a low rate of progressive renal dysfunction once the diagnosis of ARVD has been established. In the 403 patients randomized to medical therapy, a deterioration of 10% in renal function was noted over the first year, but only a further 1% renal functional deterioration occurred during the succeeding 3 years.[19] Analysis of our large cohort of ARVD patients in Salford shows that annual rates of progression to ESRD are low and have diminished over time with 3.2% in 1990

to 1995, 3.5% in 1995 to 2000, 2.3% in 2000 to 2005, and only 0.8% for patients diagnosed since 2005.[6] Those patients who progressed to dialysis had significantly more proteinuria and interestingly were less likely to have been treated with RAB or statins than the patients who did not progress, but these data are undoubtedly confounded by selection bias.

Patients with ARVD have a high mortality, which is largely related to their extensive vascular comorbidities and heart disease. ARVD patients in studies of Medicare had an annual mortality rate of 16.3% a decade ago, nearly six times greater than risk of developing ESKD, and three times greater than that of the non-ARVD population.[7] The major determinants of mortality seem to be worse level of baseline renal function,[42] greater proteinuria,[43] and the extent of extrarenal macrovascular disease. However, with modern vasculoprotective therapy, the survival of ARVD patients may be improving, and in ASTRAL, in which patients were followed until 2008, the overall annual mortality was 8%.[19] Once patients with ARVD reach dialysis, they have an even worse prognosis; in a large study of the US dialysis population, the annual mortality rate was around 35% in patients with ARVD, although this was no greater than the crude mortality of all other dialysis patients.[18]

Conversely, the presence of RAS confers a worse prognosis in patients with other vascular diseases. In patients with symptomatic PVD, those with severe RAS had a 2.9-fold increased risk of death compared to patients free of the condition, whereas the presence of RAS reduces survival of patients with CAD by about a third.[44] The severity of RAS was of prognostic significance in this study, patients with 50% RAS having a 4-year survival of 70%, compared to 48% for patients with >95% RAS.

MESENTERIC ISCHEMIA

Introduction

Mesenteric ischemia is a relatively rare condition, challenging to both diagnose and treat. There have been several advances over the last 20 years in particular regarding imaging and endovascular treatment. However, mesenteric ischemia still remains a serious clinical condition often diagnosed too late and having a high mortality. The pathology is usually atherosclerotic, and it frequently coexists with other vascular comorbidity. Increasingly, the diagnosis is being made in the elderly patient who is often a high-risk surgical candidate.

The broad classification is by the clinical presentation, that is, acute or chronic. Some cases of mesenteric ischemia are due to venous thrombosis, but these are discussed in Chapter 84.

Classification of Mesenteric Ischemia

- Acute mesenteric ischemia 50%
- Chronic mesenteric ischemia 35%
- Mesenteric venous thrombosis 5% to 15%

Pathophysiology

The gut is supplied by three arteries, which supply the foregut (celiac axis), midgut (superior mesenteric artery, SMA), and hindgut (inferior mesenteric artery, IMA), respectively. There is a common but variable anastomosis between these vessels through the pancreaticoduodenal arcade and marginal artery of Drummond. There is an additional communication between the internal iliac arteries and the IMA around the rectum. As a result of this collateral circulation, the gut can tolerate gradual occlusion of one and usually two of its three arteries without any clinical sequelae. However, complications will ensue if the collateral supply is disrupted, for example, after previous abdominal surgery, or when the arterial occlusion is abrupt, as with an acute SMA embolus. The cause of pain in visceral ischemia is probably due to lactate build-up as in other vascular beds, although it has been postulated that the pain may be due to a vascular steal phenomenon that deprives the stomach of blood.[45]

Although the vast majority of cases involve an arterial stenosis or occlusion, mesenteric ischemia can occur in the presence of normal vessels when there is a low-flow situation, such as in cardiogenic shock.

Acute Mesenteric Ischemia

Acute mesenteric ischemia affects females more often than males, and the mean age at presentation is 67 years. Patients almost always present as an "acute abdomen," and the pain is severe and said to be out of proportion to the clinical signs. There is often vomiting and diarrhea, and there may be a history of a previous embolic event or an arrhythmia (most commonly atrial fibrillation). The typical clinical course involves rapid deterioration of clinical status over a few hours with accompanying leukocytosis, acidosis, and endotoxemia. Breakdown of the mucosal gut barrier is followed by transmural necrosis. The SMA is the most frequently involved vessel. The etiology is often an embolus of cardiac origin, but thrombosis of an underlying stenosis can occur with plaque rupture. Other causes to consider include aortic dissection, when the dissection extends into a visceral vessel, or iatrogenic causes such as from a recent endovascular aortic procedure, for example, renal or aortic stenting, or ligation of the IMA during open aortic surgery. The diagnosis is made by maintaining a high index of suspicion and requesting urgent CTA or MRA. The diagnosis will be made in many cases as a result of a conventional CT scan performed for investigation of an acute abdomen (FIGURE 95.5), as the advances in imaging have made delineation of the visceral vessels simple.

Although there are reports of successful endovascular treatment, acute mesenteric ischemia remains a surgical emergency. The viability of the bowel is often in doubt, and this cannot be reliably assessed by CT at present. The goal of treatment is to reestablish antegrade flow in the occluded artery, and this may be achieved using a Fogarty embolectomy catheter. Bypass grafts may be necessary utilizing either an aortic or iliac donor site. If there is peritoneal contamination (due to loss of the mucosal gut barrier), then prosthetic grafts should be avoided. Unfortunately, there is often extensive small-bowel infarction rendering revascularization futile. Even when initial surgery appears to have been successful, there is sometimes a need to reoperate with a "second-look" laparotomy at 24 to 48 hours in order to check the bowel viability. Nonviable segments of small bowel will be resected, and some surviving patients may suffer a short-bowel syndrome and require long-term nutritional support, depending upon the extent of the resections. If the clinical presentation is very early and the clinical signs are favorable, then thrombolytic therapy may resolve the occlusion followed by stenting of any underlying stenosis.[46] However,

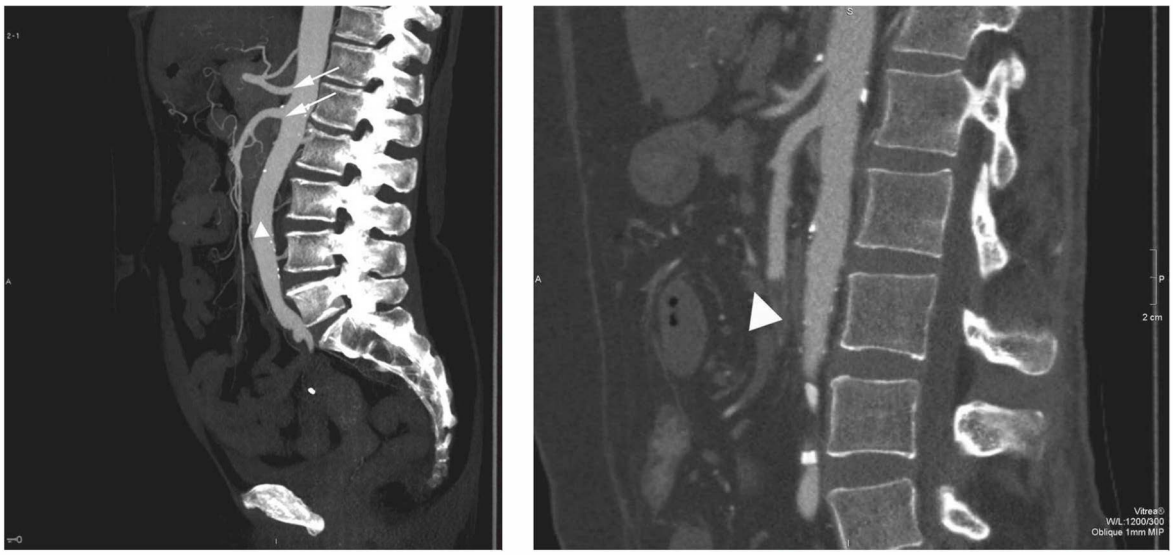

FIGURE 95.5 **A**: Normal CT angiogram in the midsagittal plane showing the celiac axis (*arrow*), SMA (*long arrow*), and IMA (*arrowhead*). All appear normal. **B**: CT angiogram in the midsagittal plane showing a large embolus in the SMA (*arrowhead*). The patient had atrial fibrillation and presented with an acute abdomen.

enthusiasm for this approach should be cautious as many emboli consist of well-organized material that is resistant to thrombolytic agents.

Chronic Mesenteric Ischemia

There are rare nonatheromatous arteritides, such as fibromuscular dysplasia, which can affect the visceral vessels and produce symptoms. However, over 95% of cases are due to atheromatous stenosis affecting the origin of the visceral arteries. The anatomical distribution is very similar to that seen with atheromatous RAS, with the plaque essentially being of aortic origin and extending into the ostium of the vessel. The median arcuate ligament syndrome is a controversial entity where the arcuate ligament of the diaphragm compresses the celiac axis. In the absence of any other vessel being involved, it should not, in theory, produce any symptoms. When it is thought to be responsible for chronic ischemia, it is necessary to surgically divide the ligament.

The presentation of chronic mesenteric ischemia is often described as "abdominal angina" as the patient suffers postprandial pain and subsequently develops a fear of eating and loses weight. Many of the patients have had a plethora of other investigations and the diagnosis is often delayed. Cross-sectional imaging is used to delineate the anatomy with either MRA or CTA (FIGURE 95.6). Although Doppler ultrasound can be used, as with renal artery disease it is operator dependent with variable results. For the reasons stated in the previous section, at least two of the three vessels should be stenosed or occluded, in patients with a convincing clinical history, in order to make a reasonably confident diagnosis of chronic mesenteric ischemia. Asymptomatic lesions rarely seem to progress to causing bowel ischemia. In a study of visceral duplex ultrasonography in 553 free-living subjects, 17.5% were found to have incidental disease of either the celiac axis or SMA. Critical mesenteric

FIGURE 95.6 Contrast-enhanced magnetic resonance angiogram in the midsagittal plane showing an occluded celiac axis (*arrowhead*) and IMA (*arrow*) and a significant stenosis of the SMA (*long arrow*). The patient presented with chronic postprandial epigastric pain typical of chronic mesenteric ischemia.

artery stenosis (MAS) was defined by the Doppler criteria; after a mean of 6.5 years, the mortality of patients with MAS was identical to those without (20%), and no deaths were attributable to intestinal infarction. MAS did not lead to development of chronic intestinal ischemia.[47]

In symptomatic patients, treatment should be aimed at improving the blood supply to the bowel by either an open surgical or an endovascular approach. There is little evidence available to help with decision making here as there have been no RCTs, and the likelihood of one being undertaken is low given the relative rarity of the condition. Many different open surgical procedures exist, and there is little consensus as to which to use. Some advocate an extensive thromboendarterectomy of the celiac and SMA origins, whereas others use some form of bypass graft either from the supraceliac or from the infrarenal aorta as the donor site. These are all major surgical procedures with considerable morbidity and reported mortality rates in the region of 5% to 10%.[48] However, the long-term patency after surgery is very good, and few patients need any further intervention, restenosis only being in the order of 10%.

An endovascular approach for the treatment of chronic mesenteric ischemia has many attractions, not least in the elderly patient with coexisting extraintestinal vascular disease. Avoidance of general anesthesia and a large abdominal incision opens up this treatment option to many patients who simply would not be surgical candidates. Initially, angioplasty was used with variable results and with a significant restenosis rate due to aortic plaque recoil. The invention of metallic stents in the late 1980s largely overcame the technical limitations of angioplasty and opened up the field of endovascular revascularization. Technical success rates vary from 88% to 100% with very low mortality and morbidity. Restenosis (usually within the stent) is not uncommon with primary patency of approximately 60%.[49] Restenosis may result in recurrent symptoms and the need for a further revascularization procedure. There have been huge efforts made by the stent industry to reduce restenosis rates in other circulations, with drug-eluting stents becoming the norm in the coronary circulation. It remains to be seen whether these benefits apply to drug-eluting stents in other arteries. Restenosis can be addressed with further endovascular options, which include angioplasty, drug-eluting angioplasty, restenting, and drug-eluting stenting.

In conclusion, the decision to perform open or endovascular revascularization is a complex one, trading off the risk of mortality and morbidity of surgery for a more durable outcome. In a young fit patient, it would be difficult to argue against an open surgical repair.

Retinal Arterial Occlusive Disease

The retina has a dual circulation. The inner retina is supplied by the central retinal artery, which is an end artery, whereas the outer retina is nourished from the choroidal circulation. Retinal artery obstructions selectively affect the inner retina only. A retinal artery obstruction is an abrupt diminution of blood flow severe enough to cause ischemia of the inner retina. Diagnosis of central retinal artery obstruction (CRAO) is straightforward when diffuse ischemic retinal whitening is present in the setting of abrupt, painless, and profound visual loss, the hallmark symptom. Examination typically reveals a visual acuity of 0.05 or worse. If a patent cilioretinal artery is present and this perfuses the fovea, normal central acuity may be permitted. The decreased blood flow results in ischemic whitening of the retina in the territory of the obstructed artery. A cherry-red spot of the macula is typical (FIGURE 95.7) and arises in this area because the nerve fiber layer is thin.[50] Fluorescein angiography shows a typical delayed arm-to-retina time and delayed arteriovenous transit, and late staining of the disc is common.

Retinal arterial obstructions are divided anatomically into central and branch, depending on the exact site of obstruction. A CRAO occurs when the blockage is within the optic nerve substance itself, and therefore the obstruction is not visible on ophthalmoscopy. Acute simultaneous obstruction of both the retinal and choroidal circulations is referred to as an ophthalmic artery obstruction. More proximal obstructions usually cause a more chronic form of visual problem—the ocular ischemic syndrome. Most CRAO result in permanent loss of vision. About one-third of patients experience some improvement in final vision, either with or without conventional treatment. On occasion, some patients experience significant restoration of normal vision. As the visual loss tends to be severe and permanent, it is fortunate that retinal artery obstructions are rare occurrences. The mean age at onset is about 60 years, with a wide range of reported ages from the first to the ninth decade of life.

Etiology of Retinal Artery Occlusions

The majority of retinal occlusions are either thrombotic or embolic in nature. As there is a strong association with systemic disease, all patients who suffer retinal artery obstructions should undergo a systemic evaluation. In the absence of local causes, this must include testing for temporal arteritis, carotid artery disease, and cardiac disease. It is important that temporal arteritis should be excluded in all patients older than 50 years of age who have a CRAO. Nevertheless, over 75% of patients with CRAO have evidence of atherosclerosis, about 60% of patients have concurrent systemic hypertension, and 25% of patients have diabetes. Less than one-third of CRAO result from emboli, and potential embolic sources are identified in about 40% of cases. However, over two-thirds of branch retinal artery obstructions are caused by emboli. Sources of emboli include hemodynamically significant ipsilateral carotid artery disease, which is present in about one-third of affected patients. An embolic source from the heart is present in <10% of patients with CRAO; however, echocardiography should be performed, especially in younger patients. Other rare associated systemic diseases include blood-clotting abnormalities.[51]

Treatment of Retinal Artery Occlusion

There is debate concerning the time period after arterial blockage before permanent visual loss occurs. Full visual recovery is possible if the blockage is removed in time. The primate retina can tolerate up to 105 minutes of ischemia before irreparable damage occurs.[52] Extrapolation to humans has led to the theory that treatment is only beneficial if commenced within 8 hours of the occlusion. Studies do suggest that shorter occlusion time results in better eventual visual recovery. The devastating nature of the condition on vision has led to a number of

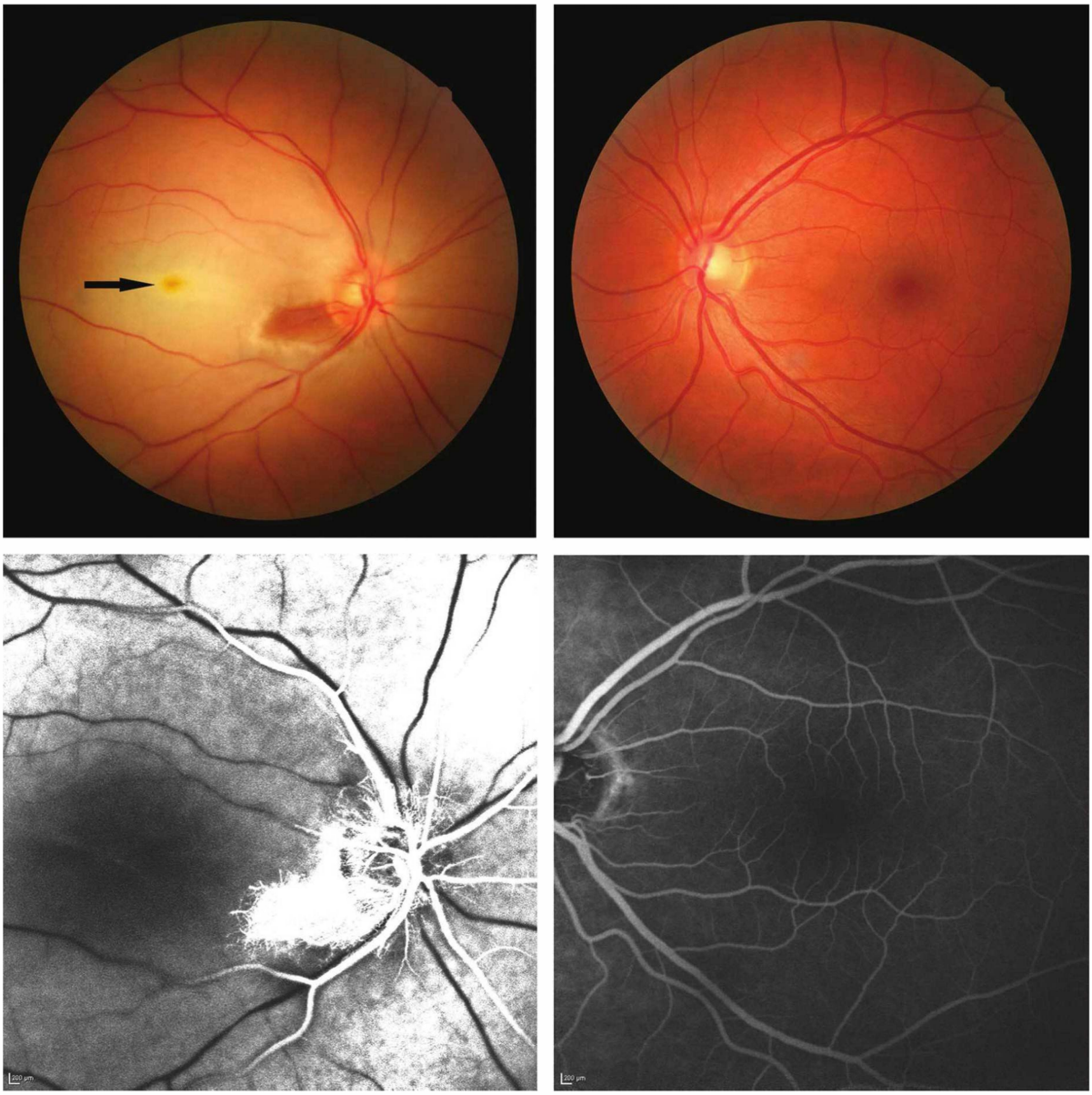

FIGURE 95.7 A 48-year-old patient with central retinal artery occlusion in the right eye. The cherry-red spot in the macula (*arrow*) and surrounding retinal edema can be seen in the fundus photography. Due to a patent cilioretinal artery, there is a perfused area in the temporal inferior segment of the retina, which can be seen with fluorescein angiography by hyperfluoresence of the dye. The left eye is normal (photography and angiography).

therapies being tried. No proven treatment exists for CRAO, but treatment strategies center around the following goals:

- To increase retinal arterial blood flow and reverse arterial obstruction
- To increase retinal oxygenation and prevent hypoxic retinal damage

The therapeutic strategies used include waiting, dilation of the artery, physical removal of the obstruction, increasing perfusion pressure by reduction of intraocular pressure, thrombolysis of the clot, antiplatelet therapy, reducing red blood cell rigidity, or use of systemic steroids. Most clinicians have used combinations of the above. No treatment is without its potential hazards, and the physician and patient must together balance the risks of these interventions with the possibility of a positive clinical effect.

An increase in retinal arterial blood flow is attempted by lowering intraocular pressure by ocular massage, paracentesis, and the administration of ocular antihypertensive medications. Medical attempts to dilate retinal arteries or block vascular spasm have been tried as well—sublingual nitroglycerin, pentoxifylline, calcium channel blockers, and β-blockers have all been used with no proof of efficacy. Few RCTs of treatment options for nonarteritic CRAO exist. One suggested a positive effect for oral pentoxifylline and the other for oral pentoxifylline

and/or enhanced external counterpulsation combined with hemodilution. However, a potentially improved retinal perfusion did not translate into better vision for the study patients. The small size of the studies and the lack of data regarding final visual recovery mean that there is no convincing evidence at present to support the routine use of pentoxifylline or of enhanced external counterpulsation in patients with CRAO. Reversal of arterial obstruction through the use of anticoagulation and fibrinolytic medications has been reported. To date, the utility of these medications has not been proved by controlled clinical trials, but anecdotal reports of success with intravenous heparin, tissue plasminogen activator, streptokinase, and urokinase do exist. In addition, an intra-arterial injection of tissue plasminogen activator, urokinase, or streptokinase during selective catheterization of the ophthalmic artery has been attempted, with reported success in some selected cases. Intra-arterial thrombolysis may produce superior visual outcomes compared with conventional treatments for CRAO when instituted in certain situations.[53] However, in the EAGLE study, 82 patients were randomized to receive conservative standard therapy or intra-arterial fibrinolysis within 20 hours of presentation with retinal artery occlusion.[54] A clinically significant improvement in best-corrected visual acuity was seen in 60% of the standard therapy patients and 57% of those receiving invasive therapy, and so the latter is not advocated as a general treatment.

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CHAPTER

96 Pathogenesis and Treatment of Biomaterial-Associated Thrombosis

MAUD B. GORBET AND MICHAEL V. SEFTON

Cardiovascular device manufacturers rely on elegant device designs and systemic pharmacologic agents to work wonders with existing materials despite their thrombogenicity. Adverse effects are minimized and existing devices are, if not risk free, providing sufficient benefit to outweigh the risks. The focus of research in biomaterials is to make better materials that pose fewer risks and offer greater benefits. Many strategies for lowering thrombogenicity have been identified, and all these strategies show a beneficial effect in at least one assay of thrombogenicity. However, the perfect material is yet to be identified.

Clinical manifestations of the bioincompatibility of cardiovascular devices are numerous[1,2]: Sudden and complete occlusion of stents[3]; acute and subacute thrombotic occlusion in medium-sized grafts (4 to 6 mm); embolic complications with artificial hearts,[4] catheters,[5] and prosthetic valves[6]; and thrombotic complications during cardiopulmonary bypass (CPB)[7] and angioplasty.[8] Large-diameter vascular grafts remain thrombogenic for many years, but fewer thrombotic complications are observed because high flows disperse the coagulation proteins. Even if the risk of thrombotic complication appears to be low (varying between 1% and 10% depending on the device), the complications may have fatal outcomes, and the cost associated with the follow-up intervention is not negligible. Furthermore, these thrombotic complications with cardiovascular devices occur, despite the use of antiplatelet and anticoagulant therapies. In the absence of nonthrombogenic materials, current devices rely on avoiding irregular flow geometries (i.e., sudden contractions, recirculation zones, and poor device streamlining) so that thrombi do not accumulate within the device.[9,10] Coupled with this is the routine use of antithrombotic agents (see Spinler et al.[11] for guidelines and Gorbet et al.[12] for review). The inherent thrombogenicity of currently existing cardiovascular devices can be illustrated by the catastrophic impact that premature (prior to 6 months) discontinuation of dual antiplatelet therapy can have on the risk of thrombosis with drug-eluting stents (DES).[13]

This chapter focuses on biomaterials and their interactions with blood. We consider how each component, including leukocytes and complement, is thought to interact with biomaterials to induce thrombosis. The role that endothelial cells and diseases such as diabetes may play in exacerbating material-induced thrombosis is also discussed.

INTRODUCTION TO BIOMATERIAL-ASSOCIATED THROMBOSIS

Overview

Cardiovascular devices (except endothelial cell–seeded vascular grafts) expose a foreign surface lacking the properties of the endothelium to the circulation. This exposure triggers a complex series of events, including protein adsorption, platelet and leukocyte activation/adhesion, and the activation of complement and coagulation; the events are highly interlinked in this simplified picture (see FIGURE 96.1).

Criteria for Nonthrombogenicity

Much of the effort in biomaterials research over the last 30 years has been directed toward the development of inert materials that do not react with platelets and coagulation factors. A number of approaches, often conflicting, have been developed. For example, there is still no consensus as to whether a surface should be hydrophilic or hydrophobic.

Thrombogenicity is defined as the ability of a material to induce or promote the formation of thromboemboli.[14] In the past, we have thought of a nonthrombogenic surface as one that prevented platelet adherence and did not shorten blood clotting time. These criteria are easily met by heparinized materials,[15] but further studies have shown that some hydrogels, even with heparin, can activate platelets, leading to high platelet consumption.[16] The latter materials promote the formation of microthrombi, although the thrombi do not attach to the materials. A clotting time measurement is a reasonable way of assessing the effect of a material on coagulation. However, this assay is likely unimportant for predicting thrombosis *in vivo* under normal and pathologic conditions.

The criteria that we use as hallmarks of nonthrombogenicity are given in Table 96.1. Rather than a clotting time, a specific thrombin production rate constant, k_p, enables the extrapolation to indicate realistic situations. Experimental results suggest that k_p is of the order of 10^{-3} per second for simple materials such as polyethylene (the same scale as mass transfer coefficients in flowing blood) but $<10^{-4}$ per second for heparinized materials.[17] According to a simple flow model (see FIGURE 96.2), only the latter low k_p materials can be expected to minimize fibrin production. This is one of the reasons heparinization and other active methods of inhibiting thrombin formation are so popular as strategies for imparting low thrombogenicity. There are, however, other criteria that must also be met. It is a requirement that platelet interactions with the surface should not lead to thrombosis. Platelet activation and consumption may therefore be better indicators of platelet incompatibility than just deposition. Furthermore, some deposition may even be desirable since these deposits appear to passivate the surface to further activation. Although a nonthrombogenic surface should not support platelet adhesion, some *ex vivo* studies[18,19] have demonstrated that even in the absence of adhesion, platelets can be "consumed." That is, the platelets can be activated by the material and prematurely removed from the

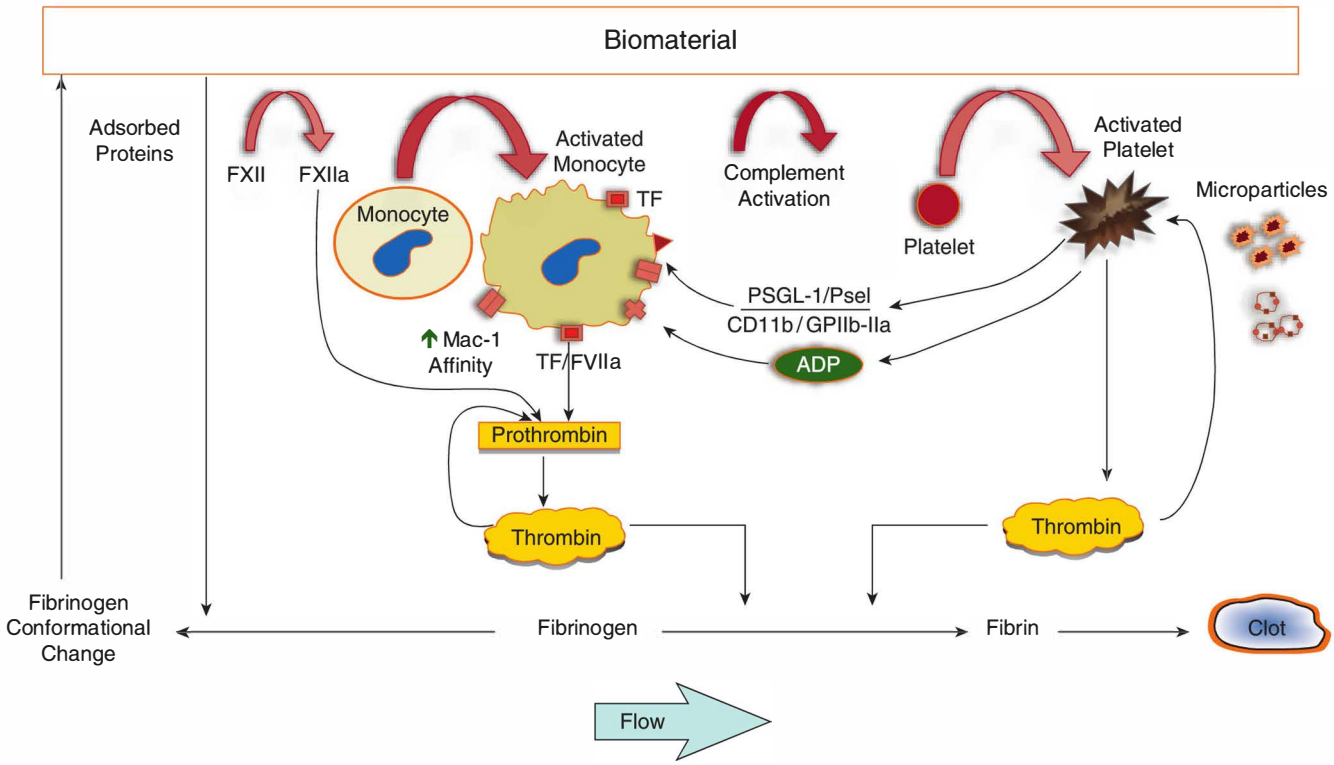

FIGURE 96.1 On or in the adsorbed protein layer, coagulation factors change their conformation or are converted into active enzymes and result in the generation of thrombin and fibrin. In parallel, platelets adhere to the surface (or adsorbed protein), become activated, change their shape, and form platelet aggregates. Both processes are linked most notably through thrombin generated on the activated platelet surface. Complement is activated on many biomaterials within the protein adsorbate and leukocytes are activated to express TF and other procoagulant activities. Flow (shear rate) is a crucial factor affecting both transport coefficients (from bulk to the surface and reverse) and the activation of leukocytes and platelets. (From Gaamangwe T, Gorbet MB, unpublished work, with permission.)

circulation (e.g., shorter platelet lifespan, decrease in systemic platelet count). This suggests that low platelet adhesion is not a sufficient criterion of *in vivo* platelet compatibility. Reduced thrombogenicity is characterized by both low platelet adhesion and low platelet activation. Leukocyte activation (and complement activation) may also be key components in thrombogenicity (see subsequent text); with further research, these factors too may become crucial parameters that define the thrombogenicity of the surface.

MECHANISTIC DETAILS

The failure to create nonthrombogenic biomaterials is directly attributable to our limited understanding of the complexity of

Table 96.1
Hallmarks of nonthrombogenicity

Conventional Wisdom	Updated Criteria
Prolonged clotting times	Thrombin production rate constant $k_p < 10^{-4}$
Few adherent platelets or thrombi	Low platelet consumption; low degree of platelet activation (e.g., MPs formation)
Spread platelets are a marker of blood incompatibility—it is better to have rounded, nonactivated platelets	Some platelet spreading (?)
Complement and leukocyte activation are not relevant to studies of thrombogenicity	Low complement and leukocyte activation (?)

(?), the validity of these criteria is subject to further verification.

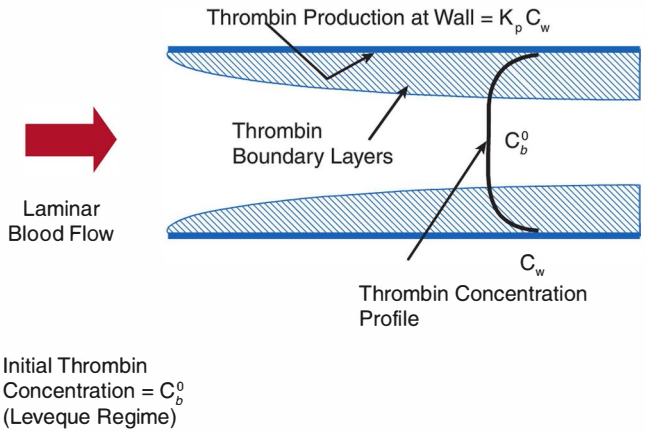

FIGURE 96.2 Model illustrating thrombin production at the surface of a tube as a balance between autoaccelerative production and mass transfer away from the wall. (From Rollasson G, Sefton MV. Measurement of the rate of thrombin production in human plasma in contact with different materials. *J Biomed Mater Res* 1992;26(5):675–693, with permission.)

biomaterial-associated thrombosis. The variety of mechanisms that are involved are summarized here.

Coagulation

In the past, the discussion of coagulation at the biomaterial–blood interface has focused on the intrinsic coagulation system and the effects of the adsorbed protein layer. Protein adsorption is certainly the first event in blood–material interactions, and adsorption of the contact-phase proteins may result in activation of the intrinsic cascade. Recent studies, however, demonstrate that tissue factor (TF) and the extrinsic coagulation system are also involved.

Early studies that were focused on protein adsorption on glass or biomaterial surfaces showed how fibrinogen is replaced over time by high molecular weight kininogen (the Vroman effect), suggesting a possible role of the intrinsic pathway in material thrombosis.[20] On the other hand, factor XII (FXII) adsorption has been observed in moderate amounts on materials used in vascular grafts[21] and hemodialysers[22] but has not been found in its activated form.[23] In some instances, *in vitro* activation of FXII and kallikrein has been reported with biomaterials;[24,25] whether this activation results in any significant coagulation is not known. Other studies have actually shown that only minute amounts of thrombin or thrombin–antithrombin complex (TAT) are generated when biomaterials are incubated with undiluted plasma alone.[26–28] Furthermore, the levels of adsorbed kallikrein and FXII on biomaterials do not correlate with TAT formation,[26] suggesting that the contact-phase proteins, by themselves, play little role in the activation of coagulation. In fact, two studies[27,29] suggested that the presence of leukocytes and/or erythrocytes is required for activation of the coagulation cascade. TAT formation on polyvinyl chloride (PVC)[27] and titanium[29] was found to be negligible in both plasma and platelet-rich plasma, whereas significant levels of TAT were observed in whole blood. *In vivo*, a small increase of FXIIa is observed during CPB,[30,31] but it appears to be in response to the surgical intervention. The establishment of extracorporeal circulation (i.e., exposure to the biomaterials of the circuit) does not further increase FXIIa levels.[31] Furthermore, no significant correlation has been observed between FXIIa and thrombin generation.[31] *In vivo* results with hemodialysis also failed to show any significant increase of FXIIa.[32] That a patient with a severe FXII deficiency showed levels of thrombin generation during CPB similar to normal patients[33] casts further doubt on the role of FXII in the initiation of coagulation with biomaterials. Taken together, these studies do not support the view that activation of the contact-phase proteins is important in the activation of coagulation by biomaterials. Rather a TF-dependent pathway of initiation for coagulation may be more important, and this is discussed in the context of leukocyte activation in subsequent text.

Platelets

Platelet activation and adhesion is known to occur during CPB and hemodialysis, as well as in vascular grafts, stents, and catheters.[2,34] The thrombotic complications associated with cardiovascular devices are clearly linked to their ability to activate platelets. Adherent platelets[35] and circulating platelet microparticles (PMPs) generated by material contact[36,37] have been shown to be procoagulant in nature. Association between platelets and leukocytes through P-selectin occurs in the presence of cardiovascular devices,[38] and such associations may directly or indirectly contribute to thrombin generation (by monocyte TF) or may participate in the removal of platelets from the circulation because several platelets can be bound to each neutrophil or monocyte. Generation of PMPs is also induced by biomaterials; when compared to balloon angioplasty alone, higher levels of PMPs have been observed within 15 minutes following coronary stenting.[39]

Following contact with the layer of adsorbed proteins on the artificial surface, platelets will either adhere or "bounce off,"[40] most likely depending on their state of activation and the ligands present at the interface.[41] Many investigators have studied the influence of surface characteristics of biomaterials (i.e., hydrophilicity/hydrophobicity, surface free energy, chemistry, charge, and roughness) on platelet adhesion *in vitro*, but the results have been contradictory and adsorbed proteins remain the crucial factor in platelet adhesion. Few platelets are found on biomaterials that preferentially adsorb albumin. Preadsorbed fibrinogen, fibronectin, vitronectin, and von Willebrand factor (vWF) support platelet adhesion *in vitro* on biomaterials. However, initial platelet adhesion on surfaces *in vitro* is mediated mainly by fibrinogen and glycoprotein (GP) IIb/IIIa.[42] No initial platelet activation is required, and binding of GP IIb/IIIa occurs through the aminoterminal of the γchain in fibrinogen.[43] A very small amount of fibrinogen appears to be necessary to support platelet adhesion (<10 ng/cm^2),[42] suggesting that a biomaterial has to be extremely resistant to protein adsorption to prevent adhesion. Such biomaterials can be characterized as "ultralow" fibrinogen-adsorbing surfaces, whereby <5 ng/cm^2 of adsorbed fibrinogen is required. Despite surface modification, such a design criterion appears difficult to meet, especially under *in vitro* conditions mimicking the *in vivo* environment (i.e., 100% plasma).[44] The role of protein in platelet adhesion on biomaterial surfaces is also highly dependent on shear rate; at low shear, platelet adhesion was reported to be dependent only on fibrinogen while at high shear both fibrinogen and vWF were reported to affect platelet adhesion.[44] Interaction with vWF

(by GP Ib/IX/V) and fibronectin (by GP IIb/IIIa) is believed to improve platelet adhesion once adherent platelets have been activated.[45–47] Adherent platelets on biomaterials become activated and undergo morphologic changes; the extent of platelet spreading or flattening is directly related to the increase in activation.[48] The state of activation of adherent platelets is also dependent on the biomaterial.[49,50] Activation of adherent platelets may result in degranulation, with the release of activation products such as β-thromboglobulin, thromboxane B_2, adenosine 5′-diphosphate (ADP) or serotonin (which can activate bulk platelets), and neutrophil-activating protein-2. In addition, adherent platelets express P-selectin (translocated to the surface during release from the α-granulae), which promotes the binding of monocytes and neutrophils. The activated adherent platelet surface supports thrombin generation (i.e., prothrombinase and tenase formation) and can also shed procoagulant microparticles (MPs) from their plasma membrane.

The absence of significant platelet adhesion does not preclude platelet activation in the bulk as shown by the generation of platelet MPs with polyvinyl alcohol (PVA) hydrogel both *in vitro*[51] and *ex vivo* in a canine shunt model.[52] In a baboon arteriovenous (AV) shunt model, the rate of platelet consumption was directly linked to the water content of hydrogels,[18] despite the absence of significant adhesion. All materials that were tested,[53] not just hydrogels, were observed to activate platelets, resulting in their premature removal from the circulation.

It is not clear whether inhibition of platelet adhesion will reduce platelet consumption or inhibit the generation of platelet MPs. It appears that the effects of antiplatelet agents vary, depending on the surface characteristics as shown by Spijker et al.[54] For example, abciximab is less effective in reducing platelet adhesion on hydrophilic surfaces than on hydrophobic surfaces. The strategies available to minimize platelet adhesion (e.g., polyethylene glycol immobilization) have not been sufficient to prevent platelet consumption.[55] The mechanism of material-induced platelet activation is often presumed to be through the generation of thrombin due to activation of the intrinsic coagulation cascade or the release of ADP from damaged red blood cells or platelets. Even in the presence of heparin, small amounts of thrombin are generated and may activate platelets. However, the inability of thrombin and kallikrein inhibitors to reduce platelet activation suggests that platelet activation is at least mediated in part by other agonists.[56] Classical pathway complement inhibitors can have some beneficial effects on platelets: pentamidine (12 mg/kg, daily, intra muscular) but neither abciximab nor low molecular weight heparin reduced PVA-induced platelet consumption in the canine AV shunt (unpublished study, Gemell CH & Sefton MV). A correlation between complement activation and thrombocytopenia has also been noted during dialysis.[57,58]

Complement activation can lead to platelet activation in many ways. Platelets possess a receptor for C1q that induces GP IIb/IIIa activation, P-selectin expression, and procoagulant activity.[59] Insertion of the complement assembly C5b-9 in platelets has also been associated with increased platelet procoagulant activity.[60]

Leukocytes

Contact with biomaterials *in vivo* activates both neutrophils and monocytes. Indicators of leukocyte activation such as L-selectin shedding and CD11b upregulation on leukocytes have been widely observed following angioplasty,[61] hemodialysis,[62] and CPB[63] (for review, see Asimakopoulos and Taylor[64]). Degranulation with the release of elastase and lactoferrin and the presence of cytokines, such as IL-1 and TNF-α, have been associated with extracorporeal circulation[65] and further demonstrate leukocyte activation. Activation of the respiratory burst is also a common response to hemodialysis.[66]

Both *in vitro*[67] and *in vivo*[68] studies have found that material-induced leukocyte activation is dependent on the exposed surface area. Monocytes express TF after coming into contact with a biomaterial.[67,69,70] Material-induced monocyte TF expression *in vitro* depends on complement activation and platelets.[71] On the other hand, the removal of platelets or the blocking of GP IIb/IIIa by monoclonal antibody (7E3) has no effect on CD11b upregulation. Contact with materials such as PVA hydrogel[72] or pellethane[73] leads to a twofold upregulation of CD11b, typical of the degree of leukocyte activation induced by phorbol esters.

Material-induced leukocyte activation results in increased adhesion to artificial surfaces. Because the biomaterial is larger than a microorganism and cannot therefore be engulfed by leukocytes, adherent neutrophils and monocytes respond with a frustrated attempt at phagocytosis, whereby these cells release their array of potent oxygen metabolites, growth factors, cytokines, and proteolytic enzymes.[74] The state of adherent leukocyte activation depends on the type of surface, the protein ligands present at the interface,[75–77] and the presence of adherent platelets.[71,78] Hydrophobic surfaces tend to promote leukocyte adhesion and activation, whereas less activation is observed in leukocytes adherent to hydrophilic surfaces.[79] *In vivo* studies have demonstrated activated leukocytes adhering to stents,[80] oxygenators,[81] and hemodialysis membranes.[82,83]

Leukocytes adhere *in vitro* to fibrinogen, fibronectin, and immunoglobulin (Ig)G adsorbed to surfaces by means of the integrin receptors CD11b/CD18 (Mac-1), CD49e/CD29 (VLA-5), and Fc receptors. Fibrinogen appears to be an important ligand in leukocyte adhesion to artificial surfaces. Monocyte adhesion correlates positively with the amount of fibrinogen adsorbed to the surface.[84] Fibrinogen is important for leukocyte adhesion *in vivo*. Fewer leukocytes are found on polymers implanted in hypofibrinogenemic mice.[85] Leukocyte binding to fibrinogen occurs by a protein kinase C–dependent pathway important for both initial and sustained adhesion. On metals such as titanium, leukocytes adhere initially through the FcγIII receptor (CD16), making IgG the ligand of preference.[86] Complement proteins[74,87] are also involved. C3a can mediate leukocyte adhesion on surfaces *in vitro* by the direct complexation with C3a receptors, whereas C5a appears to have a lesser role.[88] The iC3b is a ligand for CD11b/CD18 and is present even on low complement-activating surfaces.[89] The iC3b has been shown to play an important role in leukocyte adhesion to hemodialysis membranes.[90]

Platelets on the surface have also been reported to mediate leukocyte adhesion by the interaction between P-selectin and P-selectin GP ligand 1 and/or GP IIb/IIIa and CD11b.[91,92] Before adhesion to the biomaterials, leukocytes may roll by shedding of the L-selectin (similar to what happens on endothelial cells), but the mechanisms by which leukocytes sample a biomaterial before adhering to it have been less well characterized than with endothelial cells. Whether the mechanisms of material-induced leukocyte activation, as distinct from adhesion, are by contact with a foreign surface, by complement activation, by kallikrein,

or by platelet activation has not been fully determined. *In vitro* and *in vivo* investigations with protease inhibitors,[56,93,94] complement inhibitors,[72,95–97] and antiplatelet agents[98,99] suggest that they all play a role, but inhibition of just one pathway of activation may not be sufficient to result in a significant impact on leukocyte activation. For example, complement inhibition using sCR1 was only partially effective in reducing leukocyte activation.[91] On the other hand, a combination of sCR1 and anti–GP IIb/IIIa (which blocks platelet activation) reduced material-induced leukocyte activation to almost background levels.

Complement

Complement activation is generally treated as if it is part of the inflammatory response induced by biomaterials. For example, complement activation is known to occur during CPB and hemodialysis,[100,101] and with catheters and prosthetic vascular grafts.[102,103] It is recognized that, both in the short and in the long term, complement activation plays a role in the leukocyte-related clinical sequelae, such as leukopenia, hypotension, and pulmonary injury, which are associated with the use of cardiovascular devices.[104,105] The presence of a biomaterial is believed to activate complement by the alternative pathway. On a non-activating surface,[106] negatively charged groups such as carboxyl, sulfate, sialic acid, and bound heparin appear to promote high-affinity association between bound C3b and factor H. On the other hand, an activating surface is usually characterized by the presence of nucleophiles such as hydroxyl and amino groups. These groups will allow covalent binding of C3b and promote formation of the C3 and C5 convertase.[107] However, even in the absence of these activating groups on the surface, some biomaterials, such as polyacrylonitrile, are able to activate complement, suggesting that the mechanism of material-induced complement activation due to nucleophilic groups is not the whole story. An alternative hypothesis places emphasis on interaction of factor H with the surface; an activating material is then defined by its capacity to bind factor B rather than factor H.[107] Binding of factor H would lead to C3b inactivation by factor I and would therefore terminate the propagation of the complement cascade.

Some activating materials generate high levels of both C3b and C5b-9, whereas other materials will generate high levels of C3b but little C5b-9. The reason why the efficiency of C5 convertase formation relative to that of C3 formation differs from one activating surface to another is not well understood. However, even low amounts of C5b-9 are able to activate leukocytes,[106] and, therefore, a low terminal complement-activating material may still induce a significant inflammatory response. The question remains as to what are the appropriate levels of complement activation that the host can accept without deleterious effect.

The hypothesis that material-induced complement activation occurs exclusively by the alternative pathway has been challenged. Reports of complement activation by the classical pathway during CPB[108,109] and a delay in complement activation observed with patients deficient in C4 who are undergoing hemodialysis[110] suggest that classical activation plays a role in material-induced activation. The presence of immune complexes may allow for a rapid onset of complement activation, and then subsequently the alternative pathway may also become activated. *In vitro* studies have also demonstrated activation of the classical pathway by some biomaterials (C. H. Gemmell, *unpublished work*, 2000).[111] C1 inhibitors such as pentamidine and benzamidine are effective in lowering platelet adhesion and activation by polystyrene beads and polyethylene tubes, whereas sCR1, an inhibitor of both pathways at the level of C3, has no effect (C. H. Gemmell, *unpublished work*, 2000).[112] Pentamidine was also effective in a canine chronic shunt by eliminating the thrombocytopenia seen with a platelet-activating material (a PVA hydrogel). A role for complement in platelet activation mediated by biomaterials offers new strategies to improve the thombogenicity of surfaces. Preliminary studies with surfaces modified with amidine derivatives (which have C1 complement inhibitory properties) have shown reduced platelet adhesion.[113,114]

Although some of the consequences of complement activation are well understood, more work is needed to fully understand how a material activates complement. Controlling the differential adsorption of C3b, factor D, and factor H may be more important than preparing the so-called nonfouling, low-adsorption surfaces. The latter may lower the adsorption of all proteins, but it may be more important to alter the composition of the protein adsorbate.

Leukocyte Activation and Coagulation

Circulating monocytes and neutrophils normally roll on the endothelium and adhere to damaged or stimulated endothelial cells or adherent platelets and further contribute to localized thrombogenesis through one or more mechanisms,[12] some of which apply to biomaterials.[12,115] Leukocytes have membrane-associated procoagulant activity, which is manifested by TF expression on the cell membrane (TF-dependent coagulation pathway), by TF-independent mechanisms through factor X binding to CD11b receptors leading to factor Xa generation or fibrinogen binding to CD11b, or by binding of the prothrombinase complex on the membrane. In addition, degranulation and oxidative products have the capacity to neutralize various anticoagulant proteins and activate platelets, whereas the association between platelets and neutrophils or monocytes may lead to mutual activation and to a microenvironment protected from inhibitors.

Many investigators thought that the extrinsic pathway is not activated during blood–material interactions.[10,116,117] However, blood contact with a material represents a stimulus to induce TF expression by monocytes,[67,71] resulting potentially in blood coagulation through the extrinsic system. Material-induced TF expression on monocytes *in vitro* results in significantly shorter clotting times when compared to control.[67] The TF expression appears to be strongly dependent on the presence of platelets and increases with material surface area.[71] Following blood contact with CPB circuits or ventricular assist devices, TF expression on monocytes has been observed *in vitro*[118,119] and *in vivo*.[69,120] TF-bearing monocyte MPs have also been shown to adhere to biomaterial surfaces *in vitro* and were able to support FXa activation.[121] Leukocyte MPs have previously been observed *in vivo*, and this may represent yet another way for leukocytes to contribute to thrombogenesis. CD11b, upregulated on monocytes by CPB, has been reported to directly activate factor X[122] and platelet–leukocyte aggregates have been observed,[123] as noted in the preceding passage. The number of studies that have tried to minimize thrombus formation by the administration of drugs specifically targeted at leukocytes underscores the potential role of leukocytes in thrombogenesis.[98,124,125] Antibodies to

block leukocyte adhesion may prove to be a reasonable therapeutic approach in the prevention of thrombus formation as demonstrated in *in vivo* baboon models.[98,126,127] An *in vivo* study recently identified C5a as a significant contributor to hemodialysis-associated thrombosis via TF expression on leukocytes.[128] Complement inhibition may thus also become a successful approach to controlling thrombosis on biomaterials.

The initiation of the coagulation cascade by contact-phase activation and TF expression occur at different times after exposure of blood to a biomaterial. Because it is part of the protein adsorption "reaction," contact-phase activation occurs during the first few seconds after blood contact with a material. On the other hand, the induction of TF expression and thrombin-generating activity in monocytes requires a minimum of 60 minutes (in a healthy patient). Thrombin generation by FXIIa on materials depends on flow,[129] because of the effects of flow on mass transfer as well as direct effects of flow on platelet/leukocyte phenotype. Therefore, the relative roles of the intrinsic and extrinsic pathways in thrombin generation probably depend on both time and blood flow. More research is needed to clarify the potential contribution of leukocyte procoagulant activities to thrombogenesis and thrombotic complications associated with the use of biomaterials and cardiovascular devices.

Fluid Dynamics

Fluid dynamics affect the growth of thrombi and the deposition of fibrin. The difference between arterial and venous thrombi is one example of this effect, although the underlying mechanisms are still not well understood. Thorough reviews are available,[130,131] and we merely highlight a few key points for the purpose of completeness. Flow determines the rates of transport of cells and proteins to the surface; it can also change the level of receptor expression on platelets and leukocytes. Because platelets are an important part of the thrombus, the effect of shear on platelets has been studied extensively. Higher shear results in higher platelet and lower fibrin deposition.[132] High shear in diseased, stenotic arteries is also able to induce platelet aggregation even in the absence of any other exogenous factors.[133,134] Conflicting results have been obtained on the effect of flow on leukocyte adhesion, whereas little is known of its effect on leukocyte activation. High shear has been shown to reduce, increase, or leave unchanged leukocyte adhesion on different substrates.[135–141] These conflicting results may be explained by differences in experimental conditions, including the presence of red blood cells,[141] platelets[137–139] and plasma proteins,[137] the surface studied,[138] and the state of leukocyte activation.[140]

As for the effects of flow on the coagulation cascade, it has been a subject of very little study. Current knowledge about biomaterials is limited to factor Xa generation initiated by the extrinsic pathway and thrombin generation initiated by the intrinsic pathway. Factor Xa generation by the complex TF:VIIa increases with shear rate (and shear stress).[142,143] For thrombin generation by the intrinsic pathway, modeling has identified three types of reactions[129]: at low flow, a significant amount of thrombin is produced after a long time lag (more than 10 hours); at moderate flow, significant thrombin generation is produced in a short time (within minutes); at high flow, low levels of thrombin are produced within seconds.

Turbulent flow can be present at anastomoses, joints, and bifurcations of cardiovascular devices, and such turbulence also contributes to the observed thrombosis. It is believed to play an important role in the failure of mechanical heart valves, for example. Turbulent flow (in contrast to recirculation and stagnation zones, which may or may not have the characteristics of turbulence) results in hemolysis and/or cell activation, but the mechanisms leading to thrombus formation are still poorly understood. Platelet deposition remains the focus of most studies, but the literature on turbulent flow and thrombus formation is more limited. Nonetheless, much effort has been devoted to the design of devices so that recirculation or stagnation zones are avoided because these are known loci for thrombus growth.

Surface Modification Strategies

Many different materials are found in cardiovascular devices. Polymers such as silicone rubber, polytetrafluoroethylene (PTFE), PVC, polyethylene, or polyurethanes are used in catheters, vascular implants, ventricular assist devices, and oxygenator membranes. Stainless steel and nickel–titanium are found in heart valves, vena cava filters, and stents. Pyrolytic carbon coatings have been used on prosthetic heart valves to improve biocompatibility, whereas chemically treated natural tissues such as pig or cadaver heart valves are also implanted. To improve the blood compatibility of cardiovascular devices, various surface modifications, such as attachment of antithrombotic agents or the immobilization of polyethylene oxide (PEO), have been considered, but their success has been limited (for a complete review, see[144]). PEO treatment to reduce protein adsorption and to prevent platelet adhesion has not reduced thrombogenicity.[145] Heparin-releasing polymers (e.g., heparin ionically bound or dispersed in surfaces), polymers with immobilized heparin (covalently bound), and heparin–PEO polymer coatings have been developed.[146] However, reports on the improvement of *in vivo* biocompatibility by heparinized surfaces with CPB tubing, catheters, and coronary stents have been mixed: heparin coatings have not been shown to significantly reduce the number of postoperative complications, improve patient outcome, or reduce hospital stay.[108,147–150]

Endothelial Cells

With most cardiovascular devices, blood interacts with a bare surface and the absence of endothelial cells may have a significant impact on material thrombogenicity. Dual antiplatelet therapy for at least a year has been recommended with DES to circumvent the lack of endothelialization and delayed healing.[151–154] The controversy between DES and bare metal stents illustrates the strong relationship between material-induced inflammation and thrombosis as well as the important role that endothelial cells can play in blood compatibility. To promote endothelialization, coatings that enable what have been termed endothelial progenitor cells to adhere are being investigated in the stent industry.[155,156] New polymers for drug delivery that are more compatible to endothelial cell attachment are also being developed.[147,157]

Seeding of endothelial cells on biomaterials to enhance antithrombogenicity before implantation has been under investigation for over 30 years but remains at the research stage (for a complete review, see Pawlowski et al.[158]), although there has been clinical success in the hands of some investigators.[159] To promote adhesion and to prevent detachment upon implantation,

adsorption of adhesive proteins, such as fibronectin[160] or specific peptide sequences, such as RGD[161] or YIGSR,[162] has been explored. However, biomaterials will influence endothelial cell phenotype (for a complete review, see McGuigan and Sefton[163]), and an activated phenotype may contribute to increase leukocyte infiltration (such as through the expression of ICAM-1) or thrombogenesis (TF expression).

While a biomaterial should promote endothelialization, material-induced leukocyte activation may also affect neighboring endothelial cells. Recent studies have shown that leukocytes having contacted polyurethanes, PTFE, or polymer beads induced endothelial cell procoagulant activity.[164,165]

The Patients

Patient disease or age may also significantly affect material thrombogenicity. Increased TF expression and other procoagulant factors have been reported in diabetic patients.[166] Harrison et al.[167] showed increased activation and adhesion of mononuclear cells from type 1 and type 2 diabetics on stainless steel. Following CPB, thrombotic complications are also increased in neonates and infants when compared to older children. It has been hypothesized that the strong inflammatory reaction induced by CPB leads to TF expression on endothelial cells and resulted in a state of hypercoagulability.[168] These two examples further highlight the importance of the extrinsic coagulation system and also the interrelation between inflammation and coagulation. These provide further design criteria for the development of new materials and/or drug therapy to minimize material-induced thrombosis.

CONCLUSIONS

Given the complexity of normal hemostatic events, it is not surprising that blood contact with artificial surfaces initiates a complex response, and the mechanisms of biomaterial-associated thrombosis are not completely clear. The role of FXII is unclear, whereas that of TF has only barely been studied. The mechanisms of leukocyte and platelet activation by materials remain to be further elucidated. The timing of the events contributing to thrombin formation is also a complex issue. Both FXII activation and platelet activation are able to generate thrombin formation within minutes, whereas thrombin generation by leukocyte TF requires hours. The question of what happens over hours to days as leukocytes synthesize TF and thrombotic deposits become remodeled is almost beyond current experimental capacity. Whether thrombosis leads to passivation or embolization or some other long-term consequence is still largely unknown.

Thrombosis is viewed now more as a multicellular event rather than just a platelet event.[169] The mechanism of material-induced platelet activation is often presumed to be by the generation of thrombin due to activation of the intrinsic coagulation cascade or the release of ADP from damaged red blood cells or platelets. A role for complement in activating platelets and the potential for complement to be activated by the fibrinolytic and kinin system have rarely been considered. If complement also plays a role in activating monocytes to express TF or directly generate fibrin,[127] then this will certainly refocus strategies in order to reduce material thrombogenicity.

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CHAPTER 97

Overview of Complex Thrombohemorrhagic Disorders

THEODORE E. WARKENTIN AND VICTOR J. MARDER

This overview of "complex thrombohemorrhagic disorders" emphasizes their heterogeneous clinical presentation ranging from bleeding to microvascular and macrovascular thrombosis (see Table 97.1). These conditions have in common the potential for clinical presentation without symptoms, with bleeding or thrombotic events, or with simultaneous manifestations of hemorrhage, vascular occlusion, and organ failure. Although this disparate group of disorders has varied and distinct etiologies, they nevertheless can mimic one another. For example, a patient who presents with splenic infarction could have an underlying myeloproliferative neoplasm (MPN) or infective endocarditis with splenic artery septic emboli. The former diagnosis is suggested by an elevated platelet count, whereas thrombocytopenia suggests sepsis. Consider also the patient with dyspnea and thrombocytopenia beginning 10 days after cardiac surgery for whom the differential diagnosis includes pulmonary embolism (PE) with or without heparin-induced thrombocytopenia (HIT) and acute sepsis. The diagnosis would be clarified once results of blood cultures, radiologic imaging, and serologic testing for HIT antibodies are available.

To add further complexity, these disorders may coexist. For example, patients with antiphospholipid syndrome (APS) occasionally develop thrombotic thrombocytopenic purpura–hemolytic uremic syndrome (TTP–HUS).[1] Acral (distal extremity) limb necrosis may develop in a patient with HIT and deep vein thrombosis (DVT) who is treated with warfarin and thereby develops a variant of coumarin necrosis, venous limb gangrene.[2] Or, consider the occurrence of HIT with a platelet count nadir of 633×10^9/L that nonetheless represented a substantial decline from the baseline level of $1{,}235 \times 10^9$/L because of preexisting essential thrombocytosis (ET).[3]

The experienced clinician acquires a "feel" for the subtle differences in clinicopathologic profile among these disorders. For example, in TTP–HUS, the presence of red cell fragments (schistocytes), thrombocytopenia, and elevated lactate dehydrogenase is usually much more marked than in a patient with disseminated intravascular coagulation (DIC) associated with acute sepsis. Although patients with HIT can have red cell fragments too,[4] this is usually present only in the subgroup with more severe thrombocytopenia and overt (decompensated) DIC. Therefore, careful correlation of the laboratory and clinical picture usually points to the correct diagnosis.

CONSUMPTIVE THROMBOHEMORRHAGIC DISORDERS

The term "consumptive coagulopathy," or the common term, DIC, does not denote a discrete diagnosis but rather a heterogeneous group of disorders that have in common increased consumption of coagulation factors and/or platelets.[5] Indeed, some of the entities discussed as separate chapters (e.g., sepsis, TTP–HUS, HIT) represent forms of consumptive coagulopathy. Therefore, consumptive coagulopathies (as with anemia) must not be regarded as a diagnosis but rather as a clinicopathologic syndrome that begs the question, "what is the underlying diagnosis?"

Systemic activation of hemostasis in pathologic settings can have a wide spectrum of clinical consequences, ranging from no clinical effect, to generalized hemorrhage, to widespread microvascular thrombosis that predisposes to multisystem organ dysfunction and acral necrosis. Bleeding tends to predominate in DIC triggered by trauma, obstetrical complications, or snakebites, whereas DIC caused by HIT or metastatic adenocarcinoma tends to produce macrovascular thrombosis affecting large veins and arteries. Some malignancies, however, produce severe bleeding, such as prostate cancer with DIC and/or hyperfibrinolysis. Microvascular thrombosis often accompanies sepsis, particularly when caused by certain organisms (meningococcus, *Capnocytophaga canimorsus*) that predispose to acral limb necrosis (sepsis-associated purpura fulminans). Contributing causes of thrombosis include severe depletion of protein C and antithrombin, ongoing thrombin production, and reduced limb perfusion secondary to shock or vasopressors.[6] To further complicate the myriad clinical features, consumption may be restricted to local processes alone, such as with a vascular malformation, aortic aneurysm, or placental abruption, any of which can cause localized activation of coagulation sufficient to deplete platelets and/or coagulation factors.[7]

THROMBOTIC THROMBOCYTOPENIC PURPURA–HEMOLYTIC UREMIC SYNDROME

The duo of microangiopathic hemolysis (red cell fragmentation) and thrombocytopenia—also known as thrombotic microangiopathy, or TMA—in the absence of an alternative explanation constitutes the modern notion of TTP–HUS.[8,9] Many, if not most, patients with "idiopathic" TTP–HUS have autoantibodies that inhibit function of ADAMTS13 (a disintegrin and

Table 97.1

Bleeding and thrombosis of complex thrombohemorrhagic syndromes

Disorder	Bleeding	Microthrombosis	Macrothrombosis
Consumptive coagulopathies (DIC)	Variable, depending on cause	Variable, cerebral (mental status), renal (oliguria), skin (purpura fulminans); limb necrosis (acquired protein C depletion)	Variable, large vein and artery thrombosis with underlying adenocarcinoma or HIT, or with use of antifibrinolytic therapy
TTP–HUS	Usually no bleeding, but petechiae and ecchymoses possible with severe thrombocytopenia	Organ dysfunction (CNS, renal) due to arterial platelet–vWF microaggregates	Occasional cerebral artery (thrombotic stroke in children with congenital form)
Vasculitides	GI bleeding (if vasculitis involves GI tract, e.g., polyarteritis nodosa); dermal hemorrhage (small-vessel vasculitis); hemoptysis (pulmonary capillaritis)	Variable, "palpable purpura" is a feature of small-vessel vasculitis; renal, neurologic, and other organ dysfunction	Macrothrombosis can be mimicked by nonthrombotic vasculitis–related vessel obstruction (e.g., intimal hyperplasia in giant cell arteritis) or large-artery dissection; aneurysm-related thrombosis
APS	Bleeding uncommon; when present, usually results from associated thrombocytopenia, low factor II, or anticoagulant therapy	Microvascular thrombosis and multiple organ failure in severe cases	Venous thrombosis in unusual sites (upper limb, abdominal, renal, cerebral), PE, coronary artery, cerebral artery (stroke, TIA), retinal ischemia, nonbacterial endocarditis; hemorrhagic adrenal infarction; recurrent fetal loss (placental infarcts)
MPN	Mucocutaneous, CNS, retinal, retroperitoneal, deep tissue, postoperative, aspirin-induced	PV and ET: intracranial (headache, visual disturbances), digital ischemia, erythromelalgia	PV and ET: cerebral artery (transient ischemia or thrombotic stroke), retinal vein occlusion, cerebral sinus thrombosis, coronary (acute MI, coronary syndromes), DVT, PE, mesenteric/portal/hepatic vein thrombosis
Stem cell transplant	Mucocutaneous bleeding with severe thrombocytopenia; diffuse alveolar hemorrhage; hemorrhagic cystitis	Hepatic veno-occlusive disease; TMA	Venous thromboembolism (DVT, PE)
Sepsis	Bleeding associated with thrombocytopenia, elevated PT or aPTT	Variable, organ dysfunction and dermal/acral necrosis	Uncommon; if DVT present, predisposes to peripheral limb ischemia/necrosis
HIT	Bleeding uncommon, except during anticoagulant therapy	Usually associated with coumarin; rarely, overt consumptive coagulopathy	DVT and PE, cerebral vein thrombosis, limb artery thrombosis, cerebral (stroke), coronary (acute MI), hemorrhagic adrenal infarction
Coumarin-induced necrosis	Early skin necrosis may resemble cutaneous hematoma	Thrombosis of subdermal venules (skin necrosis), venous limb gangrene	Associated large vein thrombosis predisposes to subtending microvascular thrombosis and acral limb necrosis

MPN, myeloproliferative neoplasms; CNS, central nervous system; PV, polycythemia vera; ET, essential thrombocytosis; DVT, deep vein thrombosis; GI, gastrointestinal; MI, myocardial infarction; PE, pulmonary embolism; DIC, disseminated intravascular coagulation; HIT, heparin-induced thrombocytopenia; PT, prothrombin time; aPTT, activated partial thromboplastin time; TTP–HUS, thrombotic thrombocytopenic purpura–hemolytic uremic syndrome; vWF, von Willebrand factor; APS, antiphospholipid syndrome; TIA, transient ischemic attack.

metalloprotease with thrombospondin 1–like domains). Because ADAMTS13 proteolyzes large multimers of von Willebrand factor (vWF) under high shear stress, reduced enzyme activity predisposes patients to formation of platelet–vWF microaggregates in small arterioles (hyaline thrombi) that constitute the pathologic hallmark of TTP–HUS. Associated organ dysfunction can cause neurologic abnormalities, oliguric renal insufficiency, myocardial ischemia, and pancreatitis. The importance of recognizing TTP–HUS relates to its special treatment by plasma exchange with fresh frozen (or cryosupernatant) plasma. This therapy reduces mortality, from >90% as occurred before 1964 to <20% today.[10] Illnesses strongly resembling TTP–HUS occur with drugs (e.g., quinine, mitomycin, cyclosporine), hematopoietic stem cell transplantation, pregnancy and the postpartum period, and autoimmune disorders (e.g., systemic lupus erythematosus, APS, acute scleroderma). Sometimes, TTP begins after surgery ("postoperative TTP"), a clinical scenario that could mimic sepsis or HIT.[11]

Classic HUS refers to a disorder in which oliguric renal failure is predominant, although 15% to 25% of patients develop neurologic abnormalities. HUS usually follows a hemorrhagic colitis that most often is caused by Shiga toxin–producing *Escherichia coli* O157:H7.[12] In 2011, a central European epidemic of Shiga toxin–producing *E. coli* O104:H4 linked to consumption of sprouts[13] was associated with a high frequency (20%) of HUS.[14] Treatment of HUS is supportive, although severely affected patients may benefit from plasma exchange or, possibly, immunoadsorption.[15]

VASCULITIDES

The vasculitides consist of a heterogeneous group of inflammatory disorders that targets and damages blood vessels.[16] Different forms of vasculitis tend to involve vessels of different sizes: large-vessel vasculitis (e.g., giant cell arteritis, Takayasu arteritis, IgG4-related aortitis), medium-sized vessel vasculitis (e.g., polyarteritis nodosa, Kawasaki disease, lupus), and small-vessel vasculitis, which is subdivided into circulating antineutrophil cytoplasmic antibody (ANCA)-positive and ANCA-negative categories. ANCA-associated small-vessel vasculitis comprises Wegener granulomatosis, Churg-Strauss syndrome, and microscopic polyangiitis, whereas those not associated with ANCA include the various immune complex small-vessel vasculitides, primarily Henoch-Schönlein purpura, cryoglobulinemic vasculitis, lupus-related, infection-related, and drug reactions, among others. Rheumatoid arthritis can be associated with either large-vessel vasculitis (rheumatoid aortitis) or small/medium-sized vessels (rheumatoid vasculitis); lupus is most likely to be associated with small-vessel leukocytoclastic vasculitis but can also be associated with vasculitis affecting medium-sized vessels.

Clinical consequences of vasculitis—such as stroke or sudden blindness—can mimic acute thrombosis, although intense intimal hyperplasia (e.g., giant cell arteritis) or marked fibrous intimal thickening (e.g., Takayasu arteritis) are more probable explanations for vessel obstruction.

ANTIPHOSPHOLIPID SYNDROME

APS is a chronic acquired thrombophilia that is strongly associated with venous and arterial thrombosis. Its *sine qua non* is the presence of autoantibodies that are detected by their effect in prolonging phospholipid-dependent coagulation assays ("lupus anticoagulant") or by immunoassays that detect reactivity against cardiolipin or other phospholipids.[17] In actuality, these "antiphospholipid" antibodies are directed against membrane proteins, most often β_2-glycoprotein 1.

"Definite APS" is defined as a clinicopathologic syndrome consisting of thrombosis and/or recurrent fetal loss plus the presence of anticardiolipin antibodies, anti-β_2-glycoprotein 1, and/or lupus anticoagulant, detected on two or more occasions at least 12 weeks apart.[18] A problem in diagnosis is that some patients have other clinical manifestations (e.g., thrombocytopenia, cardiac valvulitis, neurologic or renal abnormalities, skin ulcers) referred to as "noncriteria clinical manifestations"[19]; conversely, nonpathologic antiphospholipid antibodies can be detected in many normal individuals[20] and transiently after infection[21] or use of certain drugs such as procainamide and quinidine. Seronegative APS indicates a patient with acute thrombosis who initially has negative testing for antiphospholipid antibodies that subsequently appear and persist on later testing.[22] Rare patients develop widespread thrombosis affecting large and small vessels with associated organ failure, so-called catastrophic APS.[23]

Thrombotic events are generally treated as indicated for patients without APS,[17] except that the patients with APS with spontaneous or recurrent thrombosis should receive long-term anticoagulation. An additional consideration is that monitoring heparin or warfarin can be complicated by baseline prolongation of the activated partial thromboplastin time (aPTT) or prothrombin time (PT) because of the *in vitro* effects of the antiphospholipid antibodies.

MYELOPROLIFERATIVE NEOPLASMS

Examples of "classic" MPN include the clonal stem cell disorders, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis.[24] Unlike most of the other disorders considered in this overview, patients with ET and PV typically have elevated platelet counts. Many patients evince both bleeding and thrombosis, for example, the patient with ET who has increased postoperative bleeding but also an increased risk of thrombotic stroke or myocardial infarction. Prominent microvascular disturbances in PV and ET include headache, lightheadedness, fleeting visual problems, acral paresthesias, and erythromelalgia (acral erythema, warmth, and pain), the latter a direct result of local platelet–endothelial interactions within the small vessels of the fingers and feet. Raised hematocrit levels are an important determinant of thrombotic risk in PV, whereas thrombocythemia is associated with increased risk of thrombosis in ET. Accordingly, phlebotomy (for PV) and hydroxyurea (for PV or ET) are often used to reduce thrombotic risk in appropriate patients. Lowering of elevated platelet counts by cytoreductive therapy, paradoxically, can improve bleeding symptoms by correcting acquired von Willebrand syndrome, which results from enhanced platelet–vWF interactions.[25]

STEM CELL TRANSPLANT-ASSOCIATED THROMBOHEMORRHAGIC DISORDERS

Thrombotic and hemorrhagic disorders are common in stem cell transplant recipients, primarily because of endothelial cell injury, profound thrombocytopenia, and acquired deficiencies in procoagulant and anticoagulant factors.[26]

Clinical consequences include thromboembolic events, hepatic veno-occlusive disease, and TMA. Unlike classic TTP, however, transplant-associated TMA is not characterized by reduced levels of ADAMTS13 or by anti-ADAMTS13 autoantibodies. Characteristic posttransplant bleeding syndromes include diffuse alveolar hemorrhage and hemorrhagic cystitis.

SEPSIS

Sepsis is a common cause of hemostatic abnormalities.[27] Coagulation testing generally reveals increased fibrin degradation products and thrombocytopenia, with some patients additionally evincing elevation in the PT or aPTT, reduced fibrinogen levels, or decreased activity of the coagulation inhibitors, antithrombin and protein C. Thrombocytopenia is associated with both increased bleeding risk and mortality,[28] as are increases of 1.5 times or more in the global coagulation times.[29,30] The association between bacteremia (or fungemia) and thrombocytopenia signifies that blood cultures are an appropriate investigation for many patients who develop thrombocytopenia without apparent cause. Microvascular thrombosis is believed to be at least in part responsible for multiple organ dysfunction.[27,31] The serious clinical sequelae of sepsis and associated DIC are best illustrated by meningococcemia, in which severely affected patients develop multiple organ failure, disseminated purpura (dermal infarction), and acral limb necrosis. In such patients, profound depletion of protein C is largely responsible for the microvascular thrombosis and associated tissue damage.[6] Large-vessel thrombosis such as DVT occurs less often in sepsis than does microvascular thrombosis.

HEPARIN-INDUCED THROMBOCYTOPENIA

HIT is a clinicopathologic disorder that can be defined as any clinical event best explained by platelet-activating antiplatelet factor 4 (PF4)/heparin antibodies ("HIT antibodies") in a patient who is receiving, or who has recently received, heparin.[32] The high sensitivity of ELISA-based assays for HIT antibodies means that negative tests virtually rule out the diagnosis.[33] The most common clinical event is thrombocytopenia, which is seen in at least 90% of patients, depending upon the definition of thrombocytopenia. Symptomatic thrombosis that involves large veins and arteries is common in HIT (relative risk, 12–15).[34] Microvascular thrombosis also can occur, usually as a complication of warfarin treatment.[2] HIT has a unique transient nature, in contrast to hereditary hypercoagulability states or other acquired severe prothrombotic disorders (e.g., APS, cancer-associated DIC). Indeed, the pathogenic HIT antibodies often become undetectable within a few weeks or months following platelet count recovery.[35] In patients who have a strong indication for heparin, such as needed for cardiac surgery, heparin is usually the best choice despite a history of previous HIT, provided that platelet-activating antibodies are not detectable at the time of repeat exposure.[36]

Another unusual feature of HIT is its well-defined time of onset between 5 and 10 days after the immunizing exposure to heparin.[35] Most commonly, heparin causes immunization when given during or immediately before or after a surgical procedure. This suggests that there is an interaction between heparin and perioperative events, such as release of PF4 or inflammatory events, in leading to antibody formation ("point immunization").[37] The characteristic temporal onset, the relatively common occurrence of HIT, and the proven benefit of anticoagulants for preventing or treating thrombosis associated with HIT provide a rationale for platelet count monitoring of patients at higher risk for this potentially life- and limb-threatening complication of heparin.[38]

COUMARIN NECROSIS

Coumarins are orally active vitamin K antagonists commonly given for long-term anticoagulation. Their anticoagulant effect results from reduction of the vitamin K–dependent procoagulant factors, especially prothrombin (factor II). However, on occasion, the reduction of one or both of the vitamin K–dependent natural anticoagulants—proteins C and S—is sufficiently severe, or occurs in a suitably susceptible patient, to cause microvascular thrombosis and tissue necrosis.[39] This pathophysiology reflects the key role of the protein C natural anticoagulant pathway to downregulate thrombin in the microvasculature. Coumarin necrosis generally involves a complex interplay of several factors that conspire to produce a transient disturbance in procoagulant–anticoagulant balance. For example, consider a patient with APS and postpartum DVT, who develops necrosis following high "loading doses" of warfarin and premature discontinuation of heparin.[40] The acute DVT and early stopping of heparin may lead to increased procoagulant activity, whereas the high initial warfarin dosing and the (presumably) impaired protein C anticoagulant pathway related to APS create a "perfect storm" resulting in microvascular thrombosis and skin necrosis.

There are two syndromes of coumarin necrosis: "classic" skin necrosis involving dermal and subdermal tissues and venous limb gangrene involving acral (distal extremity) necrosis, usually in a limb with DVT.[41] As a general rule, skin necrosis often occurs in a patient with chronic impairment of the protein C natural anticoagulant pathway (e.g., congenital deficiency in protein C or protein S, factor V_{Leiden}, antiphospholipid antibodies), whereas venous limb gangrene typically occurs in a patient with an acquired hypercoagulability state with prominent platelet activation, such as HIT or cancer-associated consumption coagulopathy.[2,42] However, these distinctions are not absolute: Some patients with HIT develop classic warfarin-induced skin necrosis,[43] and factor V_{Leiden} predisposes to venous limb gangrene in a patient receiving warfarin for DVT.

SUMMARY

Common to all of these disorders is the important role for the clinician to explain and, if possible, treat effectively the combination of bleeding or clotting (sometimes both in the same patient) associated with a wide spectrum of laboratory abnormalities that can involve various coagulation factors, platelet number or function, and even red and white blood cells. The complexity of each underlying condition warrants a careful analysis of cause, manifestation, and pathophysiology, and treatment must be both rational and individualized for each circumstance.

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CHAPTER

98 Consumptive Thrombohemorrhagic Disorders

MARCEL LEVI, DONALD I. FEINSTEIN, ROBERT W. COLMAN, AND VICTOR J. MARDER

Known variously as *disseminated intravascular coagulation* (DIC), *defibrination*, or the *consumption coagulopathies*, consumptive thrombohemorrhagic disorders are a heterogeneous group of disorders that can be manifested by the entire range of hemorrhagic and thrombotic pathology. Essentially, DIC comprises a clinicopathological state in which systemic activation of coagulation occurs, leading to thrombotic obstruction of small- and mid-size vessels[1,2,3,4,5] impairing blood supply and contributing to multiple-organ dysfunction (MOD). Due to ongoing activation of the coagulation system and other processes, such as impaired synthesis of platelets and coagulation proteins, depletion of coagulation factors, protease inhibitors, and platelets may occur.[6,7] This situation leads to a severe impairment of hemostasis and results in serious bleeding, in particular in patients who are at risk for major blood loss, such as perioperative patients or trauma patients. In fact, bleeding may dominate the clinical picture of a patient with DIC.

In view of these multiple, often contrasting mechanisms that occur in patients with DIC, a consensual definition of DIC had been a matter of debate. In 2001, the subcommittee on DIC of the International Society on Thrombosis and Hemostasis proposed a definition that reflects the central role of the microvascular milieu, that is, endothelial cells, blood cells, and the plasma protease system, in the pathogenesis of DIC. This definition of DIC reads as follows: "DIC is an acquired syndrome characterized by the intravascular activation of coagulation without a specific localization and arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction."[8]

The diagnosis of DIC may be hampered by the nonspecific nature of many indicators of coagulation activation, although newly developed scoring algorithms, based on readily available routine laboratory parameters, show promising diagnostic accuracy.[8,9] Owing to the complexity of the clinical presentation, the variable and unpredictable course, and the multitude of therapies given to patients with DIC, properly conducted clinical trials are extremely difficult to perform and even to devise. Management relies on limited evidence from clinical trials in combination with small studies employing surrogate outcome parameters and experience from case series, as well as an understanding of the pathophysiologic mechanisms involved, together precariously applied to the individual patient.[10]

HISTORY

One of the first reports of DIC comes from Dupuy in 1834, who described the effect of the intravenous injection of brain material in animals.[11] The animals almost immediately died, and at autopsy there were widespread clots in the circulation, presumably due to what we would now call tissue factor (TF)-dependent systemic activation of coagulation. Thirty years later, Trousseau described the thrombotic tendency and the inclination of blood to clot in patients with advanced malignant disease.[12,13] Nauyn observed gross intravascular coagulation of animal blood after infusion of hemolyzed erythrocytes,[14] and Foa and Pellacani[15] observed the same phenomenon after injection of a variety of fresh organ extracts. Mellanby recognized that the coagulation defect of DIC produced by snake venoms resulted in hypofibrinogenemia.[16,17] A more precise description of DIC and its underlying pathogenesis had to wait until 1955 when more insight into the mechanism of blood coagulation was attained and better laboratory tests had become available. Ratnoff et al.[18] describe the hemostatic abnormalities that occur in women with fetal death and amniotic fluid embolism. McKay[19] was the first to realize that DIC was an "intermediary mechanism" in many diseases. Hardaway[2] emphasized that shock and acidosis occurring in a variety of diseases could initiate clotting with consumption of fibrinogen, platelets, and other coagulation factors. Verstraete et al.[20] reported several other cases of successful heparin use in DIC and suggested that this drug might be of more general value.

CLINICAL FEATURES OF CONSUMPTIVE THROMBOHEMORRHAGIC DISORDERS

The spectrum of clinical and laboratory presentation is affected by several parameters, as listed in Table 98.1. Herein lies much of the confusion, especially because sometimes cases of so-called DIC are not disseminated, or intravascular, or even related to coagulation. These variables determine whether specific (supportive) therapy for the "consumption" is required, over and above that indicated for the underlying disease or its other complications. Table 98.2 summarizes the clinical manifestations of severe consumptive hemorrhagic disorders.

When presented with the patient with a clinical suspicion of DIC, the physician must judge whether the clinical presentation is severe enough to warrant specific therapy, and whether the coagulation disorder is likely to be a self-limiting problem that will wane on therapy for the underlying condition or will gather momentum and assume importance beyond that of the initiating stimulus. If the problem comes to the attention only as the result of a screening laboratory survey, management will depend on whether an abnormal test result is the only reflection of consumption or whether serious clinical manifestations also exist or are likely to develop. In particular, signs of ischemic organ dysfunction, macrovascular thrombosis, excessive bleeding, and fragmentation hemolytic anemia should be sought (FIGURE 98.1).

Table 98.1

Parameters that influence the clinical and laboratory presentation of consumptive thrombohemorrhagic disorders

Parameter	Variation
Tempo	Acute vs. chronic Mild vs. severe
Location	Localized vs. systemic (disseminated) Intravascular vs. extravascular
Pathologic mechanism	Tissue factor—mediated thrombin generation + impaired anticoagulant pathways + impaired plasmin formation Primary hyperfibrinolysis Isolated platelet consumption

Given that factors of tempo, location, and pathophysiology can all operate as independent variables superimposed on a variety of underlying disorders, each patient's clinical and laboratory presentation must be individually considered before rational management can be started.

Many clinical entities have been associated with DIC,[1,21] and the major conditions are listed in Table 98.3. The frequency of DIC in these diseases varies considerably, ranging from rare or doubtful according to current criteria for DIC to highly frequent (severe gram-negative sepsis). Infectious diseases and malignant disorders together account for approximately two-thirds of DIC cases in the major series.[22,23,24] Trauma was a major cause of DIC in some series, probably reflecting the specialized nature of the clinical case mix in those centers.[25]

ACUTE, SEVERE CONSUMPTION COAGULOPATHY

Acute, severe DIC could be defined as a "pathological syndrome resulting from activation (and consumption) of certain coagulant proteins, formation of thrombin, and production of fibrin thrombi."[7] Diffuse multiorgan bleeding, hemorrhagic necrosis, microthrombi in small blood vessels, and thrombi in medium and large blood vessels are common findings at autopsy, although patients who had unequivocal clinical and laboratory signs of DIC may not have consistent postmortem findings.[26,27] Conversely, some patients in whom clinical and laboratory signs were not diagnostic of DIC had typical autopsy findings.[28,29] This occasional lack of correlation among clinical, laboratory, and pathologic findings is partly due to extensive postmortem changes in the blood, for example, excessive fibrinolysis, but remains unexplained in most instances.[27] Organs most frequently involved by diffuse microthrombi are the lungs and kidneys, followed by the brain, heart, liver, spleen, adrenal glands, pancreas, and gastrointestinal organs. Most thrombi consist of fibrin monomers or polymers in combination with platelets, and activated mononuclear or other inflammatory cells and other signs of inflammatory activation.[30] In cases of long-lasting DIC, organization and endothelialization of the microthrombi are often observed. Acute tubular necrosis is more frequent than renal cortical necrosis.[26] Clinically, thrombotic occlusive events occur first, as the result of microthrombi of fibrin or platelets

Table 98.2

Clinical manifestations of severe consumptive hemorrhagic disorders

Organ	Manifestation
Skin	Purpura, bleeding from injury sites, hemorrhagic bullae, focal necrosis, acral gangrene
Cardiovascular	Shock, acidosis, myocardial infarction, cerebrovascular events, thromboembolism in all types, and caliber blood vessels
Renal	Acute renal insufficiency (acute tubular necrosis), oliguria, hematuria, renal cortical necrosis
Liver	Hepatic failure, jaundice
Lungs	Adult respiratory distress syndrome, hypoxemia, edema, hemorrhage
Gastrointestinal	Bleeding, mucosal necrosis and ulceration, intestinal ischemia
Central nervous	Coma, convulsions, focal lesions, bleeding
Adrenals	Adrenal insufficiency (hemorrhagic necrosis)

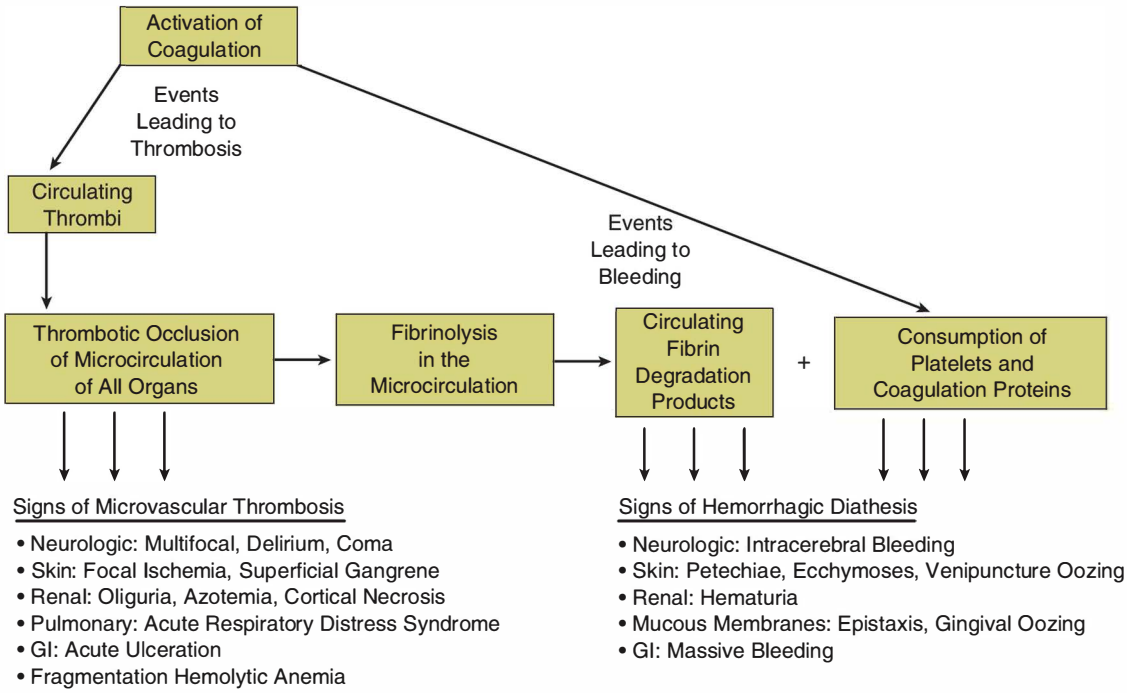

FIGURE 98.1 Sequence of events during DIC, leading to the clinical appearance of thrombotic and hemorrhagic phenomena. GI, gastrointestinal. (From Marder VJ. Microvascular thrombosis. In: Lichtman MA, ed. *The science and practice of clinical medicine*, vol 6. New York: Grune & Stratton, 1980:230–234, with permission.)

that obstruct the microcirculation of organs (FIGURE 98.1).[5] These thrombi result from clots that form either in the circulation or *in situ* in arterioles, capillaries, or venules. Circulatory obstruction produces organ hypoperfusion and even ischemia, infarction, and necrosis. The process is disseminated throughout the microcirculation; therefore, all organs are potentially vulnerable. Venous thromboembolism or arterial embolism from nonbacterial endocarditis can occur but are much more common in patients with subacute or chronic DIC, particularly in those patients with malignant disease.[23]

Renal dysfunction in patients with DIC is usually multifactorial. The conditions usually associated with DIC are frequently complicated by hypovolemia or hypotension that causes prerenal azotemia, even renal failure due to acute tubular necrosis. Thrombosis of renal afferent arterioles or glomerular capillaries by fibrin thrombi may cause ischemic renal cortical necrosis (FIGURE 98.2).

Cerebral dysfunction is most often manifested as global changes, such as an altered state of consciousness, convulsions, or coma, rather than by isolated focal lesions. Pathologic lesions affecting cerebral function may also include major vessel occlusion and subarachnoid hemorrhage, as well as multiple cortical and brainstem hemorrhages following microvascular occlusions.[31] Microthrombi, macrothrombi, emboli, and hemorrhage in the cerebral vasculature all have been held responsible for the nonspecific neurologic symptoms and signs displayed by patients with DIC.[32]

Respiratory dysfunction in DIC can manifest as transient hypoxemia in mild cases to hemoptysis, dyspnea, and chest pain caused by pulmonary hemorrhage and adult respiratory distress syndrome (ARDS) in severe cases.[33–35] Physical examination reveals rales, wheezing, and occasionally pleural friction rub, and imaging shows diffuse infiltration resulting from intra-alveolar hemorrhage. ARDS is characterized by tachypnea, auscultatory silence, hypoxemia, low lung compliance, normal wedge pressure, and "white lungs" on chest images.[36] It stems from severe damage to the pulmonary vascular endothelium, which permits egress of blood components into the pulmonary interstitium and alveoli. This situation leads to intra-alveolar hyaline membrane formation and severe respiratory insufficiency.[37] ARDS can be caused by septic shock, severe trauma, fat embolism, amniotic fluid embolism, and heat stroke, but only a fraction of patients with ARDS exhibit signs of DIC. When DIC and ARDS are simultaneously triggered, each aggravates the other.

Hepatocellular dysfunction sufficient to cause jaundice has been reported in 20% to 50% of patients with DIC,[4,25] notably in patients with infection and prolonged hypotension. Cellular necrosis around the hepatic central veins may follow shock and hypoxemia or intravascular coagulation,[26,38] leading to decreased synthesis of procoagulant and anticoagulant factors.

Adrenal cortical failure, producing the Waterhouse-Friderichsen syndrome,[39] is a consequence of hemorrhagic necrosis.

Fragmentation-type hemolysis (microangiopathy) secondary to fibrin deposition in the microvasculature may contribute to anemia[40] (FIGURE 98.3). However, overt hemolytic anemia associated with DIC occurs in only 5% to 10% of patients, and similar morphologic abnormalities occur in many other disorders not associated with fibrin formation (e.g., thrombotic thrombocytopenic purpura [TTP]).

Table 98.3

Clinical conditions that may be complicated by consumption coagulopathy

Acute, Severe Consumption Coagulopathy

- Sepsis/severe infection
- Trauma
- Heat stroke
- Snake bite
- Obstetrical conditions
  - Amniotic fluid embolism
  - Abruptio placentae
  - HELLP syndrome
- Liver disease (e.g., after peritoneovenous shunt)
- Acute hemolysis and transfusion reactions

Chronic Consumption Coagulopathy

- Malignancy
  - Solid tumors
  - Hematological malignancies
- Liver disease

Localized Intravascular Coagulation

- Vascular abnormalities
  - Kasabach-Merritt syndrome
  - Other vascular malformations
  - Aortic aneurysms

FIGURE 98.2 Kidney biopsy from a patient with sepsis-related disseminated intravascular coagulation (DIC). The picture shows intravascular fibrin in a small arteriole (*arrow*). (Photograph courtesy of Jan J. Weening, MD, Amsterdam, The Netherlands.)

Dermal ischemia and hemorrhagic skin necrosis occurs particularly in patients with acute DIC (see FIGURE 98.4), particularly those associated with severe infection, hypotension, and peripheral vasoconstriction. This complication of acute DIC is in contrast to idiopathic purpura fulminans, which typically occurs in young children who may be convalescing from an infection involving the skin and who are *not* acutely infected at the time of the appearance of purpura fulminans.[41,42]

Although the initial pathologic events are manifested by fibrin deposition in the microcirculation, the patient's initial clinical events may relate to hemorrhagic manifestations such as mucosal oozing, spontaneous ecchymoses, petechiae, and massive gastrointestinal (GI) blood loss. If the patient has had recent invasive or operative procedures, the incisions will also be vulnerable to excessive bleeding. The latter results from ongoing consumption and relatively insufficient synthesis of platelets and hemostatic factors.

Disorders Associated with Acute DIC

Infections

Virtually every type of infectious agent has been implicated in the consumptive thrombohemorrhagic disorders, usually producing mild or severe systemic intravascular coagulation, with consumption of platelets and soluble clotting factors (see also Chapter 123: *Thrombohemorhagic Complications of Sepsis*). The most flagrant examples are the Waterhouse-Friderichsen syndrome and purpura fulminans, both of which bear pathologic and histologic similarity to the experimental animal model of consumption, the Shwartzman reaction.[43] The Waterhouse-Friderichsen syndrome is most commonly seen during fulminant meningococcal sepsis, although other organisms such as *Pneumococcus* may

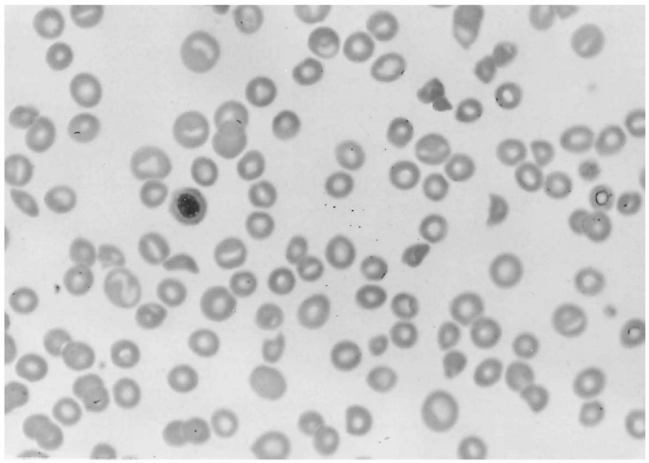

FIGURE 98.3 Microangiopathic hemolytic anemia in a patient with thrombotic thrombocytopenia purpura (TTP): A nucleated red blood cell is seen in addition to the schistocytes and other fragmented erythrocytic forms.

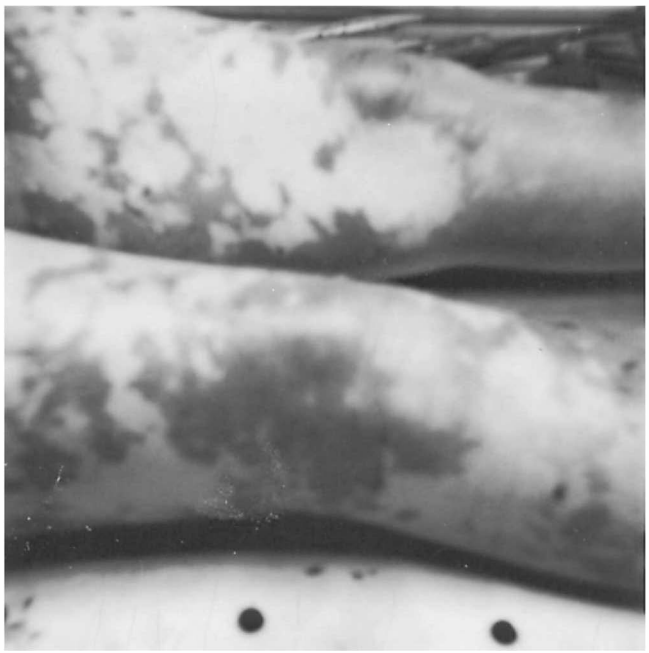

FIGURE 98.4 Patient with purpura fulminans, showing the irregular distribution of ischemic and hemorrhagic cutaneous lesions that reflect widespread occlusion of the arteriolar supply to the skin.

also cause this complication.[42,44] Widespread intravascular fibrin and platelet thrombi obstruct arterioles, capillaries, and venules of vital organs, especially causing bilateral hemorrhagic necrosis of the adrenals. This type of sepsis is associated with severe shock, which contributes significantly to high mortality. Although these clinical entities appear to be distinctive syndromes, they can be considered extreme manifestations of acute DIC. Variations in the acuteness of onset and the degree of consumption, perhaps related to the patient's age and health and the type of infectious agent, account for the wide range of clinical manifestations, including in some patients with laboratory abnormalities of consumption but without clinical signs.

Bacterial infections are among the most common causes of DIC.[1,45] Certain patients are particularly vulnerable to infection-induced DIC, such as immune-compromised hosts; asplenic patients whose ability to clear bacteria, particularly pneumococci, is impaired; and newborns whose coagulation inhibitory systems are immature. In addition, infections can aggravate bleeding and thrombosis by directly inducing thrombocytopenia, hepatic dysfunction, and shock.[46] Clinically overt DIC may occur in 30% to 50% of patients with gram-negative sepsis[47,48] and is common in patients with gram-positive sepsis.[49,50] Extreme examples of sepsis-related DIC are *Streptococcus* A toxic shock syndrome, characterized by deep-tissue infection and vascular collapse,[51] and meningococcemia characterized by extensive hemorrhagic necrosis and shock. The extent of hemostatic derangement in patients with meningococcemia correlates with prognosis.[52,53] Patients affected by *Pseudomonas aeruginosa*, *Escherichia coli*, and *Proteus vulgaris* bacteremias may have only laboratory signs of activated coagulation or may present with severe DIC, especially accompanied by shock.[54,55]

The mechanism by which DIC is elicited in *Staphylococcus aureus* bacteremia may be related to an α-toxin that activates platelets and induces IL-1 secretion by macrophages.[56] *Streptococcus pneumoniae* infection has been associated with the Waterhouse-Friderichsen syndrome particularly in asplenic patients,[57] ascribed to a capsular antigen and to antigen–antibody complex formation.[58] Clostridial bacteremia is a highly lethal disease characterized by septic shock, DIC, renal failure, and hemolytic anemia.[59]

Activation of the coagulation system has also been documented for nonbacterial pathogens, that is, viruses causing hemorrhagic fevers,[59,60] protozoa (Malaria),[61,62] and fungi.[63] Common viral infections, such as influenza, varicella, rubella, and rubeola, have rarely been associated with DIC.[64] However, purpura fulminans associated with DIC has been reported in patients with infections and either hereditary thrombophilias[65,66] or acquired antibodies to protein S.[67] Other viral infections can cause "hemorrhagic fevers" characterized by fever, hypotension, bleeding, and renal failure (see Chapter 123). Laboratory evidence of DIC can accompany Korean, rift valley, and dengue-related hemorrhagic fevers,[68–70] perhaps secondary to TF release from cells in which viruses replicate[71] and increased levels of proinflammatory cytokines.[58]

Purpura Fulminans

Purpura fulminans is a severe, often lethal form of DIC in which extensive areas of the skin over the extremities and buttocks undergo hemorrhagic necrosis.[41] The disease affects infants and children predominantly[72,73] and may occur in patients with acute bacterial infections and in patients with preexisting inherited or acquired disorders of the protein C anticoagulant pathway. Sepsis-associated purpura fulminans is much more common in children than adults,[39] *Meningococcus* being the most common (but not the sole) organism involved.[4] Onset may appear 2 to 4 weeks after a mild infection such as scarlet fever, varicella, or rubella or can accompany an acute viral or bacterial infection in patients with thrombophilia affecting the protein C pathway.[52,72] The initial presentation in sepsis-associated purpura fulminans is often that of overwhelming acute infection with hypotension and peripheral hypoperfusion; in contrast, in idiopathic purpura fulminans, the blood pressure is well preserved. The skin necrosis in sepsis-associated purpura fulminans often begins in the distal extremities (FIGURE 98.4), with subsequent proximal progression, or involves the entire body in a patchy distribution.[74] Infants who are homozygous for protein C or S deficiency may be born with skin necrosis and intravascular coagulation (hereditary purpura fulminans).[75,76] Patients who are heterozygotes for protein C or S deficiency may develop skin necrosis when placed on warfarin therapy.[77] Protein C or S levels may be low because of liver dysfunction, consumption, or, rarely, secondary to an acquired inhibitor.[78]

Trauma or Massive Tissue Necrosis

The acute coagulopathy accompanying severe trauma may complicate severe head injury, massive soft tissue trauma, multiple fractures and fat embolism, gunshot wounds, and extensive burns.[79] In a study of 16 patients with head injury who had DIC and came to autopsy within 4 days of injury, Kaufman et al.[80] noted necrosis and bleeding, most notably in the brain and lungs, and microthrombi in the central nervous system, liver, lungs, kidneys, and pancreas. Thrombi were less prevalent with

longer delays after injury, attesting to a probable fibrinolytic response in the microcirculation to dissolve these thrombi. Head injury in children is also associated with abnormal clotting tests in 71% of cases, and mortality is fourfold greater in patients with DIC than in those without DIC.[81] The high mortality rate is associated with increased bleeding and laboratory evidence of DIC and is the foundation for prognostic scoring systems.[81,82,83]

Extensive exposure of TF to blood and hemorrhagic shock probably are the immediate triggers of acute coagulopathy, but cytokines likely play a pivotal role. In fact, the changes in cytokine levels are virtually identical in trauma patients and septic patients.[84] The levels of TNF-α, IL-1β, plasminogen activator inhibitor-1 (PAI-1), circulating TF, plasma elastase released from neutrophils, and soluble thrombomodulin all can be elevated in patients with signs of DIC, predicting multiple-organ failure (MOF) (ARDS included) and death.[85,86] Careful monitoring of laboratory signs of DIC, reduced fibrinolytic activity, and perhaps low antithrombin (AT) levels also are useful for predicting the outcome of such patients.[87]

The coagulopathy can be aggravated in patients with severe trauma who require massive blood replacement because stored blood components are diluted and do not contain sufficient amounts of viable platelets and factors V and VIII. Moreover, activation of fibrinolysis may aggravate bleeding and hypotension.[88–91]

The time interval between trauma and medical intervention correlates with the development and magnitude of DIC. Experience during wars proved that fast evacuation and prompt medical care reduce the risk of DIC.[92–94]

TF exposed to blood at sites of burned tissue, the systemic inflammatory response syndrome induced by the burn, and superimposed infections can trigger DIC.[95] Bleeding, laboratory tests indicative of DIC, and vascular microthrombi in biopsies of undamaged skin have been described in patients with extensive burns.[96] Significant local consumption of fibrinogen and platelets occur in burned areas.[97] Laboratory signs of DIC are associated with organ failure, and the extent of protein C and AT deficiencies correlates with poor outcome.[96] A clinicopathologic study of 139 patients who died during treatment for a severe burn disclosed that 18% had cerebral infarctions caused by septic arterial occlusions or DIC and 4% had intracranial hemorrhage.[98]

Heat Stroke

Heat stroke is frequently complicated by consumption coagulopathy.[99,100] James Wellstead published in 1841 his book "Travels to the City of the Caliphs" (currently known as Baghdad) and vividly described that on an extremely hot day in the Persian Gulf the decks of the ship Liverpool resembled a slaughterhouse, so numerous were the bleeding patients.[101,102] Heat stroke is a syndrome characterized by a rise in body temperature to over 42°C that follows collapse of the thermoregulatory mechanism. Predisposing factors include high environmental temperature, strenuous physical activity, infection, dehydration, and lack of acclimatization.[103,104] Extensive hemorrhage, unclottable blood, and venous engorgement were found as early as 1841 in postmortem examination of patients who died of heat stroke.[102] Investigations have confirmed that a severe hemorrhagic diathesis and MOF often accompany heat stroke.[95,105–107] Diffuse fibrin deposition and hemorrhagic infarctions are found in fatal human cases. DIC associated with profound fibrin(ogen)olysis is evident in patients with heat stroke. The possible triggers of DIC in patients with heat stroke include endothelial cell damage and TF released from heat-damaged tissues.[105]

In a series of 18 critically ill patients from Paris with heat stroke during the 2003 heat wave in Western Europe,[107] patients showed very high levels of IL-6 and IL-8, a striking activation of white blood cells (β2-integrin upregulation and increased reactive oxygen species) and evidence of systemic activation of coagulation. There was a correlation between the extent of inflammation and coagulation activation and clinical severity of the heat stroke. Patients have been categorized as nonbleeders, bleeders without DIC but with slight consumption of hemostatic factors, and bleeders with typical signs of DIC.[99] Prompt cooling and support of vital functions have substantially reduced the high mortality that was commonly observed in early studies.[108]

Snakebite

The bite of certain snakes, especially vipers and rattlesnakes, causes a coagulopathy that is highly reminiscent of DIC, although it is a matter of debate whether this condition can be truly classified as DIC.[17] Prominent among these species are the *Vipera*, *Echis (E. carinatus* or *E. coloratus)*, *Aspis*, *Crotalus*, *Bothrops*, and *Agkistrodon*. Venoms of these snakes contain enzymes or peptides that exert a variety of activities[109–111]: (a) thrombin-like activity, cleaving fibrinopeptide A from the Aα chain of fibrinogen, (b) activation of prothrombin even in the absence of calcium ions, (c) activation of factors X and V, (d) fibrinogenolysis, (e) thrombocytopenia by platelet aggregation, (f) inhibition of platelet aggregation, (g) activation of protein C, and (h) endothelial damage cells. Victims of snake bites rarely experience excessive bleeding or thromboembolism, in spite of the serious derangements in hemostasis and findings that are sometimes consistent with DIC.[112–114]

Several species of snakes belonging to the Viperidae family produce venoms that have a wide range of activities affecting hemostasis. The symptoms are characterized by local tenderness and swelling, mild bleeding from the wound or from venipunctures, occasional hypotension without shock, and transient mild oliguria. The major symptoms and signs related to envenomation are vomiting, diarrhea, apprehension, hypotension, local swelling, ischemia, and necrosis. Treatment for victims of snake bites consists of immediate immobilization, administration of antivenom and fluids, and other general measures to preserve vital functions. Local incisions, cooling, and application of tourniquet should be avoided.[109] Some patients begin to recover spontaneously after 1 to 2 days and are completely normal within 1 week, whereas others demonstrate incoagulable blood for more than 3 weeks.[114,115] Active secondary fibrinolysis accounts for the rapid recovery of renal microcirculatory obstruction. Chugh et al.[116] documents severe illness after viper bites, with 45 of 157 patients having acute renal failure, and death occurring in eight of 10 with bilateral renal necrosis and in four of 23 with "less severe acute tubular lesions."

Acute Obstetric Complications

Since 1971, when DeLee[117] reported a state of "temporary hemophilia" in patients with premature separation of the placenta and a macerated dead fetus, it has been evident that a consumptive thrombohemorrhagic state is observed in a wide range of obstetric complications, including *abruptio placentae*, retained dead fetus, amniotic fluid embolism, saline-induced or septic

abortion, and occasionally toxemia. The site of consumption may be limited to a single extravascular locale (the uterus), disseminated in the blood, or present in both locations; the pathologic mechanism may involve coagulation, fibrinolysis, or both simultaneously; and the broadest range of tempo may occur, from the fulminant onset of an *abruptio placentae* to the chronic, low-grade effects of the retained dead fetus syndrome.

Pregnancy is associated with a baseline hypercoagulable state characterized by increased levels of procoagulant factors, low protein S levels, and reduced fibrinolytic activity.[118,119] Pregnancy predisposes patients to DIC for at least four reasons: (a) a hypercoagulable state, manifested by low-grade thrombin generation, with elevated levels of fibrin monomer complexes and fibrinopeptide A; (b) reduced fibrinolytic activity because of increased plasma levels of PAI-1; (c) decline in the plasma free protein S; and (d) during labor, leakage of TF from placental tissue into the maternal circulation. DIC may be difficult to diagnose during pregnancy because of the high initial levels of coagulation factors such as fibrinogen, factor VIII, and factor VII,[120,121] but progressive reductions in these factors can help confirm or exclude the diagnosis. Thrombocytopenia may be particularly helpful in determining whether DIC is present, provided other causes are excluded.[122]

Abruptio Placentae

Placental abruption is a leading cause of perinatal death.[123] Older multiparous women or patients with one of the hypertensive disorders of pregnancy are at highest risk. The severe hemostatic failure accompanying abruptio placentae is the result of acute DIC following the introduction of large amounts of TF into the circulation from the damaged placenta and uterus.[124] Amniotic fluid activates coagulation *in vitro*, and the degree of placental separation correlates with the extent of DIC, suggesting that leakage of thromboplastin-like material from the placental system is responsible for DIC. Abruptio placentae occurs in 0.2% to 0.4% of pregnancies, but only 10% of these cases are associated with DIC.[119,122] Although in the classic study by Pritchard and Brekken[119] only 38% of patients had hypofibrinogenemia (<150 mg/dL), most patients had a relatively low fibrinogen for that stage of pregnancy. Rapid volume replacement and evacuation of the uterus is the treatment of choice.[123] Transfusion of cryoprecipitate, fresh-frozen plasma, and platelets should be given for profuse bleeding, but blood components may not be necessary in less severe cases, as coagulation factors increase rapidly following delivery. Heparin or antifibrinolytic agents are not indicated.

Amniotic Fluid Embolism

Amniotic fluid embolism was firmly established as a syndrome by the clinical and histopathologic changes described by Steiner and Lushbaugh[125] in 1941. Its incidence is 1:8,000 to 1:80,000 live births and is responsible for 10% of maternal deaths in the United States.[126] Patients predisposed to amniotic fluid embolism are multiparous women whose pregnancies are postmature and women undergoing a tumultuous labor. Amniotic fluid is introduced into the maternal circulation through tears in the chorioamniotic membranes, rupture of the uterus, and injury of uterine veins.[126] The trigger of DIC probably is TF present in amniotic fluid.[127,128] The mechanical obstruction of pulmonary blood vessels by fetal debris, meconium, and other particulate matter in the amniotic fluid enhances local fibrin–platelet thrombus formation and fibrinolysis. The extensive occlusion of the pulmonary arteries and an acute anaphylactoid response reminiscent of severe systemic inflammatory response syndrome provoke sudden dyspnea, cyanosis, acute cor pulmonale, left-ventricular dysfunction, shock, and convulsions. In patients who survive the initial episode, excessive bleeding follows in 40% of cases, usually after a latent period of 0.5 to 4 hours, and the great majority have laboratory evidence of consumption.[126] In the National Registry for amniotic fluid embolism (46 patients), cardiac arrest occurred in 87% and mortality was 61%, but only 15% of patients survived neurologically intact.[129] Seventy-nine percent of fetuses survived, but only 50% were neurologically unimpaired.

Hemorrhage is particularly severe from the atonic uterus, puncture sites, and GI tract. The best prospect for decreasing mortality lies in early termination of parturition in patients at high risk and prevention of hypertonic and tetanic uterine contractions during labor. When the syndrome is recognized, immediate termination of pregnancy under pulmonary and cardiovascular support is essential. Vigorous supportive therapy directed toward gas exchange with mechanical respiratory assistance and fluid and blood replacement to treat severe hypotension are essential. In patients in whom continued entry of amniotic fluid debris may be contributing to further intravascular clotting, heparin is a reasonable adjunct to therapy.[121] When bleeding is the major manifestation, heparin may increase bleeding, especially if adequate replacement therapy for consumed clotting factors has not been achieved. In addition to replacement therapy for treatment of dangerous bleeding, fibrinolytic inhibitors may be helpful, but the same precautions regarding their use apply as in other settings of DIC.

Septic Abortion

In contrast to the relatively mild clinical state of saline-induced abortion, septic abortion heralds the most fulminant type of severe DIC. In a group of more than 6,000 patients who had undergone abortion, 16% were infected, 4% of whom had shock and DIC, leading to death in one-half of these seriously affected patients.[130] Any of a large number of bacterial organisms, both aerobic and anaerobic, may be the inciting agent; a combination of severe hypotension and DIC complicates the clinical picture. Once established, the clinical symptoms, laboratory manifestations, and principles of therapy are as for any severe infection leading to shock, with the added consideration that evacuation of the uterus and sometimes hysterectomy are required to eliminate the infection. These patients can be the most refractory to therapy, and vigorous attention must be paid to proper antibiotic choice, maintenance of blood volume and electrolyte balance, and replacement of deficient hemostatic factors. The use of heparin in these circumstances is controversial, and there is little evidence of its benefit. As with other circumstances of acute, severe DIC, fibrinolytic inhibitors should be withheld so as not to impair the physiologic fibrinolytic response in the microcirculation of ischemic organs.

Acute Fatty Liver

Acute fatty liver of pregnancy is a rare disorder that occurs during the third trimester of pregnancy,[131] which can lead to hepatic failure, encephalopathy, and maternal and fetal death.[132–135] In 15% to 20% of cases, acute fatty liver of pregnancy is associated with fetal homozygosity or compound heterozygosity

for long-chain acyl-CoA dehydrogenase (LCAD) deficiency,[136] one mutation (G1528C) accounting for 65% to 90% of cases. The precise mechanism by which LCAD deficiency in the fetus causes the severe liver disease in the heterozygous mother is unclear. The typical histologic feature of acute fatty liver disease of pregnancy is microvesicular fatty infiltration of the liver, which is characterized by severe liver dysfunction, renal failure, hypertension, and DIC.[133,137] Exceedingly low levels of AT and other laboratory signs of DIC were observed in a series of 28 patients, but no definite clinical benefit from AT concentrate infusion was achieved.[137] The primary therapy for these patients is early delivery and supportive care, which yield a maternal survival of 90% and perinatal survival of more than 85%[133,138] Pancreatitis is a potentially lethal complication of acute fatty liver of pregnancy.[139]

Preeclampsia and "Hemolysis, Elevated Liver Enzymes, and a Low Platelet Count" (HELLP Syndrome)

The existence of DIC as a constant feature in patients with preeclampsia or eclampsia is controversial.[19,140,141] A critical analysis concluded that the thrombocytopenia in preeclamptic patients stems from endothelial injury rather than DIC,[140] but, other investigators provide evidence for DIC,[142,143] including a good correlation between the clinical severity and abnormalities in platelet counts and fibrin(ogen) degradation products.[141] The syndrome of hemolysis (H), elevated liver enzymes (EL), low platelet count (LP), and severe epigastric pain is a complication of pregnancy-induced hypertension.[144] Seventy percent of the cases occur during the third trimester of pregnancy and 30% occur during the postpartum period.[145] Hemolysis, elevated liver enzymes, and a low platelet count (HELLP) syndrome occurs more often in whites, multipara, and women older than 35 years.[146] Liver biopsy shows fibrin deposition, and laboratory tests are consistent with DIC in a significant proportion of patients.[145,147,148] Hepatic imaging in 33 patients revealed subcapsular hematomas in 13 and intraparenchymal hemorrhage in 6.[149] Although the majority of patients do not have laboratory evidence of DIC, a significant proportion have a low fibrinogen level (<300 mg/dL), which frequently is associated with evidence of abruptio placentae. MOD manifested by acute renal failure, ascites, pulmonary edema, and severe hemorrhage resulting from DIC may develop, leading to significant maternal and perinatal mortality. Management of patients with HELLP syndrome consists of supportive care, careful monitoring, and blood component replacement therapy.[150] With few exceptions, immediate delivery, not necessarily by Caesarian section, is indicated. HELLP syndrome tends to recur in subsequent gestations.[151]

Liver Disease

Advanced liver disease may result in chronic low-grade DIC (see below). However, complications of acute or chronic liver disease may trigger episodes of an acute coagulopathy, most notably in patients with peritoneovenous (LeVeen or Denver) shunts or fatty liver of pregnancy. As reviewed by LeVeen et al.,[152] peritoneovenous shunting has a mortality of <1% in "uncomplicated cirrhosis," and postoperative coagulopathies can largely be avoided. However, a substantial number of patients develop DIC shortly after shunt placement. Ragni et al.[153] detected DIC in 10 of 11 such patients, and Rubinstein et al.[154] found DIC after 14 of 27 shunt procedures and a greater tendency for this complication in patients with cirrhosis.

Acute Hemolysis and Transfusion Reactions

Acute hemolysis such as that after the transfusion of 500 mL or more of incompatible blood (major mismatch) can produce a hemorrhagic diathesis characterized by laboratory changes of hypofibrinogenemia and thrombocytopenia.[2] This pattern is compatible with acute DIC, with variable effects of intravascular thrombi on renal function depending on the acuteness and degree of defibrination and the additional presence of hypovolemia or hypotension. However, massive hemolysis (other than that associated with a major mismatch) does not uniformly produce DIC, as Mannucci et al.[155] observed in 28 patients with glucose-6-phosphate dehydrogenase deficiency who were exposed to fava beans. The DIC secondary to massive hemolysis is self-limiting, and in the absence of serious end-organ damage, patients improve rapidly with supportive measures. Treatment depends on maintenance of blood volume and organ perfusion and on blood replacement with compatible erythrocytes. DIC that accompanies incompatible blood transfusion may result from extensive antigen–antibody reaction causing release of elastase and TNF-α from neutrophils and activation of monocytes that express TF and complement, leading to assembly of the membrane attack complex inflicting damage to endothelial cells.[156,157]

CHRONIC OR SUBACUTE CONSUMPTION COAGULOPATHY

In contrast to the acutely ill patient with complicated, severe DIC, other patients may have mild or protracted clinical manifestations of consumption or even subclinical disease manifest by only laboratory abnormalities.[4,21,158] The clinical picture of subacute to chronic DIC is exemplified by the retained dead fetus syndrome, and the recalcitrant chronic hypercoagulability that may accompany malignancy. Mucin-producing adenocarcinomas may also present acutely with major vessel occlusions, nonbacterial thrombotic endocarditis with systemic arterial embolization and infarction, as well as with acute, severe DIC (Trousseau syndrome).[159] In patients with chronic DIC, laboratory manifestations are extremely variable. Some patients have all of the classic abnormalities of thrombocytopenia, hypofibrinogenemia, elevated fibrin degradation product (FDP) levels, and prolonged partial thromboplastin time and prothrombin time (PT), whereas other patients have thrombocytopenia only or virtually normal laboratory results, owing to increased synthesis of coagulation proteins and effective microvascular fibrinolysis.

Clinical Conditions Associated with Subacute or Chronic Consumption Coagulopathy

Neoplasia

Solid Tumors

Trousseau was the first to describe the propensity to thrombosis of patients with cancer and cachexia,[152] and evidence for malignancy-related primary fibrinolysis and/or DIC has since been provided.[12,160,161] In 182 patients with malignant disorders described by Sack et al. in 1977,[155] excessive bleeding was recorded in 75 cases, venous thrombosis in 123, migratory thrombophlebitis in 96, arterial thrombosis in 45, and arterial embolism resulting from nonbacterial thrombotic endocarditis in 31; 12 patients had venous thrombosis, endocarditis, and

bleeding. The overall incidence of DIC in consecutive patients with solid tumors in one series was 7%.[162]

Solid tumor cells may express TF, which forms a complex with factor VII(a) to activate factors IX and X, and a cancer procoagulant (CP), a cysteine protease with factor X-activating properties.[163,164] In breast cancer, TF is expressed by vascular endothelial cells as well as by the tumor cells,[165,166] and also is involved in tumor metastasis and angiogenesis.[167–169] CP can be found in extracts of neoplastic cells but also in the plasma of patients with solid tumors.[170,171] Interactions of P- and L-selectins with mucin from mucinous adenocarcinoma can induce formation of platelet microthrombi and probably constitute a third mechanism of cancer-related thrombosis.[172] Depending on the rate and quantity of exposure or influx of shed vesicles from tumors containing TF, nonovert or overt DIC develops.[173–175] For instance, a patient may be asymptomatic or present with venous thromboembolism if the tumor cells expose or release TF slowly or intermittently, and the ensuing utilization of fibrinogen and platelets is compensated by increased production of these components. Conversely, massive thrombosis or severe bleeding may supervene in a patient whose circulation is deluged by TF.[162,164] Another mechanism by which tumor cells may contribute to DIC involves fibrinolytic proteins.[176,177] Many malignant cells express urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), but most tumors induce a hypofibrinolytic state. Virtually all pathways that contribute to DIC are driven by cytokines, for example, IL-6, which induces TF expression on cells.[178] Fibrinolysis and microvascular physiological anticoagulant pathways are mostly dependent on TNF-α.[179–181] On the contrary, anti-inflammatory cytokines such as IL-10 inhibit DIC.[182–184]

Patients with solid tumors are vulnerable to additional triggers of DIC that can aggravate thromboembolism and bleeding.[161] Risk factors include advanced age, metastatic spread, and chemotherapy or antiestrogen therapy,[176,185] and triggers include septicemia and immobilization. Unusual clinical thrombotic states, such as recurrent venous thrombi, multiple limb thrombosis, hemorrhage after reasonable doses of anticoagulation, gangrene secondary to arterial emboli, thrombocytosis, phlegmasia cerulea dolens and venous gangrene, combined thrombosis and bleeding, arterial and venous thromboemboli, or all of these in a single patient, suggest an occult neoplasm. Patients with carcinoma of the prostate may have hemorrhagic manifestations that dominate the clinical picture, and this could reflect production of fibrinolytic activators as well as procoagulants.[186] The tendency of some tumor cells to produce such clot-dissolving activity may be reflected by prolonged localized bleeding after biopsy of the tumor.[187] Direct thrombin inhibitors such as hirudin or argatroban are probably more effective than heparin for application to the hospitalized patient. Fondaparinux, low molecular weight heparin, or unfractionated heparin are reasonable agents for prolonged outpatient therapy.

Leukemia

In 161 consecutive patients presenting with acute myeloid leukemia, DIC was diagnosed in 52 (32%), and in acute lymphoblastic leukemia, DIC was diagnosed in 15% to 20%.[188] The incidence of DIC in acute leukemia patients might increase during induction chemotherapy.[189]

In patients with acute promyelocytic leukemia (APL), DIC is present in more than 90% of patients at the time of diagnosis or after initiation of chemotherapy.[24,187,190] APL is characterized by intracytoplasmic inclusions and dense granules in promyelocytes, and the hemostatic disturbance is related to interaction of the malignant cells with endothelial cells.[170,188] APL cells express TF and the CP that can initiate coagulation, and they release IL-Iβ and TNF-α, which downregulate endothelial thrombomodulin, thereby compromising the protein C anticoagulant pathway. APL cells also express increased amounts of annexin II, which augments conversion of plasminogen to plasmin. Although hemorrhagic phenomena predominate, venous as well as arterial thromboembolic complications occur, with autopsy evidence of diffuse thrombosis reported in 15% to 25% of patients.[191] The majority show a prolonged PT, hypofibrinogenemia, elevated FDP levels, and a shortened fibrinogen half-life, even in patients without clinically identifiable disease.[190] Both TF and fibrinolytic proteases can be demonstrated in promyelocytic subcellular components, but the former predominates in both *in vitro* analyses and clinical presentation.[188] u-PA and tPA have been found within APL cells in addition to low plasma PAI and α_2-plasmin inhibitor levels.[190] In addition, protease activity stemming from leukocyte elastase can also cause increased fibrinogen catabolism, degrade several clotting factors, and increase fibrinolysis.[187,192] This clinical disorder may present with serious hemorrhagic manifestations due to DIC and fibrin(ogen)olysis, further complicated by severe thrombocytopenia secondary to both the intravascular coagulation and decreased thrombopoiesis. Before the use of all-*trans*-retinoic acid (ATRA), anthracycline-based induction therapy induced complete remission in 50% to 60% of patients, but a significant number died early from intracranial hemorrhage.[193] Control of the leukemic process determines the patient's ultimate response. A consumptive disorder may be induced or exacerbated by cell lysis during chemotherapy, but the coagulopathy associated with APL has dramatically decreased with the introduction of ATRA. The coagulopathy rapidly diminishes over 2 to 6 days,[192] and the net effect of ATRA is to reduce prothrombotic potential and hemostatic consumption.[194,195] ATRA, used for induction and maintenance therapy of APL, inhibits the deleterious effect of APL cells *in vitro* and *in vivo* and has reduced the frequency of hemorrhagic death.[170,192]

Liver Disease

A major role for a consumptive thrombohemorrhagic process in patients with liver disease is controversial. For example, histopathologic changes compatible with DIC are uncommon in patients with liver disease, as reported in an autopsy study showing only four of 184 cases of acute and chronic liver disease with microthrombi in more than one organ.[196] However, the absence of fibrin thrombi in patients with chronic liver disease may be a result of a systemic fibrinolytic state. The similarities between the hemostatic defects observed in patients with liver disease and in patients with DIC are striking and have evoked an ongoing controversy as to whether DIC contributes to hemostatic derangements associated with liver disease.[197]

Observations that support the hypothesis that DIC accompanies hepatic disorders include a shortened half-life of radiolabeled fibrinogen and prolongation of fibrinogen half-life by administration of heparin[198,199]; failure of replacement therapy to significantly increase the levels of hemostatic factors (suggesting continuous consumption); and increased blood levels of D-dimer, thrombin-antithrombin (TAT) complexes, and fibrinopeptide A, all consistent with ongoing thrombin generation.[196,200,201]

Observations that argue against the hypothesis that DIC accompanies liver diseases include an extremely low incidence (2.2%) of microthrombosis in the tissues of patients who die of liver disease and causes other than, or inconsistent with, DIC for the deranged findings in liver disease.[196] Examples of alternative explanations include acquired dysfibrinogenemia to explain a prolonged thrombin time,[202] reduced synthesis to explain low levels of coagulation factors and inhibitors,[203] and primary fibrinogenolysis induced by decreased clearance of t-PA. Kinetic data show increased rather than decreased factor VIII levels,[204] loss of fibrinogen into extravascular spaces that contributes to apparently excessive consumption,[158] and normal plasma survival of fibrinogen and plasminogen.[205] Another hypothesis maintains that patients with liver disease usually do not present with DIC but are extremely sensitive to the various triggers of DIC because of their impaired capacity to clear procoagulants and to synthesize essential components of the coagulation, inhibitory, and fibrinolytic systems. Patients with primary or metastatic liver disease who undergo a peritoneovenous shunt operation for severe ascites are more likely to develop DIC than are patients with ascites who undergo the same procedure because of other causes.[206]

What, then, should be the approach to patients with liver disease and bleeding without an apparent local cause? First, possible underlying causes of DIC should be considered and identified, and then a hemostatic profile should be examined at frequent intervals in order to detect any dynamic changes that may be helpful in recognizing DIC. The sensitive assays that reflect thrombin generation (TAT complex and prothrombin fragments 1.2) or concomitant thrombin and plasmin generation (D-dimer) may help establish the diagnosis of DIC in a patient with liver disease.[207]

Retained Dead Fetus Syndrome

Retained dead fetus syndrome exists if the fibrinogen concentration falls below normal for the stage of pregnancy in the context of intrauterine fetal death. This syndrome is a slowly developing, compensated or low-grade form of DIC. Its incidence increases with longer periods of fetal retention to as much as 35% of patients with a duration of 5 weeks or longer.[208] Although an occasional patient appears to have an acute primary fibrinolytic response to the dead fetus, the evidence strongly suggests that intravascular coagulation is the sole mechanism for the defibrination in most patients. As expected in patients with slowly developing consumption, the secondary fibrinolytic response keeps pace with the formation of fibrin thrombi, and organ obstruction and ischemia are not part of the clinical picture. The low fibrinogen and platelet counts do not usually cause serious bleeding, but patients are at risk for postpartum hemorrhage if hemostatic defects are not corrected. As with other obstetric consumptive complications, the essence of treatment is to evacuate the uterus. However, in the rare instance of a twin pregnancy with a single fetal death and consequent DIC, heparin treatment can reverse DIC and save the second twin.[209]

LOCALIZED INTRAVASCULAR COAGULATION

Certain disorders are associated with the consumption of platelets and clotting factors in strictly defined, localized anatomic sites. Laboratory studies are frequently characterized by thrombocytopenia, hypofibrinogenemia, and elevated levels of FDPs or D-dimer. Occasionally, this condition may cause bleeding. However, because the consumption is usually localized, distant microvascular occlusion and organ ischemia do not occur. Two risks of not recognizing the limited disorder are inappropriate therapy for a presumed "disseminated" state and failing to recognize the form and location of a potentially reversible underlying disorder.

Aortic Aneurysm

Localized consumption of platelets and fibrinogen in aortic aneurysms can produce not only striking laboratory abnormalities but even symptomatic bleeding.[210,211] Although few patients with aneurysm demonstrate overt hemorrhagic manifestations, this complication may be particularly associated with acute extensions and enlargements of the aneurysm.[212] In some cases, dramatic manifestation of spontaneous bleeding episodes is associated with radiographic evidence of an expanding aneurysmal mass or with the sudden onset of severe pain indicative of dissection. In a series of patients with aortic aneurysm, 40% had elevated levels of fibrin(ogen)-degradation products, but only 4% had significant bleeding and laboratory evidence of DIC.[210] Several factors predispose patients with aortic aneurysms to the development of DIC: a large surface area, dissection, and expansion of the aneurysm.[212] Clinical and laboratory signs of DIC should be carefully sought in patients with an aortic aneurysm because bleeding may seriously complicate surgical repair of the aneurysm.[212,213] The initiation of localized and generalized intravascular coagulation can be ascribed to activation of the TF pathway by the abundant amounts of TF present in atherosclerotic plaques.[214] When patients present with significant bleeding or when surgery is planned, hemostatic defects should be sought and corrected.[215] Stent grafting, which is a common procedure for repair of aortic aneurysms, was complicated by DIC and death in two patients, of whom one had cirrhosis and the other underwent a lengthy procedure.[216] However, a study of 31 such patients failed to detect DIC following stent grafting of thoracic aneurysms.[217] Other anticoagulants have not been systematically evaluated in this condition.

Hemangiomas

Kasabach and Merritt[218] first described giant cavernous hemangiomas as a cause of a hemorrhagic diathesis. Primarily a disorder of infants and children, these hemangiomas are usually benign tumors that may enlarge or evolve into a convoluted mass of vascular channels that sequester and consume platelets and fibrinogen. This progression has been clearly demonstrated by physiologic studies in which the half-life of platelets is shortened and radioactive platelets and fibrinogen accumulate in the tumor.[219] Within the hemangioma, consumption of platelets and fibrinogen occurs because of localized intravascular clotting, but the channels are patent because of local, reactive fibrinolysis.[220,221] Conceivably, concomitant local activation of the coagulation pathway and release of large amounts of t-PA by the abnormal endothelium lining the tumor vessels occur. Microangiopathic hemolytic anemia and laboratory signs of DIC and fibrinolysis have been demonstrated in patients with giant hemangiomas.[222] Accelerated growth of these hemangiomas in infants is associated with augmented consumption of hemostatic factors, and treatment utilizes irradiation, glucocorticoids, and interferon. However, medical treatment is often ineffective, and surgery can be dangerous because of problems in achieving

effective hemostasis. Patients are usually managed conservatively, unless cosmetic corrections are needed or symptoms of bleeding result from severe thrombocytopenia and hypofibrinogenemia. The mass occasionally regresses spontaneously, or it may shrink after radiation or laser therapy.[219] Antithrombotic treatment with either antiplatelet agents or heparin has been attempted in order to correct the platelet count and fibrinogen level and alleviate bleeding symptoms. This rationale of first correcting the hemostatic defect by blocking the local consumption has been used in anticipation of, and in preparation for, surgical removal of the tumor under the safety of a normal hemostatic system. Regression of the tumor may occur by thrombosing the vascular channels by administering a fibrinolytic inhibitor (ε-aminocaproic acid [EACA] or tranexamic acid).[223]

Systemic Versus Localized Consumption Coagulopathy

Marked differences in the procoagulant response and underlying pathogenetic pathways exist between cells and tissues.[224] First, localization of coagulation activity may relate to a cell-specific gene expression, for example, inflammatory mediators enhance PAI-1 gene expression in a complex and tissue-specific way,[225] and the von Willebrand factor (vWF) promoter contains cell-specific elements.[226] Second, the tissue environment determines whether specific gene transcription occurs.[227] In mice with disturbances in the plasminogen–plasmin system subjected to hypoxia, the formation of fibrin is induced and is particularly evident in the lungs.[228] In contrast, these same mice respond to endotoxemia with fibrin deposition in the microvasculature of the kidney in particular. Similarly, mice with a functional thrombomodulin deficiency have a marked increase in pulmonary fibrin deposition after hypoxic challenge.[229] In addition, when mice with a functional defect in the thrombomodulin gene were challenged with endotoxin in sublethal amounts, fibrin formation was apparent in the lungs, but not in other organs.[230] Last, organs may markedly differ in their endothelial cell response toward inflammation and injury. In general, endothelial cells play a central role in the coagulation response upon systemic inflammation,[231] being directly involved in the initiation and regulation of thrombin generation, and in the inhibition of fibrin removal. Endothelial cells may express TF, the main initiator of coagulation, and physiological anticoagulant pathways, such as AT, the protein C system, or tissue factor pathway inhibitor (TFPI). Proinflammatory cytokines mediate effects on endothelial cells, which themselves may also express cytokines, thereby amplifying the coagulative response.[178] Although not completely clear, various organs may differ in all these endothelial cell-related factors influencing local coagulation activation and fibrin deposition.

CONSUMPTION RELATED TO ANTITHROMBOTIC OR HEMOSTATIC MEDICATION

Therapeutic Defibrination

Defibrinating enzymes such as ancrod (Arvin) from *Agkistrodon rhodostoma* and defibrase from *Bothrops atrox* have been purified for therapeutic defibrination and treatment of patients with venous thromboembolic disease.[115] The rationale for therapy is that plasma fibrinogen is decreased by conversion to noncross-linked fibrin, which is in turn lysed without causing any vascular occlusion or organ ischemia, leaving the patient with enough fibrinogen for normal hemostasis but sufficiently decreased to deter propagation of a thrombus. Results show that an antithrombotic state is as safe and effective as heparin.[232]

Heparin-Induced Thrombocytopenia

Heparin-induced thrombocytopenia (HIT) (see Chapter 108) is a drug-induced immune reaction that causes thrombocytopenia, usually 5 days or more after heparin therapy is started or sooner in the presence of antibody.[233,234] The pathophysiologic mechanism is the induction of antibody to a heparin–platelet factor 4 complex. The antigen–antibody complex activates platelets by binding to the platelet FcγIIa receptor and activates the endothelium by binding to platelet factor 4–proteoglycan complexes. Platelet procoagulant microparticles are released, and platelet aggregation, a variable degree of thrombocytopenia, endothelial cell activation with resultant expression of TF, and venous and/or arterial thrombosis may follow. Multifocal venous and arterial thrombosis may simulate DIC because thrombocytopenia, elevated D-dimer levels, and hypofibrinogenemia can be present.

Prothrombin Complex Concentrates and Recombinant Factor VIIa

Concentrates of vitamin K–dependent clotting factors (prothrombin complex concentrates, PCCs) are used for the treatment of patients with congenital or acquired deficiencies of prothrombin or factors VII, IX, or X or (in activated form) with acquired inhibitors against factors VIII or IX.[235] Some preparations contain thrombogenic material in addition to unactivated coagulation factors, including factors VIIa, IXa, and Xa, any of which may contribute to thrombotic complications.[236,237] The modern preparations of nonactivated PCCs are probably not associated with systemic activation of coagulation.[238]

A recombinant preparation of human factor VIIa has shown significant efficacy in hemophilic patients with inhibitors to factor VIII or IX and in patients with hereditary factor VII deficiency.[239,240] The agent is also used for many other (off-label) indications in situations characterized by excessive blood loss, in particular elderly patients, and very high doses of recombinant factor VIIa may provoke thrombotic complications.[241]

Coumadin

Coumarin (see Chapter 109) anticoagulation initiated in patients with partial (heterozygous) deficiency of protein C or protein S may produce the "coumarin necrosis" syndrome or defibrination as the result of a more rapid decrease in the vitamin K–dependent inhibitors than in the vitamin K–dependent coagulation factors.[242] In patients with HIT, Coumadin-induced thrombosis may result in an exaggerated limb ischemic syndrome (venous gangrene).[243]

CONSUMPTION IN THE NEONATE

Newborns have a limited capacity to cope with triggers of DIC, because of their decreased ability to clear soluble fibrin and activated factors, their decreased fibrinolytic potential because of a

low plasminogen level, and their limited capacity to synthesize coagulation factors and inhibitors.[244,245] Criteria for diagnosis of DIC in newborns are different from those for diagnosis in adults.[246] Important to consider are the physiologic hemostatic findings common at this age, which include low levels of the vitamin K–dependent factors, reduced AT and protein C levels, and prolonged thrombin time (see Chapter 119). Laboratory evidence of DIC in the newborn is based on the progressive decline of hemostatic parameters; thrombocytopenia; and reduced levels of fibrinogen, factor V, and factor VIII.[244,247,248]

DIC occurs in sick neonates and particularly in those who are premature. More than one underlying cause usually can be identified in newborns with DIC, the most frequent underlying conditions being sepsis, hyaline membrane disease (respiratory distress syndrome), asphyxia, necrotizing enterocolitis, intravascular hemolysis, abruptio placentae, and eclampsia.[245,249] Bleeding from multiple sites is the most common manifestation of DIC in newborns, with intracranial hemorrhage being the most life-threatening condition. Clinical manifestations of DIC may not be apparent in 20% of neonates;[247] so a high index of suspicion in patients at risk is essential.

Rare cases of fulminant neonatal DIC may result from the deficiency of coagulation inhibitors, especially protein C. Such cases are seen in homozygous deficiency states and are variations of the striking thrombotic tendency that is usually manifested as multiple venous thrombi. A relative deficiency of fibrinolytic response in the neonate may reflect a decreased plasma plasminogen concentration, which could contribute to thrombotic occurrences.[247,250] Treatment depends on provision of the missing inhibitor by plasma or purified protein products, complemented by long-term anticoagulant therapy.

INITIATING PATHWAYS

TF plays a central role in the initiation of inflammation-induced coagulation in DIC,[71] and blocking TF activity completely inhibits inflammation-induced thrombin generation in experimental models of endotoxemia or bacteremia.[251,252] Most cells constitutively expressing TF are found in tissues not in direct contact with blood, such as the adventitial layer of larger blood vessels. TF is exposed to blood upon disruption of the vascular integrity, or when cells present in the circulation, such as monocytes, are triggered to express TF. *In vivo* expression is dependent on IL-6 generation, inhibition of which abrogates TF-dependent thrombin generation in experimental endotoxemia.[178,253] In severe sepsis, monocytes, stimulated by proinflammatory cytokines, express TF, which leads to systemic activation of coagulation.[254] Even in experimental low-dose endotoxemia in healthy subjects, a 125-fold increase in TF mRNA levels in blood monocytes can be detected.[255] A potential alternative source of TF may be endothelial cells and polymorphonuclear cells, and it is hypothesized that TF from these sources is shuttled between cells through microparticles derived from activated mononuclear cells.[256] However, it is unlikely that cells other than monocytes synthesize TF in substantial quantities.[254,257]

ROLE OF THROMBIN AND PLATELETS

Consumptive processes reflect the multiple actions of thrombin. Thrombin proteolytically cleaves fibrinopeptides from fibrinogen to produce fibrin monomers that either combine with fibrinogen to form soluble complexes or polymerize to form fibrin thrombi. Thrombin converts factor XIII to an active transamidase that cross-links fibrin and renders it more resistant to fibrinolysis.[258] Fibrin can also be cross-linked to other proteins such as antiplasmin, which also contributes to its plasmin resistance (see Chapter 17).

Thrombin binds to specific platelet receptors at low concentrations and initiates shape change, aggregation, and secretion. Thrombin-stimulated platelets lack both dense granules and α-granules and show functional defects, so even if the platelet count exceeds 100,000/μL, the bleeding time may be prolonged. The platelet contribution to prothrombin activation is in part due to platelet factor V bound to the platelet surface, which serves as a receptor for factor Xa. Thrombin stimulates the platelet to release (from α-granules) platelet factor V, which is then bound to the platelet membrane. Thrombin potentiates the coagulation cascade by increasing the activity of factors V and VIII; however, its continued action leads to their degradation by activated protein C (APC). Therefore, thrombin itself accounts for decreases in fibrinogen, platelets, and factors II, V, VIII, and XIII in acute consumption.[259] The decrease in other clotting factors, such as factors IX and X, is due to rapid clearance of activated clotting factors *in vivo*.

Importantly, thrombin acts at the crossroad of coagulation and inflammation by binding to protease-activated receptors (PARs), a family of the transmembrane domain, G-protein–coupled receptors.[260] PARs serve as their own ligand, and proteolytic cleavage by an activated coagulation factor leads to exposure of a neo–amino-terminus that activates the same receptor and initiates transmembrane signaling. PAR-1, -3, and -4 are thrombin receptors, whereas PAR-2 does not bind thrombin but (along with PAR-1) can be activated by the TF–VIIa complex, factor Xa, and trypsin. PARs are localized on endothelial cells, mononuclear cells, platelets, fibroblasts, and smooth muscle cells, and binding of thrombin to these cellular receptors may induce cytokine and growth factor production, thereby promoting the inflammatory response.[261,262]

TF expression on monocytes is markedly stimulated by platelets and granulocytes in a P-selectin–dependent reaction.[263] This effect may be the result of nuclear factor kappa B activation induced by binding of activated platelets to neutrophils and mononuclear cells.[174] This cellular interaction also markedly enhances the production of Il-1b, Il-8, MCP-1, and TNF-α.[264] The expression of P-selectin on the activated platelet membrane will mediate the adherence of platelets to endothelial cells and leukocytes.

ROLE OF COAGULATION INHIBITORS

Failure of natural inhibitory (anticoagulant) mechanisms, the protein C system, TFPI, and AT predisposes to or aggravates hypercoagulable states by allowing fuller expression of thrombin activity.[265,266] Therefore, adequate amounts of natural anticoagulants are necessary to dampen or modulate increased procoagulant activity. Because of increased utilization, enhanced degradation (due to elastase released from activated neutrophils), and possibly decreased production, AT and protein C levels are decreased in patients with DIC.[267]

The protein C–related inhibitor system is dependent *in vivo* on surface-oriented reactions, specifically that of thrombomodulin and endothelial protein C receptor (EPCR) on endothelial

cells, and acts to facilitate the degradation (inactivation) of factors Va and VIIIa.[268,269] In this complex molecular system, thrombin forms a bimolecular complex with the transmembrane protein thrombomodulin, at which point it loses its fibrinogen-clotting, factor XIII–activating, and platelet-stimulating activities. However, the thrombin–thrombomodulin complex can convert the inactive plasma precursor, protein C, to an active form (APC). The EPCR not only accelerates the activation of protein C several fold, but also serves as a receptor for APC, and binding of APC to this receptor may amplify its anticoagulant and anti-inflammatory effects.[270] In turn, protein APC in the presence of the facilitating cofactor protein S degrades factors Va and VIIIa to inactive forms, thereby negatively influencing the thrombotic tendency. Apart from these effects, in DIC a resistance toward APC may occur by other mechanisms, which are partly dependent on a sharp increase in factor VIII levels (released from endothelial cells), but partly occur by yet unidentified mechanisms.[271]

Levels of TFPI in patients with DIC vary,[272–274] but the potential role of TFPI in preventing DIC was provided by experiments in rabbits in which pretreatment with anti-TFPI antibody sensitized the animals to more striking laboratory and tissue evidence of DIC.[275] Moreover, in an animal model of *E. coli*–induced DIC, recombinant TFPI decreased the coagulopathy and mortality, and endothelium-targeted delivery of TFPI inhibited intravascular thrombosis in endotoxin-treated mice.[276,277]

Thrombin activity is neutralized by two inhibitory systems, which to different degrees depend on the interaction of plasma proteins with the endothelial surface. AT and heparin cofactor II neutralize thrombin slowly by complex formation (see Chapters 18 and 45). However, such neutralization occurs at markedly accelerated rates in the presence of heparin, albeit at much higher concentrations for heparin cofactor II than for AT.[278] Heparin exerts its facilitative effect in solution, for example, after administration for therapeutic purposes or as membrane-associated molecules (heparans) that contribute to the antithrombotic properties of the vascular endothelium. In addition, AT neutralizes factor IXa and Xa. During DIC, AT levels are markedly decreased owing to impaired synthesis, degradation by elastase from activated neutrophils, and, most importantly, consumption as a consequence of ongoing thrombin generation.[231] Proinflammatory cytokines can also cause reduced synthesis of glycosaminoglycans on the endothelial surface, which also contributes to reduced AT function.[279,280]

ROLE OF PLASMIN

Plasmin is a potent proteolytic enzyme capable of digesting fibrin and fibrinogen and other clotting proteins, including factors Va and VIIIa. The balance between thrombin and plasmin determines whether the clinical picture is thrombin predominant, characterized by thrombosis, organ ischemia, and bleeding, or plasmin dominant, characterized by bleeding. Increased thrombin generation unbalanced by increased plasmin formation leads to microvascular fibrin deposition, causing organ ischemia and depression of organ dysfunction.[281] If plasmin is formed in sufficient quantities in the circulation, it produces degradation products of fibrinogen, which also inhibit fibrin polymerization and degrades clotting factors V and VIII. In combination with low plasma fibrinogen concentration, low levels of plasma factors V and VIII, and thrombocytopenia, FDPs contribute to bleeding manifestations.

In experimental models of DIC, plasmin formation is initially activated but subsequently inhibited, because of an increased release of PAI-1 by endothelial cells.[252,282] In a study of 69 DIC patients (31 with MOF), higher levels of t-PA antigen and PAI-1 and low levels of α_2-antiplasmin (presumably due to consumption caused by initial plasmin generation) were observed in patients with DIC and MOF compared to DIC patients without MOF.[283] This finding supports the conclusion that fibrinolysis is an important mechanism in preventing MOF. Experiments in mice with targeted disruptions of genes encoding components of the plasminogen–plasmin system confirm that fibrinolysis plays a major role in inflammation. Mice with a deficiency of plasminogen activators have more extensive fibrin deposition in organs when challenged with endotoxin, whereas PAI-1–knockout mice, in contrast to wildtype controls, have no microvascular thrombosis upon endotoxin administration.[228]

TAFI, like PAI-1, may play a role in impeding fibrinolysis and in augmenting formation of microvascular thrombi. Studies in a DIC cohort demonstrated high levels of TAFI proportionate to thrombin generation in such patients, particularly in those with infection-associated DIC.[284] Hence, TAFI may contribute (along with PAI-1) to microvascular thrombosis-induced ischemia in organs resulting in MOF.

In some patients, in particular those with APL and advanced adenocarcinoma, an exceptional type of DIC, characterized by a marked hyperfibrinolysis, may occur. The clinical presentation of severe bleeding associated with laboratory findings of a low fibrinogen level, very high levels of fibrin split products and fibrinogen degradation products, and massive consumption of plasminogen and α2-antiplasmin (leading to inordinate high levels of plasmin–α2-antiplasmin complex levels) support that notion.[193] The precise pathogenesis of this hyperfibrinolysis has, however, not been elucidated. Plasma levels of physiological plasminogen activators (such as u-PA and t-PA) cannot explain the massive plasminogen activation nor is a role of leucocytic elastase-mediated hyperfibrinolysis likely. Some studies pointed to a potential newly described receptor for fibrinolytic proteins, annexin II, which is expressed on the surface of leukemic cells in patients with APL that could explain the hyperfibrinolysis.[285] This hypothesis, however, has not been confirmed so far.

LABORATORY TESTS AND THE DIAGNOSIS OF DIC

Because DIC is associated with a variety of underlying disorders, the laboratory manifestations in a given patient are variable and depend not only on features of DIC but also on those due to the underlying disease. In some patients, DIC is of little clinical significance, and the diagnosis is established by laboratory tests, while in other patients, the clinical severity of DIC is striking and obscures the underlying disease. Given this variability, it is not surprising that criteria for the diagnosis of DIC are inadequate.

No single laboratory test is sensitive or specific enough to allow a definite diagnosis of DIC (Table 98.4).[10,286]. However, some sophisticated laboratory tests, for example, thrombin–antithrombin complex and prothrombin fragment 1.2, are

Table 98.4

Routine laboratory value abnormalities in DIC

Test	Abnormality	Causes Other Than DIC Contributing to Test Result
Platelet count	Decreased[a]	Sepsis, impaired production, major blood loss, hypersplenism
PT	Prolonged	Vitamin K deficiency, liver failure, major blood loss
aPTT	Prolonged	Liver failure, heparin treatment, major blood loss
FDPs	Elevated	Surgery, trauma, infection, hematoma
Protease inhibitors[b]	Decreased	Liver failure, capillary leakage

[a]Note that in pure hyperfibrinolytic states the platelet count may be normal.
[b]For example, protein C, AT, protein S.

Table 98.5

Diagnostic algorithm for the diagnosis of overt DIC[a]

1. Presence of an underlying disorder known to be associated with DIC?
   If no: do not use this algorithm
2. Score global coagulation test results
   - Platelet count *(>100 = 0; <100 = 1; <50 = 2)*
   - Level of fibrin markers (soluble fibrin monomers/FDPs) *(no increase: 0; moderate increase: 2; strong increase: 3)*[b]
   - Prolonged PT *(<3 s = 0; >3 s but <6 s = 1; >6 s = 2)*
   - Fibrinogen level *(>1.0 g/L = 0; <1.0 g/L = 1)*
3. Calculate score
4. If ≥5: compatible with overt DIC; repeat scoring daily
   If <5: suggestive (not affirmative) for nonovert DIC; repeat next 1–2 d

[a]According to the Scientific Standardization Committee of the International Society of Thrombosis and Haemostasis (Taylor FBJ, Toh CH, Hoots WK, et al. Towards definition, clinical and laboratory criteria, and a scoring system for disseminated intravascular coagulation. *Thromb Haemost* 2001;86(5):1327–1330).
[b]In validation studies, a moderate increase was above the upper limit of normal but <5× upper limit, and a strong increase was >5× upper limit of normal.

sensitive toward ongoing activation of coagulation pathways, and soluble fibrin in plasma is one of the best parameters for detecting ongoing DIC.[287–290] FDPs may be detected by specific ELISAs or by latex agglutination assays, allowing rapid and bed-side determination,[291] but these assays do not discriminate between degradation products of cross-linked fibrin and fibrinogen.[292,293] The specificity of FDP is limited, and conditions, such as trauma, recent surgery, inflammation or active major vessel occlusion, are associated with elevated FDPs.

Tests that are aimed at the detection of neoantigens on degraded cross-linked fibrin, the best example of which detects an epitope related to cross-linked γ-chains (D-dimer), distinguish cross-linked fibrin from fibrinogen or fibrinogen-degradation products,[294] but from patients with venous thromboembolism, surgery, inflammatory conditions, or serous effusions may have elevated levels.[291,295]

In routine practice, simple laboratory tests in conjunction with clinical considerations are used for establishing the diagnosis of DIC. The simple tests include platelet count, PT, fibrinogen level, and fibrin-related markers, such as FDP or D-dimer. Caution should be exercised when using these laboratory parameters in the algorithms described below, because an underlying disease by itself can cause an abnormality. For example, impairment of hemostasis and/or thrombocytopenia unrelated to DIC can arise from hepatic disease and from marrow involvement by leukemia. Conversely, elevated levels of some hemostatic components that may occur during pregnancy may obscure the presence of DIC. The laboratory criteria are, however, arbitrary, and no consensus exists. For example, because fibrinogen levels increase as an acute-phase reactant in infection, fibrinogen levels may be normal when DIC complicates the illness and fibrinogen levels can be normal in up to 57% of patients with DIC.[286] In contrast, in DIC complicating severe liver disease, a low fibrinogen level may be caused by decreased synthesis as well as by increased consumption. Similar problems may arise using other criteria of DIC, such as thrombocytopenia in the presence of splenomegaly, low protein C in the presence of liver dysfunction, increased factor VIII in the presence of infection, and elevated FDPs in the presence of systemic fibrinolysis.

A scoring system, utilizing simple laboratory tests, has been reviewed by the subcommittee on DIC of the International Society on Thrombosis and Haemostasis,[8] and a five-step diagnostic algorithm to calculate a DIC score is summarized in Table 98.5. Tentatively, a score of 5 or more is compatible with DIC, whereas a score of <5 may be indicative but is *not* affirmative for nonovert DIC. By using receiver-operating characteristic curves, an optimal cutoff for a quantitative D-dimer assay was determined, thereby optimizing sensitivity and the negative predictive value of the system.[296] Prospective studies show that the sensitivity of the DIC score is 93%, and the specificity is 98%.[9,83] The severity of DIC according to this scoring system is related to the mortality in patients with sepsis.[82] Linking prognostic determinants from critical care measurement scores such as Acute Physiology and Chronic Health Evaluation-II to DIC scores is an important means to assess prognosis in critically ill patients. In addition, certain biochemical indicators of organ dysfunction may imply a DIC risk. For example, serial assessment of arterial lactate is a reliable prognostic indicator of DIC development among patients with the systemic inflammatory response syndrome.[297]

Criteria for less overt DIC are more difficult to establish.[8,298] In the algorithm for nonovert DIC, the global coagulation tests are scored as with the overt DIC algorithm; however, when scoring by the algorithm is being serially repeated, improvement in any laboratory test confers a negative score (rather than a

zero or neutral score). This "trend" scoring allows longitudinal assessment of the patient's status and evaluation of therapy.[299,300] Measurements of several markers for assessing the risk of progression from nonovert to overt DIC and prediction of MOD are potentially valuable. For example, impaired fibrinolysis may play an important role in MOD resulting from DIC of sepsis.[281] Therefore, assaying PAI-1, plasmin–antiplasmin complexes, or TAFI in septic patients may be important, and other markers, such as APC and ADAMTS 13 (vWF protease), may help identify individuals with microangiopathy who are not prone to worsening disease.[301,302]

TREATMENT

Because of the heterogeneity of the underlying disorders causing DIC and the great variability of the manifestations of DIC in a given patient, management is controversial.[1] The cornerstone of treatment is prompt, vigorous treatment of the underlying disease and aggressive support measures. After identifying the cause of DIC and promptly instituting appropriate specific and aggressive supportive therapy for the underlying disease, questions must be asked to determine whether replacement of depleted hemostatic factors is necessary and whether interruption of DIC with anticoagulants should be seriously considered.

Bleeding. Does the patient have a low fibrinogen level, LP, or low clotting factor levels? If so, is the patient actively bleeding? If the answer to the second question is no and the patient does not require surgery or an invasive procedure, replacement therapy is probably unnecessary.[303,304] In contrast, if the patient is actively bleeding or requires an invasive procedure, it probably will be necessary to replace the deficient hemostatic factors, which usually means cryoprecipitate, fresh-frozen plasma, PCC (if available), and platelets. The argument that replacement therapy may "fuel the fire" and cause thrombosis in patients with active DIC is theoretically possible, but only on occasion has this been proved to occur.[305] However, replacement therapy is most effective in patients with very low levels of platelets and fibrinogen, in whom DIC is self-limiting or has run its course. A common problem is the failure to increase the level of hemostatic factors when the patient is bleeding because of ongoing DIC. In these cases, it may be necessary to replace the hemostatic factors under cover of anticoagulant therapy, however contraintuitive this may seem. The effect of replacement therapy should be monitored by platelet and fibrinogen levels after completing transfusion to determine whether further replacement therapy is necessary.

Thrombosis. Does the patient have clinical evidence of fibrin deposition, such as overt venous thromboembolism, dermal necrosis, oliguria, or confusion? If so, active inhibition of thrombin by anticoagulant therapy is probably indicated.[10] Patients with purpura fulminans show a rapid progression of ecchymotic areas of skin to hemorrhagic bullae and hemorrhagic ischemic necrosis, and anticoagulation reduces mortality from 90% to 18%.[306] A similar clinical picture occurs in patients with bacteremia and sepsis, with patchy hemorrhagic skin lesions, progressive symmetric digital gangrene, and even gangrene of the extremities.[243] Heparin is indicated but unfortunately is often given after frank gangrene is already present. Therefore, when dermal or acral ischemia is first noted, heparin or other anticoagulant should be given to interrupt the DIC, along with bactericidal antibiotic and aggressive supportive therapy. Although ischemia secondary to DIC has been proposed as a major cause of organ dysfunction, including ARDS and acute renal failure, fibrin deposition *per se* may not represent the direct cause of these problems.[10,149] ARDS occurs in the absence of DIC, and renal failure in patients with DIC can be due to causes other than renal cortical necrosis. Therefore, it is not surprising that heparin therapy may not curtail organ damage in a given patient.

Disorders that May Require Heparin Anticoagulation

Retained Dead Fetus Syndrome

Retained dead fetus syndrome is defined by the presence of a dead fetus associated with evidence of DIC, which is usually low grade, chronic, and frequently compensated.[119] The major goals of therapy are to deliver the fetus and to prevent excessive bleeding at the time of delivery. If the patient presents with hypofibrinogenemia, intravenous heparin (and fibrinogen replacement) is effective in increasing the fibrinogen level to normal before elective delivery.[142]

Giant Hemangiomas (Kasabach-Merritt Syndrome)

Giant hemangiomas (Kasabach-Merritt syndrome) are benign childhood tumors that may progressively enlarge and cause localized consumption of platelets and fibrinogen.[219] Surgical removal can cause massive bleeding and should be avoided in most cases. If the patient requires an elective surgical procedure for reasons other than removal of the tumor, heparin or cryoprecipitate and platelet transfusions can be used before the procedure in an attempt to normalize the platelet count and fibrinogen level. In some patients, the tumor itself has enlarged to such an extent that spontaneous bleeding ensues because of low fibrinogen or platelet levels. In these patients, an attempt to thrombose the vascular channels with selective infusions of EACA or tranexamic acid has led to significant regression of the tumor and control of the localized consumptive process.[223]

Aortic Aneurysm

Localized coagulation may occur in the aneurysm, with laboratory evidence of compensated or decompensated intravascular coagulation.[307–309] In the latter case, the patient may present with spontaneous bleeding or a history of a bleeding tendency associated with minor trauma or invasive procedures. In patients without an aneurysmal leak who undergo elective surgery, heparin may normalize hemostatic factors before surgical repair.[212] In contrast, patients who have an aneurysmal leak associated with evidence of consumption require emergency surgery, in which case heparin is contraindicated and hemostatic levels of fibrinogen and platelets should be achieved with cryoprecipitate and platelet transfusions. Patients with symptomatic bleeding require prompt treatment, because significant recent extension has probably occurred.

Solid Tumors

DIC in patients with solid tumors is usually low grade and chronic, but clinical features are widely heterogeneous.[32,161] Patients may present with very serious disease, with excessive bleeding, venous thromboembolism, nonbacterial

thrombotic endocarditis with or without arterial thromboembolism, microangiopathic hemolytic anemia, or any combination thereof. In contrast, DIC may be manifested by laboratory abnormalities only with no clinical features. Interruption of clinically evident DIC with anticoagulation may provide significant benefit. In the study by Sack et al.,[310] 29 of 48 such patients had improvement of hemostatic measurements and 19 patients had recurrent symptoms after heparin was discontinued. Prolonged outpatient therapy with a glycoseaminoglycan is effective. The newly developed oral anti-IIa and anti-Xa agents have not been evaluated in this clinical setting so far.

APL is often dominated by DIC and hemorrhagic complications.[24,188] DIC probably results from the release of procoagulant material (TF and cysteine protease) or procoagulant-stimulating activity (IL-1) from the abnormal promyelocytes, and frequently the DIC worsens during induction chemotherapy (without ATRA).[311] Fibrinolytic activity is also increased in this disorder owing to cellular u-PA and perhaps leukocyte elastase.[312] Because initial induction therapy now includes ATRA, the coagulopathy disappears within 2 to 6 days, and the use of heparin, fibrinolytic therapy, or both is unnecessary. However, if the patient is unresponsive to ATRA, either primarily or in relapse, and chemotherapy is deemed necessary, the use of heparin therapy should be considered as an adjunct to aggressive transfusion therapy. Although most of the previous data strongly suggested that heparin therapy is beneficial in APL, they are nevertheless equivocal.[313] In studies in which heparin prophylaxis was not used, the incidence of fatal hemorrhage (usually intracerebral) was 16% to 63%; the incidence in studies in which heparin was used was 0% to 44%.[188,195] Although the literature shows fewer hemorrhagic deaths (20.7% vs. 14.6%) in patients treated with heparin, no prospective or well-controlled studies have been reported, and transfusion support varies between trials. In summary, DIC associated with APL should be managed with aggressive blood component replacement in an attempt to keep the platelet count >50,000/μL and the fibrinogen level >150 mg/dL. If the patient responds to ATRA, the DIC usually subsides rapidly over a few days, but if the patient requires chemotherapy, aggressive hemostatic replacement therapy may be difficult to achieve. In those patients, the addition of low-dose heparin may help in achieving reasonable platelet and fibrinogen levels. If evidence of systemic fibrinolysis as measured by a low α_2-plasmin inhibitor level is found, EACA or tranexamic acid can be used either as a simple agent or added to heparin if DIC is also present. Usually after a week of induction chemotherapy, the DIC progressively subsides and heparin and fibrinolytic inhibitor can be discontinued; platelet transfusions are needed until normal thrombopoiesis returns.

When Heparin is Probably Not Helpful

In most instances of consumption coagulopathy, heparin therapy has not been helpful and occasionally has been harmful. Acute forms of DIC may be either brief or end with prompt treatment of the underlying disease, in which case it is difficult to evaluate the contribution of heparin. Snakebite, heatstroke, rhabdomyolysis, massive head trauma, and acute hemolysis secondary to an incompatible blood transfusion all may result in acute consumption, and the evidence for heparin benefit is mostly from individual case reports, so heparin therapy remains controversial.[45,314,315]

Sepsis

For a more extensive discussion of the supportive treatment of the coagulopathy in sepsis, please see Chapter 123. Corrigan's review of patients with septic shock and DIC found no difference in survival between patients receiving (24%) and not receiving (21%) heparin.[316] The major determinant of survival in these patients is probably the presence or absence of shock and not the presence or absence of DIC. There is less debate, however, on the use of low-dose prophylactic heparin to prevent thromboembolic complications in patients with severe illness. In a large trial in patients with severe sepsis, there was a slight, but nonsignificant benefit, of low-dose heparin on 28-day mortality,[317] likely related to the prevention of thrombotic complications rather than an effect on DIC.

Complications of Pregnancy

Abruptio Placentae

Although renal cortical necrosis with demonstrable intravascular fibrin occurs in a few patients with *abruptio placentae*, the course of the illness is usually favorable when adequate support measures are taken. Most important is that blood be given to correct hypovolemia and hypotension, improve renal perfusion, and prevent acute tubular necrosis or renal cortical necrosis. With the use of this therapy alone, vaginal delivery is usually accomplished without hemorrhagic complications, and rapid regeneration of plasma fibrinogen follows.[121] If hysterotomy is necessary or episiotomy is performed during vaginal delivery, supplementation with fibrinogen (cryoprecipitate) and platelets is reasonable to provide normal hemostasis. Heparin is contraindicated before surgery, because this could contribute to increased bleeding during vaginal delivery or hysterotomy.

Amniotic Fluid Embolism

Amniotic fluid embolism is an acute fulminant catastrophic event that induces pulmonary vascular obstruction. Sudden death follows in as many as 60% of the patients; in the remaining patients, a brief latent period (30 minutes to 4 hours) is followed by evidence of excessive bleeding from venipuncture sites and from the site of placental separation.[126] Because of its rapid course, the effectiveness of various treatment modalities has not been carefully assessed. Vigorous supportive therapy directed toward gas exchange with mechanical respiratory assistance and fluid and blood replacement to treat severe hypotension are essential. When bleeding becomes the major manifestation, however, the use of heparin may accentuate the bleeding, especially if adequate replacement therapy for consumed clotting factors has not been achieved. If intense systemic fibrinolysis complicates DIC, fibrinolytic inhibitors may be useful in patients refractory to replacement therapy to control serious bleeding. However, the same precautions regarding antifibrinolytic use apply as in other settings of DIC.

Toxemia of Pregnancy and HELLP Syndrome

Although sensitive parameters of thrombin generation are increased in toxemia of pregnancy and patients with the HELLP syndrome, and patients with severe toxemia may have fibrin thrombi in the microvasculature,[144,318–320] overt DIC occurs infrequently.[143] Therefore, it is not surprising that no significant

benefit of heparin therapy in toxemia of pregnancy (or the HELLP syndrome) has been established; in fact, the risk of intracranial bleeding in patients with severe, uncontrolled hypertension makes the use of anticoagulants dangerous. Therapy is directed primarily at controlling the eclamptic state with antihypertensive agents, magnesium sulfate, and rapid termination of the pregnancy. Because HELLP syndrome usually reverses approximately 48 hours after delivery, the cornerstone of treatment is prompt delivery of the infant.[144] If the syndrome persists beyond 72 to 96 hours, plasmapheresis and exchange with fresh-frozen plasma has been used, albeit with equivocal results.[134]

Other Treatment Modalities

Antithrombin and Other Thrombin Inhibitors

Replacement of AT with fresh-frozen plasma or AT concentrate with or without heparin should neutralize excess thrombin and dampen or moderate the intravascular coagulation process.[259] This approach should be particularly effective in patients with DIC associated with hepatic failure, because of decreased synthesis and increased consumption of AT. Although AT concentrates shorten the duration of DIC in some patients with fatty liver of pregnancy, they are not helpful in patients with DIC due to peritoneovenous shunts.[321] A low level of AT in a patient with severe infection or septic shock is a sign of poor prognosis.[322,323] In experimental animal models of sepsis and DIC, infusion of AT decreased the degree of DIC and mortality, particularly if administered before the induction of sepsis and in very high doses.[324] AT may have an anti-inflammatory effect in animal models.[325,326] A series of small clinical trials showed a modest reduction in mortality in AT-treated patients, but no trial reached statistical significance,[327] and a large-scale, multicenter, randomized controlled trial showed no significant reduction in mortality of patients with sepsis who were treated with AT.[328] Interestingly, *post hoc* subgroup analyses indicated some benefit in patients with DIC who did not receive concomitant heparin, but this observation needs prospective validation.[329] Hirudin and other direct thrombin inhibitors have shown efficacy in animal models, but trials of clinical efficacy in DIC have not yet been performed.[330–333]

Activated Protein C

APC, in the presence of protein S as a cofactor, inactivates factors Va and VIIIa. In a study by Taylor et al., APC prevented the coagulopathy and lethal effect of *E. coli*.[334] APC administered to rats given endotoxin reduced the pulmonary vascular injury and hypertension,[335] changes that were independent of its anticoagulant effect. A phase III trial of APC concentrate in patients with severe sepsis was prematurely stopped because of efficacy in reducing mortality,[336] all-cause mortality at 28 days being 24.7% in the APC group versus 30.8% in the control group (19.4% relative risk reduction). Presumably, the mortality advantage provided by APC was mediated by its ameliorating effect on DIC, which in turn decreased the occurrence of organ failure. Patients with DIC had a greater survival benefit from APC than did patients without DIC, [82] (relative risk reduction 38% vs. 18% in patients with sepsis without DIC). APC is relatively more effective in higher disease severity groups, and a prospective trial in septic patients with relatively low disease severity did not show any benefit of APC.[337]

Fibrinolytic Inhibitors

EACA or tranexamic acid should not be used in most patients with DIC because inhibition of the fibrinolytic system may lead to widespread fibrin deposition in the microcirculation, resulting in ischemic organ dysfunction or failure. In patients with pathologic fibrinogenolysis or in those in whom DIC is associated with excessive fibrinolytic activity, however, lysine analogues may be helpful.[4,10] In addition, in patients with amniotic fluid embolism after the episode of DIC has subsided, the patient may continue to bleed because of intense fibrinolytic activity, in which case EACA or tranexamic acid can be helpful in controlling bleeding.

SUMMARY OF TREATMENT

Table 98.6 summarizes the general principles of the treatment of DIC. The treatment of DIC is extremely complex because DIC complicates many different disorders and may have a variety of clinical manifestations. In addition, well-controlled studies are lacking regarding various methods of management in relatively homogeneous groups of patients. Therefore, treatment continues to be controversial and probably will remain so. Any careful clinical and laboratory observations in groups of patients with the same underlying disorder are extremely helpful in elucidating the natural history of the process and its effects on the patient, and in clarifying whether certain therapeutic interventions may be helpful. The continued accumulation of careful clinical studies will help in determining the proper management of DIC in patients with the same underlying disorder and in given individual situations.

Table 98.6

Treatment of acute, severe DIC

Modality	Rationale	Details	Expectations
Life-support measures	Self-evident	Fluids, blood, respiratory care, pressors, etc.	Maintain cardiac output, gas exchange, electrolyte balance, etc.
Treating the underlying disorder	Correct the cause of DIC	Dependent on the primary diagnosis	Inhibit or block the complicating pathologic mechanism of DIC in parallel with the response (if any) of the disorder
Antithrombotic agents	Block microthrombus formation	Heparin by continuous IV infusion (initial dose 7.5 U/kg/h), monitor with fibrinogen and platelet levels; continue as long as the predisposing clinical state persists	Prevent fibrin formation; tip the balance within the microcirculation toward physiologic fibrinolysis; allow reperfusion of the skin, kidneys, and brain
Transfusion	Reestablish normal hemostatic potential	Infuse platelets and fibrinogen (cryoprecipitate); repeat as indicated by laboratory and clinical observation	Platelet count and fibrinogen should increase significantly; bleeding should diminish and stop during an interval of hours to several days
Transfusion + heparin	Restore normal hemostasis if transfusion of hemostatic factors fails to achieve significant increment in factor levels	Infuse platelets and cryoprecipitate 2 h after starting continuous heparin infusion (7.5 U/kg/h); repeat as indicated by laboratory and clinical observation	Platelet count and fibrinogen should increase significantly if consumption is blocked; bleeding should diminish; if bleeding increases, discontinue heparin
Fibrinolytic inhibitors	Block excessive fibrinolysis and the accumulation of degradation products in blood; protect hemostatic plugs	For adults, EACA: loading dose, 4–6 g, then 1 g q12h for a limited duration (up to 48 h), adjusted dose with renal failure	Bleeding ceases rapidly but keeps vascular channels occluded with thrombus; dangerous if the thrombotic process was not previously treated with heparin

DIC, disseminated intravascular coagulation; IV, intravenous; AT-III, antithrombin III.

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CHAPTER

99

The Thrombotic Thrombocytopenic Purpura and Hemolytic Uremic Syndromes

JAMES N. GEORGE

Thrombotic thrombocytopenic purpura (TTP)[1,2] and the hemolytic uremic syndrome (HUS)[3,4] were described initially as distinct disorders. Although they may be distinct in their classic presentations, TTP in adults with acquired, severe ADAMTS13 deficiency and the diarrhea-associated typical HUS in children, in many patients this distinction is neither apparent nor important. TTP is often said to be associated with more severe neurologic abnormalities and HUS with more severe renal failure. However, many adult patients with acquired, severe ADAMTS13 deficiency have neither severe neurologic abnormalities nor renal failure,[5,6] and some children with typical HUS whose primary abnormality is acute renal failure have severe neurologic abnormalities.[7–9] There are multiple other syndromes that are described as TTP or HUS in addition to these two classic disorders in which the distinction between TTP and HUS is similarly unclear. Table 99.1 summarizes the common clinical nomenclature of these syndromes.[6]

Pathologic abnormalities are similar in both TTP and HUS. The characteristic histologic features of thrombi in terminal arterioles and capillaries are described as thrombotic microangiopathy (TMA) (Table 99.1).[10,11] FIGURE 99.1 demonstrates the characteristic pathologic features of systemic TMA in a patient with acquired, severe ADAMTS13 deficiency. TMA can also occur in multiple other disorders, such as malignant hypertension, scleroderma, antiphospholipid antibody syndrome, acute renal allograft rejection, and severe preeclampsia and the hemolysis, elevated liver enzymes, and a low platelet count (HELLP) syndrome[10]; in these disorders the TMA is usually restricted to the kidney. Microangiopathic hemolysis with red-cell fragmentation may result from the turbulence and high shear stress of circulation through partially occluded arterioles.[12] Therefore, the clinical features of all disorders associated with TMA are similar. All disorders associated with TMA may resemble the clinical features of TTP and HUS. This can make clinical decisions of definition, diagnosis, and treatment of the TTP and HUS syndromes difficult.

The definition of TTP has changed since the classic review in 1966 that summarized all previously reported cases.[13] This review defined a pentad of clinical features that became the cornerstone of diagnosis of TTP for many years: (a) microangiopathic hemolytic anemia, (b) thrombocytopenia, (c) neurologic symptoms and signs, (d) renal function abnormalities, and (e) fever. In that review,[13] the diagnosis of TTP was supported by pathologic demonstration of hyaline thrombi in 93% of patients; 90% of the patients died. In the past 30 years, the availability of curative plasma exchange treatment[14,15] has created an urgency for establishing the diagnosis of TTP, which in turn has resulted in less stringent diagnostic criteria.[6,15] Now only microangiopathic hemolytic anemia and thrombocytopenia without another clinically apparent cause are sufficient to establish the diagnosis of TTP.[6,15] Decreased stringency of diagnostic criteria has inevitably led to an increased frequency of diagnosis[16] and to a broader clinical spectrum of disorders for which plasma exchange treatment is considered. A current classification of presenting clinical features and disease associations of the TTP and HUS syndromes is presented in Table 99.2.

This chapter focuses on TTP in adults, initially describing adults with acquired, severe ADAMTS13 deficiency (defined as <10% activity) (Table 99.2). Clinical characteristics of the TTP and HUS syndromes that are associated with other apparent etiologies are discussed in subsequent sections.

TTP ASSOCIATED WITH ACQUIRED, SEVERE ADAMTS DEFICIENCY

Although patients with acquired, severe ADAMTS13 deficiency (activity <10%) are considered to be the "classic" TTP, they have represented only 23% of all 299 patients who were treated with plasma exchange for an initial clinical diagnosis of TTP or HUS in the Oklahoma TTP-HUS Registry, 1995 to 2010, and who had measurement of ADAMTS13 activity immediately before plasma exchange was begun (FIGURE 99.2).[5,6] Only 48% of 114 patients with idiopathic TTP and severe ADAMTS13 deficiency.[6] This observation emphasizes the heterogeneity of the TTP and HUS syndromes. Although patients with acquired, severe ADAMTS13 deficiency constitute only a minority of all patients with these syndromes, their pathogenesis, clinical features, and outcomes are well described and represent the basis for understanding the mechanisms of microvascular thrombosis as well as the evaluation and management of patients with clinically suspected TTP.

Pathogenesis

von Willebrand factor (vWF) abnormalities are central to the pathogenesis of TTP in patients with acquired, severe ADAMTS13 deficiency.[17–21,22] vWF is secreted by endothelial cells into the plasma where it is processed into smaller multimers that do not spontaneously interact with circulating platelets (see Chapter 13). ADAMTS13, an enzyme present in normal plasma, processes vWF by proteolysis, which is the principal mechanism in this respect. vWF may also be processed

Table 99.1

Nomenclature of the TTP and HUS syndromes

Name	Definition
Pathologic Descriptive Term	
TMA	The characteristic histologic abnormality of both TTP and HUS. Describes the microvascular features (swelling of endothelial cells and the subendothelial space) associated with thrombosis. TMA may also occur in many other disorders, such as malignant hypertension, antiphospholipid antibody syndrome, systemic lupus erythematosus, and preeclampsia.
Acquired Clinical Syndromes	
TTP	The appropriate term for all adults with microangiopathic hemolytic anemia and thrombocytopenia with or without renal failure or neurologic abnormalities and without an alternative etiology. Children without renal failure are also described as TTP. Includes but is not limited to patients with severe ADAMTS13 deficiency (<10%). In some adults with predominant acute renal failure, such as with quinine sensitivity or *E. coli* O157:H7 infection, the comprehensive term, TTP–HUS, is often used.
Typical HUS	Children with a syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure with a diarrhea prodrome caused by enteric infection with *E. coli* O157:H7 or other Shiga toxin–producing bacteria.
aHUS	A syndrome of microangiopathic hemolytic anemia, thrombocytopenia, and renal failure without a diarrhea prodrome. May be caused by pneumococcal infections or acquired disorders of complement regulation.
Congenital Clinical Syndromes	
Upshaw-Shulman syndrome	Caused by congenital deficiency of ADAMTS13.
aHUS	Associated with congenital deficiencies of complement regulatory factors, resulting in unrestrained complement activation.

Adapted from George JN. How I treat patients with thrombotic thrombocytopenic purpura. *Blood* 2010;116:4060–4069.

following secretion by reduction of disulfide bonds that link the vWF dimers.[23–25] In 1982, Moake et al. observed in the plasma of patients with chronic relapsing (congenital) TTP[26] unusually large vWF multimers (ULvWF), which were comparable in size to vWF initially secreted by endothelial cells. These ULvWF multimers can directly agglutinate platelets when the vWF structure is altered by the high shear stress that occurs in arterioles.[20] In 1996 to 1998, the origin of ULvWF was documented to be a severe deficiency of ADAMTS13 activity.[17,18,27–30] In patients with acquired TTP, autoantibodies inhibit ADAMTS13 function by interacting with multiple domains, particularly the Cys-rich and spacer domains.[31] In some patients with apparently acquired, severe ADAMTS13 deficiency, no inhibitory autoantibodies are detectable. Autoantibodies may have been neutralized by previous transfusions,[5] or autoantibodies may impair ADAMTS13 function by accelerating clearance from plasma or by impairing binding to the endothelial cell surface rather than by neutralizing enzymatic activity.[32]

Undetectable ADAMTS13 activity is not always associated with evidence for disease. Approximately 20% of patients have persistent severe deficiency of ADAMTS13 activity following recovery from an acute episode of TTP.[5] These observations are similar to those of the absence of the TTP phenotype in *Adamts13*$^{-/-}$ mice[33,34] and suggest that absence of ADAMTS13 may be only one of several components causing acute episodes of TTP. However in baboons, it has been demonstrated that absence of ADAMTS13 activity is sufficient to initiate the clinical features of TTP.[22]

Risk Factors for TTP Associated with Acquired, Severe ADAMTS13 Deficiency

Similar to other thrombotic and autoimmune disorders, multiple risk factors may contribute to the onset of TTP. Similar to systemic lupus erythematosus (SLE),[35,36] middle age, female gender, and black race are important risk factors.[36,37,38] Although it had been postulated that blood group O may be protective against TTP because it is associated with lower plasma vWF levels[39–43] and a faster rate of vWF proteolysis by ADAMTS13,[42,44] blood group O is actually a risk factor for TTP associated with acquired, severe ADAMTS13 deficiency.[45] This observation suggests a novel hypothesis that higher basal levels of normal-sized vWF in people with non-O blood groups may compete with ULvWF for platelet binding, reducing the risk for ULvWF-mediated formation of platelet thrombi and providing partial protection from ULvWF-mediated thrombosis in patients with non-O blood groups. This hypothesis is consistent with previous observations that elevated plasma vWF levels are not a risk factor for the occurrence of the features of TTP in *Adamts13*$^{-/-}$ mice.[33]

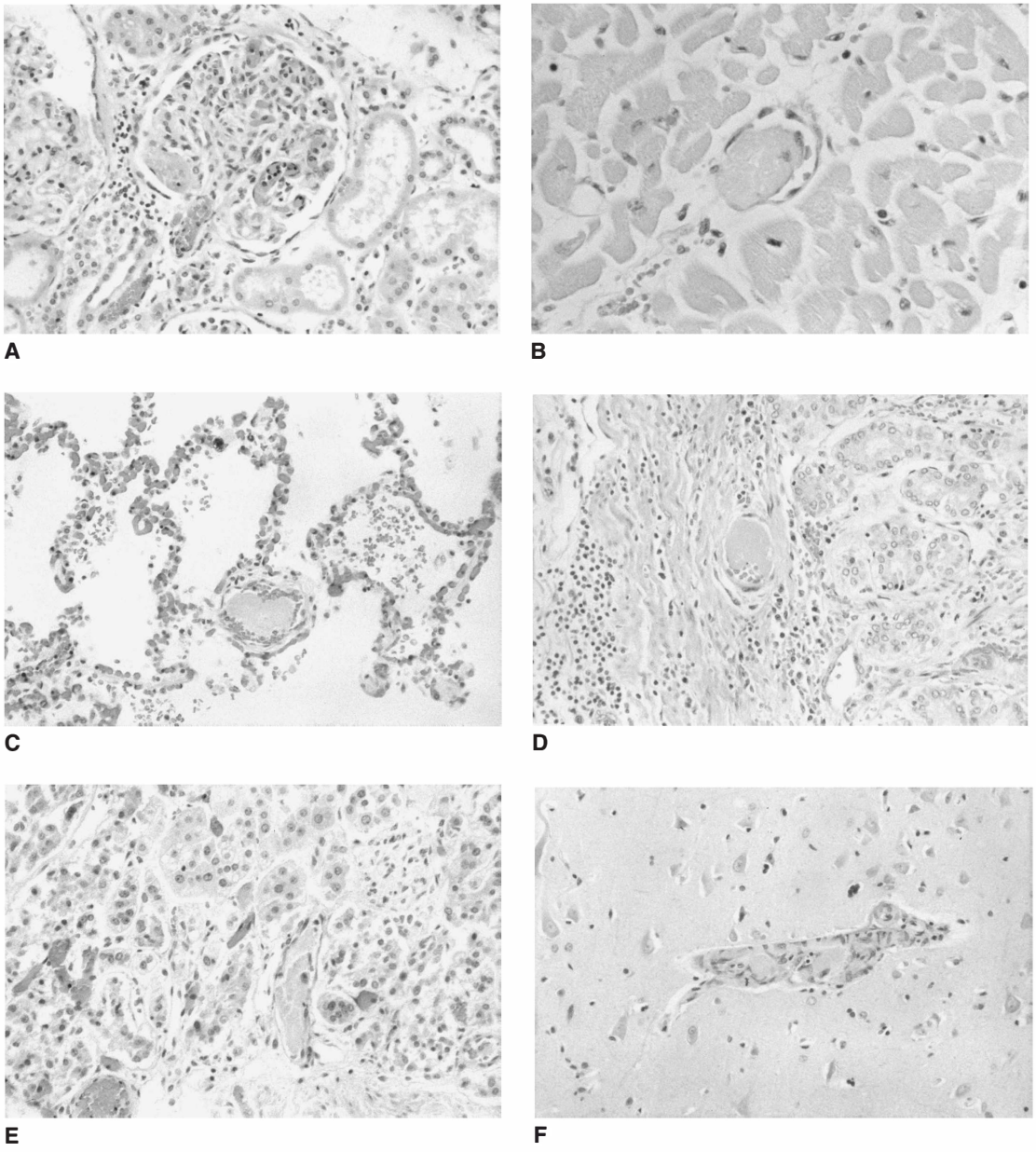

FIGURE 99.1 Systemic microvascular thrombi in TTP. These autopsy tissue sections are from a previously healthy young man who became ill with 3 days of abdominal pain, nausea, and vomiting followed by severe confusion. Clinical features on admission to the hospital were platelet count 11 × 10^9/L, hematocrit 21%, LDH 2,231 U/L, creatinine 1 mg/dL, urinalysis with proteinuria and hematuria, and no fever. He received three plasma exchange treatments plus corticosteroids but died with grand mal seizures and cardiopulmonary arrest 3 days after hospital admission. Subsequent analysis of serum collected before the initial plasma exchange treatment demonstrated ADAMTS13 activity <5% with an ADAMTS13 inhibitor of >2 Bethesda units. Tissue sections demonstrate **(A)** kidney afferent arteriole and glomerular capillary thrombi, **(B)** myocardial thrombi, **(C)** pulmonary thrombi, **(D)** gastric submucosal thrombi, **(E)** adrenal thrombi, **(F)** cerebral thrombi, and **(G)** thrombi in the choroid plexus. (Photographs by Zoltan Laszik, MD, Department of Pathology, University of Oklahoma.)

Table 99.2

Clinical features of the TTP and HUS syndromes

Syndrome	Clinical Features
Acquired Disorders	
ADAMTS13 activity <10%	Considered to be the "classic" TTP. Autoimmune ADAMTS13 deficiency occurs primarily in adult women with increased relative frequency among blacks. Acute episodes may be triggered by pregnancy or infections and may be associated with SLE or other autoimmune disorders. One-third has no neurologic abnormalities; acute renal failure and fever rare; immunosuppressive treatment required in addition to PEX. Relapse may occur in 41%.
Idiopathic TTP, ADAMTS13 activity ≥10%	Etiology unknown. Some may be associated with abnormalities of complement regulation. Compared to patients with ADAMTS13 activity <10%, these patients are older, more often have renal failure, and do not relapse. PEX appropriate because clinical distinction from patients with ADAMTS13 <10% is uncertain.
Typical HUS	Primarily occurs in children <5 years old and accounts for 90%–95% of childhood HUS; also occurs in adults. Preceded by diarrhea, often bloody, and manifested by acute renal failure. Severe neurologic abnormalities uncommon in children but more common in adults. Children managed by supportive care; PEX appropriate for adults because clinical distinction from patients with ADAMTS13 <10% is uncertain. Relapse does not occur.
aHUS	Patients in whom HUS is not preceded by diarrhea. Accounts for 5%–10% of childhood HUS; also occurs in adults. PEX may be appropriate. Relapse may occur.
Drug-induced: acute, immune mediated	Quinine is the most common and the only one with clearly documented etiology, caused by drug-dependent antibodies to platelets, endothelial cells, and other cells. Characterized by a sudden onset of systemic symptoms and anuric acute renal failure. PEX appropriate because drug etiology may be uncertain. Also reported with other drugs.[a]
Drug-induced: dose-dependent toxicity	Mitomycin C, gemcitabine, and cyclosporine are the most commonly reported drugs. Also associated with drugs inhibiting VEGF. Insidious onset with predominant renal failure. Reversibility and role of PEX are uncertain.
Congenital Disorders	
Upshaw-Schulman syndrome (ADAMTS13 deficiency)	Initial episodes may occur at any age; recurrent acute episodes are common. Plasma infusion is sufficient treatment; prophylactic plasma infusion may be required.
Deficiencies of complement regulatory factors (aHUS)	Described as aHUS since acute renal failure is predominant without a diarrhea prodrome. Initial acute episodes may occur at any age. PEX may be appropriate. Relapse may occur.

[a]Other drugs that have been associated with acute and apparently immune-mediated TTP and HUS syndromes are clopidogrel, ticlopidine, and trimethoprim-sulfamethoxazole.
PEX, plasma exchange treatment; SLE, systemic lupus erythematosus; VEGF, vascular endothelial growth factor.

In addition to demographic risk factors, there are multiple acquired risk factors that can be associated with or perhaps even trigger acute episodes of TTP in susceptible patients. Pregnancy is associated with episodes of both congenital and acquired TTP.[46–48] The increased risk for TTP during pregnancy may be related to the decreased ADAMTS13 plasma levels during the third trimester,[49] which are more severe when obstetric complications such as HELLP syndrome occur.[50] However, most pregnancies following recovery are uncomplicated.[47] Inflammatory conditions, such as pancreatitis,[51] infections,[52] and surgery,[53] may also trigger acute episodes of TTP.

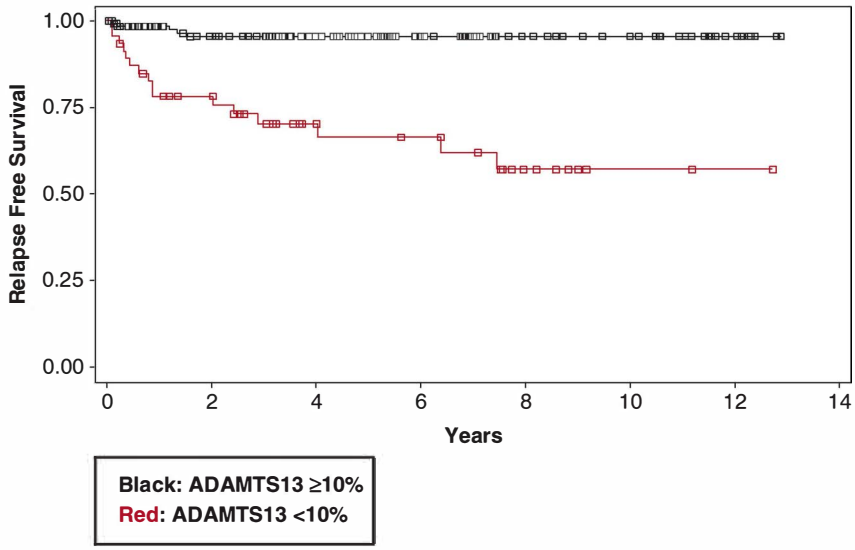

FIGURE 99.2 Kaplan-Meyer analysis of time to relapse for patients with ADAMTS13 activity <10% and patients with ADAMTS13 activity ≥10% at the time of their initial diagnosis of TTP. Data are from all 47 surviving patients who had initially presented with ADAMTS13 activity <10% and all 136 surviving patients with ADAMTS13 activity ≥10% who presented to the Oklahoma TTP–HUS Registry, 1995 to 2008. Median follow-up of all 47 patients with ADAMTS13 activity <10% was 7.5 years; median follow-up of 26 living patients with ADAMTS13 activity <10% who had not relapsed was 4.6 years. (Adapted from Kremer Hovinga JA, Vesely SK, Terrell DR, et al. Survival and relapse in patients with thrombotic thrombocytopenic purpura. *Blood* 2010;115:1500–1511.)

Clinical and Laboratory Features

Validity of the minimal diagnostic criteria of microangiopathic hemolytic anemia and thrombocytopenia, in the absence of another clinically apparent cause,[6,15] is supported by the presenting features of patients who have TTP associated with acquired, severe ADAMTS13 deficiency. Many of these patients have no neurologic or renal function abnormalities.[6] The occurrence of the "classic pentad" of diagnostic features described nearly 50 years ago[13] now rarely occurs, and then only in atypical patients[6] see Table 99.3. Serum LDH levels are typically very high, reflecting severe hemolysis and also the ischemic injury of multiple organs.[54] Case reports have described three patients who had recovered from TTP associated with severe ADAMTS13 deficiency and subsequently had stroke symptoms that were attributed to TTP even in the absence of thrombocytopenia and microangiopathic hemolytic anemia.[55,56] The role of TTP as the etiology of the stroke symptoms was postulated because of the presence of severe ADAMTS13 deficiency and apparent response to plasma exchange treatment.[55,56] The occurrence of neurologic abnormalities in the absence of microangiopathic hemolytic anemia and thrombocytopenia is probably rare, but the possibility is an important clinical concern.

Treatment

Plasma exchange has profoundly affected the prognosis of TTP, decreasing the mortality from >90% before 1964[13] to approximately 20% now.[5,15] Although plasma exchange has remarkable benefit, it also has serious risks including death from hemorrhage or sepsis in 2% to 3% of patients.[57] It has been postulated that plasma exchange is effective by removing autoantibodies to ADAMTS13 as well as replacing ADAMTS13. However, plasma exchange also appears to benefit patients who do not have severe ADAMTS13 deficiency. Among patients with idiopathic TTP, the survival of patients treated with plasma exchange is the same in patients with and without severe ADAMTS13 deficiency.[6] In patients who have severe, acquired ADAMTS13 deficiency, glucocorticoid therapy is required in addition to plasma exchange to achieve a durable remission.[6] Glucocorticoid therapy is typically given as prednisone, 1 mg/kg/d (or the equivalent dose of parenteral preparations). Glucocorticoids are continued until the platelet count is normal and plasma exchange is stopped, and then tapered and discontinued over several weeks. In patients who are severely ill with neurologic complications, short courses of larger doses of parenteral glucocorticoids, such as methylprednisolone, 500 to 1,000 mg/d for 3 days, may be used. In patients with exacerbations and relapses, more intensive immunosuppression with rituximab in addition to glucocorticoids may be required.[5,6,58,59] Rituximab is used in the standard regimen used for other indications, 375 mg/m^2 weekly for 4 weeks.

Long-Term Outcomes

Persistent, subtle cognitive abnormalities may occur in most patients following recovery from TTP[60,61] (Table 99.4). Patients commonly report problems with memory, concentration, and endurance. These patients are not disabled; they have returned to their normal lives and occupations, but daily activities are more difficult.

Relapse rarely occurs in patients who do not have severe ADAMTS13 deficiency.[5] Among patients with acquired severe ADAMTS13 deficiency, the estimated risk for relapse is 41% at 7.5 years; relapses are most common during the first year after recovery.[5] The presence of severe ADAMTS13 deficiency during

Table 99.3

Presenting clinical features and outcomes of 65 consecutive patients with their first episode of ttp associated with adamts13 activity <10%

Clinical Features and Outcomes	Number of Patients (%)
Clinical Category	
Idiopathic	51 (79)
Pregnant/postpartum	3 (5)
Bloody diarrhea prodrome	2 (3)
Allogeneic HSCT	1 (1)
Additional disorder: SLE, Sjögren syndrome	3 (5)
Alternative disorder: systemic infection	4 (6)
Alternative disorder: systemic malignancy	1 (1)
Symptoms and Signs	
Gastrointestinal (nausea, vomiting, diarrhea)	45 (69)
Neurologic abnormalities	
Major (coma, stroke, seizure, focal abnormalities)	23 (35)
Minor (confusion, headache)	20 (31)
None	22 (34)
Laboratory data	**Median (range)**
Hematocrit (%)	25 (15–43)
Platelet count ($\times 10^3/\mu L$)	16 (2–124)
LDH (U/L)	1,247 (256–3,783)
Creatinine (mg/dL)	1.1 (0.7–4.6)
Outcomes	**Number of Patients (%)**
Death	13 (20)
Relapse (in 52 survivors)	18 (35)

Data from all 65 consecutive patients with ADAMTS13 activity <10% presenting to the Oklahoma TTP–HUS Registry, 1995 to 2009. Assignment of patients to clinical categories has been previously described (From Vesely SK, George JN, Lämmle B, et al. ADAMTS13 activity in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome: relation to presenting features and clinical outcomes in a prospective cohort of 142 patients. *Blood* 2003;101:60—68, Ref. 115). Additional disorders are defined as a distinct, concomitant disorder, such as systemic lupus erythematosus; alternative disorders are defined as a new diagnosis discovered after plasma exchange for TTP has begun and which explains the presenting clinical features, such as sepsis, systemic malignancy, or malignant hypertension. When an alternative disorder is diagnosed, plasma exchange treatment is stopped because it is assumed that the initial diagnosis of TTP was incorrect.
HSCT, hematopoietic stem cell transplantation.
Adapted from George JN. How I treat patients with thrombotic thrombocytopenic purpura. *Blood* 2010;116:4060–4069.

remission does not appear to indicate a risk for relapse.[5] The increasing use of rituximab for management of acute episodes may decrease the occurrence of relapses.[5,58,59] Survival with a relapse episode is better than with an initial episode because of prompt recognition and treatment.[5]

Because pregnancy is a recognized risk factor for triggering acute episodes of TTP,[62] pregnancy is often assumed to be associated with a high risk for a relapse. This assumption seemed to be supported by case reports of subsequent pregnancies associated with recurrent TTP,[47] but case reports may preferentially report dramatic events rather than uncomplicated pregnancies. In the Oklahoma TTP–HUS Registry, TTP has recurred in only 6 of 51 subsequent pregnancies (data from Ref. 47 updated through December 31, 2010).

Since severe, acquired ADAMTS13 deficiency is an autoimmune disorder, there may be an increased risk for development of other autoimmune disorders. This potential risk is also suggested by the similar demographic features of patients with severe, acquired ADAMTS13 deficiency and SLE.[6,36]

CONGENITAL TTP

In patients with congenital TTP, multiple homozygous or compound heterozygous mutations of the *Adamts13* gene can cause deficient ADAMTS13 activity.[63,64] These patients are very rare. It has been estimated that only approximately 100 patients have been identified and confirmed worldwide (Kremer Hovinga, *personal communication*). The clinical features of congenital

Table 99.4

Long-term outcomes following recovery from TTP associated with ADAMTS13 activity <10%

Outcome	Comments
Cognitive abnormalities	Problems with memory, concentration, and fatigue are common and persistent but rarely impair normal activities and careers.
Relapse	Estimated risk at 7.5 years—41%. Most relapses occur in the first year after recovery. ADAMTS13 deficiency during remission may not predict risk for relapse. Although pregnancy is recognized as a risk factor for acute episodes of TTP, most subsequent pregnancies are not associated with relapse.
Other autoimmune disorders	Serologic abnormalities characteristic of SLE are common; risk for development of overt SLE or other autoimmune disorders may be increased.

TTP are extremely variable. Although all identified patients have severely deficient ADAMTS13 activity, variation in residual enzyme activity may be important for modifying clinical features.[46,65] Whether these mutations result in type I deficiencies, with no detectable ADAMTS13 antigen, or type II deficiencies, with circulating ADAMTS13 that has defective activity, has not yet been reported. Some patients have severe microangiopathic hemolysis and hyperbilirubinemia at birth.[66,67] In other families, women have been asymptomatic until near term of their first pregnancy[48] and male siblings have remained asymptomatic.[46] Although microangiopathic hemolysis and thrombocytopenia are initially predominant, end-stage renal failure may ultimately occur.[66–68] These patients may also have severe cerebrovascular ischemic events.[29] In patients with congenital TTP, intermittent plasma infusion to replace ADAMTS13 is sufficient therapy.[65,69,70] A plasma infusion regimen of 10 mL/kg every 3 weeks is usually sufficient to achieve a safe, minimum ADAMTS13 activity level of 5%.[65] Patients with recurrent symptoms may need more frequent plasma infusions. Observations in patients with congenital ADAMTS13 deficiency have documented that the half-life of ADAMTS13 in plasma is 2 to 3 days and that the minimum plasma activity sufficient to prevent microangiopathic abnormalities is 5%.[65,70] Recombinant ADAMTS13 is being developed for treatment of TTP. The initial population for clinical trials will be patients with congenital TTP, but these trials have not yet begun.

OTHER TTP AND HUS SYNDROMES

Shiga Toxin–Producing *Escherichia Coli*

Typical HUS of young children follows acute enteric infection caused by *E. coli* serotypes (predominantly *E. coli* O157:H7) that produce Shiga toxin. The association between Shiga toxin–producing *E. coli* O157:H7 and HUS was first documented in 1983.[71] Although outbreaks traced to undercooked beef, unpasteurized milk, juices, fruits, vegetables, and water[72–76] are dramatic and widely publicized, almost all cases are sporadic rather than associated with an outbreak.[77,78] Progression of *E. coli* O157:H7 infection to HUS is estimated to be 2% to 7% in sporadic cases but up to 30% in some epidemics.[72,74,79] The peak age of infection is 6 months to 5 years, suggesting that older children and adults may acquire immunity to the Shiga toxin,[72–74] but Shiga toxin–induced TTP–HUS can occur at any age.[80]

The clinical presentation is characteristically dominated by acute abdominal pain with diarrhea that becomes overtly bloody. If HUS develops, oliguria follows the occurrence of bloody diarrhea.[81] In adults, the clinical presentation is similar, but the course is more severe, with a high rate of severe neurologic abnormalities and death.[80] Although the standard of care for children with typical HUS is supportive care, plasma exchange is appropriate for adults because of the severe clinical course and also because TTP associated with acquired, severe ADAMTS13 deficiency can also present with bloody diarrhea.[6,82] In patients with acquired, severe ADAMTS13 deficiency, microvascular thrombosis in the colon can cause hemorrhagic enterocolitis that is pathologically indistinguishable from the colitis caused by Shiga toxin.[82]

Congenital HUS can be caused by abnormalities of complement regulation resulting in uncontrolled complement activation.[83] These patients often have recurrent episodes of renal failure associated with microangiopathic hemolytic anemia and thrombocytopenia. In adult women, syndromes previously described as postpartum acute renal failure have now been identified as associated with genetic abnormalities of complement regulation.[84] The recurrent episodes distinguish these patients from patients with Shiga toxin, diarrhea-associated HUS. Because of the absence of a diarrhea prodrome, these patients are described as having atypical HUS (aHUS). Many different factors that can control complement activation have been reported to be altered in aHUS.[83,85] For children with aHUS in whom an abnormality of complement regulation is suspected, current opinion suggests that plasma exchange should be used.[85,86]

Drug Hypersensitivity

TTP can be an idiosyncratic, acute adverse reaction to a drug, mediated by drug-dependent antibodies to platelets, neutrophils, endothelial cells, and perhaps also other cells.[87,88] Because of the predominance of acute renal failure, the term TTP–HUS

is often used to describe these patients.[89] Quinine is the most common cause of immune-mediated drug-induced TTP–HUS,[6,89] and it is the only drug associated with TTP–HUS in which the etiology has been supported by recurrent acute illness with recurrent exposure to quinine[89] or by documentation of drug-dependent, platelet-reactive antibodies (unpublished data from the Blood Center of Wisconsin [the largest resource for drug-dependent antibody testing in the United States[90]], *personal communication*, Aster). The presenting clinical features are characteristic: sudden onset of severe symptoms including oliguria, severe thrombocytopenia, and microangiopathic hemolytic anemia.[89] Patients are treated with plasma exchange because the clinical features were consistent with TTP, the quinine-associated etiology is often not initially recognized, and the mortality is high.[89]

Other drugs have been reported to cause acute episodes of TTP–HUS,[91] but their causal association is uncertain. In some patients who have taken ticlopidine or clopidogrel, a severe deficiency of ADAMTS13 activity and the presence of an inhibitor of ADAMTS13 has been identified,[92,93] but no drug dependence of the inhibitor was demonstrated. It is possible that these patients had idiopathic TTP caused by acquired, severe autoimmune ADAMTS13 deficiency and that exposure to ticlopidine and clopidogrel was coincidental and not involved in pathogenesis. Although TTP–HUS associated with chemotherapeutic agents is typically a dose-dependent toxicity, acute and presumed immune-mediated TMA has been reported with oxaliplatin.[94] Oxaliplatin-dependent, platelet-reactive antibodies have been documented in patients with acute oxaliplatin-induced thrombocytopenia[95] but not yet in patients with acute TTP–HUS syndromes.

Thrombotic Microangiopathy Caused by Dose-Dependent Drug Toxicity

Chemotherapeutic agents and calcineurin inhibitors can cause dose-dependent renal toxicity resulting in TMA. Chronic and cumulative dose dependence of the toxicity has been documented for mitomycin C[96] and gemcitabine.[97–99] One patient has been reported with a toxic overdose of deoxycoformycin causing acute, severe TMA.[100] The calcineurin inhibitors, cyclosporine and tacrolimus, can also cause dose-dependent renal toxicity resulting in TMA.[101,102] For patients with TMA associated with dose-dependent drug toxicity, stopping the suspected drug may be all that is required, but patients with TMA associated with mitomycin C and gemcitabine may have persistent, severe renal failure.[96,98] Plasma exchange has no documented benefit.[98,103]

Thrombotic Microangiopathy Following Allogeneic Hematopoietic Stem Cell Transplantation

Patients who developed clinical features of TMA following allogeneic hematopoietic stem cell transplantation (HSCT) were previously described as TTP and treated with plasma exchange.[104,105] However, substantial experience failed to document effectiveness of plasma exchange treatment[106]; therefore the name of this syndrome has been changed to HSCT-associated TMA to avoid consideration of plasma exchange and other treatments used for TTP.[107,108] There are no reports of autopsies demonstrating systemic microvascular thrombosis characteristic of TTP in patients with HSCT-associated TMA, further supporting the distinction between HSCT-associated TMA and TTP.[106,109] Although many complications of HSCT may mimic TMA[110] and many diverse etiologies may contribute to TMA following HSCT,[111] HSCT-associated TMA is considered to be a specific syndrome complicating allogeneic HSCT.[107,108,109,111,112] In addition to the calcineurin inhibitors, cyclosporine and tacrolimus, a potentially important etiology of HSCT-associated TMA is sirolimus, which is also used for graft-versus-host disease prophylaxis.[113] Sirolimus, which inhibits vascular endothelial cell growth factor (VEGF), has been associated with an increased incidence of HSCT-associated TMA.[113] Bevacizumab, which also inhibits VEGF, can also cause renal TMA.[114] These clinical observations have been supported by experimental observations that VEGF inhibition, caused by gene targeting in mice to delete VEGF from renal podocytes, can cause glomerular injury resulting in TMA.[114]

CONCLUSIONS

Because of the absence of explicit diagnostic criteria, the diagnosis of the TTP and HUS syndromes remains based on clinical judgment and is often uncertain. Future studies need to provide more precision for the diagnosis. Plasma exchange is effective treatment for patients with severe, acquired ADAMTS13 deficiency, but it is a treatment with significant morbidity. Survival rates for TTP have not changed in 20 years since the introduction of plasma exchange. Future studies need to develop treatment regimens that are more effective as well as safer and more accessible.

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CHAPTER

100 Vasculitides

JAMES R. STONE AND JOHN H. STONE

The vasculitides comprise a heterogeneous group of disorders characterized by inflammation that targets and damages blood vessels. Vasculitis may be caused by infection or be a result of a direct attack on the vascular wall by the body's immune system. This latter situation, the noninfectious vasculitides, will be covered here. Emphasis will be placed on classification, molecular mechanisms, and pathobiology, with specific digressions into treatment and clinical management where appropriate.

CLASSIFICATION OF VASCULITIS

The classification of vasculitis is an extremely important issue, since it forms the basis for how we approach these disorders in terms of diagnosis, patient management, and even research. It should be understood that the vasculitides have the potential to be as diverse as the complexity of the immune system itself, and thus no classification system is likely to be perfect in all situations. Nonetheless, defining specific vasculitic conditions has significant value. The ideal classification scheme would be one that is based largely on specific molecular mechanisms, predicts prognosis accurately, and guides treatment reliably. Given our limited understanding of many of the vasculitides, this ideal classification is not currently possible. However, great strides have been made utilizing a combination of clinical, laboratory, and pathologic findings.

Multiple diagnostic schemes have been proposed for subclassifying vasculitis in the clinical setting based often on limited information.[1,2] However, the most widely applied scheme for classifying the vasculitides into distinct pathologic entities was developed at the Chapel Hill Consensus Conference.[3] A key feature of the Chapel Hill System is to first consider the sizes of the vessels that are involved, resulting in the general categories of large-vessel vasculitis, medium-sized vessel vasculitis, and small-vessel vasculitis. Large vessels in this context refer to the aorta, arch branches, and distributing arteries in the extremities and head. Medium-sized vessels refer to small- and medium-sized arteries throughout the body, which includes both distributing and intraparenchymal arteries. It is a common point of confusion that in this context small arteries are defined as medium-sized vessels rather than small blood vessels. Small vessels refer to arterioles, capillaries, and venules. Of course, many vasculitides can involve more than one size of vessel. By Chapel Hill criteria, large-vessel vasculitides may involve medium-sized vessels, and small-vessel vasculitides may also involve medium-sized vessels. However, a vasculitis defined as a medium-sized vessel vasculitis should not involve small vessels.

GIANT CELL ARTERITIS

Giant cell arteritis (GCA), one of the most common vasculitides, primarily affects individuals above the age of 50. Based on clinical presentations, the prevalence of GCA is as high as 0.2% for people over the age of 50 and of European descent.[4,5] A large autopsy-based study reported that the prevalence of GCA may be as high as 1.7% in older patients, suggesting that the majority of cases of GCA are subclinical.[6] Pathologically, GCA is characterized by granulomatous inflammation that contains numerous activated epithelioid macrophages, which often coalesce to form giant cells.[7,8] This granulomatous component typically directly targets elastic laminae within the vessel walls of medium-sized and large arteries. There is also usually an accompanying lymphocytic or lymphoplasmacytic inflammatory component, which may be primarily restricted to the adventitia.[9] While almost any artery can be affected, GCA is most commonly seen in the arteries of the head and neck, particularly the superficial temporal arteries (FIGURE 100.1), and in the aorta (FIGURE 100.2). In GCA, there is injury to the arterial wall that leads to the formation of intense intimal hyperplasia. In fact, luminal obstruction is most often secondary to intimal hyperplasia rather than overt thrombosis. In medium-sized vessels, overt necrosis is typically absent or minimal compared with that seen in polyarteritis nodosa (PAN) and granulomatosis with polyangiitis (GPA). Thus, in this regard, GCA can often be viewed as a relatively chronic insidious vasculitis.

Patients often present with fever, headache, visual changes, and elevated nonspecific serum inflammatory markers, such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP).[10] Jaw claudication is a relatively specific but less sensitive symptom of GCA.[11] GCA also has a known association with polymyalgia rheumatica, which causes musculoskeletal pain generally focused on the shoulder and hip girdles. Biopsy of the superficial temporal arteries can confirm the diagnosis. Glucocorticoids can prevent permanent vision loss from obstruction of the posterior ciliary, ophthalmic, or retinal arteries. Other complications of GCA include ischemic necrosis of the scalp and tongue, formation of aortic aneurysms, and life-threatening aortic dissection. Approximately 15% to 20% of patients with GCA experience permanent intracranial ischemic injury, such as blindness and cerebrovascular accidents.[10] The specific etiology of GCA remains elusive.

TAKAYASU ARTERITIS

Takayasu arteritis is a large-vessel vasculitis that primarily affects young women of Asian or Middle Eastern descent. In contrast to GCA, at the time of diagnosis the patients are usually <50 years old, but patients of almost any age may be affected. There is a striking female predominance, with a female to male ratio of 9:1.[12] In North America, the annual

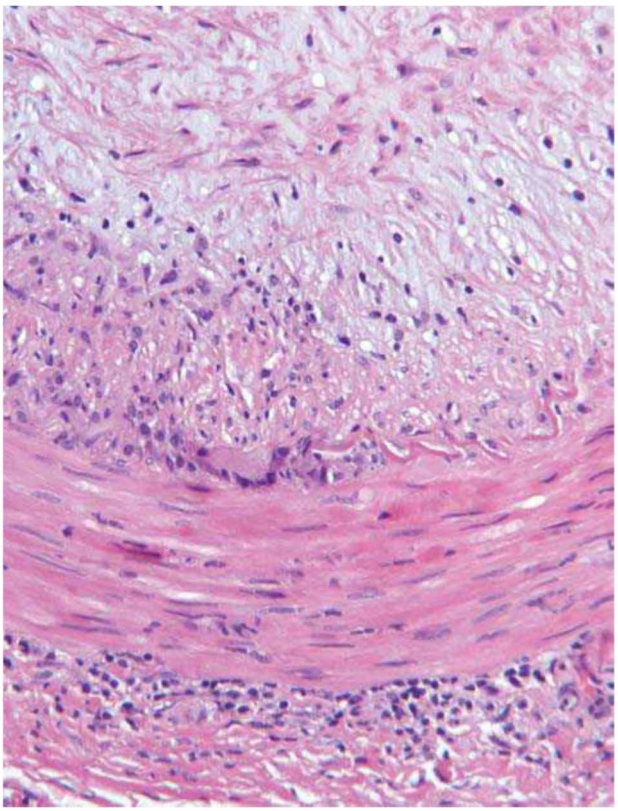


FIGURE 100.1 Giant cell arteritis. Temporal artery biopsy from a 74-year-old woman showing active GCA. There is granulomatous inflammation with a giant cell at the internal elastic lamina. A lymphoplasmacytic infiltrate is present in the adventitia **(bottom)**, and there is extensive intimal hyperplasia **(top)**.

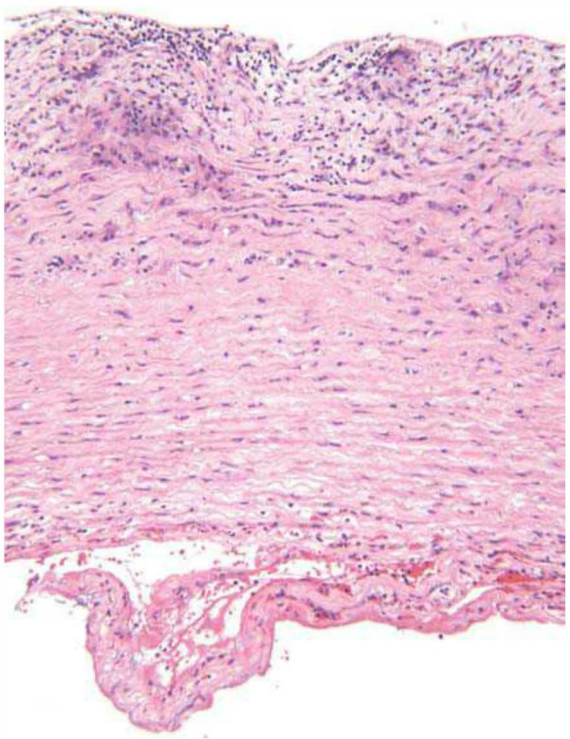


FIGURE 100.2 Giant cell aortitis with aortic dissection. An 87-year-old woman with a history of cranial GCA presented with aortic dissection. Upon surgical repair of the dissection, the aorta demonstrated active giant cell aortitis. Shown is a histologic section of the inner half of the dissected aorta. There is granulomatous inflammation with giant cells **(top)** and the dissection channel with fibrin **(bottom)**.

incidence is about 2 per million per year, making Takayasu arteritis 10- to 50-fold less common than GCA in this region.[13] In its complete presentation, Takayasu arteritis is a disease with two distinct phases. There is an acute phase characterized by fever, weakness, weight loss, arthralgias, and myalgias. This phase is followed by an occlusive phase weeks to years later. The occlusive phase is characterized by vascular occlusion most characteristically of the aortic arch branches, resulting in diminished or absent pulses and bruits on physical examination. Between the acute and occlusive phases, there may be an intervening asymptomatic phase. In addition to the aorta and arch branches, Takayasu arteritis is known to involve the vertebral arteries, renal arteries, coronary arteries, and pulmonary arteries in a significant minority of cases. While occlusive disease is most common, aneurysms also develop in a minority of patients. Fundic abnormalities including vascular tortuosity, microaneurysms, and arteriovenous shunts are also seen in a significant minority of cases.[12] Serum inflammatory markers such as the ESR and CRP are often elevated in the acute phase, but may be normal in the occlusive phase.

The pathologic features of Takayasu arteritis are granulomatous inflammation within the vessel wall and an associated lymphocytic or lymphoplasmacytic component. The disease has a predilection for the adventitia and outer half of the media.[13,14] Giant cells are not uncommon, and compact granulomas may be observed. There is usually marked adventitial scarring (FIGURE 100.3). The pathologic features overlap with those of GCA. However, compared with GCA, in Takayasu arteritis there is more severe adventitial scarring, there is more severe involvement of the outer half of the media compared with the inner half, and in some cases there are compact granulomas. In GCA, typically the inner half of the media is more affected than the outer half, adventitial scarring is less prominent, and compact granulomas are only rarely observed. In Takayasu arteritis, there is marked fibrous intimal thickening, which is responsible for the vascular occlusion (FIGURE 100.4).

Patients are most often treated with glucocorticoids to induce remission, but second-line agents may include cyclophosphamide, azathioprine, or methotrexate.[15] With treatment, the 15-year survival is approximately 80%.[12] The etiology of Takayasu arteritis remains unclear; however some studies suggest an association with infectious diseases, particularly mycobacterial infections.[16]

RHEUMATOID VASCULITIS

Rheumatoid arthritis (RA) is a common chronic destructive inflammatory disease, which primarily affects the joints. Approximately 1% of the population is afflicted, most

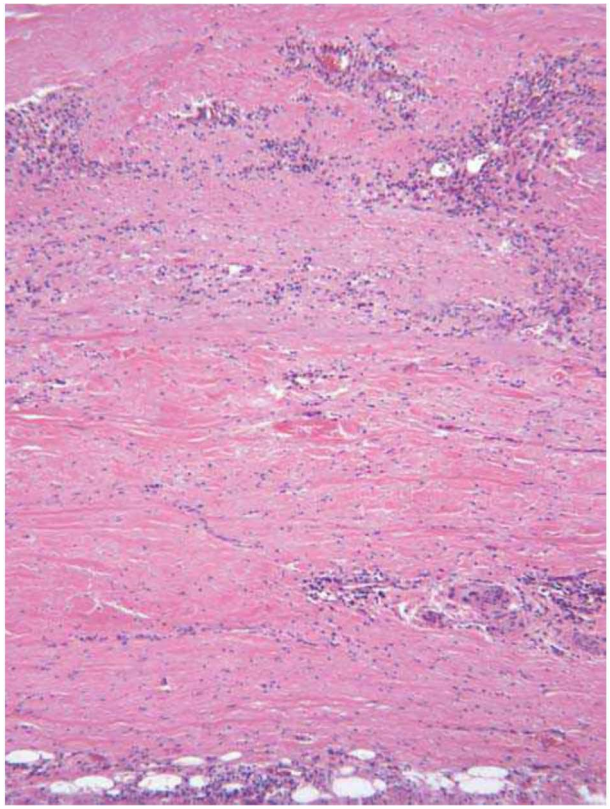


FIGURE 100.3 Takayasu aortitis. A 34-year-old woman with a history of Takayasu arteritis underwent surgical repair of an ascending aortic aneurysm. Histologic assessment of the resected aorta showed extensive granulomatous inflammation in the wall of the aorta **(top)** with marked adventitial scarring **(bottom)**.

FIGURE 100.4 Takayasu arteritis, occlusive aisease. Autopsy performed on a 61-year-old woman with a long history of Takayasu arteritis revealed occlusion of the left common carotid artery with fibrous intimal thickening. There was also marked scarring of the vessel wall, and focal recanalization of the lumen.

commonly middle-age to older adults. Women are more susceptible to the disease than are men, by a ratio of approximately 2.5 to 1. Although joints are the most common organs involved, other sites of involvement include the skin and internal organs. RA is felt to be largely due to a defect in inducing or maintaining tolerance to self, resulting in immune reactivity to multiple systemic autoantigens.[17] Both genetic factors, such as HLA-DRB1 alleles, and environmental factors, such as cigarette smoking and other respiratory exposures, contribute to disease susceptibility.[18] Relatively specific serum tests for RA include the presence of autoantibodies to the Fc portion of the immunoglobulin heavy-chain IgG (rheumatoid factor) and autoantibodies to cyclic citrullinated peptides, both of which may be present in patients prior to the onset of arthritis.

The joint pathology of RA is characterized by perivascular inflammation comprised of lymphocytes, plasma cells, and macrophages. There may be extensive destruction of the articular surface with formation of a characteristic inflammatory pannus comprised of chronic inflammatory cells, reactive synovium, granulation tissue, and activated fibroblasts. The most characteristic extra-articular pathologic feature of the disease is the rheumatoid nodule. Rheumatoid nodules are a type of caseating granuloma, consisting of a central zone of necrotic tissue surrounded by a zone of granulomatous inflammation, comprised of numerous large epithelioid macrophages, lymphocytes, and plasma cells. Macrophage giant cells are often present. This granulomatous inflammation frequently has a palisading appearance, in which the macrophages align perpendicular to the border of the necrotic zone. Rheumatoid nodules are seen in the skin in roughly 25% of patients with RA and are less commonly seen in internal organs including the spleen, lungs, pericardium, cardiac valves, and aorta (FIGURE 100.5).

A minority of patients with RA develop an associated vasculitis, which may involve primarily small- and medium-sized

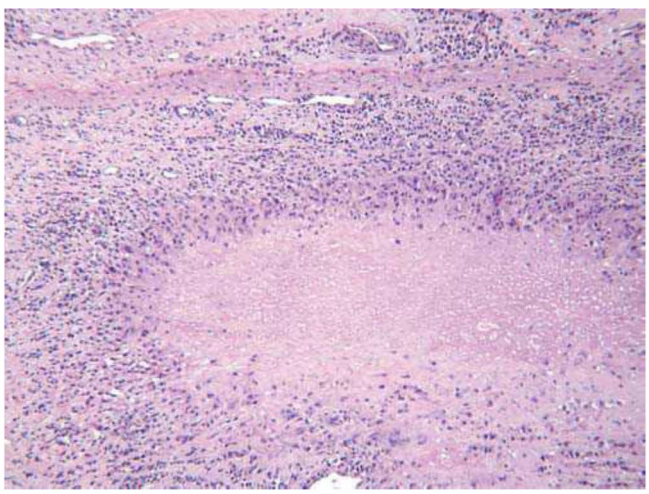


FIGURE 100.5 Rheumatoid valvulitis. A 55-year-old woman with a long history of RA underwent aortic valve replacement due to aortic regurgitation. Histologic examination of the resected valve revealed extensive inflammation and classic rheumatoid nodules.

vessels (rheumatoid vasculitis) or large arteries including the aorta (rheumatoid aortitis). These complications are typically encountered in patients with more severe disease and with cutaneous rheumatoid nodules. The onset of rheumatoid vasculitis typically occurs after many years of joint disease when the arthritis is "burned out." Rheumatoid vasculitis is characterized by lymphoplasmacytic inflammatory infiltrates within small- to medium-sized vessels with destruction of the vessel wall.[19,20] The infiltrate is often most florid in the adventitia. Fibrinoid necrosis and neutrophils may also be present. In a large autopsy series, rheumatoid aortitis was present in 5% of autopsies of patients with RA.[21] In rheumatoid aortitis, the lymphoplasmacytic infiltrate is accompanied by granulomatous inflammation, and actual rheumatoid nodules are present in the media or adventitia of the aorta in about half of the cases. The small- and medium-sized vessel rheumatoid vasculitis manifests in patients as primarily skin ulcers, nailfold lesions, or neuropathies. Rheumatoid aortitis can result in aortic aneurysms or potentially fatal aortic dissection.

Patients with RA are treated primarily to relieve joint pain and prevent joint destruction. Traditional approaches employed a variety of immunosuppressive medications, including methotrexate, hydroxychloroquine, and glucocorticoids. Newer therapies include antibodies targeting tumor necrosis factor (TNF), the interleukin-6 receptor, or the B-lymphocyte surface marker CD20.

IgG4-RELATED AORTITIS

IgG4-related aortitis is a recently identified form of large-vessel vasculitis, which occurs in the setting of IgG4-related systemic disease (IgG4-RSD). IgG4-RSD is a systemic sclerosing disease, which most commonly afflicts older men.[22] It is characterized by the presence of lymphoplasmacytic inflammation with an unusually high proportion of plasma cells expressing IgG4, and by the presence of fibrosis or scarring. The disorder was first recognized to involve the pancreas and is often referred to as autoimmune pancreatitis. IgG4-RSD can involve diverse tissues throughout the body including the liver, thyroid gland, kidneys, salivary glands, lymph nodes, the orbits, the meninges, the retroperitoneum, the mediastinum, the pericardium, and the aorta. A relatively specific serum test for IgG4-RSD is an elevated level of IgG4 within the serum. It is not currently known if the IgG4 itself is actually pathogenic or is a marker of an aberrant immunologic reaction.

IgG4-related aortitis is characterized by lymphoplasmacytic inflammation within the aortic wall.[23,24–27] In contrast to many other forms of aortitis, granulomatous inflammation is not typically present. In IgG4-related aortitis, the majority of the plasma cells express IgG4, whereas in giant cell aortitis and Takayasu arteritis, typically <30% of the plasma cells express IgG4. In some cases of IgG4-related aortitis, the lymphoplasmacytic infiltrate shows a marked adventitial distribution. Such cases may have been referred to in the past as "inflammatory aneurysm" or periaortitis. Other cases of IgG4-related aortitis show considerable infiltration within the media of the aorta itself (FIGURE 100.6). As in other tissue sites, the lymphoplasmacytic inflammatory infiltrate is typically accompanied by fibrosis, which may have a storiform pattern. In the aorta, involvement of adventitial venules leads to an obstructive phlebitis. However, occlusion of small adventitial arteries, endarteritis obliterans, which is a hallmark of syphilitic aortitis, is not typically seen in IgG4-related aortitis. IgG4-related aortitis most commonly results in aortic aneurysm formation; however aortic dissection and aneurysm rupture may also occur.

Patients typically show response to glucocorticoids, including normalization of serum IgG4 levels. In patients with an incomplete response to glucocorticoids, a more complete response can be elicited by B-lymphocyte depletion using antibodies targeting the B-lymphocyte CD20 surface antigen.[28]

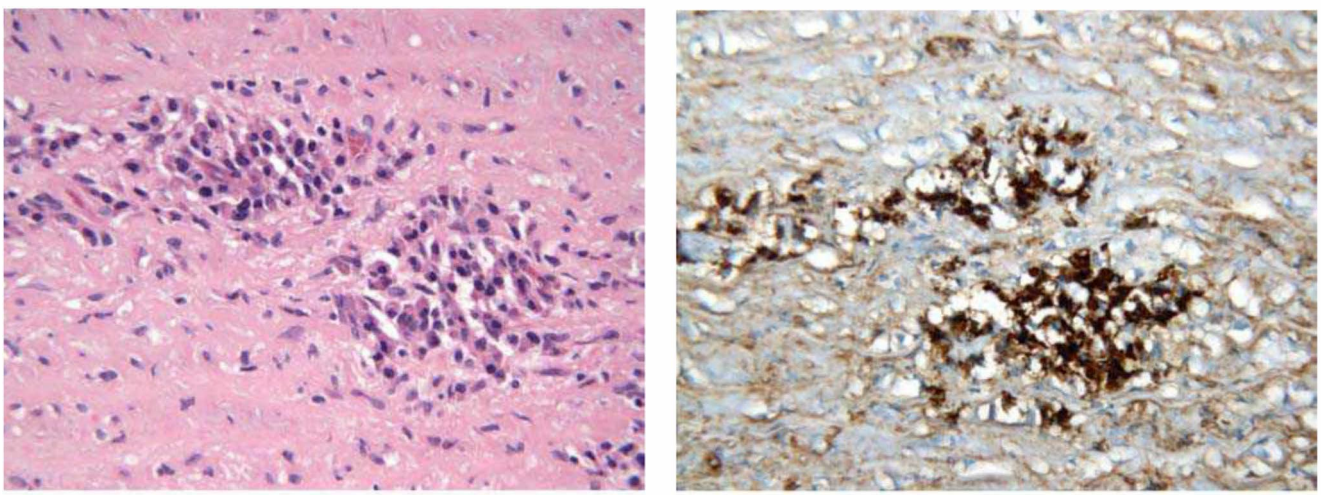


FIGURE 100.6 IgG4-related aortitis. Shown is a histologic section of an aorta from a 65-year-old man undergoing surgical repair of an aortic dissection. There is infiltration of the aortic wall by plasma cells **(A)**, the majority of which stain for IgG4 **(B)**. The patient was found to have IgG4-RSD. (See Stone JH, Khosroshahi A, Hilgenberg A, et al. IgG4-related systemic disease and lymphoplasmacytic aortitis. *Arthritis Rheum* 2009;60(10):3139–3145.)

POLYARTERITIS NODOSA

PAN is a rare necrotizing vasculitis of small- and medium-sized arteries that primarily afflicts middle-aged adults. By Chapel Hill criteria, PAN does not involve small vessels such as arterioles, capillaries, and venules, and it is not associated with the presence of antineutrophil cytoplasmic antibody (ANCA), which would signify other conditions, particularly microscopic polyangiitis (MPA), GPA, or eosinophilic granulomatosis with polyangiitis (EGPA). Understanding PAN has been complicated over the years by changing definitions. Previously in some cases, both ANCA-associated vasculitides and immune-complex vasculitides (ICVs) have been reported as being PAN. However if the small-vessel vasculitides are excluded, then true systemic PAN is a rare disorder. There is a clear association between hepatitis B virus (HBV) infection and PAN. Approximately 7% of cases of PAN are associated with HBV.[29] The annual incidence of PAN is roughly 1 to 2 per million in populations not hyperendemic for HBV.

PAN is a systemic segmental necrotizing vasculitis with a predilection for the distributing arteries in the abdomen. PAN tends to spare the lungs and aorta. The vascular inflammation can result in either aneurysm formation or arterial occlusion due to thrombosis with subsequent ischemia of distal tissues. Arterial rupture or dissection may also occur. Presenting symptoms are often nonspecific and include malaise, fever, weight loss, myalgias, and arthralgias. About half of patients experience vasculitic neuropathy, which may manifest as a mononeuritis multiplex with both sensory and motor features. Mesenteric artery involvement can lead to ischemic enteritis/colitis, bowel infarction and perforation, and abdominal hemorrhage. In addition to the systemic form of the disease, PAN can also present as an isolated self-limited disorder typically involving the skin or genitourinary tract, particularly the testes.[30]

Pathologically, PAN is characterized by a mixed inflammatory infiltrate composed of lymphocytes, macrophages, neutrophils, and eosinophils (FIGURE 100.7). There is typically marked necrosis of the vessel wall in which the necrosis acquires a homogenous eosinophilic character resembling fibrin (fibrinoid necrosis). Granulomatous inflammation is not typically present. About half of the patients with PAN can be managed with high-dose glucocorticoids, but the remaining patients will typically require treatment with cytotoxic agents. Patients with HBV-associated PAN can be treated effectively with antiviral agents and only short courses of immunosuppression, sometimes accompanied by plasma exchange.

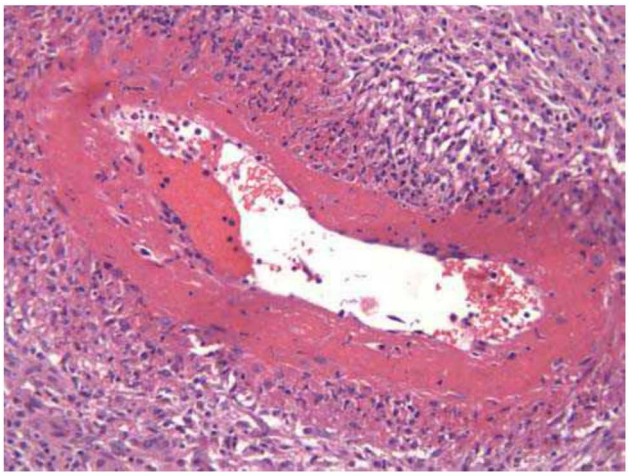


FIGURE 100.7 PAN. A 50-year-old man presented with testicular pain and unilateral inhomogeneous echotexture in the testis by ultrasound. Histologic examination of the orchiectomy specimen revealed necrotizing medium-sized vessel vasculitis with extensive fibrinoid necrosis typical of PAN. The serum was negative for ANCA, and clinically the vasculitis was limited to the testis.

KAWASAKI DISEASE

Kawasaki disease is an acute febrile illness with an associated necrotizing vasculitis of medium-sized arteries that primarily afflicts infants and young children, preferentially those of Eastern descent. The incidence ranges from approximately 10 per 100,000 children younger than 5 years of age in the United States up to 200 per 100,000 children <5 years of age in Japan.[31] The patients typically present with a constellation of signs that may include fever, conjunctivitis, erythema of the mouth and oropharynx with a strawberry tongue, red swollen hands and feet, a macular or morbilliform rash involving the trunk and extremities, and cervical lymphadenopathy.

Up to one-fourth of untreated patients develop a necrotizing vasculitis of medium-sized vessels that preferentially involves the coronary arteries. The coronary vasculitis may lead to thrombotic coronary artery occlusion and myocardial infarction or the formation of coronary artery aneurysms, which may rupture and lead to sudden death. Kawasaki disease is the most common cause of acquired heart disease in children in the United States. The vasculitis of Kawasaki disease is characterized pathologically by a transmural inflammatory infiltrate, which is predominantly lymphohistiocytic with varying numbers of plasma cells, neutrophils, and eosinophils. In some cases, the plasma cells primarily secrete IgA,[32] in contrast to the typical vascular inflammatory infiltrates in which the plasma cells show an IgG predominance. Recent provocative studies have provided evidence that the antibodies generated by these IgA-secreting plasma cells recognize viral-like cytoplasmic inclusions within the repertory epithelium of patients with acute Kawasaki disease, supporting the long-held belief that the disorder is triggered by an infectious agent.[33]

Patients with Kawasaki disease are often effectively treated with intravenous immunoglobulin and aspirin. A primary goal of treatment is to prevent or reduce coronary artery injury, which is responsible for a large proportion of the fatal complications of the disease. However, patients effectively treated for the acute phase of the disease remain at increased risk for development of premature coronary artery disease.[34]

SMALL-VESSEL VASCULITIS

The small-vessel vasculitides comprise a group of disorders characterized by a neutrophil-predominant or leukocytoclastic vasculitis involving small vessels; that is, arterioles, capillaries, and venules. The neutrophils often show degeneration or leukocytoclasia. There is typically injury to the vessel wall, which may take the form of fibrinoid necrosis, and the vessel often becomes obstructed due to thrombosis. Hemorrhage from injured vessels is a common occurrence.

The small-vessel vasculitides are divided into those associated with the circulating ANCA and those not associated with ANCA.[35,36] Those not associated with ANCA are associated with immune-complex deposition and are thus referred to as the ICVs. Immune complexes activate complement, which leads to the formation of chemotactic factors that attract neutrophils. In contrast, the ANCA-associated vasculitides are considered pauciimmune small-vessel vasculitides, as they are not associated with significant immune-complex deposition. In these conditions, the ANCA itself may be pathogenic or at least play a role in amplification of the inflammatory response.

The ANCA-associated small-vessel vasculitides include three principal disorders: GPA, CSS, and MPA. While the immune-complex small-vessel vasculitides can include essentially any condition that results in immune-complex formation, the principal disorders are Henoch-Schönlein purpura (HSP), cryoglobulinemic vasculitis, and drug reactions. Other causes of ICV include systemic lupus erythematosus (SLE), inflammatory bowel disease, infections, and paraneoplastic phenomena.[37,38]

Given the similarity of the vascular inflammation in all of these conditions, it is not surprising that many of the manifestations of these disorders are very similar. As examples, palpable purpura due to involvement of the superficial epidermal capillary plexus, hemoptysis due to pulmonary capillaritis, and hematuria due to glomerulonephritis are common to most forms of small-vessel vasculitis, particularly the ANCA-associated disorders. In addition, all small-vessel vasculitides are also capable of involving small- and medium-sized arteries and causing aneurysms and thrombotic obstructions.[39,40] There are, however, distinct differences between these various conditions.

GRANULOMATOSIS WITH POLYANGIITIS

GPA (formerly Wegener granulomatosis) and the other ANCA-associated vasculitides are small-vessel vasculitides with a predilection for involving the lungs and kidneys.[41,42,43] These conditions are associated with circulating antibodies either to proteinase-3, which stains neutrophils in a cytoplasmic pattern (C-ANCA), or to myeloperoxidase, which stains neutrophils in a perinuclear pattern (P-ANCA). Patients with GPA usually have ANCA directed against proteinase-3, often referred to as PR3-ANCA, and much less commonly ANCA directed against myeloperoxidase (MPO-ANCA).[44] GPA is an uncommon disease with an annual incidence of approximately 1 per 100,000 in European countries, afflicting a wide age range from children to the elderly.[45] In addition to lung and kidney involvement, GPA may involve any organ, but has a propensity to involve the upper respiratory tract and the ocular orbits. Chronic destruction of the tissue in the nose can result in a characteristic saddle-nose deformity (FIGURE 100.8).

Pathologically, GPA is characterized by both a small-vessel neutrophilic vasculitis and granulomatous inflammation, which is largely extravascular, but in unusual cases can cause a granulomatous vasculitis. GPA likely begins as a granulomatous disorder in the respiratory tract before becoming a disseminated small-vessel vasculitis.[46] The granulomatous component is composed primarily of activated macrophages with scattered macrophage giant cells, and often contains areas of necrosis, which may occur as discrete collections of neutrophils (microabscesses). The granulomatous component can cause destructive mass lesions in the orbits, airways, and chest cavity.

With treatment, the 10-year mortality for patients with GPA is around 12%.[42] These patients have traditionally been managed with a combination of glucocorticoids and cyclophosphamide.[41,42,47] However, treatment with antibodies that target CD20 on B cells (Rituximab) has recently been shown to be as effective as cyclophosphamide in inducing remissions and preventing relapses.[48]

EOSINOPHILIC GRANULOMATOSIS WITH POLYANGIITIS

EGPA (formerly Churg-Strauss syndrome) is an ANCA-associated vasculitis closely related to GPA. A key distinction between the two conditions is that patients with EGPA typically have asthma or allergic rhinitis and also demonstrate an elevated level of eosinophils circulating in the blood.[49,50,51] Also in EGPA, only about 40% of patients develop ANCA compared with >90% of patients with severe GPA and in EGPA the ANCA is primarily MPO-ANCA rather than PR3-ANCA. EGPA is a rare disease with an annual incidence of 2 per million per year and preferentially affects middle-aged adults.

There are distinct phases in EGPA with a prodromic phase consisting of allergic rhinitis or asthma, which is followed by a phase in which eosinophils are found within tissues along with granulomatous inflammation.[52] This is followed years later by a systemic small-vessel vasculitis primarily affecting the kidneys, skin, peripheral nerves, and lungs and less commonly other organs including the heart and brain. The pathology of EGPA is similar to that of GPA, except that in some cases increased numbers of eosinophils are identified. Patients are often managed with glucocorticoids, with addition of cyclophosphamide in severe cases.

MICROSCOPIC POLYANGIITIS

MPA is an ANCA-associated small-vessel vasculitis, which lacks the granulomatous inflammation found in patients with GPA and EGPA. It was previously regarded as a variant of PAN and was also previously referred to as microscopic periarteritis and microscopic polyarteritis. About 75% of patients with MPA have ANCA, which is predominantly MPO-ANCA.[53] Like the other ANCA-associated vasculitides, MPA frequently affects the kidneys and lungs. Kidney involvement often takes the form of rapidly progressive glomerulonephritis. In addition, skin involvement, nerve involvement (mononeuritis multiplex), and arthralgias and myalgias are also common in MPA. Medium-sized vessel involvement with microaneurysm formation occurs occasionally in MPA.

The annual incidence ranges from 2 to 12 per million per year in Europe.[54] MPA afflicts primarily middle-aged to older adults. Patients are typically treated with cyclophosphamide and glucocorticoids to induce remission. However, as with GPA, treatment with antibodies that target CD20 on B cells (Rituximab) has been shown to be as effective as cyclophosphamide in inducing remissions and preventing relapses in patients with MPA.[48]

HENOCH-SCHÖNLEIN PURPURA

HSP is an immune-complex small-vessel vasculitis due to formation of IgA1-containing immune complexes.[55,56,57] HSP is more common in children than adults and is the most common vasculitis of childhood, with an annual incidence of 10 per

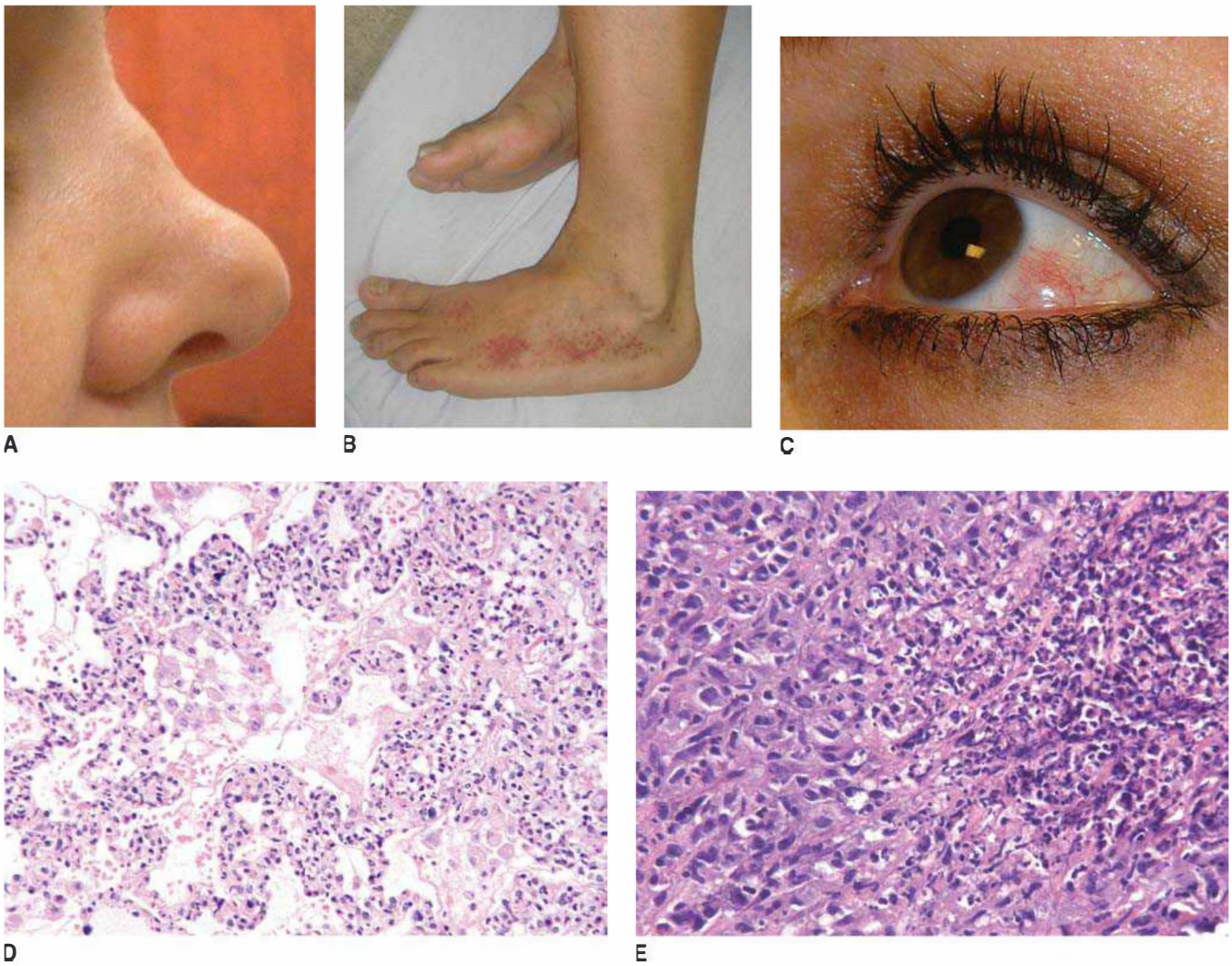


FIGURE 100.8 GPA. Shown are the gross and histologic manifestations of granulomatosis with polyangiitis. **A**: Saddle-nose deformity has a predilection for occurring in young women with GPA whose disease beyond the respiratory tract may be quite limited. **B**: Cutaneous vasculitis occurs in GPA, but is less commonly than in immune-complex vasculitis. **C**: Episcleritis can herald a disease flare, but also can continue in the absence of evidence of active disease in other organ systems. **D**: Pulmonary capillaritis may present as hemoptysis. **E**: Granulomatous inflammation with microabscess formation **(right)** is a classic pathologic feature of GPA, and in this case resulted in an intrathoracic mass surrounding the pulmonary artery.

100,000 children per year. In about 50% of cases, its appearance is preceded by an upper respiratory tract infection, suggesting an infectious trigger in some cases. In other cases, environmental triggers (such as medications) are suspected. The classic presentation includes skin involvement (palpable purpura), abdominal pain due to gastrointestinal involvement, and arthritis. Renal involvement with glomerulonephritis may also occur, particularly in older children and adults, but pulmonary hemorrhage is only rarely seen. The gastrointestinal involvement results in hematemesis or occult bleeding, and in rare occasions massive gastrointestinal hemorrhage. About half of patients will have elevated levels of IgA in the blood.

Pathologically, HSP displays the features of a neutrophilic small-vessel vasculitis detailed above (FIGURE 100.9). In addition, by immunofluorescence microscopy, IgA immune complexes are present in the acute vascular lesions. The disease is often self-limited lasting about 4 weeks. In about a third of cases, there will be recurrences over the next 4 to 6 months. Glucocorticoids have not been shown to significantly alter the disease course, and treatment is usually supportive.[55] The most common long-term complication is renal failure secondary to nephritis.

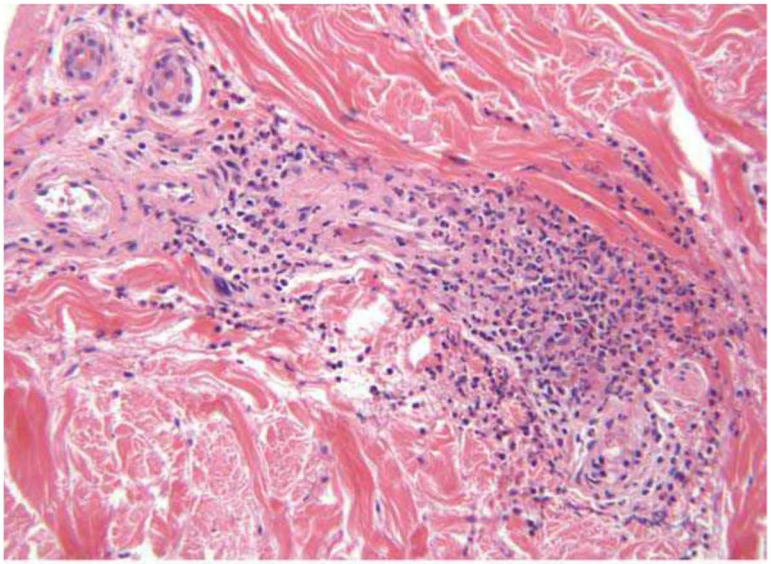
CRYOGLOBULINEMIC VASCULITIS

Cryoglobulins are circulating immunoglobulins that precipitate when serum is cooled below 37°C and dissolve upon rewarming. Type I cryoglobulins are composed of monoclonal immunoglobulins and are typically encountered in the setting of a lymphoproliferative disorder. Type II cryoglobulins are composed of polyclonal IgG and monoclonal IgM rheumatoid factor, while type III cryoglobulins are composed of polyclonal IgG and polyclonal IgM rheumatoid factor. Type II and type III

A

B

C



D

FIGURE 100.9 Immune-complex small-vessel vasculitis. **A**: A patient with a type II cryoglobulinemia not associated with hepatitis C infection developed confluent palpable purpura. In immune-complex-mediated forms of cutaneous vasculitis, the purpura is frequently confluent and diffuse, sometimes extending to the trunk and upper extremities in addition to its usual location on the lower extremities. **B**: This patient developed hypersensitivity vasculitis following treatment with naf-cillin. There was extensive palpable purpura with necrotic centers covering the upper and lower extremities and trunk. The patient also developed interstitial nephritis. **C**: HSP has a tendency to induce pustular skin lesions. This patient underwent routine presurgical prophylaxis with cefazolin, which led to palpable purpura. She then received another dose of cefazolin 10 days after the first, which resulted in an exuberant pustular rash over the lower and upper extremities. Light microscopy and immunofluorescence studies of the skin biopsy were diagnostic of HSP. The rash resolved following a short course of prednisone. **D**: A skin biopsy from a man with HSP shows leukocytoclastic vasculitis, with numerous neutrophils in and around small vessels with vascular injury.

cryoglobulins are both referred to as mixed cryoglobulins. Mixed cryoglobulinemia has been associated with multiple infectious, rheumatologic and neoplastic conditions including hepatitis C virus (HCV), human immune deficiency virus, endocarditis, Sjögren syndrome, SLE, and non-Hodgkin lymphoma.[58,59] If no underlying condition is identified, then the patient is considered to have essential mixed cryoglobulinemia. About 10% of patients with mixed cryoglobulinemia will develop cryoglobulinemic vasculitis due to deposition of immune complexes, primarily within small vessels.

Up to 90% of patients with cryoglobulinemic vasculitis are chronically infected with HCV.[60] About half of patients with HCV have circulating mixed cryoglobulins, but only 5% to 10% will develop cryoglobulinemic vasculitis. The vasculitis most commonly affects the skin, kidneys, nerves, and joints. These patients will often have additional laboratory abnormalities, particularly low levels of complement C4 and a positive rheumatoid factor. HCV-associated cryoglobulinemic vasculitis is often treated with antiviral agents, particularly interferon-α and ribavirin. There is now increasing interest in combining antiviral therapies with B-cell depletion strategies, because these two treatment approaches appear to have synergistic effects.[61]

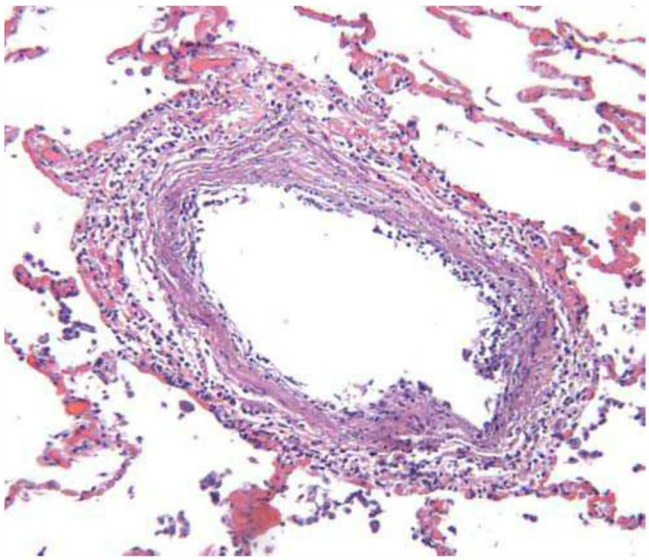


FIGURE 100.10 Medium-sized vessel vasculitis in lupus. Shown is a small pulmonary artery from an autopsy of a patient who died from florid SLE. There is a lymphocytic infiltrate in and around the vessel with necrosis of the vessel wall.

DRUG-INDUCED VASCULITIS

Drug-induced small-vessel vasculitis, often referred to as hypersensitivity vasculitis, is the most common small-vessel vasculitis in adults.[62] The disorder is triggered by the administration of a pharmaceutical drug. Agents that have been implicated are diverse and include antibiotics, propylthiouracil, hydralazine, allopurinol, *D*-penicillamine, phenytoin, isotretinoin, and methotrexate.[63–65] The drugs themselves are typically too small to serve as antigens for immune-complex formation. It is most likely that in some cases the drugs become linked to proteins, and that the drug–protein complex serves as an antigen for immune-complex formation. In other cases, drugs appear to induce the formation of ANCA, which may itself be pathologic without extensive immune-complex formation.[66]

By far, the most common clinical manifestation of drug-induced vasculitis is skin involvement, a cutaneous leukocytoclastic vasculitis resulting in palpable purpura. Less commonly, other organs including kidneys, lungs, liver, and the gastrointestinal tract may be involved. Pathologically, drug-induced vasculitis shows the features of a neutrophilic small-vessel vasculitis detailed above. By immunofluorescence microscopy, immune complexes primarily composed of IgM or IgG are often present in the acute vascular lesions,[67] in contrast to the IgA immune complexes seen in HSP. Immune complexes are not typically observed when drug-induced ANCA is present. Drug-induced vasculitis is often effectively treated by discontinuation of the drug. In severe cases, immunosuppression with glucocorticoids and even cyclophosphamide may be necessary.

MEDIUM-SIZED VESSEL VASCULITIS IN LUPUS

SLE is a systemic autoimmune disorder associated with the generation of autoantibodies. Due to the propensity for immune-complex formation, small-vessel leukocytoclastic vasculitis is a not uncommon feature of patients with SLE.[68] However, much less commonly, patients with SLE may develop a distinct form of vasculitis affecting primarily medium-sized vessels; that is, small- to medium-sized arteries.[69–74] This vasculitis most commonly involves the brain, kidneys, and gastrointestinal tract and less commonly the other internal organs. In contrast to the small-vessel immune-complex vasculitis, the medium-sized vessel vasculitis seen in patients with SLE is predominantly lymphocytic with fewer numbers of macrophages and neutrophils (FIGURE 100.10) and thus is primarily a lymphocytic vasculitis. This medium-sized vessel vasculitis typically shows necrosis of the vascular wall, which may have a fibrinoid appearance, and luminal thrombus may be present.

The key pathologic differential diagnosis includes PAN and ANCA-associated vasculitis particularly MPA. Medium-sized vessel vasculitis in SLE tends to show less overt fibrinoid necrosis than PAN. In addition, in the medium-sized vessel vasculitis associated with lupus, the inflammatory infiltrate is more lymphocyte predominant than typically seen in PAN, in which the inflammatory infiltrate is more mixed. MPA can involve medium-sized vessels; however, the composition of the inflammatory infiltrate is primarily neutrophilic rather lymphocytic. In addition, MPA has a closer relationship to the presence of ANCA than has been established for medium-sized vessel vasculitis in lupus.

BEHÇET DISEASE

Behçet disease is an idiopathic inflammatory disorder characterized by genital ulceration, oral ulcers (aphthous stomatitis), and uveitis. The condition predominantly afflicts young to middle-aged adults of Mediterranean, Middle Eastern, and Eastern descent. Prevalence rates range from approximately 1 per 100,000 in Europe up to 300 per 100,000 in Turkey. Vascular involvement occurs in about one-third of patients.[75] The vasculitis in Behçet disease may involve any size of vessel and may

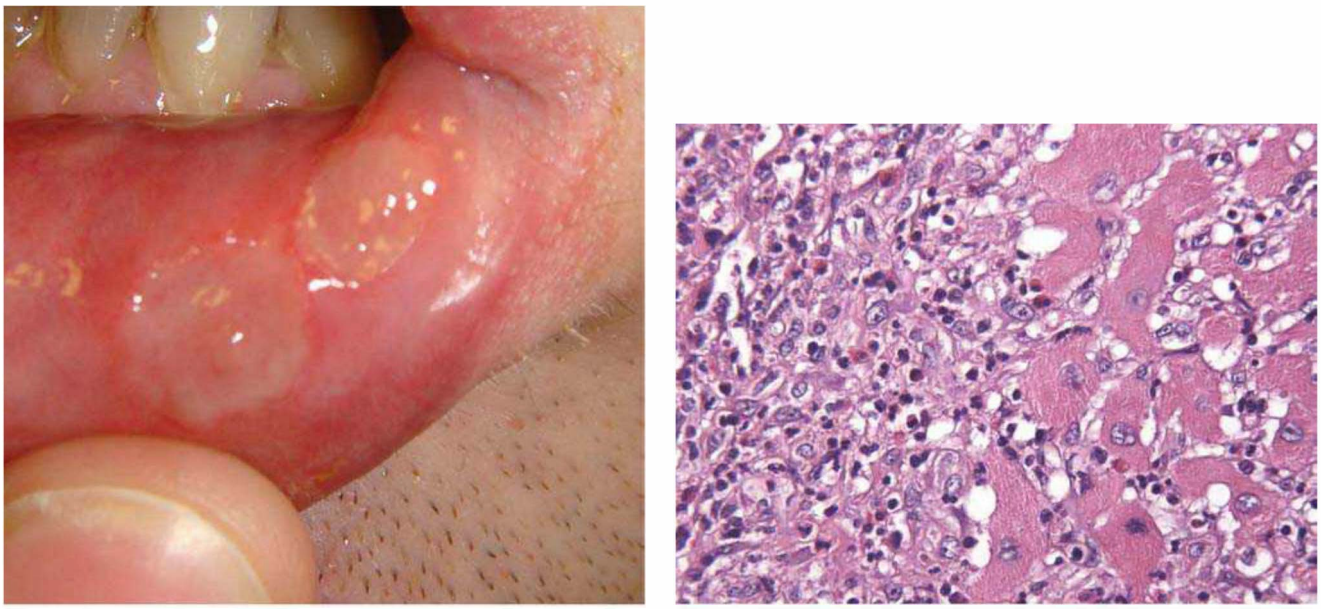


FIGURE 100.11 Behçet disease. **A**: Oral aphthous ulcers are present on the lower lip. **B**: A 16-year-old boy with Behçet disease was found to have a right-ventricular cardiac mass, which on biopsy showed mixed inflammation infiltrating into the myocardium. (See Adams EE, Aluquin VP, Bingham CA, et al. Cardiac tumor in a juvenile onset Behçet disease: case report and review of the literature. *Pediatr Cardiol* 2010;31(2):277–279.)

involve both arteries and veins. Arterial involvement may lead to aneurysms, pseudoaneurysms, or less commonly stenoses. Venous involvement results characteristically in venous thrombosis and in some cases pulmonary emboli. Involvement of the right ventricle of the heart may also cause luminal thrombosis and pulmonary emboli.

The vascular inflammation in Behçet disease ranges from an entirely mixed pattern in the heart and aorta (FIGURE 100.11) to a predominantly lymphocytic pattern in, for example, the central nervous system.[76–79] Granulomatous inflammation is not typically present. Since venous clots in these patients are thought to be due to inflammation, patients with venous thrombosis are often treated with glucocorticoids, azathioprine, and targeted inhibitors of TNF.

COGAN SYNDROME

Cogan syndrome is a rare inflammatory condition characterized by inflammation of the eye and inner ear that afflicts primarily young to middle-aged adults, with an average age of onset of around 30 years.[80,81] Only a few hundred cases have been reported, and both genders are afflicted approximately equally. The inflammation in the inner ear can result in tinnitus, vertigo, and often deafness. The inflammation affecting the eye is typically an interstitial keratitis; although, scleritis, iritis, uveitis, chorioretinitis, or even optic neuritis may be seen. About half of patients with Cogan syndrome will have systemic manifestations, primarily headache, arthralgias, fever, or myalgias. About 10% to 20% of patients with Cogan syndrome develop some form of vasculitis. In these patients, the aorta is most commonly involved, which result in aortic aneurysms or aortic dissection. Medium-sized vessel PAN-like vasculitis and small-vessel leukocytoclastic vasculitis have also been reported in patients with Cogan syndrome.[80] Involvement of medium-sized arteries such as the coronary arteries and cerebral arteries can lead to arterial occlusion with subsequent myocardial infarction or stroke, respectively.

Detailed descriptions of the pathology of the large-vessel vasculitis in Cogan syndrome are sparse.[82,83] The aortitis of Cogan syndrome is typically an acute necrotizing aortitis, containing either a mixed inflammatory infiltrate with neutrophils or even a neutrophil-predominant inflammatory infiltrate (FIGURE 100.12). Thus, the histologic pattern of inflammation in Cogan aortitis appears to differ substantially from both the granulomatous pattern seen in GCA, Takayasu arteritis, and rheumatoid aortitis and the lymphoplasmacytic pattern seen in IgG4-RSD.

There are currently no specific laboratory tests for Cogan syndrome, and the diagnosis is usually based on clinical signs and symptoms. The vestibuloauditory and ophthalmologic symptoms are typically treated with glucocorticoids.

CONCLUSION

The vasculitides are a heterogeneous group of related but distinct disorders, with characteristic clinical presentations, laboratory abnormalities, and histopathologic features. While our understanding of these diseases continues to grow, there is much yet to be accomplished in terms of developing effective treatments for these conditions. Future progress in treating these disorders will likely entail elucidating and targeting the specific pathophysiologic mechanisms underlying these conditions.

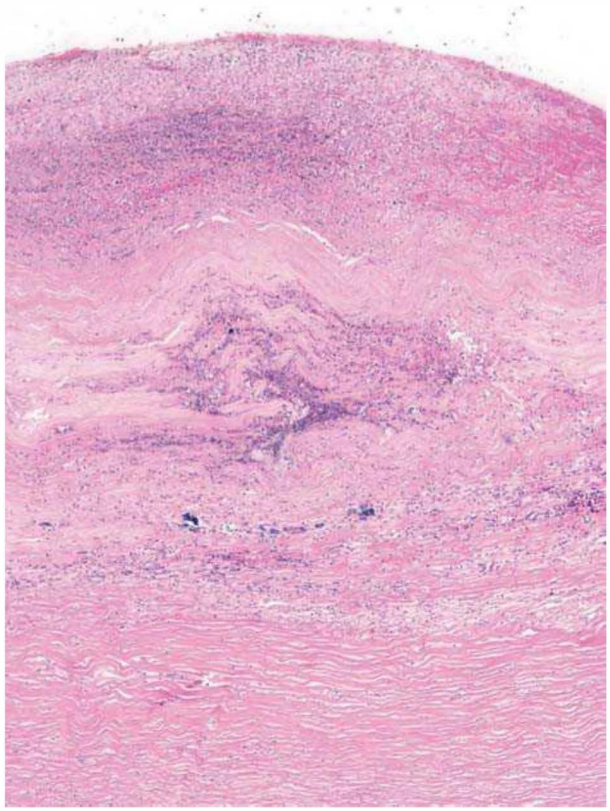


FIGURE 100.12 Aortitis in Cogan syndrome. A 17-year-old girl with Cogan syndrome underwent surgical repair of a thoracoabdominal aortic aneurysm. (See Romain PL, Miller SW, Oas JG, et al. A 17 1/2-year-old girl with a thoracoabdominal aneurysm. *N Engl J Med* 1999;340(8):635–641.) Histologic examination of the resected aorta revealed a severe aortitis comprised of a mixed inflammatory infiltrate containing numerous neutrophils with extensive necrosis. Marked adventitial fibrosis was also present **(bottom)**.

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Full Reference Lists can be found online

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CHAPTER

101 The Antiphospholipid Syndrome

JACOB H. RAND AND LUCIA R. WOLGAST

HISTORICAL BACKGROUND

The antiphospholipid syndrome (APS) is an autoimmune thrombophilic condition that is defined by a constellation of clinical and laboratory manifestations. In retrospect, antiphospholipid (aPL) tests were first described in 1952 with the reports of the biologic false-positive serologic test for syphilis (STS)[1] and an inhibitor of the partial thromboplastin time.[2] In 1957, an association among the biologic false-positive STS, a circulating anticoagulant, and recurrent pregnancy loss was described.[3] The absence of bleeding and a paradoxical association between these coagulation inhibitors and thrombosis were recognized in 1963.[4] The term *lupus anticoagulant* (LA) for these inhibitors of phospholipid-dependent coagulation reactions was introduced,[5] because most of the initial patients appeared to have systemic lupus erythematosus (SLE), although this finding was not sustained by later studies. In the 1980s, immunoassays were developed for anticardiolipin (aCL) antibodies,[6,7] and a new "syndrome of thrombosis, abortion, and neurologic disease"[8] was named the "anticardiolipin syndrome,"[9] subsequently revised to the "antiphospholipid syndrome."[10] In the early 1990s, it was discovered that the antibodies in this autoimmune thrombotic condition did not recognize phospholipids without a protein cofactor identified as β_2-glycoprotein I (β_2GPI)[11,12] and that other phospholipid-binding proteins[13] could also fulfill this role. These antibodies were therefore referred to in literature as being "cofactor-dependent," or "β_2GPI-dependent" aPL antibodies, in contrast to the antibodies generated in patients with syphilis and other infections that recognized phospholipid directly and classified as "cofactor independent." As explained below, it is now accepted that β_2GPI itself is the major target of antibodies in APS.

CURRENT DEFINITION—DIAGNOSTIC CRITERIA AND THEIR LIMITATIONS

The current investigational criteria for APS (Table 101.1), referred to as the Sydney Criteria,[14] were not intended as absolute requirements for the clinical diagnosis. Rather, they were meant to characterize patients whom investigators could agree have "definite APS" for clinical studies." These criteria include histories of documented thrombosis and/or defined pregnancy complications together with specific laboratory tests. However, the syndrome is incompletely understood, and many patients appear to have other clinical abnormalities (Table 101.2) that are referred to as "noncriteria clinical manifestations."[15] Also, as described in the historical background above, the current laboratory assays used for defining the syndrome were derived from empiric observations and were not designed to measure disease mechanisms. Newer laboratory tests that have been developed but not yet accepted by consensus are referred to as "noncriteria laboratory tests." To complicate matters, some patients may have typical clinical manifestations of APS but entirely negative aPL antibody assays and/or LA test, a situation referred to as seronegative APS.[16] Rare patients with aPL antibodies may develop disseminated thrombosis in large and small vessels with resulting multiorgan failure, known as catastrophic antiphospholipid syndrome (CAPS). Preliminary criteria that define CAPS for clinical studies have been published (Table 101.3).[17]

PREVALENCE AND ANTIGENIC SPECIFICITIES OF "ANTIPHOSPHOLIPID ANTIBODIES"

A significant proportion of patients with venous thrombosis have elevated levels of aPL antibodies, with prevalence rates estimated at 5% to 30% for any aPL antibody, 1% to 16% for LA, and 4% to 24% for aCL antibodies. In arterial thrombosis, the prevalence is 0.1% to 10% for aCL antibodies and 0.02% to 0.3% for anti-β_2GPI antibodies. In patients with recurrent spontaneous pregnancy losses, the prevalence is estimated to be 11% for LA and 29% for aCL antibodies.[18] The prevalence of aPL antibodies in the asymptomatic "normal" population has generally been estimated at 3% to 10%,[19,20] with a prevalence of 1% to 5% for LA, 1% to 5% for aCL antibodies, and 3% for anti-β_2GPI antibodies.[18] In one group of healthy young women, who served as controls in a study, 18% had elevated aCL antibodies and 13% tested LA positive.[21] aPL antibodies that occur after infections are generally not associated with thrombosis.[22]

A significant proportion of SLE patients have elevated aPL antibodies; estimates range between 12% and 30% for aCL antibodies and 15% and 34% for LA antibodies.[23] aPL antibodies have been associated with virtually all other autoimmune conditions.[24–32] In some patients, the presence of aPL antibodies may herald the development of SLE; one study reported that 18% of SLE patients had positive aCL antibodies prior to diagnosis.[33]

Antigenic Targets for aPL Antibodies

The main antigen targeted by the APS disease process, β_2GPI, is a 50-kd glycoprotein comprised of five 60–amino acid domains with homology to other members of the complement control protein superfamily[34] (FIGURE 101.1). The biologic function of β_2GPI has not yet been established. β_2GPI binds to apoptotic cells and may aid their clearance.[35,36] The protein also binds to platelet microvesicles and promote their phagocytosis by macrophages.[37] β_2GPI binds as well to oxidized low-density lipoprotein (LDL) and may promote its clearance.[38]

Table 101.1

Sydney investigational criteria for the diagnosis of the APS[a]

Clinical

- Vascular thrombosis (one or more episodes of arterial, venous, or small-vessel thrombosis). For histopathologic diagnosis, there should be no evidence of inflammation in the vessel wall
- Pregnancy morbidities attributable to placental insufficiency, including (a) three or more otherwise unexplained recurrent spontaneous miscarriages, before 10 weeks of gestation, (b) one or more fetal losses after the 10th week of gestation, (c) stillbirth, and (d) episode of preeclampsia, preterm labor, placental abruption, intrauterine growth restriction, or oligohydramnios that are otherwise unexplained

Laboratory

- Medium- or high-titer aCL or anti-β_2GPI IgG and/or IgM antibody present on two or more occasions, at least 12 weeks apart, measured by standard ELISAs
- LA in plasma, on two or more occasions, at least 12 weeks apart, detected according to the guidelines of the ISTH SSC Subcommittee on Lupus Anticoagulants and Phospholipid-Dependent Antibodies

"Definite APS" is considered to be present if at least one of the clinical criteria and one of the laboratory criteria are met

aCL, anticardiolipin; aPL, antiphospholipid; β_2GPI, β_2-glycoprotein I; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin.
[a]Modified from Miyakis et al. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome. *Thromb Haemost* 2006;4:295–306.

Table 101.2

Clinical manifestations associated with the APS

Systemic vascular thrombosis	DVT of lower extremity, thoracic, abdominal, or pelvic veins, pulmonary embolism, arterial thrombosis with downstream ischemia and infarction
Reproductive manifestations	Recurrent fetal loss, preeclampsia, abruption placentae, miscarriage, prematurity, intrauterine fetal demise, intrauterine growth restriction, and oligohydramnios
Neurologic manifestations	Stroke, TIA, *migraines, seizures, chorea, Guillain-Barrè Syndrome, transient global amnesia, dementia, diabetic peripheral neuropathy, and orthostatic hypotension*
Cardiovascular manifestations	Thrombotic occlusion of nonatherosclerotic coronary artery, thrombotic myocardial infarction, *coronary artery disease, premature atherosclerosis, atherosclerotic myocardial infarction, peripheral vascular disease, valvular abnormalities*
Pulmonary manifestations	Pulmonary hypertension attributable to thrombotic occlusion, *other pulmonary hypertension, acute diffuse alveolar damage*
Hepatic and gastrointestinal manifestations	Esophageal necrosis with perforation, intestinal ischemia/infarction, pancreatitis, colonic ulceration, acalculous acute cholecystitis attributable to biliary vascular occlusion, mesenteric venoocclusive disease, *primary biliary cirrhosis*
Renal manifestations	Thrombosis of renal arteries or veins, renal infarction, *APS nephropathy, renal artery stenosis, minimal change disease/focal segmental glomerulonephritis, membranous nephropathy, mesangial C3 nephropathy, pauciimmune crescentic glomerulonephritis*
Skin manifestations	Ulcerations attributable to microvascular infarction, *livedo reticularis, skin ulcerations, skin necrosis, necrotizing vasculitis, livedoid vasculitis, thrombophlebitis, erythematous macules, purpura, ecchymoses, painful nodules, subungual splinter hemorrhages, Anetoderma, discoid lupus erythematosus, cutaneous T-cell lymphoma*
Retinal abnormalities	Retinal vein occlusion, cilioretinal artery occlusion, optic neuropathy
Other organ manifestations	Acute adrenal failure due to bilateral adrenal hemorrhagic infarction, *osteonecrosis without histologic evidence for microvascular occlusion or vasculitis, acute sensorineural hearing loss*
Other coagulation abnormalities	*Thrombocytopenia, ITP, hypoprothrombinemia, protein S deficiency, acquired APC resistance, acquired inhibitors to specific coagulation factors, and AvWS*

Manifestations that are not included in consensus-based diagnostic criteria are italicized.

Table 101.3

Proposed criteria for the classification of CAPS

1. Evidence of involvement of three or more organs, systems, and/or tissues[a]
2. Development of manifestations simultaneously or in less than a week
3. Confirmation by histopathology of small-vessel occlusion in at least one organ or tissue[b]
4. Laboratory confirmation of the presence of aPL antibodies (LA and/or aCL antibodies)[c]
Definite CAPS
• All four criteria
Probable CAPS
• All four criteria, except for only two organs, systems, and/or tissues involvement
• All four criteria, except for the absence of laboratory confirmation at least 6 weeks apart due to the early death of a patient never previously tested for aPL prior to the CAPS event
• Criteria 1, 2, and 4
• Criteria 1, 3, and 4 and the development of a third event in more than a week but less than a month, despite anticoagulation

[a]Usually, clinical evidence of vessel occlusions, confirmed by imaging techniques when appropriate. Renal involvement is defined by a 50% rise in serum creatinine, severe systemic hypertension (N180/100 mm Hg), and/or proteinuria (N500 mg/24 h).
[b]For histopathologic confirmation, significant evidence of thrombosis must be present, although, in contrast to Sydney criteria, vasculitis may coexist occasionally.
[c]If the patient had not been previously diagnosed as having an APS, the laboratory confirmation requires that the presence of antiphospholipid antibodies must be detected on two or more occasions at least 6 weeks apart (not necessarily at the time of the event), according to the proposed preliminary criteria for the classification of definite APS.
Modified from Asherson RA, Cevera R, de Groot PG, et al. Catastrophic antiphospholipid syndrome: international consensus statement on classification criteria and treatment guidelines. *Lupus* 2003;12:530–534.

Anti-β_2GPI antibodies from different patient samples are heterogeneous in their epitope specificities and recognize all five domains of the protein. IgG antibodies against an epitope comprising Gly40-Arg43 in the domain I of β_2GPI have been reported to have a stronger correlation with thrombosis than antibodies against other epitopes.[39] Recent data have shed light on interesting changes in the conformation of β_2GPI that may have a role in the APS disease process. Circulating plasma β_2GPI was found to have a closed circular conformation due to the affinity of a portion of carboxyterminal domain V for the protein's aminoterminal domain I, where the phospholipid binding site is located.[40] The affinity for anionic phospholipids derives from (a) cationic residues from its aminoterminus that have affinity for anionic polar heads of phospholipids and (b) a hydrophobic loop which inserts into the lipid bilayer. Binding to anionic phospholipid membranes requires the protein to open from its circular conformation, which exposes an epitope in domain I that had been cryptic in the circular conformation (FIGURE 101.1).

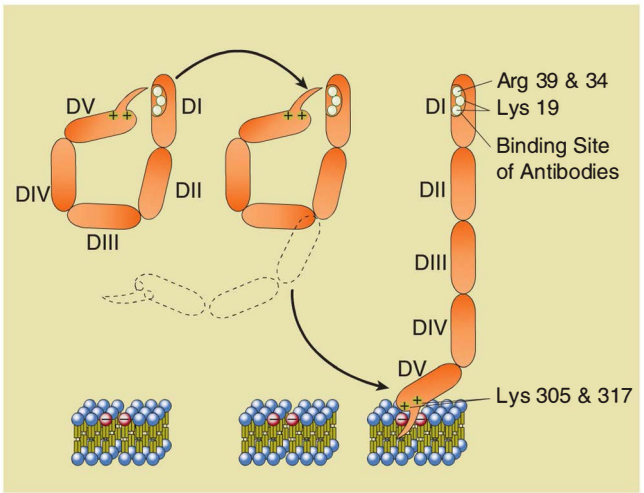

FIGURE 101.1 Schematic representation of β_2GPI conformational change. (Reprinted from Rand JH. A snappy new concept for APS. *Blood* 2010;116:1193–1194, with permission. Inside Blood commentary on Agar C, van Os GM, Morgelin M, et al. β_2-Glycoprotein I can exist in 2 conformations: implications for our understanding of the antiphospholipid syndrome. *Blood* 2010;116:1336–1343, with permission. Professional illustration by Paulette Dennis.)

Several other antigenic targets for aPL antibodies include prothrombin, coagulation factor V, protein C, protein S, annexin A2, annexin A5, high and low molecular weight kininogens, heparin, factor VII/VIIa,[41–44] oxidized LDLs,[45] vimentin complexed to cardiolipin,[46] lyso(bis)phosphatidic acid,[47] sulfatides, and acidic glycosphingolipids.[48]

PATHOPHYSIOLOGIC MECHANISMS

Overview

A host of mechanisms have been postulated to explain the thrombotic tendency in APS (Table 101.4). The APS disease entity is probably composed of subcategories that are attributable to specificities of the particular antibodies. aPL antibodies are highly heterogeneous even in individual patients, and these may exert different effects in different experimental

Table 101.4

Proposed pathogenic mechanisms of APS

I. Inhibition of endogenous anticoagulant and fibrinolytic mechanisms
A. Disruption of the annexin A5 anticoagulant shield
B. Inhibition of the protein C pathway—decreased activation of protein C, barrier of APC proteolysis of factor Va and VIIIa, prevention of protein C and EPCR binding
C. Interference with thrombin regulation—enhanced thrombin generation, decreased thrombin inactivation
D. Inhibition of heparinoids, TFPI, and inactivation of factor IXa
E. Interference with fibrinolysis via annexin A2, β_2GPI cofactor activity, autoactivation of XIIa, direct inhibition of plasmin, and increase of PAI-1
II. Activation of platelets
A. Activation of platelets—via ApoER2 and via β_2GPI-PF4 interaction
B. Interference of vWF-mediated platelet adhesion
III. Injury and activation of endothelial cells and monocytes
A. Direct injury
B. Signaling via annexin A2/TLR4/ApoER2-inducing proadhesive prothrombotic phenotype
IV. Complement activation

systems. In an effort to find additional clues, some investigators have recently pursued proteomic studies to identify candidate proteins that might be involved in the APS disease process.[49]

Inhibition of Endogenous Anticoagulant and Fibrinolytic Mechanisms

Disruption of the Annexin A5 Anticoagulant Shield

Annexin A5 is highly expressed by a variety of cells serving barrier functions including endothelial cells, where the protein binds to the surfaces and inhibits thrombin formation.[50] It is also expressed on placental trophoblasts on the apical membranes of the syncytiotrophoblasts that interface with the maternal blood circulation.[51] Annexin A5 forms two-dimensional crystal lattices over phospholipid bilayers[52,53] that shield them from availability for phospholipid-dependent coagulation enzyme reactions[52] (FIGURE 101.2). The protein appears to be important for maintaining placental blood circulation; pregnant mice treated with antiannexin A5 antibodies developed placental necrosis, fibrosis, and pregnancy loss.[54]

There is significant evidence that aPL antibodies interfere with the annexin A5 binding and crystallization and thereby promote coagulation reactions; this has been demonstrated through immunohistochemical studies of placental tissues,[55] cell culture experiments,[56] and atomic force microscopic imaging[52] (FIGURE 101.3). The aPL antibody-mediated disruption of annexin A5 correlates with antibody recognition of the epitope on domain I of β_2GPI, which in turn has been correlated with significantly increased risk of thrombosis.[39,57]

Inhibition of the Protein C Pathway

aPL antibodies can interfere with the protein C pathway (see Chapter 19) in several ways (Table 101.4). Antibodies against protein C and against protein S have been described,[58–60] with high levels of antiprotein C IgM correlating with increased risk of venous thrombosis.[60] Antibodies against β_2GPI may crossreact with activated protein C (APC),[61] and APS patients often exhibit acquired APC resistance,[62] an effect that has also been correlated with anti-β_2GPI domain I antibodies.[63,64] Antiendothelial antibodies in patients with APS [65,66] may interfere with the positioning of protein C on the endothelial protein C receptor (EPCR) or bind to thrombomodulin and block the activation of protein C by thrombin. Antithrombomodulin antibodies can also interfere with the activation of protein C in SLE patients.[67,68] Antiprothrombin antibodies from APS patient also interfere with the protein C pathway by reducing availability of thrombin for binding to thrombomodulin.[69,70] Antibodies against EPCR have been identified in pregnant APS patients and associated with increased risk of fetal death.[71]

Interference with Thrombin Regulation

It has been proposed that antibodies against noncatalytic epitopes on prothrombin may provoke thrombosis by crosslinking prothrombin molecules, thus increasing their density and thrombin generation on the surface of endothelial cells.[72–74] aPL antibodies and antiprothrombin antibodies can also interfere with inactivation of thrombin by antithrombin.[75] Some antiprothrombin antibodies may themselves display a catalytic function and proteolyze prothrombin to generate a thrombin-like activity.[76]

Inhibition of Heparinoids, TFPI, and Inactivation of Factor IXa

aPL antibodies crossreact with heparin and heparinoid molecules and inhibit the acceleration of antithrombin activity.[77] APS patients can have autoantibodies against tissue factor pathway inhibitor (TFPI) that reduce its activity.[78] Some aPL antibodies interact with factor IXa and impair its inactivation by antithrombin.[79]

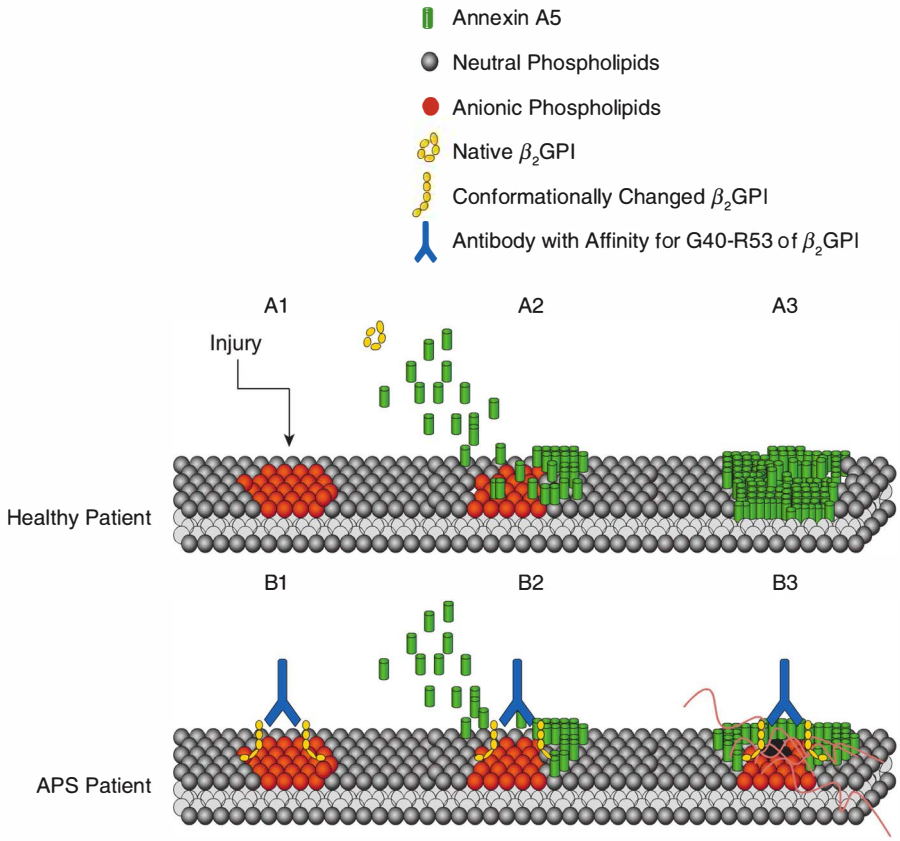

FIGURE 101.2 Proposed mechanism for thrombosis in APS. Proposed mechanism of anti-D1 IgG-mediated disruption of annexin A5 on cell surface. (*A1–A3*) Annexin A5 anticoagulant activity in absence of APS. When injury exposes anionic phospholipids on the cell surface (*A1*), annexin A5 assembles over damaged areas of endothelium, creating a shield over the endothelial surface and inhibiting anionic phospholipid-dependent coagulation reactions. (*B1–B3*) Annexin A5 disruption in the presence of APS. In the presence of antibodies against the G40-R43 epitope on domain I of β_2GPI, there is a conformational change and dimerization of β_2GPI (*B1*), and anti-β_2GPI/β_2GPI complexes displace annexin A5 on anionic phospholipid surfaces (*B2*). This creates gaps in the annexin A5 shield, exposing more anionic phospholipids to coagulation reactions and thrombosis (*B3*). (Modified from de Laat B, Wu XX, van Lummel M, et al. Correlation between antiphospholipid antibodies that recognize domain I of β$_2$-glycoprotein I and a reduction in the anticoagulant activity of annexin A5. *Blood* 2007;109(4):1490–1494.)

Interference with Fibrinolysis

aPL antibodies can interfere with fibrinolysis in several ways. Antibodies against annexin A2, an endothelial surface receptor for tissue plasminogen activator and plasminogen, have been reported,[80] and these can interfere with binding of plasminogen and tissue-plasminogen activator (t-PA) and thereby reduce plasmin formation and fibrinolysis.[81,82] Monoclonal antibodies derived from APS patients can directly interfere with plasmin activity.[83] β_2GPI is a cofactor for t-PA-mediated activation of plasminogen,[84] and antibodies against β_2GPI can also interfere with this activity. Anti-β_2GPI antibodies can inhibit the autoactivation of factor XII, with ensuing reductions of kallikrein and urokinase.[85] Also the elevated plasminogen activator inhibitor-1 (PAI-1) levels observed in women with APS may impair fibrinolysis.[58]

Platelet Activation

aPL antibodies can activate platelets via signaling through ApoER2′, a member of the LDL-receptor family, which binds β_2GPI via domain V.[86] IgG-mediated dimerization of β_2GPI and binding to ApoER2′ increases the sensitivity of platelets to agonists of aggregation.[87] Antibodies directed against domain I of β_2GPI can also activate platelets by binding to platelet GPIbα, which activates the phosphatidylinositol 3-kinase/Akt pathway.[88] The aPL antibody-mediated interactions with ApoER2′ and with GPIbα may be related *in vivo* since ApoER2′ has been shown to complex with GPIbα on the platelet surface.[89] β_2GPI has been shown to dampen platelet adhesion by interfering with the platelet–von Willebrand factor (vWF) interaction, and aPL antibodies may interfere with this effect, thereby promoting platelet adhesion.[90] The majority of a cohort of APS patients with neurologic manifestations were found to have activated platelets in their blood by flow cytometry measurement of CD62 positivity.[91]

Binding of anti-β_2GPI antibodies to β_2GPI complexed to homotetramers of platelet factor-4 (PF4), each of which can bind two molecules of β_2GPI, activates platelets.[92] Whereas heparin-induced thrombocytopenia and thrombosis (see Chapter 108) is associated with antibodies against PF4 that activate platelets via their Fc domains, immune complexes of anti-β_2GPI antibodies with PF4/β_2GPI activate platelets through their F(ab)2 domains.

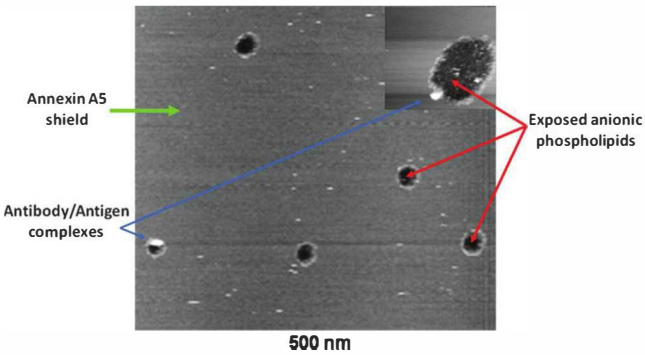

FIGURE 101.3 Disruption of annexin A5 shield by monoclonal aPL antibodies and β_2GPI. Atomic force microscopy picture showing the effect of aPL mAB IS3 on a preformed annexin A5 crystal. The figure demonstrates the smooth lipid bilayer covered by the annexin A5 crystals, disrupted by antibody–β_2GPI complexes (*white circles*) and exposing anionic phospholipids (*black holes*) to coagulation factors and accelerated coagulation. (Modified from Rand JH, Wu XX, Quinn AS, et al. Human monoclonal antiphospholipid antibodies disrupt the annexin A5 anticoagulant crystal shield on phospholipid bilayers: evidence from atomic force microscopy and functional assay. *Am J Pathol* 2003;163(3):1193–1200.)

Injury and Activation of Endothelial Cells and Monocytes

aPL antibodies can injure and/or activate cultured vascular endothelial cells.[93–96] An indicator that this may occur *in vivo* is that circulating levels of endothelial-derived microparticles are increased in the blood of patients with LAs.[97] Antibody binding to β_2GPI on the endothelial surface can also trigger signaling events that increase expression of tissue factor and adhesion molecules. This appears to occur with the involvement of annexin A2, which serves as a receptor for β_2GPI on vascular endothelium.[98] Binding of anti-β_2GPI, which cross-links the β_2GPI–annexin A2 complex, can activate signaling pathways through the Toll-like receptor-4 (TLR-4) adaptor molecule, also called myeloid differentiation factor 88.[99] This leads to the upregulation of the transcription factor and nuclear factor-κB (NF-κB) and subsequent increase in the release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, IL-8, and surface expression of tissue factor and cell adhesion molecules (FIGURE 101.4). A mutation in murine TLR-4 that results in loss of ability to bind lipopolysaccharide (LPS) attenuated the prothrombic state in wildtype mice injected with aPL antibodies.[100] However, a direct interaction between TLR-4 and anti-β_2GPI/β_2GPI complexes has yet to be confirmed. This endothelial activating effect has also been described for antibodies that bind annexin A2 directly,[81,101]— that is, without the involvement of β_2GPI.

Apolipoprotein E Receptor 2 on the Endothelial Surface

ApoER2′, whose role on platelets was described above, may also serve as receptor for anti-β_2GPI/β_2GPI complexes on endothelial cells[102] and monocytes,[103] where it can also trigger tissue factor and cell adhesion molecule expression (FIGURE 101.4). Evidence from ApoER2′$^{-/-}$-knockout mice has supported this mechanism in mediating the prothrombotic effects of aPL antibodies.[104] In accordance with this hypothesis, a soluble binding domain I of ApoER2′ that interfered with the binding of dimerized β_2GPI to ApoER2′ inhibited the effect of aPL antibodies on wildtype, that is, ApoER2′$^{+/+}$, mice.[104]

Activation of Complement Cascade

There is evidence from an animal model that complement activation can play a role in the APS disease process. Blockade of complement activation using a C3 convertase inhibitor or genetic deletion of C3 protected mice from pregnancy complications induced by aPL antibodies.[105,106,107] These effects on pregnancy appeared to be due to stimulation of tissue factor expression by myeloid cells[108] and appear to involve protein-activated receptor-2 signaling.[109] Those results suggested the interesting concepts that thrombin can be pathogenic via both its stimulation of fibrin formation and via its stimulation of signaling pathways, and that complement activation can be pathogenic in two ways—via both direct injury and downstream signaling.

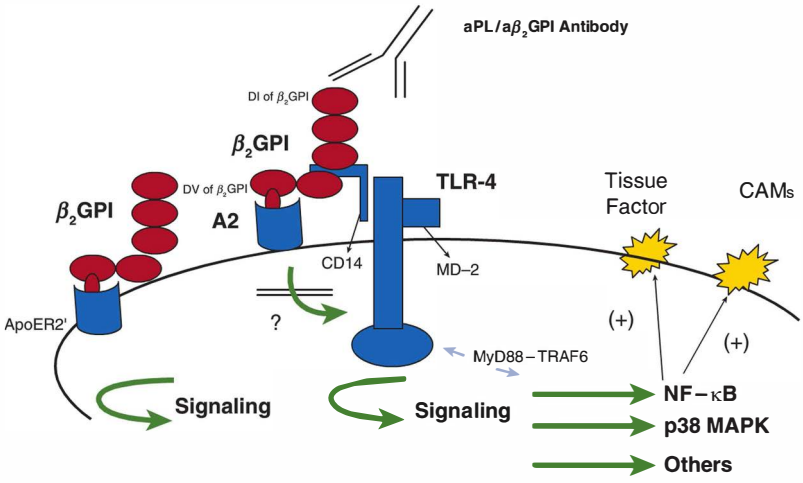

FIGURE 101.4 β_2GPI receptors on the endothelial cell membranes and their signaling pathways. Anti-β_2GPI/β_2GPI complexes bind through domain V of β_2GPI to ApoER2 and annexin A2 (A2) to activate downstream signaling pathways and activate p38 mitogen-activated protein kinase and NF-κB, leading to the upregulation of tissue factor and cellular adhesion molecules and a proinflammatory/prothrombotic phenotype. Note: annexin A2 does not have a transmembrane domain; therefore other membrane proteins such as TLR-4 and ApoER2 may act as accessory molecules. (Modified from Romay-Penabad Z, Montiel-Manzano MG, Shilagard T, et al. Annexin A2 is involved in antiphospholipid antibody-mediated pathogenic effects in vitro and in vivo. *Blood* 2009;114:3074–3083.)

Insights into the Pathogenesis of Pregnancy Complications in APS

Although there has been some controversy whether the pregnancy complications of APS are attributable to increased coagulation, a significant body of evidence supports this concept. Prothrombin activation fragment F1.2, a marker for thrombin generation, is increased in pregnant patients with aPL antibodies and a previous history of pregnancy losses, compared to control healthy aPL antibody-negative pregnant women.[110] Histologic abnormalities occur in many, but not all, placentas of APS patients.[111] Evaluation of placentas from aPL-positive women revealed that about half of women with aPL antibodies but without a prior history of fetal loss had evidence for uteroplacental vascular pathology, about half had evidence of thrombotic occlusion, and about one-third had chronic villitis and/or decidual plasma cell infiltrates.[112,113] These events may be due to the effects of aPL antibodies on placental annexin A5, by the triggering of tissue factor expression by aPL antibodies, and as described above by complement activation.

aPL antibodies have been implicated in mechanisms that do not involve thrombotic or inflammatory pathways. The antibodies can directly target the maternal decidua and affect the expression of integrins and cadherins interfering with decidual adhesion and invasion.[114] aPL antibodies were found to impair endometrial differentiation and reduce expression of complement regulatory proteins (DAF/CD55).[115] The antibodies may impact upon endometrial endothelial cell angiogenesis by reducing vascular endothelial growth factor (VEGF) and matrix metalloproteinases as well as NF-κB DNA–binding activity.[116] aPL antibodies may be involved in defective placentation; they were found to bind to trophoblast monolayers and reduce heparin-binding epidermal growth factor, which is important for blastocyte implantation, in placental tissue.[117]

Genetic Aspects of the APS Disease Process

Genetic factors are likely to be important determinants of susceptibility to development of aPL antibodies and APS. Several cases of familial APS have been described.[118,119] One study of seven families with 30 individuals, who met consensus criteria for APS, concluded that the inheritance pattern of aPL antibodies appeared to be autosomal dominant.[120] In other studies, genes in the major histocompatibility complex (MHC) have been associated with autoimmune disorders including specific MHC antigens. In patients with SLE, HLA-DPB1 alleles have been associated with anti-β_2GPI.[121]

Investigators have begun to apply genomic studies to identify specific pathogenic mechanisms. A recent genomic study done on peripheral blood mononuclear cells of aPL antibody-positive patients reported a gene-expression pattern that appeared to correlate with a predisposition toward developing thrombosis[122]. Some of the genes that were associated with APS encoded proteins are known to be involved in thrombosis—for example, apolipoprotein E (ApoE), coagulation factor X, and thromboxane–while others had no known connection to prothrombotic mechanisms.

CLINICAL MANIFESTATIONS OF THE ANTIPHOSPHOLIPID SYNDROME

Systemic Vascular Thrombosis

Although patients may present with venous and/or arterial thromboembolism in virtually any portion of the vasculature, the majority tend to have venous thromboembolic events. In one study, 59% of patients had thrombi limited to the venous circulation, 28% had solely arterial thrombi, and 13% had both types of events.[123] Deep vein thrombosis (DVT) of the lower extremities was the most common finding, occurring in about half of the patients; other sites of venous thrombotic events included pulmonary embolism, thoracic veins (superior vena cava, subclavian vein, or jugular vein), and abdominal or pelvic veins.[123] As in patients without APS, thrombosis may occur spontaneously or in the presence of risk factors such as estrogen hormone replacement therapy, oral contraceptives, pregnancy, the postpartum state,[124,125] vascular stasis, surgery, or trauma. Some patients with venous thrombosis—but generally not with arterial thrombosis[126]—also have concurrent hereditary thrombophilic conditions.[126–129] The most significant risk factor for a future thromboembolic event is having a clinical history of a thromboembolic event. The risk of recurrence increases to approximately 30% in patients with a first episode of venous thromboembolism (VTE) and aCL antibodies during 4 years of observation from the initial event.[130] The risk of recurrence and death appears to be increased in patients who have increased levels of antibodies[130,131] and with the presence of an LA. Antibodies against β_2GPI domain I significantly increase the risk of thrombosis, compared to antibodies and/or LA that are domain I independent.[39]

Catastrophic Antiphospholipid Syndrome

Rare patients develop a *catastrophic* form of APS, with widespread vascular occlusions and a high mortality (Table 101.3).[132] An inciting cause, such as surgery or infection, is suspected in about half of the patients. CAPS is defined by severe multiorgan ischemia/infarction, usually with concurrent microvascular thrombosis. Patients can present with massive VTE, along with respiratory failure due to acute respiratory distress syndrome and diffuse alveolar hemorrhage, stroke, abnormal liver enzymes, renal impairment, adrenal insufficiency, and cutaneous infarction. For recent information on the clinical characteristics of CAPS, the interested reader is referred to (http://www.med.ub.es/mimmun/forum/caps.htm) "The Registry of the European Forum on Antiphospholipid Antibodies for Patients with CAPS." At the time of writing, this registry included 280 patients of whom 72% were females, with a mean age of 37 (range 11 to 60); 46% had primary APS—that is, without other autoimmune disorders—and 40% had SLE. In about half of the patients, CAPS was the initial presentation for APS or thrombosis. Seventy percent of the patients had noninflammatory thrombotic microangiopathy as a main pathologic finding. In contrast to APS, which usually manifests as thrombosis in a large vein or artery, CAPS patients also had a susceptibility to intra-abdominal thrombotic complications affecting the kidneys, adrenal glands, splenic, intestinal, and mesenteric or pancreatic vasculature. Dermatologic abnormalities such as livedo reticularis, purpura, and necrosis occur in about half of CAPS patients. Finally, CAPS patients were more likely to have evidence of infarction at unusual sites—for example, testicular/ovarian infarction, prostate necrosis, acalculous cholecystitis, bone marrow infarction, esophageal rupture, gastric ulceration, colonic ulcerations, thrombotic pancreatitis, and adrenal infarction.[133] Although the mortality of the disorder is high, patients who recover from CAPS rarely experience a recurrence.

Pediatric Antiphospholipid Syndrome

APS is increasingly recognized to be a significant cause of thrombosis in children[134] and, as with adults, diverse clinical features are common. A European registry recently reviewed the initial 121 registered cases of pediatric APS and found thrombotic manifestations similar to those experienced by adults. Differences between primary and secondary APS were noted in the pediatric registry, with the primary APS patients being younger and having a higher frequency of arterial thrombotic events and the secondary APS patients being older and having a higher frequency of venous thrombotic events.[135] CAPS appears to be much less common in children than in adults.[136]

Organ Systems Targeted in APS

Reproductive Manifestations

The prevalence of aPL antibodies among general obstetric populations is 5% or less, and most of these aPL antibody-positive patients are asymptomatic.[137] Approximately 16% to 38% of obstetric patients with recurrent fetal losses (usually defined as three or more) have elevated aPL antibodies. Pregnant women with elevated aPL antibodies had significantly more obstetric complications than aPL antibody-negative pregnant women.[138–140] Women with obstetric APS typically present with a history of recurrent miscarriages, half of which occur in the first trimester. Pregnant patients with APS are also more prone to developing DVT during pregnancy or the puerperium. Rarely, pregnant patients develop CAPS.[141,142]

The best predictor for pregnancy loss in a patient who tests positive for aPL antibodies appears to be the clinical history of events including previous pregnancy loss, pregnancy complications attributable to placental insufficiency, or thrombosis, rather than the degree of laboratory abnormality.[131,143] A recent study showed that aPL antibody-positive women with an unexplained early loss prior to 10 weeks have a higher risk of complications in their second pregnancy.[144] Patients who have positivity of more than one assay—the so-called multipositivity—are more likely to have antepartum and postpartum DVTs[145] and an increased likelihood of poor neonatal outcome.[140]

Regarding the question whether aPL antibodies might contribute to early reproductive failure (i.e., infertility), there is a consensus that there is no relationship.[146] The Practice Committee of the American Society for Reproductive Medicine has stated that, "the assessment of aPL antibodies is not indicated among couples undergoing IVF, and therapy is not justified on the basis of existing data."[146] The reader should be aware that while this is a general consensus that is backed by evidence[147] and a practice guideline, the issue is not totally settled; there are some data contradicting this,[148] and some studies suggest that aPL antibodies may impair trophoblastic differentiation, proliferation, and migration and lead to recurrent implantation failure and infertility.[149–151]

Neurologic Manifestations

Stroke or transient ischemic attack (TIA) was the initial presentation in 30% of adults with APS in a large European cohort[152] and in 18% of Latin American adults with APS.[153] In the European cohort, stroke/TIA was the event that recurred most frequently and contributed to a significant proportion of deaths.[152] A multinational lupus cohort showed that aPL antibody-associated stroke accounted for 12% of deaths.[154] Stroke is also a frequent complication in CAPS; the European CAPS registry reported cerebral manifestations in 62% of patients, and these were responsible for 13% of deaths.[155]

The diagnosis of APS should be suspected when TIAs or strokes occur in younger patients, particularly when the usual risk factors such as hypertension, hypercholesterolemia, diabetes mellitus, and smoking are absent.[156] Case–control and prospective studies have shown strong associations between aPL antibody positivity or LA positivity and ischemic strokes in young adults,[157,158] with a remarkably high odds ratio (OR) of 43 for positive LA tests and ischemic strokes in this population.[158] Younger patients with stroke were also more likely to have venous occlusion,[159] with 7% of children in a multinational childhood registry presenting with cerebral sinus venous thrombosis.[135]

There are some conflicting data regarding the relative significance of the various aPL assays in stroke. The first prospective analysis by the AntiPhospholipid Antibody Stroke Study (APASS) group reported an association between elevated levels of aCL antibodies and risk for stroke but not for other thromboembolic events.[160] Patients who tested positive for both aCL and LA showed a trend to developing more thromboocclusive events than patients who tested negative for both (32% vs. 24%, $P = 0.07$).[160] However, the same group later reported that elevated aCL antibodies were not significantly associated, but rather that positive LA and elevated antibodies against β_2GPI were the most significant risk factors for stroke.[161] In the Risk of Arterial Thrombosis In relation to Oral contraceptives (RATIO) study, women with LA positivity and other modifiable risk factors such as smoking or use of oral contraceptives had an increased risk for ischemic strokes.[158] The presence of cardiac valvular lesions is also a significant risk factor for stroke in APS patients,[162] and this together with the high prevalence of cardiac valvular abnormalities in APS (see *Cardiovascular manifestations*) is the reason why clinicians should consider echocardiography in patients with APS.

Other aPL antibody-associated neurologic abnormalities include dementia, which is usually attributable to multiple infarcts, and also manifestations not usually attributable to thrombosis such as migraines, seizures, chorea, Guillain-Barrè syndrome, transient global amnesia, peripheral neuropathy, and orthostatic hypotension.[163] In the pediatric APS registry, migraines (7%), chorea (4%), and epilepsy (3%) were the most common nonthrombotic neurologic manifestations.[135]

Recurrent seizures are more prevalent in SLE patients with persistently elevated aCL antibodies—compared to those without elevated aCL antibodies or with antibodies that are occasionally elevated—and may be associated with ischemic changes.[164] aPL antibody positivity has also been associated with cognitive impairment in SLE, especially in the areas of attention, psychomotor speed, and executive abilities.[164,165]

The diagnosis of multiple sclerosis in patients with aPL antibodies can be problematic since elevated aCL antibodies occur frequently in patients diagnosed with multiple sclerosis, and these appear to be independent of any risk for thrombosis or stroke.[166] However, it has been proposed that aPL antibodies may themselves cause demyelination by directly damaging white matter tracts and basal ganglia structures and thereby account for multiple sclerosis-like features and movement disorders.[167]

Cardiovascular Manifestations

aPL antibodies have been associated with increased susceptibility to coronary artery disease,[168] particularly premature atherosclerosis.[169,170] aPL antibodies appear to be a risk factor for adverse outcomes following all coronary revascularization procedures, interventional and surgical,[168] and for restenosis after percutaneous transluminal coronary angioplasty.[171,172]

The LA was the aPL laboratory parameter that correlated best with myocardial occlusion in a large multicenter population-based case–control study—the RATIO study, mentioned above—with an OR of 5, which increased to 22 in women who used oral contraceptives and to 34 in women who smoked.[158] In that study, elevated anti-β_2GPI antibodies correlated with a mild increase in stroke but not with myocardial infarction. Antiprothrombin and aCL antibodies did not correlate with an increased risk of arterial thrombosis,[158] although antiprothrombin antibodies correlated with increased risk of myocardial infarction in middle-aged men.

Peripheral vascular disease has also been associated with the presence of elevated aPL antibodies. Approximately one-third of patients with peripheral arterial disease undergoing bypass grafting procedures had elevated aPL antibody levels that were mostly aCL antibodies.[173] In that study, aPL-positive patients did not have an increased risk for reocclusion compared to the aPL antibody negatives; however, both groups received anticoagulant therapy.

About 35% of patients with the primary APS have cardiac valvular abnormalities detectable by echocardiography.[174] Conversely, about 20% of patients with cardiac valvular diseases have elevated aPL antibodies compared to about 10% of matched controls.[175] Valvular abnormalities were reported in about half of patients with the combination of SLE and aPL antibodies; these abnormalities include leaflet thickening, vegetations, regurgitation, and stenosis.[176] The mitral valve is mainly affected, followed by the aortic valve.[177] In a prospective follow-up of 89 patients with severe, nonspecific valvular heart disease, thromboembolic events were significantly more frequent in the aPL antibody-positive group than in the aPL antibody-negative group; however, the presence of aPL antibodies was not an independent risk factor for thromboembolic events.[178] There was one dissenting study that did not find a relationship between increased aCL antibodies and valvular abnormalities in patients with SLE.[179]

Pulmonary Manifestations

aPL antibodies have been described in patients with pulmonary hypertension.[180] A multiinstitutional study of 687 patients with chronic thromboembolic pulmonary hypertension reported that aPL antibodies were a significant risk factor.[181] In one prospective trial of 38 consecutive patients with precapillary pulmonary hypertension, approximately 30% had aPL antibodies with various phospholipid specificities.[182]

APS patients may also bleed into the acinar portion of the lung and develop diffuse alveolar hemorrhage. Biopsies of those cases reveal microvascular thrombosis, septal thickening due to edema, neutrophilic septal infiltrates, and extravasation of red blood cells into the alveolar spaces. Since acute diffuse alveolar hemorrhage can occur in anticoagulated APS patients, it is thought that the lesions may be due to vasculitis rather than thrombosis.[183]

Hepatic and Other Gastrointestinal Manifestations

The liver is often affected in APS, mainly via occlusion of hepatic and biliary blood vessels.[184] aPL antibody levels are also frequently elevated in patients with a variety of chronic liver diseases. In a prospective study of patients with liver disease, approximately half of patients with alcoholic liver disease and one-third of patients with chronic hepatitis C virus had elevated aPL antibody levels. The frequency was even higher in patients with severe cirrhosis.[185] The aPL antibodies seen in viral hepatitis are usually cofactor independent.[22] Hepatitis C is an exception in that some patients present with "true" autoimmune aPL antibodies; the most common features reported were intra-abdominal thrombosis and myocardial infarction.[186]

Other gastrointestinal manifestations of APS include esophageal necrosis with perforation, intestinal ischemia and infarction, pancreatitis, and colonic ulceration. Primary biliary cirrhosis,[187] acute acalculous cholecystitis with gallbladder necrosis,[188,189] and giant gastric ulceration have been associated with APS.[190] APS has been reported in patients with mesenteric inflammatory venoocclusive disease[191] and with mesenteric and portal venous obstruction.[192]

Renal Abnormalities

Patients with APS may present with vasoocclusive manifestations such as renal artery stenosis and/or thrombosis, renal infarction, and renal vein thrombosis and with nonthrombotic manifestations, such as glomerulonephritis.[193,194] An entity named "APS nephropathy" has been described, which consists of a vasoocclusive disease of small-size intrarenal vessels and features fibrous intimal hyperplasia, focal cortical atrophy, and thrombotic microangiopathy.[195] Two forms of this vascular nephropathy, acute and chronic, have been described. The acute form resembles other thrombotic microangiopathies, such as hemolytic uremic syndrome or thrombotic thrombocytopenic purpura. The chronic form is often clinically silent and consists of a vasoocclusive process that develops at all levels of the renal vasculature, from the main renal artery, its branches, to arterioles, to glomerular capillaries, and renal veins.

Several nonthrombotic types of renal lesions have been identified in APS patients. A review of 29 consecutive renal biopsies from patients with primary APS described 20 cases of APS nephropathy and identified nine cases with other distinct pathologic features;[194] these included membranous nephropathy, minimal-change disease/focal segmental glomerulonephritis, mesangial C3 nephropathy, and pauciimmune crescentic glomerulonephritis.

Skin Manifestations

Skin manifestations can be the first sign of APS in some patients.[196] The cutaneous manifestations of APS have recently been reviewed.[197,198] Livedo reticularis is relatively common, occurring in 24% of a series of 1,000 aPL patients,[193] and occasionally presents in a necrosing form.[199] It is usually widespread and can localize on nonadjacent areas on limbs, trunk, and buttocks. The prevalence is higher in APS that is associated with SLE compared to primary APS, in women compared to men, and in patients with high levels of aCL antibodies. Livedo reticularis may be associated with other manifestations of APS such as cerebral or ocular ischemic arterial events (OR 10.8; 95% CI 5.2 to 22.5), seizures (OR 6.5; 95% CI 2.6 to 16), arterial events (OR 6; 95% CI 2.9 to 12.6), heart valve abnormalities detected

on echocardiography (OR 7.3; 95% CI 3.6 to 14.7), and arterial systemic hypertension (>160/90 mm Hg) (OR 2.9; 95% CI 1.5 to 5.7).[200]

Skin ulcerations associated with skin necrosis are a common presenting feature of APS, occurring in 4% of cases.[200] Necrotizing vasculitis, livedoid vasculitis, thrombophlebitis, cutaneous ulceration and necrosis, erythematous macules, purpura, ecchymoses, painful skin nodules, subungual splinter hemorrhages, anetoderma (macular atrophy), discoid lupus erythematosus, and cutaneous T-cell lymphoma have all been reported.

Retinal Abnormalities

The diagnosis of APS retinopathy should be suspected in patients with diffuse retinal vasoocclusion, particularly when characterized by involvement of arteries and veins, neovascularization at presentation, and symptoms of systemic rheumatologic disease.[201] Elevated aPL antibody levels were present in 5% to 33% of patients with retinal vein occlusion.[202,203] Cilioretinal artery occlusion,[204] optic neuropathy,[205] and severe vasoocclusive retinopathy[206] have been described in patients with APS.

Other Organ Systems

Acute adrenal failure secondary to bilateral infarction of the adrenal glands has been reported as the first manifestation of primary APS.[207] Adrenal hemorrhage has been reported.[208] aPL antibodies have been associated with osteonecrosis.[209,210] Sudden acute sensorineural hearing loss in patients with SLE or lupus-like syndromes has been described as a manifestation of APS.[211]

Thrombocytopenia and Bleeding in APS

Thrombocytopenia occurs in a large fraction of patients with APS but is rarely significant enough to cause bleeding complications or to affect anticoagulant therapy.[212,213] Most cases appear to be immune mediated. According to one study, the majority of patients with APS and thrombocytopenia had antibodies against the platelet membrane glycoproteins, GPIIb/IIIa and/or GPIb/IX complexes.[214] However, in another study, no correlation was found between the presence of antibodies against platelet GPIIb/IIIa, GPIb/IX, and thrombocytopenia, and the eluted platelet antibodies did not have any LA activity.[215] Conversely, aPL antibodies and antibodies against platelet membrane glycoproteins were present simultaneously in about 70% of patients with immune-mediated thrombocytopenia (ITP).[216] In a prospective cohort study, the 5-year, thrombosis-free survival of aPL antibody-positive ITP patients was significantly less than aPL antibody negatives at 39% and 98%, respectively;[217] this also indicates that thrombocytopenia itself is not protective against thrombosis in these patients.

When patients with APS exhibit a bleeding tendency, the presence of a concurrent coagulopathy must be considered. Acquired hypoprothrombinemia has been reported with severe bleeding.[218,219] This diagnosis may be missed when abnormal coagulation screening tests are attributed only to the LA effect; therefore the plasma prothrombin level should be checked when the prothrombin time is prolonged. Other causes of bleeding in the setting of APS include acquired thrombocytopathies, thrombocytopenia (see above), acquired inhibitors against specific coagulation factors, such as factor VIII, and the acquired von Willebrand syndrome (AvWS). The mechanism for AVWS in autoimmunity has not been established, and this deficiency of vWF is rarely associated with a demonstrable inhibitor.[220]

LABORATORY DIAGNOSIS

Two classes of assays are used for the detection of aPL antibodies: (a) coagulation assays, in which LAs are detected by the inhibition of phospholipid-dependent coagulation reactions, and (b) immunoassays, in which aPL antibodies against phospholipids and protein cofactors are detected most commonly by enzyme-linked immunosorbent assay (ELISA).

aCL IgG and IgM assays are the most sensitive, but least specific assays; anti-β_2GPI IgG and IgM assays are more specific but less sensitive, and LA assays, of which the dilute Russell Viper Venom Time (dRVVT) is the most common, are the least sensitive but the most specific.[220a]

Lupus Anticoagulants

The LA phenomenon is one of the most intriguing aspects of APS.[221,222] There are several forms of the LA test, all of which are intended to report aPL antibody-mediated inhibition of phospholipid-dependent blood coagulation reactions.[223] These include modifications of the activated partial thromboplastin time (aPTT) test with LA-sensitive and LA-insensitive reagents, the kaolin clotting time (KCT), the dRVVT, the tissue thromboplastin inhibition time, the hexagonal phase array test, and the platelet neutralization procedure. The results of LA tests can be so variable that even specialized laboratories may disagree on the results.[224]

Despite these limitations, the presence of the LA activity is more predictive and more specific for the occurrence of thrombosis or pregnancy loss than the aCL ELISA assays, both in lupus patients[225] and in others. In a systematic literature review, 12 of 12 studies showed significant associations between LA and thrombosis, with ORs ranging from 5.7 to 9.4,[226] while only 15 of 28 studies showed significant associations between aCL antibodies and thrombosis. In the APASS study,[160] positivity for both LA and aCL antibodies, but not for aCL antibodies alone, predicted a higher risk for recurrent thromboocclusive events in patients with first ischemic stroke. In the RATIO study,[158] the presence of LA was a major risk factor for arterial thrombotic events in young women including an OR 5.3 for myocardial infarction and 43.1 for ischemic stroke, up to 22 and 201, respectively, for women on oral contraceptives, and 34 and 87, respectively, for cigarette smokers. There was no increased risk of myocardial infarction with anti-β_2GPI antibody positivity and only a mild increased risk (OR 2.3) for ischemic stroke. Neither aCL antibodies nor antiprothrombin antibodies increased the risk of myocardial infarction or stroke.[158]

The Antiphospholipid Antibodies Subcommittee of the International Society of Thrombosis and Hemostasis has prioritized appropriateness of testing for LA into the following categories:[227]

- *Low appropriateness* includes older patients with venous or arterial thromboembolism.
- *Moderate appropriateness* includes asymptomatic patients with an incidentally found prolonged aPTT and patients with recurrent spontaneous early pregnancy loss or provoked VTE.
- *High appropriateness* includes patients with unprovoked and unexplained VTE, arterial thrombosis in young patients (<50 years of age), thrombosis at unusual sites, late pregnancy loss, and any thrombosis or pregnancy morbidity in patients with autoimmune diseases.

Detailed specific guidelines have been proposed for standardizing all of the aspects of LA testing from blood collection

through centrifugation, preservation, test performance, and interpretation.[228] Two different LA tests based on different assay principles are required. The dRVVT is specific for detecting LA in patients at high risk of thrombosis.[229] aPTT testing with silica as an activator and low phospholipid content is the second test of choice.[230,231] Kaolin or ellagic acid are not recommended as activators, nor are the dilute prothrombin time assays based on snake venoms such as Ecarin and Textarin, and KCT.[228]

Assays Used for the Detection of Antiphospholipid Antibodies

Dilute Russell Viper Venom Time

The dRVVT uses Russell viper venom in a system containing limiting quantities of diluted rabbit brain phospholipid. Russell viper venom directly activates coagulation factor X, leading to the formation of a fibrin clot. LA prolongs dRVVT by interfering with assembly of the prothrombinase complex, and the prolongation is reversed by adding excess phospholipid. Anticoagulant therapy with heparin, warfarin, or direct thrombin inhibitors can yield falsely abnormal test results (Table 101.5).

Activated Partial Thromboplastin Time Tests

Prolongation of the aPTT detects some LAs, and prolonged aPTT in otherwise healthy individuals is most frequently due to an LA.[232] When the aPTT is prolonged and not "correctable" by mixture with normal plasma, an LA should be suspected, especially if the patient does not have bleeding symptoms. The LA needs to be differentiated from inhibitors of specific coagulation factors and from anticoagulants such as heparin. The clinician should check whether the aPTT normalizes when an LA-insensitive aPTT reagent is used, when the assay is performed with hexagonal phase array phospholipids, or when the assay is performed using frozen washed platelets as the source of phospholipid, a procedure referred to as the *platelet neutralization procedure*. The clinician should be aware that in rare patients, both types of anticoagulants—that is, LA and specific coagulation factor inhibitors–may coexist and yield a confusing laboratory picture.

Anticardiolipin Antibody Immunoassays

The aCL immunoassay was initially developed to quantify the biologic false-positive syphilis test.[6] In the aCL ELISA, the amount of binding of IgG- and IgM-aCL is expressed in IgG phospholipid units (GPL) or IgM phospholipid units (MPL) units, one unit representing the cardiolipin-binding activity of 1 μg/mL of affinity-purified aPL antibodies from reference sera. Correlation of aCL antibody levels determined in reference sera by different laboratories has been problematic, particularly when aCL antibodies are measured using different commercial ELISA kits.[233]

Most patients with APS are identified by elevated levels of aCL antibodies, a test with high sensitivity but poor specificity, especially in asymptomatic patients. There is an interesting seasonal variability, with more healthy people having increased aPL antibodies in the winter than in the summer months.[234]

High levels of aCL antibodies are associated with increased risk of venous thrombosis, whereas any elevated level is associated with myocardial infarction or stroke. During a 10-year follow-up of patients with elevated levels of aCL antibodies, approximately 50% of patients who presented with the antibodies but without clinical manifestations of APS subsequently developed APS.[235] Women with IgM antibodies, IgG aCL antibody titer <20 IgG binding units, and no LA do not appear to be at risk for APS.[236] In contrast, women with an IgG aCL antibody titer >20 binding units or a positive LA were more likely to develop complications.[236] A meta-analysis on aPL antibodies in women with recurrent fetal losses that reviewed 25 studies[237] showed significant correlation with the presence of increased aCL IgG; however, the highest OR was seen with the LA.

With respect to stroke, elevated levels of aCL antibodies of IgG or IgM isotype were reported to be significant risk factors.[238] aPL antibodies also are an independent risk factor for stroke in young women.[21]

Anti-β_2GPI Antibody Immunoassay

β_2GPI is the major antigenic target for aPL antibodies.[239] Although β_2GPI antibodies are usually present in conjunction with abnormal aCL and antiphosphatidylserine antibodies, some patients with APS present solely with antibodies to β_2GPI.[240,241] Despite their reported higher specificity for APS (98%), β_2GPI antibodies alone cannot be relied upon for the diagnosis because of their low sensitivity (40% to 50%) and interlaboratory variability,[242,243] and concurrent testing for both antibodies and LA is advised.

In a systematic literature review, 34 of 60 studies, of which none were prospective, showed significant associations between anti-β_2GPI antibodies and thrombosis.[244] Of the 10 studies that included a multivariate analysis, only two confirmed that IgG anti-β_2GPI antibodies were independent risk factors for venous thrombosis.

Multipositivity for aPL Tests and Clinical Risk

Strong positivity for more than one of the aPL assays correlates with a significant increased risk for clinical events. Multipositivity for aPL antibodies was associated with antenatal and postnatal DVTs,[145] a higher probability of poor neonatal outcome,[140] and a cumulative incidence of recurrent thromboembolism of 44.2% after 10 years.[245]

Overall, in patients with thromboembolic events and/or pregnancy morbidities, the data indicate that triple-positive patients (LA positive, aCL IgG or IgM positive, and anti-β_2GPI IgG or IgM positive) should be considered an especially high-risk population.[246]

Annexin A5 Resistance Assay

The annexin A5 resistance (A5R) assay and the others described below are not included in the consensus investigational criteria for diagnosing APS. This is the first functional test that was designed to identify a specific pathogenic mechanism—the aPL antibody-mediated disruption of annexin A5 crystallization on phospholipid surfaces. The failure of annexin A5 to sufficiently prolong coagulation times because of the presence of aPL antibodies is referred to as "A5R." A study of 96 patients demonstrated significantly lower values in APS patients with a history of thrombosis than in aPL antibody-positive patients with no thrombosis history, aPL antibody-negative patients with a thrombosis history, or healthy controls (FIGURE 101.5).[247]

Antidomain I of β_2GPI Immunoassay

This nonconsensus test measures IgG antibodies against domain I of β_2GPI. A multicenter study of 442 patients, who tested positive for anti-β_2GPI antibodies, reported that the detection of

Table 101.5

Assays that detect aPL antibodies

Assay	Test Type	Description	Results	Limitations
dRVVT	Sensitive test for detecting inhibition of phospholipid-dependent coagulation	Uses snake venom that activates factor X and limiting quantity of phospholipid, complete panel requires confirmation with mixing of test plasma with normal plasma and with neutralization with excess phospholipid	Interpretative result—positive or negative—based on ratio of clotting time results in the absence and presence of excess phospholipid. Significant shortening of clotting time by excess phospholipid is referred to as "confirmatory test"	Potential false-positives in patients who are anticoagulated or have other coagulation abnormalities
aPTT	PTT panel for detecting LA	Compares results with LA-sensitive aPTT reagent versus LA-insensitive aPTT reagent, correction with hexagonal phase array phospholipids, or platelet neutralization procedure	Interpretative result—positive or negative, based on difference between the two aPTT results	Potential false-positives or false-negatives in patients who are anticoagulated or have other coagulation abnormalities
aCL antibody IgG and IgM	Immunoassays done by ELISA or fluorescent bead assays	Cardiolipin is the target antigen, but requires source of β_2GPI for APS specificity	GPL or MPL units	Potential false-positives with some infectious diseases, and with polyclonal hypergammaglobulinemias, and issues with precision and interlaboratory agreement
Anti-β_2GPI IgG and IgM	Immunoassays done by ELISA or fluorescent bead assays	β_2GPI as the target antigen	GPL or MPL units	Potential false-positives with some infectious diseases, with polyclonal hypergammaglobulinemias, and issues with precision and interlaboratory agreement
Other Assays				
A5R	Coagulation-based assay	Annexin A5 is added to prolong coagulation time. Failure to sufficiently prolong coagulation time by annexin A5 in the presence of aPL antibodies indicates resistance	Expressed as ratio of coagulation times in the presence and absence of annexin A5	Under current investigation

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Table 101.5

Assays that detect aPL antibodies (*Continued*)

Antidomain I of β_2GPI IgG	Immunoassay done by ELISA	Identifies IgG antibodies against a specific amino acid sequence, G40-R30, within domain I of β_2GPI	OD units	Under current investigation
Other immunoassays Antiprothrombin antibody	Immunoassay done by ELISA	Identifies antibodies to target antigen	GPL or MPL units	Under current investigation
Antiphosphatidylserine antibody immunoassay				
IgA antibodies to cardiolipin and β_2-GPI			Arbitrary units	

specific antidomain I IgG antibodies more strongly correlated with thrombosis and obstetric complications than when using the standard anti-β_2GPI antibody assays. Antidomain I IgG antibodies were present in plasma of 55% of patients, of which 83% had a history of thrombosis (OR 3.5) and 57% had pregnancy complications (OR 2.4).[57]

Antiprothrombin Antibody Immunoassay

Prothrombin is considered the second major target for aPL antibodies after β_2GPI. In a systematic literature review, 17 of 46 studies showed significant associations between antiprothrombin antibodies and thrombosis.[244] Of the eight studies that included a multivariate analysis, two confirmed that antiprothrombin antibodies were independent risk factors for thrombosis, and three other studies showed that antiprothrombin antibodies added to the risk borne by LA or aCL antibodies. Overall, there was no association found between antiprothrombin antibodies and risk of thrombosis.

However, in a possible improvement over the standard antiprothrombin assay, a new nonconsensus test for antibodies against the phosphatidylserine–prothrombin complex (aPS/PT) was closely associated with APS and LA,[248] and IgG aPS/PT antibodies were highly prevalent in APS patients compared to patients with other diseases with an OR of 12.8.[249]

Antiphosphatidylserine Antibody Immunoassay

It was hypothesized that immunoassays for antibodies against phosphatidylserine, which is exposed on syncytialized cells, apoptotic cells, and activated platelets, may be more relevant pathophysiologically than those that use cardiolipin, which is normally present in mitochondrial membranes. Experimental animal models indicate that antiphosphatidylserine antibodies are pathogenic to the trophoblast, and there is a relationship between antiphosphatidylserine antibodies and pregnancy complications,[148,250–252] and in arterial thrombosis antiphosphatidylserine antibodies have been shown to correlate more specifically with APS than aCL antibodies.[253,254] However, recent studies have shown no relationship between antiphosphatidylserine antibodies and pregnancy complications.[255–258]

IgA Antibodies to Cardiolipin and β_2-glycoprotein I

There have been case reports of patients with elevated IgA aCL antibodies with APS, but the prevalence of true positivity to IgA aCL antibodies is extremely low.[259] The international consensus statement on APS did not include aPL antibodies of IgA isotype (either aCL or anti-β_2GPI) in the consensus laboratory criteria for APS classification based on the opinion that these autoantibodies lack specificity and do not add to the diagnostic value of testing for the other aCL and anti-β_2GPI isotypes.[14,260,261] There are, however, some data that suggest that IgA anti-β_2GPI antibodies may be significant. A recent retrospective case–control study identified records of 56 patients with elevated IgA anti-β_2GPI, but normal results for all of the other aPL laboratory parameters, and matched them with aPL-negative controls.[262] About half of the cases had SLE; interestingly, the investigators found a significant increase in thromboembolic events that was restricted to the IgA-positive patients who also had SLE.[262]

TREATMENT OF PATIENTS WITH ANTIPHOSPHOLIPID ANTIBODIES

Thrombosis

Patients with a venous thrombotic event and confirmed positive aPL antibodies should generally be treated with warfarin to a target INR of 2.0 to 3.0.[263,264] There is controversy about whether the same treatment applies to arterial thrombosis or whether a higher intensity of INR > 3.0 should be used.[265] There is a general agreement that patients with spontaneous or recurrent thrombosis should be treated long-term, but there is uncertainty on duration of anticoagulant treatment of patients with provoked—for example, trauma, surgery, stasis, airplane travel,

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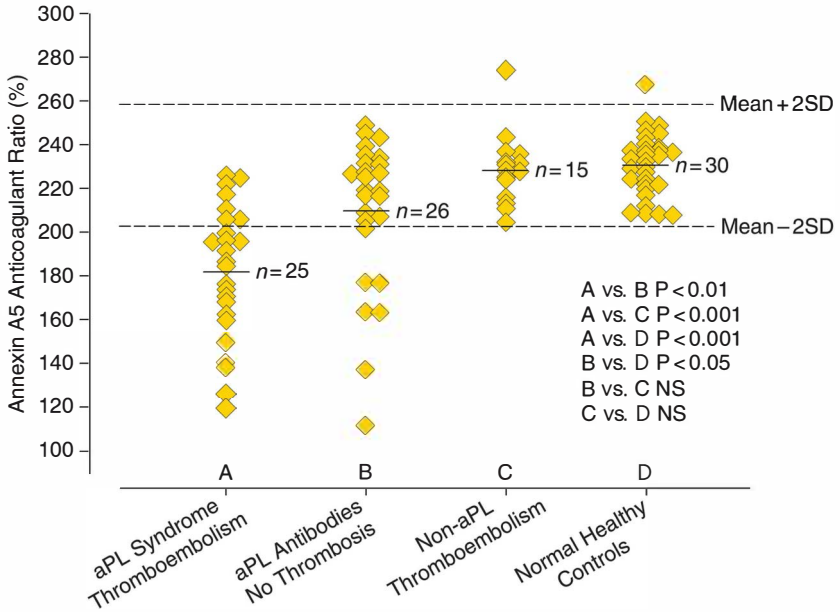

FIGURE 101.5 Reduction of annexin A5 anticoagulant effect—"A5R"—in plasmas from patients with APS. The mean annexin A5 anticoagulant ratio for the APS with thromboembolism group (*A*) was significantly decreased compared to the aPL antibody-positive patients who did not have a history for thrombosis (*B*), the aPL antibody-negative thromboembolism group (*C*), and the healthy control group (*D*). The mean ratio for the plasmas from the aPL antibody-positive patients who did not have a history for thrombosis (*B*) also was significantly reduced compared with the healthy control group (*D*). *Broken lines* indicate values included within mean ± 2SD of normal healthy controls. (From Rand JH, Wu XX, Lapinski R, et al. Detection of antibody-mediated reduction of annexin A5 anticoagulant activity in plasmas of patients with the antiphospholipid syndrome. *Blood* 2004;104:2783.)

pregnancy, or estrogens—or single thrombotic events. The last American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines made a Grade 1A recommendation for long-term anticoagulant therapy for all VTE patients—that is, not limited to APS–with a first unprovoked event without an increased risk for bleeding and who could achieve good anticoagulant monitoring.[266]

Stroke

There is controversy about the appropriate antithrombotic treatment of APS-associated stroke. A fair summary of the current views is that most hematologists and rheumatologists treat APS stroke no differently from other thrombotic manifestations of APS—that is, with anticoagulant therapy—whereas neurologists feel that evidence supports treatment with aspirin. The APASS study concluded that treating with aspirin (325 mg) alone is equivalent to anticoagulation but without the hemorrhagic risks.[160] Guidelines issued in 2006 from the American Heart Association/American Stroke Association stated that antiplatelet therapy is reasonable for APS cerebrovascular events and specified anticoagulant therapy for patients who also had APS manifestations in other organ systems.[267] The ACCP Guidelines made a Grade 1A recommendation for treatment of all noncardioembolic strokes or TIAs with aspirin over anticoagulation but qualified that there is disagreement about specific populations that included APS.[268]

Pregnancy Complications

Women with recurrent pregnancy losses or other pregnancy complications attributable to APS should be treated with a prophylactic dose of heparin along with aspirin 81 mg daily.[269] Although most clinical studies were done with unfractionated heparin (UFH) (at a usual dose of 5,000 units subcutaneously q12h), most clinicians treat with low molecular weight heparins because of the pharmacokinetic advantages and the hope of reducing adverse effects associated with UFH. About a month prior to the estimated delivery date, patients are usually switched to UFH, which is withheld when labor begins or, for induced or cesarean section deliveries, about 24 hours prior to delivery.[270,271] Anticoagulation is then resumed for about 6 weeks because of the increased risk of VTE during the puerperium.[270]

Catastrophic Antiphospholipid Syndrome

As described above (see Section "Clinical Manifestations of the Antiphospholipid Syndrome"), CAPS is a devastating disorder that continues to have a high mortality of around 50%.[272] In view of this and the lack of sufficient response to conventional anticoagulant therapy, these patients need to be aggressively treated. The main causes of death are stroke (27.2%), followed by cardiac involvement (19.8%) and infection (19.8%).[273] According to the CAPS Registry,[274] anticoagulation plus corticosteroids was the most common therapy used, followed by anticoagulation plus corticosteroids

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plus plasma exchange and/or intravenous IgG (IVIG). The highest recovery rate was obtained by the combination of anticoagulation, corticosteroids, and plasma exchange (78%), followed by a combination of anticoagulation, corticosteroids, plasma exchange, and/or IVIG (69%). The higher rate of the use of the last combination of treatment seems to be the main explanation for the significant reduction in mortality rate to 30% in CAPS.

Asymptomatic Antiphospholipid Antibody-Positive Patients

In general, otherwise healthy and asymptomatic patients who are found to have abnormal aPL laboratory tests should not be treated with prophylactic anticoagulation. Although there is not yet evidence on this, in our opinion anticoagulant therapy may be considered for rare asymptomatic patients who may be at extraordinarily high risk for future thromboembolism. These would include individuals with convincing strong family histories for thromboembolic complications of APS who themselves manifest significant laboratory abnormalities, patients with SLE who have significant multipositive aPL laboratory abnormalities, and patients who have additional significant risk factors for thrombosis (e.g., severe valvular heart disease).[178] A study of nearly 100 patients showed no benefit from treating asymptomatic aPL antibody-positive patients with aspirin.[275] However, in asymptomatic aPL antibody-positive patients with SLE, primary prophylaxis with aspirin and hydroxychloroquine (HCQ) appeared to reduce the frequency of thrombotic events.[276] The utility of HCQ for treating APS will need to be established in randomized, controlled trials.

Although there is no evidence from clinical trials that prophylactic treatment with aspirin benefits asymptomatic aPL antibody-positive patients, some authors recommend this approach in persistently aPL antibody-positive patients, in aPL antibody-positive patients with SLE, and in patients with obstetric APS.[277] During periods of increased thrombotic risk (surgery and/or prolonged immobilization), heparin prophylaxis is warranted, but the optimal type and duration of treatment is still unknown. American Society of Hematology evidence-based guidelines stress the importance of individual assessment of thrombotic risk in aPL antibody-positive patients and that reversible thrombotic risk factors should also be addressed.[278]

Unproven Treatments

Hydroxychloroquine

HCQ, a synthetic antimalarial drug, has several anti-inflammatory and antithrombotic effects.[279–281] Recently, HCQ was shown to inhibit the formation of anti-β_2GPI IgG–β_2GPI complexes[282] and to reverse the aPL antibody-mediated disruption of annexin A5 crystallization.[283] In a cohort of SLE patients, HCQ treatment was associated with a decreased risk of thrombosis,[284] and a cross-sectional study indicated that HCQ was protective against thrombosis in asymptomatic aPL antibody-positive patients.[285] Randomized, controlled trials will be needed to confirm whether this drug is useful for treating APS or asymptomatic individuals who are considered to be at high risk for recurrence.

Statins

A clinical trial is currently underway investigating the use of fluvastatin in APS patients (ClinicalTrials.gov Identifier: NCT00674297). In a preliminary analysis of nine APS patients treated with fluvastatin, seven out of nine patients showed significant decreases in proinflammatory/prothrombotic markers such as VEGF, soluble tissue factor, and TNF-α serum titers.[286]

Rituximab

Limited case reports have shown rituximab, an anti-CD20 chimeric monoclonal antibody, to be effective in treating thrombocytopenia and hemolytic anemia in aPL antibody-positive patients. It has not yet been established whether rituximab is effective against aPL antibody-mediated thrombosis. An open-label, phase IIa, pilot study is currently underway to evaluate the safety and effectiveness of rituximab in aPL antibody-positive patients with anticoagulant resistant manifestations such as persistent thrombocytopenia, autoimmune hemolytic anemia, cardiac valve disease, chronic skin ulcers, aPL nephropathy, and/or cognitive dysfunction (ClinicalTrials.gov Identifier: NCT00537290).

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CHAPTER

102

Myeloproliferative Neoplasms and Thrombohemorrhagic Complications

AYALEW TEFFERI

The term "myeloproliferative disorder" (MPD) was first introduced by Dameshek[1] in 1951 to emphasize the clinicopathologic interrelationship between essential thrombocythemia (ET), polycythemia vera (PV), primary myelofibrosis (PMF), chronic myeloid leukemia (CML), and erythroleukemia (di Guglielmo's syndrome). Between 1967 and 1981, Fialkow et al. showed that the "MPDs" were also biologically interrelated on the basis of being clonal stem cell disorders with involvement of both myeloid and lymphoid lineage.[2–9] In the interim, several other myeloid malignancies have been described and shown to display clinicopathologic as well as biologic attributes that are similar to those of Dameshek's "MPD." Furthermore, there is now plenty of evidence to consider MPDs as myeloid malignancies and the name has accordingly been changed to "myeloproliferative neoplasms (MPNs)."

The World Health Organization (WHO) system recognizes five categories of myeloid malignancies including acute myeloid leukemia (AML), myelodysplastic syndromes (MDSs), MPNs, MDS/MPN overlap, and *PDGFR*/*FGFR1*-rearranged myeloid/lymphoid neoplasm with eosinophilia (Table 102.1).[10] "*BCR-ABL1*-negative MPN" is an operational subcategory of MPN that includes PV, ET, and PMF.[11] All three disorders are characterized by stem cell–derived clonal myeloproliferation and the presence of somatic mutations involving primarily *JAK2* and to a lesser extent *MPL*, *LNK*, *CBL*, *TET2*, *ASXL1*, *IDH*, *IKZF1*, *EZH2*, *DNMT3A*, *TP53*, or *SF3B1* mutations.[12–15,16,17] None of these mutations appear to garner the disease specificity or pathogenetic relevance otherwise displayed by *BCR-ABL1*, in CML.[12]

EPIDEMIOLOGY

Incidence figures for ET, PV, and PMF vary across studies but a population-based report from Olmsted county, Minnesota, USA provided estimates of 2.5, 1.9, and 1.5/100,000, respectively.[18,19] The corresponding figures for median age at diagnosis were 72, 67, and 70 years. All three *BCR-ABL1*-negative MPNs are rare in children and there is a slight male predominance in PMF and female predominance in ET.[20–22] A higher disease incidence involving all three MPNs has been suggested in persons of Jewish ancestry.[23–27] In general, there is no hard evidence that links any of these MPNs with environmental toxins.

Clinical Features

Most patients with ET are currently diagnosed incidentally and in an asymptomatic state.[28] However, symptoms and signs of disease are usually present when either PV or PMF is diagnosed.[29,30] Thrombohemorrhagic manifestations are the most frequent life threatening events in both PV and ET, and include microvascular disturbances (headache, lightheadedness, visual symptoms, palpitations, atypical chest pain, acral paresthesias, erythromelalgia), thrombotic episodes (stroke, transient ischemic attacks, retinal vein occlusion, cerebrovascular vein thrombosis, myocardial infarctions, angina pectoris, pulmonary embolism, abdominal large vein thrombosis, extremity deep vein thrombosis, digital ischemia, gangrene), and bleeding complications (central nervous system, retinal, mucosal, gingival, gastrointestinal, retroperitoneal, cutaneous, deep tissue, post-surgical, aspirin induced).[28,31–33] Bleeding in both PV and ET is exacerbated by the use of aspirin and other nonsteroidal antiinflammatory drugs.

Microvascular symptoms, especially erythromelalgia, are the result of small vessel platelet–endothelium interaction with associated inflammation and transient thrombotic occlusion.[34] Erythromelalgia represents acral erythema, warmth, and pain (FIGURE 102.1).[35] Young women with ET may present with recurrent first trimester miscarriages.[36] Newly diagnosed or untreated patients with PV might display plethora (a red and congested facial complexion), palmar erythema, and sausage-shaped distention of retinal veins. They might also experience symptoms of hyperviscosity that include head fullness, dizziness, flushing, visual disturbances, tinnitus, epistaxis, dyspnea, and increased blood pressure.[37–39] Nonvascular symptoms in PV include pruritus and constitutional symptoms including fatigue, malaise, and night sweats. PV-associated pruritus occurs in more than 50% of patients and is usually exacerbated by taking a bath.[40] Other symptoms and signs of PV include gout, splenomegaly, and early satiety.

The typical clinical presentation in PMF includes progressive anemia, severe constitutional symptoms, and marked splenomegaly.[30] Hepatosplenomegaly in PMF is secondary to extramedullary hematopoiesis (EMH). EMH in PMF may also occur in nonhepatosplenic tissue including lymph nodes, pleura (effusion), peritoneum (ascites), lung (interstitial process), and the paraspinal and epidural spaces (spinal cord and nerve

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Table 102.1

WHO classification of myeloid malignancies

1. Acute myeloid leukemia (AML) and related precursor neoplasms[a]
2. Myeloproliferative neoplasms (MPNs)
2.1. Classic MPN
2.1.1. Chronic myelogenous leukemia, *BCR-ABL1* positive (CML)
2.1.2. Polycythemia vera (PV)
2.1.3. Primary myelofibrosis (PMF)
2.1.4. Essential thrombocythemia (ET)
2.2. Nonclassic MPN
2.2.1. Chronic neutrophilic leukemia (CNL)
2.2.2. Chronic eosinophilic leukemia, not otherwise specified (CEL-NOS)
2.2.3. Mastocytosis
2.2.4. Myeloproliferative neoplasm, unclassifiable (MPN-U)
3. Myelodysplastic syndromes (MDSs)
3.1. Refractory cytopenia[b] with unilineage dysplasia (RCUD)
3.1.1. Refractory anemia (ring sideroblasts <15% of erythroid precursors)
3.1.2. Refractory neutropenia
3.1.3. Refractory thrombocytopenia
3.2. Refractory anemia with ring sideroblasts (RARS; dysplasia limited to erythroid lineage and ring sideroblasts ≥15% of bone marrow erythroid precursors)
3.3. Refractory cytopenia with multilineage dysplasia (RCMD; ring sideroblast count does not matter)
3.4. Refractory anemia with excess blasts (RAEB)
3.4.1. RAEB-1 (2%–4% circulating *or* 5%–9% marrow blasts)
3.4.2. RAEB-2 (5%–19% circulating *or* 10%–19% marrow blasts *or* Auer rods present)
3.5. MDS associated with isolated del(5q)
3.6. MDS, unclassifiable
4. MDS/MPN
4.1. Chronic myelomonocytic leukemia (CMML)
4.2. Atypical chronic myeloid leukemia, *BCR-ABL1* negative
4.3. Juvenile myelomonocytic leukemia (JMML)
4.4. MDS/MPN, unclassifiable
4.4.1. Provisional entity: Refractory anemia with ring sideroblasts associated with marked thrombocytosis (RARS-T)
5. Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*,[c] *PDGFRB*,[c] or *FGFR1*[c]
5.1. Myeloid and lymphoid neoplasms with *PDGFRA* rearrangement
5.2. Myeloid neoplasms with *PDGFRB* rearrangement
5.3. Myeloid and lymphoid neoplasms with *FGFR1* abnormalities

[a]AML-related precursor neoplasms include "therapy-related MDS" and "myeloid sarcoma."
[b]Either mono- or bicytopenia: hemoglobin level < 10 g/dL, absolute neutrophil count < 1.8×10^9/L, or platelet count < 100×10^9/L. However, higher blood counts do not exclude the diagnosis in the presence of unequivocal histologic/cytogenetic evidence for MDS.
[c]Genetic rearrangements involving platelet-derived growth factor receptor α/β (*PDGFRA*/*PDGFRB*) or fibroblast growth factor receptor 1 (*FGFR1*).

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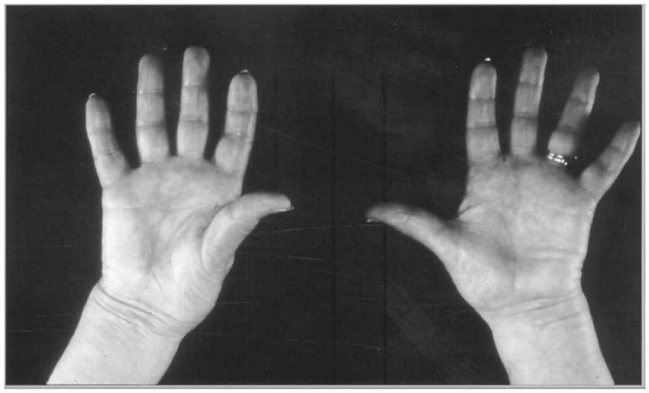

FIGURE 102.1 Erythromelalgia represents painful, erythematous discoloration of the hands or toes associated with warmth and burning sensation.

root compression). Constitutional symptoms in PMF include profound fatigue, weight loss, night sweats, and low-grade fever. Weight loss in PMF consists of mostly muscle mass wasting. Other disease manifestations include peripheral edema, diarrhea, early satiety, portal hypertension, and splenic infarcts.[41,42]

PATHOGENESIS

MPNs Are Clonal Stem Cell Diseases with Recurrent Somatic Mutations

As early as the 1970s and 1980s, the use of glucose-6-phosphate dehydrogenase (G-6-PD) isoenzyme analysis has suggested that patients with MPN including ET,[9] PV,[4] and PMF[7] have clonal hematopoiesis that originates at the stem cell level and may involve both myeloid and B lymphoid lineage.[43] This early observation has been supported by more recent investigations using X-linked DNA[44,45] and transcript[46] analysis in informative females. The disease-causing events in *BCR-ABL1*-negative MPN remain elusive. Beginning in 2005, several somatic mutations involving primarily *JAK2* and to a lesser extent *MPL*, *LNK*, *CBL*, *TET2*, *ASXL1*, *IDH*, *IKZF1*, *EZH2*, *DNMT3A*, *TP53*, or *SF3B1* have been described.[12–15,16,17] However, none of these mutations appear to garner the disease specificity or pathogenetic relevance otherwise displayed by *BCR-ABL1*, in CML.[12] Among the aforementioned mutations, *JAK2* and *MPL* have been more closely associated with MPN.

*JAK2*V617F results from a somatic G to T mutation involving *JAK2* exon 14, which leads to nucleotide change at position 1849 and the substitution of valine by phenylalanine at codon 617.[47] The mutation affects the JH2 domain and is believed to derail its kinase-regulatory activity. *JAK2*V617F-mediated transformation is believed to require co-expression of type 1 cytokine receptor and leads to STAT5/3 activation.[48–51] Also, *JAK2*V617F might have an epigenetic effect through nuclear translocation of the mutant molecule and direct phosphorylation of histone H3.[50] *JAK2*V617F induces PV-like phenotype in mouse transplantation models[52] and this observation has been further confirmed by a recent report of an inducible *JAK2*V617F knock-in mouse model where both heterozygous and homozygous mutation expression induced PV-like disease, with the latter causing a more aggressive phenotype with myelofibrosis (MF).[53] Such experimental data along with the fact that virtually all patients with PV carry a *JAK2* mutation[54] suggest a cause–effect relationship with erythrocytosis.[55–59] *JAK2*V617F homozygosity is infrequent in ET and its frequent occurrence in PV has been ascribed to mitotic recombination, possibly facilitated by *JAK2*V617F-induced genetic instability.[60]

*JAK2*V617F-positive MPN has been associated with older age at diagnosis (ET and PMF), higher hemoglobin level (ET and PMF), leukocytosis (ET and PMF), and lower platelet count (ET).[61] A higher mutant allele burden has been associated with pruritus (PV and PMF), higher hemoglobin level (PV), leukocytosis (PV, ET, and PMF), and larger spleen size (PV, ET, and PMF).[62–66] However, the mere presence of *JAK2*V617F or increased mutant allele burden does not appear to affect survival or leukemic transformation.[66–72] *JAK2*V617F allele burden increases with time in PV and PMF,[63,65,73] but not in ET.[66] Current evidence is not conclusive with regard to the relationship between *JAK2*V617F and thrombosis.[65–68,74–76] *JAK2* exon 12 mutations are relatively specific to *JAK2*V617F-negative PV.[55] *JAK2* exon 12 mutation–positive PV patients are often heterozygous for the mutation and are characterized by predominantly erythroid myelopoiesis, subnormal serum erythropoietin (Epo) level, and younger age at diagnosis.[55,77,78] The clinical course of these patients appears to be similar to that of patients with *JAK2*V617F-positive PV.[56,78,79]

MPL (Myeloproliferative Leukemia Virus oncogene) is located on chromosome 1p34. Gain-of-function germline *MPL* mutations have been associated with familial thrombocytosis (S505N) that is, interestingly, associated with an MPN-phenotype including splenomegaly, MF, and an increased risk of thrombosis.[80] An *MPL* single nucleotide polymorphism (G1238T) that results in a K39N substitution is found in approximately 7% of African Americans and is associated with higher platelet counts.[81] Somatic *MPL* mutations are rare and their occurrence is largely limited to patients with MPN. *MPL*W515L results from a G to T transition at nucleotide 1,544 (exon 10) resulting in a tryptophan to leucine substitution at codon 515. *MPL*W515L was first described in patients with *JAK2*V617F-negative PMF and induces a PMF-like disease with thrombocytosis in mice.[82] Subsequently, *MPL*W515K and other exon 10 *MPL* mutations (e.g., *MPL*W515S and *MPL*S505N) were described in ET and PMF with mutational frequencies that range from 3% to 15%.[82–87]

MPL-mutated ET has been associated with older age, lower hemoglobin level, higher platelet count, microvascular symptoms, and a higher risk of post-diagnosis arterial thrombosis.[87,88] The presence of *MPL* mutation did not appear to affect survival, fibrotic, or leukemic transformation.[87] *MPL*-mutated PMF has been associated with the female gender, older age, lower hemoglobin level, and a higher likelihood of becoming transfusion-dependent.[86] MPL mutations do not affect survival or leukemic transformation in PMF.[89]

MPNs Are Associated with Bone Marrow Stromal Reaction

Bone marrow histology in MPN, primarily in PMF,[90,91] but also to some extent in both PV[92,93] and ET,[94–96] displays excess collagen fibrosis, new bone formation, and angiogenesis.[95,97–101] Bone marrow fibroblasts in MPN are polyclonal and participate in the altered cellular as well as extracellular concentrations of various

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fibrogenic and angiogenic cytokines.[102–105] The latter are believed to derive from clonal megakaryocytes and/or monocytes and include platelet-derived growth factor (PDGF), transforming growth factor-β, basic fibroblast growth factor, and vascular endothelial growth factor. The current assumption is that these as well as other cytokines mediate the bone marrow stromal reaction in MPN.[102–104] Furthermore, increased tissue levels of pathogenetic cytokines might be promoted by an altered interaction between megakaryocytes and neutrophils that is promoted by increased expression of P-selectin by the former.[106] Similarly, neutrophil-derived elastase and other enzymes might contribute to the abnormal peripheral blood egress of myeloid progenitors in PMF.[107,108]

MPNs Are Associated with Endogenous Myeloid Colony Growth

Endogenous (spontaneous, growth factor-independent) *in vitro* erythroid or megakaryocyte colony formation is not seen in either normal subjects or reactive myeloproliferation.[109] Epo-independent erythroid proliferation is primarily seen in PV,[110–112] but is also seen in a proportion of patients with ET[113–116] or PMF.[114,117] Furthermore, endogenous colony growth is also manifested by granulocyte[118] and megakaryocyte[119] progenitors. Such growth factor-independence has not been attributed to mutations in ligand receptor[120,121] or receptor-associated signal transducer molecules.[122]

In Vitro Myeloid Growth in MPN is Associated with Growth Factor Hypersensitivity

Growth factor hypersensitivity under *in vitro* conditions has been demonstrated for both PV and ET. Erythroid cells in PV are hypersensitive to a variety of cytokines including insulin-like growth factor (IGF-1),[123] stem cell factor,[124] granulocyte–monocyte colony-stimulating factor,[125] interleukin-3 (IL-3),[126,127] and Tpo.[128] A similar growth factor hypersensitivity to IL-3[129] or Tpo[130] has also been suggested in ET. The consistently observed IGF-1 hypersensitivity of erythroid cells in PV has been attributed to alterations in IGF-1 binding proteins.[131,132] The receptors for both Epo[120,133,134] and Tpo[121,135] have been examined in patients with MPN and found to be intact.

MPNs Are Associated with Decreased Megakaryocyte/Platelet Mpl Expression

Decreased megakaryocyte/platelet Mpl expression was first reported in ET[136] and subsequently in PV[137] and other MPNs.[138,139] In ET, the decreased surface expression of Mpl has been associated with reduced *c-Mpl* transcription.[136,140] In PV, the particular defect was traced to a post-translational hypoglycosylation of the Mpl protein with derailment of membrane localization.[141]

Pathogenesis of Bleeding Diathesis in MPN

Platelet morphology in MPN is variable and includes both small and giant forms.[142] These platelets are often hypogranular, and on electron microscopy display decreased granular contents involving both δ (serotonin, adenine nucleotides) and α (PDGF, β-thromboglobulin, platelet factor 4) specific storage granules.[143–148] This acquired storage pool deficiency as well as altered surface expression of hemostatic molecules is believed to contribute to the prohemorrhagic platelet defects in MPN.

In vitro observed hemostatic abnormalities in MPN include poor platelet aggregation in response to various platelet agonists (i.e., thrombin, ADP, epinephrine, collagen, thromboxane A_2 (TXA_2), platelet-activating factor),[149–152] abnormally low intra-platelet levels of adenine nucleotides and serotonin,[153] reduced platelet factor X-activating activity,[154] defective platelet lipid peroxidation,[155] impaired binding to fibrinogen as a result of decreased glycoprotein IIb/IIIa expression,[156] decreased adrenergic receptor expression that could explain the often observed absent epinephrine response and mechanistically involve impaired signal transduction,[157,158] and acquired von Willebrand syndrome (AvWS).[159] Similarly, quantitative radiolabeled as well as flow cytometric studies in PV and ET have revealed decreased expression of platelet glycoprotein receptors Ib and IIb/IIIa.[156,160,161]

AvWS occurs in more than a third of the patients with MPN and has been associated with a bleeding diathesis.[162,163] Several investigators have established a relationship between the platelet count and loss of large von Willebrand factor (vWF) multimers in plasma of MPN patients.[164–166] The finding that large vWF multimers in plasma decrease progressively at increasing platelet counts of $>1{,}000 \times 10^9$/L is in agreement with epidemiologic studies in ET showing an increased bleeding risk when the platelet count exceeds this level.[31,167–169] The functional deficiency of MPN-associated acquired von Willebrand disease (AvWD) may not be apparent when measuring vWF:Ag and FVIII levels alone, as these are typically maintained within the normal range.[159,170]

The assays used to assess vWF function (collagen binding activity [VWF:CBA] or ristocetin cofactor activity [vWF:RCoA]) reveal that vWF function declines relative to the vWF:Ag with increasing platelet counts.[171–173] This phenomenon is not restricted to MPN, but has also been described among patients with reactive thrombocytosis (RT).[164] Therefore, the observed loss of large multimers in ET and PV is likely an effect of the absolute platelet number, rather than an effect of dysfunctional clonal platelets. Further evidence of the importance of platelet number is provided by the observed resolution of the clinical bleeding complications and the AvWD in ET and PV patients following platelet cytoreduction with myelosuppressive therapy or platelet pheresis.[159,164,170,172] Similarly, resolution of RT also restores the large multimers and, as a result, vWF function among affected patients.[164]

Studies of patients with ET and PV have demonstrated that the reduction in the levels of large vWF multimers is the result of increased turnover *in vivo*.[172] Increased clearance of large vWF multimers in MPN has been proposed by some investigators to be the result of enhanced binding to platelets. However, the binding of vWF to single platelets from patients with either ET or RT was not shown to be different from that of normal controls.[174] Instead, enhanced proteolytic degradation of vWF is the more likely mechanism.[159,175–178] Regardless, the relationship between extreme thrombocytosis and decreased large vWF multimers suggests a key role for the number of circulating platelets in mediating this process.[164] Platelet in excess may facilitate the interaction between platelet surface receptor GPIb and vWF,

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thus inducing the appropriate conformational changes in vWF that allows ADAMTS 13 to gain access to its cleavage site.

Pathogenesis of Thrombotic Diathesis in MPN

Thrombosis is often multifactorial in etiology. MPNs have an inherent predisposition to thrombosis driven by the underlying clonal proliferation, and this risk may be escalated in association with thrombogenic stimuli that contribute to thrombosis in the general population. As such, the role of hereditary and acquired cardiovascular and thrombophilic risk factors has been studied in relation to their contribution to MPN-related vascular complications.

In regards to PV, hematocrit level is the major determinant of thrombosis and the most effective treatment strategy in this regard has been phlebotomy.[179] The relationship between thrombosis, hematocrit level, and *in vitro* parameters of blood viscosity and tissue perfusion is complex. For example, although hematocrit has been shown to be the major determinant of whole blood viscosity *in vitro*,[180] flow dynamics in blood vessels is different than that predicted by *in vitro* experiments and, in addition, the strong relationship between hematocrit and oxygen transport substantially influences *in vivo* blood viscosity.[180] For example, *in vivo*, increased hematocrit level is associated with decreased cerebral blood flow rate[181] not because of the change in viscosity but that of arterial oxygen content.[182] At low shear rates, which is comparable to flow in large veins, the endothelial displacement of platelets, leukocytes, and other proteins from axial migration of red cells might enhance thrombogenic interaction between endothelial cells and platelets.[183] At high shear rates, which is comparable to flow in arterioles and other small vessels, red cell-derived platelet aggregants, such as ADP, might contribute to thrombosis.

Although the reduction in hematocrit level by phlebotomy addresses the above mentioned potentially detrimental, hematocrit-associated conditions,[183,184] it does not abolish the risk of thrombosis in PV. Therefore, factors other than hematocrit (platelets, leukocytes, endothelial cells, coagulation proteins) might contribute to thrombosis risk in both PV and other MPNs. In this regard, potentially thrombogenic, qualitative cell defects have been described and include a diminished response of platelet adenylate cyclase to prostaglandin D2 (a physiologic inhibitor of platelet aggregation),[185] an increased baseline platelet production of TXA_2 (a platelet aggregant),[186,187] and abnormal *in vivo* activation of leukocytes,[188] endothelial cells,[188,189] and platelets.[187,189] Furthermore, previous reports of widespread activation in coagulation proteins,[188] reduced levels of physiologic anticoagulants (ATIII, proteins C and S),[190,191] and decreased fibrinolytic activity[192] that may be partly secondary to increased plasma levels of plasminogen activator inhibitor-1[193] suggest a baseline prothrombotic state in these disorders.

Spontaneous platelet activation in MPN has been demonstrated by measurement of plasma and urinary levels of arachidonate metabolites (TX B_2 and α-granule proteins [PDGF, β-thromboglobulin, platelet factor 4]), and accounts for the noted acquired storage pool disorder.[144–147] Additional evidence comes from the assessment of platelet surface membrane expression of markers of platelet activation (with monoclonal antibodies to P selectin and the activated fibrinogen receptor, GPIIb/IIIa) by flow cytometry.[161] It is possible that this abnormal activation state may be in part due to MPN-specific defects in arachidonic acid metabolism, which result in abnormal and sustained spontaneous TXA_2 generation in PV and ET.[187,194,195] This observed endogenous TXA_2 generation in PV and ET was found to be almost completely suppressible with low-dose aspirin.[187,195] Clinically, this effect has been shown to correlate with resolution of microvascular thrombosis.[34,196] The pathogenesis of the enhanced TXA_2 production in MPN is unknown, but may involve preferential shunting of arachidonate to the cyclooxygenase pathway because of concurrent lipoxygenase deficiency.[194] However, earlier studies suggested that MPN patients with lipoxygenase deficiency might display a hemorrhagic rather than thrombotic phenotype.[194]

DIAGNOSIS AND LABORATORY INVESTIGATION

Essential Thrombocythemia

According to the 2008 WHO system (Table 102.2),[10] a working diagnosis of ET is considered in the presence of persistent non-RT and after the diagnostic exclusion of CML, PV, MDS, and PMF (including "prefibrotic" PMF). Accordingly, one has to first entertain the possibility of RT from various causes (Table 102.3) during the initial evaluation of all such patients. In this regard, patient history and physical examination are crucial and often adequate to answer the specific question. Additional help is readily available from routine laboratory tests, including serum ferritin (to consider the possibility of iron deficiency), peripheral blood smear (to look for Howell-Jolly bodies and other markers of hyposplenism), and C-reactive protein or similar acute phase reactants (to consider the possibility of occult inflammation or malignancy).[197] In addition, rare cases of hereditary thrombocytosis (activating mutation of *MPL* or *TPO*) have been described, and must be kept in mind while evaluating a patient with either life-long history of thrombocytosis or a family history of the same.[12] If neither patient history nor the aforementioned laboratory tests suggest RT, *JAK2*V617F mutation screening is indicated and its presence confirms the presence of an underlying MPN, but its absence does not rule out the possibility since up to 40% of patients with ET might be *JAK2*V617F-negative.[198] It is also important to note that other *JAK2*V617F-positive MPN (or MDS/MPN) can mimic ET in their presentation; these include prefibrotic PMF[199] and refractory anemia with ring sideroblasts with marked thrombocytosis (RARS-T).[200] Therefore, bone marrow examination is often necessary to make an accurate morphologic diagnosis of ET and distinguish it from other myeloid neoplasms, especially from prefibrotic PMF; megakaryocytes in ET are large and mature-appearing whereas those in prefibrotic PMF display abnormal maturation with hyperchromatic and irregularly folded nuclei (FIGURE 102.2).[201] A recent large international study confirmed the prognostic relevance of distinguishing ET from prefibrotic PMF.[202] In the absence of *JAK2*V617F, the possibility of CML is readily addressed by *BCR-ABL1* mutation screening, but it is also to be noted that megakaryocytes in CML (small and hypolobulated) are easily distinguished from those of ET (FIGURE 102.3).[203]

Polycythemia Vera

The diagnosis of PV should be suspected in the presence of either an increased hemoglobin level (Hb >18 gm/dL in a male white or the corresponding level in the opposite sex or in the

Table 102.2

WHO diagnostic criteria for PV, ET, and PMF

	2008 WHO Diagnostic Criteria		
	Polycythemia Vera[a]	**Essential Thrombocythemia**[a]	**Primary Myelofibrosis**[a]
Major criteria	**1** Hgb > 18.5 g/dL (men) > 16.5 g/dL (women) *or*[b]	**1** Platelet count ≥ 450 × 10^9/L	**1** Megakaryocyte proliferation and atypia[c] accompanied by either reticulin and/or collagen fibrosis, *or*[d]
	2 Presence of *JAK2*V617F or *JAK2* exon 12 mutation	**2** Megakaryocyte proliferation with large and mature morphology.	**2** Not meeting WHO criteria for CML, PV, MDS, or other myeloid neoplasm
		3 Not meeting WHO criteria for CML, PV, PMF, MDS, or other myeloid neoplasm	**3** Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of reactive marrow fibrosis
		4 Demonstration of *JAK2*V617F or other clonal marker *or* no evidence of RT	
Minor criteria	**1** BM trilineage myeloproliferation		**1** Leukoerythroblastosis
	2 Subnormal serum Epo level		**2** Increased serum LDH level
	3 EEC growth		**3** Anemia
			4 Palpable splenomegaly

[a]PV diagnosis requires meeting either both major criteria and one minor criterion *or* the first major criterion and two minor criteria. ET diagnosis requires meeting all four major criteria. PMF diagnosis requires meeting all three major criteria and two minor criteria.
[b]*Or* Hgb or Hct >99th percentile of reference range for age, sex, or altitude of residence *or* RCM >25% above mean normal predicted *or* Hgb > 17 g/dL (men)/ >15 g/dL (women) if associated with a sustained increase of ≥2 g/dL from baseline that can not be attributed to correction of iron deficiency.
[c]Small to large megakaryocytes with aberrant nuclear/cytoplasmic ratio and hyperchromatic and irregularly folded nuclei and dense clustering.
[d]Or In the absence of reticulin fibrosis, the megakaryocyte changes must be accompanied by increased marrow cellularity, granulocytic proliferation and often decreased erythropoiesis (i.e., prefibrotic PMF).
BM, bone marrow; Hgb, hemoglobin; Hct, hematocrit; Epo, erythropoietin; EEC, endogenous erythroid colony; WHO, World Health Organization; CML, chronic myelogenous leukemia; PV, polycythemia vera; PMF, primary myelofibrosis; MDSs, myelodysplastic syndromes; LDH, lactate dehydrogenase.

presence of a different ethnic origin) or a PV-associated clinical feature (pruritus, erythromelalgia, splenomegaly, splanchnic vein thrombosis, increased leukocytosis or thrombocytosis). In the absence of the above scenario, a repeat complete blood count (CBC) in 3 months is all that is needed. Otherwise, one should further pursue the diagnosis of PV.

As is the case with ET, PV is currently diagnosed according to the WHO system (Table 102.2).[10] Other causes of polycythemia include secondary (usually Epo-mediated) and apparent (spurious or relative) polycythemia. Traditionally, this was accomplished through the Polycythemia Vera Study Group (PVSG) criteria, which required the demonstration of an elevated red cell mass (RCM).[204] At present, we seldom use either the PVSG criteria or RCM measurement and instead depend on the clinical presentation, *JAK2*V617F mutation screening, serum Epo level, and bone marrow histology in order to make a working diagnosis of PV.

FIGURE 102.3 provides a practical diagnostic algorithm for PV and other MPNs. The laboratory detection of *JAK2*V617F is highly sensitive (97% sensitivity), and virtually 100% specific for distinguishing PV from other causes of increased hematocrit.[205,206] The possibility of false positive or false negative mutation test result is effectively addressed by the concomitant measurement of serum Epo level, which is expected to be subnormal in more than 85% of patients with PV.[207] A subnormal serum Epo level in the absence of *JAK2*V617F mandates additional mutational analysis for *JAK2* exon 12 mutation in order to capture some of the approximately 3% of PV patients who are *JAK2*V617F negative.[56] Bone marrow examination is not essential for the diagnosis of PV, because patients who otherwise fulfill the diagnostic criteria for PV are labeled as having PV even if they display substantial bone marrow fibrosis (Table 102.2).[77]

Primary Myelofibrosis

The first clue to the diagnosis of PMF is a myelophthisic peripheral smear (the presence of nucleated red blood cells, granulocyte precursors, and teardrop-shaped erythrocytes). However, it should be noted that a myelophthisic smear may also be associated with either another myeloid malignancy with bone marrow fibrosis or a nonfibrotic bone marrow infiltrating process, including metastatic cancer, granulomatous infection, and lymphoma. Therefore, a careful morphologic evaluation of the bone marrow is necessary before making the diagnosis of PMF, and cytogenetic tests and fluorescence in situ hybridization (FISH)

Table 102.3

Causes of thrombocytosis

Primary Thrombocytosis	Reactive Thrombocytosis
• Essential thrombocythemia	• Infection
• Polycythemia vera	• Tissue damage
• Myelofibrosis with myeloid metaplasia (overt)	• Chronic inflammation
• Myelofibrosis with myeloid metaplasia (cellular phase)	• Malignancy • Rebound thrombocytosis
• Chronic myeloid leukemia	• Renal disorders
• Myelodysplastic syndrome	• Hemolytic anemia
• Acute leukemia	• Post-splenectomy • Blood loss

Buss DH, Cashell AW, O'Connor ML, et al. Occurrence, etiology, and clinical significance of extreme thrombocytosis: a study of 280 cases. *Am J Med* 1994;96:247–253; Chen HL, Chiou SS, Sheen JM, et al. Thrombocytosis in children at one medical center of southern Taiwan. *Acta Paediatr Taiwan* 1999;40:309–313; Chuncharunee S, Archararit N, Ungkanont A, et al. Etiology and incidence of thrombotic and hemorrhagic disorders in Thai patients with extreme thrombocytosis. *J Med Assoc Thai* 2000;83(Suppl 1):S95–S100; Robbins G, Barnard DL. Thrombocytosis and microthrombocytosis: a clinical evaluation of 372 cases. *Acta Haematol* 1983;70:175–182; Santhosh-Kumar CR, Yohannan MD, Higgy KE, et al. Thrombocytosis in adults: analysis of 777 patients. *J Intern Med* 1991;229:493–495; Yohannan MD, Higgy KE, al-Mashhadani SA, et al. Thrombocytosis. Etiologic analysis of 663 patients. *Clin Pediatr (Phila)* 1994;33:340–343.

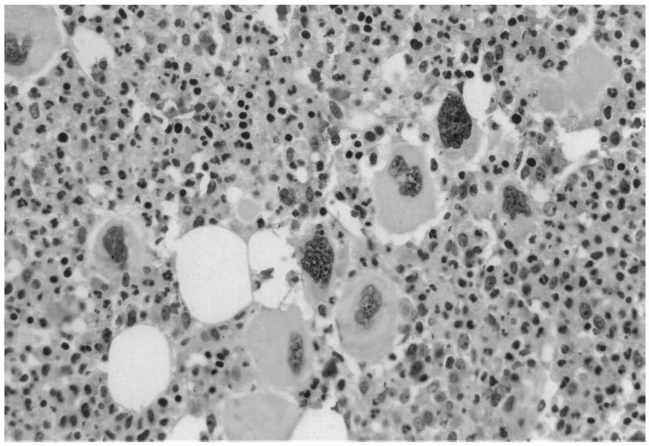

FIGURE 102.2 Megakaryocyte clusters in MPDs.

for bcr/abl should always be done to rule out fibrotic CML. Similarly, it is important to know that both MDS and other myeloid entities can be accompanied by bone marrow fibrosis, and the input from an experienced clinical pathologist is essential to distinguish these disorders from PMF.

Typical PMF is characterized by the presence of dysplastic megakaryocytic hyperplasia, collagen and reticulin fibrosis, osteosclerosis, and intra-sinusoidal hematopoiesis. In prefibrotic PMF, the degree of bone marrow fibrosis may be minimal, but

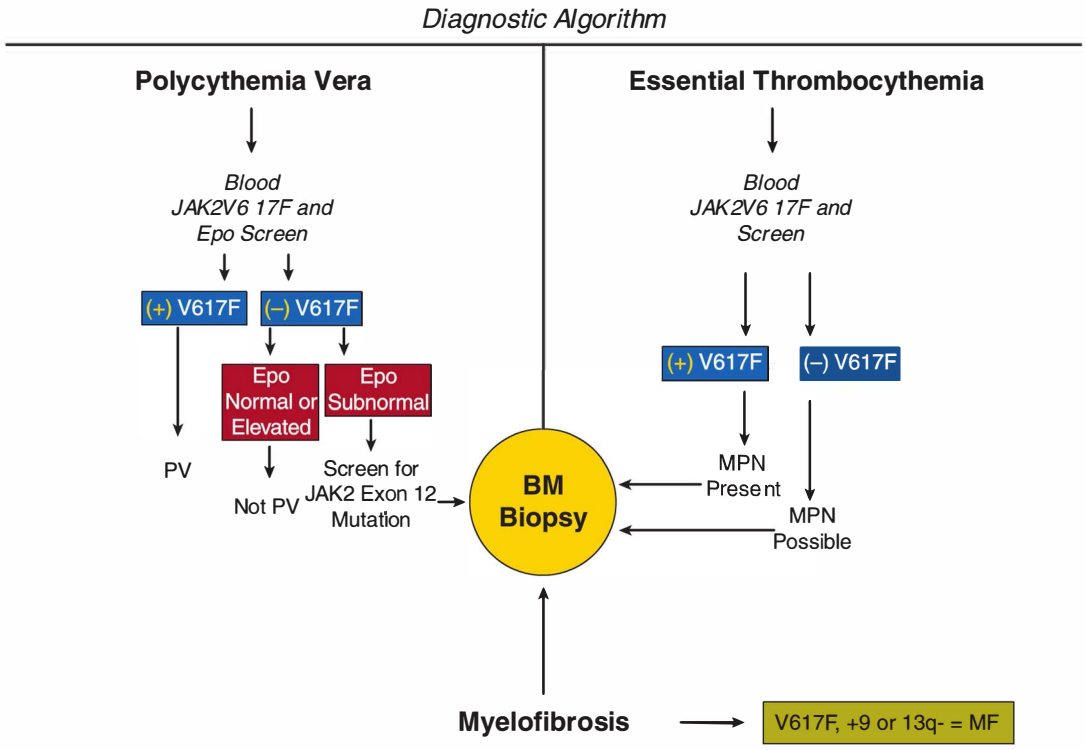

FIGURE 102.3 A practical algorithm for the diagnosis of polycythemia vera (PV), ET, and PMF. BM, bone marrow; MPN, myeloproliferative neoplasm.

splenomegaly, myelophthisis, and increased lactate dehydrogenase are often present. Recurrent cytogenetic abnormalities are seen in approximately a third of chemotherapy naïve patients with PMF.[208] The diagnosis of post-PV or post-ET MF should adhere to criteria recently published by the International Working Group for MPN Research and Treatment (Table 102.3).[209]

Coagulation and Platelet Function Tests in Myeloproliferative Disorders

Routine coagulation studies, including activated partial thromboplastin time (APTT), prothrombin time (PT), thrombin time (TT), and fibrinogen, are generally normal in both PV and ET. Although AvWS is associated with MPN, unlike the case with congenital vWD, the condition is usually associated with normal factor VIII levels, and thus will not be detected on the basis of a prolonged APTT.[170] Despite evidence of platelet dysfunction in ET and PV, as assessed by platelet aggregation studies, a prolonged bleeding time is seen only in a minority of cases (range, 7% to 26%).[31,210–212] Prolonged bleeding time in MPN has been attributed to an underlying defect in primary hemostasis (primary platelet dysfunction or AvWD), which is exacerbated by aspirin therapy.[213]

Platelet aggregation studies are frequently abnormal in MPN (demonstrating either or both hypo- and hyperfunction).[31,214,215] In general, platelet hypofunction (decreased aggregation with one or more agonists) is the predominant finding.[169] The most commonly reported abnormalities include decreased primary and secondary aggregation patterns to either epinephrine or ADP and decreased response to collagen, in descending order of frequency, with generally normal responses to arachidonic acid.[169] In a review of several studies in MPN patients, decreased aggregation responses to one, two, or all of these agonists were noted in 57%, 39%, and 37%, respectively.[210] Unlike the commonly encountered aspirin-type release defect in which the primary wave in response to epinephrine is maintained, but no secondary wave is obtained, complete absence of response to epinephrine is characteristic of MPN.[210]

Spontaneous platelet aggregation is another characteristic finding in MPN, but has not been correlated with clinical bleeding or thrombosis.[31,214] The possibility that whole blood-based platelet aggregation tests might provide a better clinical correlation has been touted, but remains to be confirmed by additional studies.[216]

ACQUIRED AND HEREDITARY THROMBOPHILIA AND MPN

The contribution of well-established risk factors of cardiovascular disease (hypertension, smoking, hypercholesterolemia, diabetes mellitus) have been assessed in MPN by multiple studies with some conflicting results, possibly reflecting the size of the studies and number of patients with the risk markers of interest included. On multivariate (hypertension, smoking, hypercholesterolemia, diabetes) analysis, one large retrospective study of ET patients (n = 148) demonstrated only the independent contribution of hypercholesterolemia, and another (n = 132), the independent association with smoking only.[28,217] The latter finding had been shown in an earlier, smaller (n = 46) study.[218] However, other large retrospective cohort studies including these variables could not confirm these associations.[31,212,219,220]

Over the last two decades there has been a vast expansion in knowledge of hereditary and acquired conditions contributing to thrombosis.[221] These include the congenital deficiencies of natural anticoagulants (antithrombin III, protein C, and protein S), genetic mutations (factor V Leiden, prothrombin G20210A, and MTHFR mutations), and acquired conditions (anticardiolipin antibodies and/or lupus anticoagulants). Several recent studies have explored the contribution of these to the occurrence of thrombotic events in MPN. Two prospective studies evaluated ET patients to determine the allele frequencies of factor V Leiden, prothrombin G20210A, and MTHFR mutations among those with and without thrombotic complications.[222,223] No significant correlation was found in either study, although both were limited by sample size (n = 43 and n = 42), respectively.

A more recent larger, yet retrospective study of 304 patients with PV (n = 178) and ET (n = 126), demonstrated a significant difference in the prevalence of the factor V Leiden mutation in patients with (16%), compared to those without (3%), a history of venous thrombotic events.[224] No such relationship was seen with regards to arterial thrombosis, as would be expected with this mutation.[225] This study did not investigate the other common thrombophilic polymorphisms (prothrombin G20210A, MTHFR) or acquired states (antiphospholipid syndrome), known to effect the phenotypic expression of prothrombotic disease.[224]

Several studies have confirmed the presence of elevated homocysteine levels among patients with MPN.[226–228] Elevated homocysteine levels may be hereditary (homozygosity for MTHFR mutation) or acquired (folic acid, vitamin B_{12} or B_6 deficiencies, renal failure), and is a recognized risk factor for both arterial and venous thrombotic disease.[229] However, no association with the homozygosity for the MTHFR mutation could be confirmed in these studies, leading investigators to postulate (and in study confirm) that the acquired increase in homocysteine may be the result of vitamin B deficiencies, possibly related to the persistent hyperproliferative hematopoiesis of these disorders. With regards to clinical impact of these observations, an association with arterial thrombotic disease was demonstrated by only one of the aforementioned studies,[226] but not the others.[227,228]

Antiphospholipid antibodies as measured either by a clot-bases assay [lupus anticoagulant] or ELISA are established risk factors for both arterial and venous thrombotic disease.[230] Several investigators have reported an increased prevalence of antiphospholipid antibodies in ET.[231,232] One of these studies suggested an association between the observed antiphospholipid antibodies and thrombosis.[231] However, the small sample sizes of patients included and lack of follow-up testing to confirm persistence (and thus significance) of the detected antibodies precludes any firm conclusions be made of these findings.

PROGNOSIS AND TREATMENT IN POLYCYTHEMIA VERA AND ESSENTIAL THROMBOCYTHEMIA

Survival in WHO-defined ET is near-normal[202] and median survival in contemporary PV is approximately 14 years.[233] Risk factors for shortened survival in ET and PV include history of thrombosis, leukocytosis, and advanced age.[202,233] Leukocytosis has also been associated with leukemic[233] and *JAK2*V617F allele burden with fibrotic[234] transformation in PV.[234] Current treatment for PV or ET is based on risk of thrombosis and designed

Table 102.4

Risk stratification in PV and ET and risk-adapted therapy

Risk Categories	Essential Thrombocythemia	Polycythemia Vera	Management During Pregnancy
Low-risk without extreme thrombocytosis (age < 60 y *and* no thrombosis history)	Low-dose aspirin	Low-dose aspirin + phlebotomy	Low-dose aspirin + phlebotomy if PV
Low-risk with extreme thrombocytosis (platelets > 1,000 × 10^9/L)	Low-dose aspirin *provided* ristocetin cofactor activity >30%	Low-dose aspirin *provided* ristocetin cofactor activity >30% + phlebotomy	Low-dose aspirin *provided* ristocetin cofactor activity >30% + phlebotomy if PV
High-risk (age ≥ 60 y *and/or* presence of thrombosis history)	Low-dose aspirin + hydroxyurea	Low-dose aspirin + phlebotomy + hydroxyurea	Low-dose aspirin + phlebotomy if PV + IFN-α
High-risk disease that is refractory or intolerant to hydroxyurea	Low-dose aspirin + IFN-α (age < 65 y) or busulfan (age ≥ 65 y)	Low-dose aspirin + phlebotomy + IFN-α (age < 65 y) or busulfan (age ≥ 65 y)	Low-dose aspirin + phlebotomy if PV + IFN-α

to prevent thrombotic and hemorrhagic complications.[235] Age ≥60 years and history of thrombosis are the two risk factors used to classify patients with PV or ET into low (0 risk factors) and high (1 or 2 risk factors) risk groups (Table 102.4).[233,236–238]

Controlled studies have confirmed the antithrombotic value of low-dose aspirin in PV[239] and hydroxyurea in ET.[240] The European collaboration study on low-dose aspirin in polycythemia[239] enrolled 518 patients with PV in a double-blind randomized trial to low-dose aspirin (100 mg daily) or placebo. Treatment with aspirin did not increase the incidence of major bleeding but instead reduced the risk of combined endpoints for "nonfatal myocardial infarction, nonfatal stroke, or death from cardiovascular causes" and "nonfatal myocardial infarction, nonfatal stroke, pulmonary embolism, major venous thrombosis, or death from cardiovascular causes."[241] Cortelazzo et al.[240] randomized 114 mostly high-risk ET patients to receive hydroxyurea ($n = 56$) or not ($n = 58$). After 27 months of follow-up, the incidences of thrombotic complications were 3.6% for hydroxyurea and 24% for no hydroxyurea.

Aspirin therapy is also considered to be adequate, and potentially useful in preventing complications during pregnancy, especially in *JAK2*V617F-positive cases.[242–244] First-trimester spontaneous miscarriage rate in ET or PV (>30%)[36,245,246] is significantly higher than the 15% rate expected in the control population and does not appear to be influenced by specific treatment.[36] Late obstetric complications as well as maternal thrombohemorrhagic events are relatively infrequent and platelet count usually decreases substantially during the second and third trimesters.[247] The use of aspirin in both PV and ET requires caution, especially in the presence of extreme thrombocytosis (platelet count >1,000 × 10^9/L), which promotes the development of AvWS.

No other drug therapy has been shown to be superior to hydroxyurea in the management of high-risk patients with ET or PV. In a randomized ET trial comparing hydroxyurea to anagrelide,[248] 809 high-risk patients were given low-dose aspirin plus either anagrelide or hydroxyurea. Hydroxyurea was better in terms of reducing the risk of arterial thrombosis, major bleeding, and fibrotic progression. Anagrelide performed better in preventing venous thrombosis. Similarly, in a nonrandomized study by the PVSG, treatment with hydroxyurea was associated with a lower incidence of early thrombosis compared to a historical cohort treated with phlebotomy alone (6.6% vs. 14% at 2 years), and the incidence of AML in patients treated with hydroxyurea, compared to a historical control treated with either chlorambucil or radiophosphorus, was significantly lower (5.9% vs. 10.6% vs. 8.3%, respectively, in the first 11 years of treatment).[249] Other studies have confirmed the low incidence of AML in PV patients treated with hydroxyurea (1% to 5.6%).[250–252]

Several drugs are available for use in hydroxyurea-refractory patients with ET or PV, including anagrelide, interferon-alpha (IFN-α), busulfan, pipobroman, and P32. The leukemogenic potential of P32 is now well established whereas pipobroman was recently similarly implicated.[253] In contrast, favorable outcome has been reported in single arm studies using oral busulfan.[254,255] In 65 busulfan-treated patients with PV followed between 1962 and 1983, median survival was 19 years in patients whose disease was diagnosed before age 60 years.[254] Only two patients (3.5%) treated with busulfan alone developed acute leukemia. A similar percentage (3%) developed the complication in another study involving ET patients.[256] The safety and efficacy of busulfan treatment in ET was recently underlined by a long-term study of 36 patients above 60 years of age[257]; no instances of AML or other malignancies were documented after a median follow-up of 72 months.

IFN-α therapy has been shown to control erythrocytosis, thrombocytosis, splenomegaly, and pruritus in the majority patients with PV or ET.[258] Two recent studies of pegylated INF-α (~90 μg SC weekly) in PV and ET reported hematologic remissions of approximately 80% accompanied by decrease in *JAK2*V617F allele burden (complete molecular remission rate of 5% to 10%).[259,260] In one of the two studies,[259] 77 cases were evaluable after a median follow up of 21 months, and 76% and 70% of patients with ET or PV, respectively, achieved a complete hematologic remission, mostly in the first 3 months; side effects

were recorded in 96% of the patients, and 22% had discontinued treatment.

Despite the availability of effective antithrombotic therapy in ET and PV, thrombotic events persist; in a recent international study of 891 patients with WHO-defined ET followed for a median of 6.2 years,[261] the rate of fatal and nonfatal thrombotic events was 1.9% patient-years, nonfatal arterial events 1.2% patient-years, and venous events 0.6% patient-years. In the same study, analysis that included 180 patients with prefibrotic PMF revealed that major bleeding during follow-up occurred in 6% of patients with WHO-ET and 12% of those with PMF, at a rate of 0.79% and 1.39% patients per year, respectively.[262] Predictors of bleeding included diagnosis of PMF, leukocytosis, previous hemorrhage, and aspirin therapy. The analysis restricted to patients with WHO-ET confirmed previous hemorrhage and aspirin as independent risk factors.

PROGNOSIS AND TREATMENT IN MYELOFIBROSIS

Current prognostication in PMF is based on the Dynamic International Prognostic Scoring System (DIPSS)-plus system (Table 102.5).[263] DIPSS-plus utilizes eight independent predictors of inferior survival: age >65 years, hemoglobin <10 g/dL, leukocyte count $>25 \times 10^9$/L, circulating blasts ≥1% and presence of constitutional symptoms, unfavorable karyotype (i.e., complex karyotype or sole or two abnormalities that include +8, −7/7q-, i(17q), inv(3), −5/5q-, 12p- or 11q23 rearrangement), red cell transfusion need, and platelet count $<100 \times 10^9$/L. The four DIPSS-plus risk categories based on the aforementioned eight risk factors are low (no risk factors), intermediate-1 (one risk factor), intermediate-2 (two or three risk factors), and high (four or more risk factors) with respective median survivals of 15.4, 6.5, 2.9, and 1.3 years.[263]

There is no evidence to support the value of specific therapy in asymptomatic patients with low or intermediate-1 risk disease (Table 102.5). MF-associated anemia is usually treated with androgens, prednisone, danazol, thalidomide ± prednisone, or lenalidomide ± prednisone.[264] Erythropoiesis stimulating agents are not used because they are ineffective in transfusion-dependent patients and could exacerbate splenomegaly. Response rates to each one of the aforementioned drugs are in the vicinity of 15% to 25%, and response durations average about 1 to 2 years. Lenalidomide works best in the presence of del(5q31). First-line therapy for MF-associated splenomegaly is hydroxyurea, which is effective in reducing spleen size by half in approximately 40% of patients. Both thalidomide and lenalidomide might improve splenomegaly and thrombocytopenia in some patients.

PMF patients with high or intermediate-2 risk disease should be considered for investigational drug therapy or allogeneic stem cell transplant (ASCT).[264] In considering ASCT, one should be acutely aware of the risks involved. In one of the largest studies of ASCT in PMF,[265] 5-year disease-free survival and treatment-related mortality were 33% and 35% for matched related and 27% and 50% for unrelated transplants, respectively. Of note, outcome did not appear to be favorably affected by reduced intensity conditioning. Several experimental drugs are currently being evaluated in MF and other related MPNs. So far, pomalidomide and JAK inhibitor ATP mimetics have shown the most promise. Pomalidomide is a second generation immunomodulatory drug, and in a phase-2 randomized study, 25% of patients with anemia responded to the drug used alone (2 mg/d) or in combination with prednisone (0.5 or 2 mg/d).[266] JAK2 inhibitor ATP mimetics that are currently in clinical trials include ruxolitinib (recently FDA approved), SAR302503, CYT387, lestaurtinib, SB1518, AZD1480, BMS911543, LY2784544, and XL019 (*clinicalTrials.gov*).

Ruxolitinib is a JAK1/JAK2 inhibitor. In a phase-1/2 study,[267] adverse events included thrombocytopenia, anemia, and a "cytokine rebound reaction" upon drug discontinuation, characterized by acute relapse of symptoms and splenomegaly.[268] Among all evaluable patients, 44% experienced ≥50% decrease in palpable spleen size. Improvement in constitutional symptoms (fatigue, pruritus, abdominal discomfort, early satiety, night sweats, and exercise tolerance) and weight gain were seen in the majority of patients. A major reduction in proinflammatory cytokines was documented and coincided with improvement in constitutional symptoms. These results were confirmed by two randomized studies comparing ruxolitinib with either placebo or best supportive care.

The aforementioned palliative effect of ruxolitinib on splenomegaly and constitutional symptoms is shared by other JAK inhibitors. SAR302503 therapy was associated with

Table 102.5

Risk stratification and risk-adapted therapy in PMF

DIPSS-Plus[42] Risk Groups PMF	Median Survival	Management PMF
Low-risk (*No risk factors*[a])	~15.4 y	Observation or conventional drugs[b]
Intermediate-1 risk (*1 risk factor*[a])	~6.5 y	Observation or conventional drugs[b] or experimental drugs
Intermediate-2 risk (*2 or 3 risk factors*[a])	~2.9 y	Allo-SCT *or* experimental drugs
High-risk (*≥4 risk factors*[a])	~1.3 y	Allo-SCT *or* experimental drugs

[a]DIPSS-plus[42] uses eight risk factors for inferior survival: age > 65 y, hemoglobin < 10 g/dL, leukocyte count > 25×10^9/L, circulating blasts ≥ 1%, presence of constitutional symptoms, presence of unfavorable karyotype, platelet count < 100×10^9/L and presence of red cell transfusion need. Please note that a transfusion-dependent patient automatically has two risk factors because of transfusion need (one risk point) and hemoglobin < 10 g/dL (one risk point).
[b]Androgen preparations or thalidomide with prednisone for anemia; hydroxyurea for symptomatic splenomegaly.
DIPSS, Dynamic International Prognostic Scoring System.[42]

gastrointestinal symptoms, but drug withdrawal syndrome was less frequent. Interestingly, 39% of patients with a baseline *JAK2*V617F allele burden of >20% experienced a ≥50% decrease in mutant allele burden. CYT387 therapy was less likely to cause anemia but was associated with grade 1 peripheral neuropathy in a third of the patients. In the absence of experimental drug therapy opportunities and in nontransplant candidates, hydroxyurea-refractory splenomegaly is managed by splenectomy or splenic irradiation. Nonhepatosplenic EMH is effectively treated with low-dose radiotherapy.

MANAGEMENT AND PREVENTION OF THROMBOHEMORRHAGIC COMPLICATIONS

Platelet count reduction to below 1 million/μL is the most effective means of controlling symptomatic, MPN-associated AvWS, and this is best accomplished by the use of cytoreductive chemotherapy.[159,165,213] The main drug of choice in this regard is hydroxyurea.[240] Platelet apheresis for MPD-associated AvWS is indicated when extreme thrombocytosis is accompanied by an urgent need to acutely reduce the platelet count.[159,165,213] The procedure has often been employed in the setting of postsplenectomy thrombocytosis because of the associated high risk of hemostatic morbidity.[213,269] Because the beneficial effect of platelet pheresis is generally brief and repeated procedures are often required, it is recommended that cytoreductive therapy be initiated as soon as possible to provide long-term control of the platelet count.

Desmopressin, during the treatment of MPN-associated AvWS, has been shown to increase both vWF:Ag and function.[159,165,176,213] However, due to the increased vWF proteolysis observed in association with MPN-associated thrombocytosis, the half-life of functional vWF is decreased, rendering the administration of desmopressin generally of limited value. Furthermore, in view of reports of thrombotic complications (stroke and myocardial infarction) among patients with increased thrombotic risk (elderly, cardiovascular disease) treated with desmopressin, administration of this agent to patients with MPN may not be prudent.[270,271] Similar problems may arise from the administration of vWF-containing plasma products (such as cryoprecipitate or vWF-containing concentrates).[213]

To minimize aspirin-associated bleeding diathesis in MPN, low-dose aspirin (75 to 100 mg/d) is administered with or after a meal and concurrent therapy with a proton-pump inhibitor is considered for patients with gastric symptoms.[272] In the presence of extreme thrombocytosis (platelet count >1,000 × 10^9/L), it is recommended that clinically significant AvWS be ruled out before the institution of aspirin therapy. If aspirin therapy is indicated in the particular instance, cytoreduction is necessary to reduce the platelet count to below 800 × 10^9/L, which usually corrects the AvWS, before instituting aspirin therapy.

Thrombotic episodes in patients with MPN are managed in the usual manner with standard dose and schedule of systemic anticoagulant therapy. However, systemic anticoagulation alone is not sufficient, and myelosuppressive therapy should be instituted to further reduce the risk of recurrent thrombosis in patients with either PV or ET.[273] Because of the potential for increased bleeding risk, especially in patients with MPN,[274] long-term warfarin therapy requires close monitoring of both the international normalized ratio, in view of potential drug interactions and dietary influences, and the platelet count to avoid overlooking inadvertent thrombocytopenia due to excessive myelosuppression. The use of aspirin in combination with oral anticoagulant therapy increases the risk of bleeding. However, no studies have been performed addressing the combined use of these agents in MPN.

Patients with MPN have a higher risk of morbidity and mortality when undergoing surgical procedures. These risks are substantially increased in the setting of splenectomy, a procedure often performed in for management of symptomatic splenomegaly due to portal vein thrombosis in MPN.[275–277] Splenectomy in MF typically leads to extreme thrombocytosis postprocedure in the absence of appropriate platelet suppression. In one series of 223 patients undergoing splenectomy for MF, postoperative thrombocytosis developed in 49 patients (22%) and 9 (18%) died as a result of either thrombosis (6 patients) or gastrointestinal bleeding (3 patients).[275] Therefore, MPN patients undergoing surgery should first have their disease well controlled (i.e., hematocrit level below 45% and platelet count below 400 × 10^9/L), and the prophylactic use of hydroxyurea should be considered before splenectomy.

Daily platelet counts should be performed in the immediate postoperative period. Aspirin therapy should be withheld for at least 1 week before elective surgery as well as when elective anticoagulation is required perioperatively. Although there are no prospective studies describing the efficacy of prophylactic anticoagulation for patients with MPN, such therapy might be instituted in view of the increased risk of postoperative thrombosis, for patients without known contraindications. However, such patients must be followed closely for the development of hemorrhage. Aspirin can be restarted 24 hours after surgery if no excessive bleeding has occurred or is anticipated by the surgeon. Aspirin can also be restarted 24 hours after stopping heparin prophylaxis in cases where this is prescribed.

CONCLUSIONS

Low-dose aspirin (40 to 100 mg/d) is the cornerstone of therapy in most low-risk patients with PV or ET, provided there are no major contraindications. In PV, phlebotomy is required to keep the hematocrit <45% but a higher target (50%) might be acceptable in aspirin-treated patients. Pregnant patients or women of childbearing potential are managed in the same general manner. In the presence of extreme thrombocytosis, screening for ristocetin cofactor activity is advised and consideration be given to withhold aspirin therapy if the result shows <30% activity. High-risk patients with PV or ET should receive hydroxyurea in order to minimize their risk of thrombosis. The dose of hydroxyurea is titrated to keep platelet count in the normal range. PV or ET patients who are either intolerant or resistant to hydroxyurea are effectively managed by INF-α (pegylated preparations preferred) or busulfan. I no longer use anagrelide for the treatment of PV or ET because of its reported association with increased risk of arterial thrombosis, major bleeding, and fibrotic progression.[248]

Low or intermediate-1 risk asymptomatic patients with PMF can be observed without any therapeutic intervention. Specific therapy is considered only in the presence of symptoms. First-line drugs of choice for anemia include thalidomide + prednisone, an androgen preparation, or danazol. I use lenalidomide in the presence of del(5q) or in case of treatment failure with thalidomide, danazol, or androgens. First-line drug of choice for symptomatic splenomegaly is hydroxyurea. Considering the lack of effective drug therapy in PMF, the risk of transplant-related

complications might be justified in those patients in whom median survival is expected to be <5 years. Nontransplant candidates are best managed with experimental drug therapy.[278–281] I have yet to be satisfied by the value of any currently available JAK inhibitor and strongly advise patients to continue participating in clinical trials.

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Full Reference Lists can be found online

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CHAPTER

103

Thrombotic and Hemorrhagic Complications Associated with Stem Cell Transplantation

YONA NADIR AND BENJAMIN BRENNER

Thrombotic and hemorrhagic complications are common in stem cell transplantation (SCT) recipients. Endothelial cell (EC) injury is a dominant contributing factor to the hemostatic impairments related to administration of chemotherapy[1] and growth factors,[1] use of intravenous catheters, and graft-versus-host reaction.[2] As bone marrow endothelial progenitor cells are one of the sources for endothelial regeneration, the ability to repair the endothelial damage during SCT is limited. Profound thrombocytopenia is another major contributor to bleeding manifestations. Refractoriness or severe reactions to platelet transfusions, common in patients previously exposed to blood product transfusions during chemotherapy, predispose them to life-threatening bleeding events. A number of alterations in procoagulant, anticoagulant, and, to a lesser extent, fibrinolytic factors have been described. They include prohemorrhagic reduction of factor VII and factor X, as well as tissue-type plasminogen activator (t-PA) increase, simultaneous to prothrombotic decrease of protein C, antithrombin, and plasminogen and increase of fibrinogen, von Willebrand factor (vWF), and plasminogen activator inhibitor (PAI-1).[3–9] Major changes in the coagulation system have been established to be predisposing risk factors for hemostatic complications in SCT patients.

In a retrospective analysis of 364 allogeneic and 83 autologous transplants, 83% of patients presented with at least one hemostatic complication as early as the start of conditioning therapy and throughout a 4- to 10-year follow-up.[10] Thromboembolic events, hepatic venoocclusive disease (VOD), and thrombotic microangiopathy (TMA) were most frequently observed in allogeneic transplant recipients, with an incidence of 15%, 4.7%, and 14.6%, respectively, leading to an increased overall mortality.[10] The majority of bleeding episodes occurred within the first 4 weeks after transplantation and were relatively mild. However, 27% of the patients hemorrhaged severely, mostly in the gastrointestinal tract and intracranial, doubling the overall mortality of SCT recipients. Bleeding was strongly associated with prolonged thrombocytopenia and graft-versus-host disease (GVHD).[10]

This chapter deals primarily with thrombotic and hemorrhagic conditions that are specific for SCT. Bleeding resulting from thrombocytopenia and venous thrombosis (e.g., deep vein thrombosis, pulmonary emboli) occurs in SCT patients as well as in many other medical conditions where patients are hospitalized and are not specific. The chapter is organized into two sections (thrombotic and hemorrhagic), each of which considers two special complications. Etiology and treatment of the complications are summarized in Table 103.1.

THROMBOTIC COMPLICATIONS

Site-Specific Microcirculation Thrombosis

The two most common thrombotic manifestations related to SCT, VOD, and TMA are characterized by microcirculation thrombosis. The endothelium synthesizes both anticoagulants and procoagulants. On the anticoagulant side, ECs express tissue factor pathway inhibitor (TFPI), heparan sulfate, thrombomodulin (TM), endothelial protein C receptor (EPCR), t-PA,[11,12,13] ecto-ADPase, prostacyclin, and nitric oxide (NO). On the procoagulant side, ECs synthesize PAI-1, vWF, protease-activated receptors, and tissue factor. Importantly, endothelial derived anticoagulant and procoagulant molecules are not expressed uniformly in the vasculature. For example, TFPI is predominantly expressed in the capillary endothelium, EPCR in large veins and arteries, endothelial NO synthase on the arterial side of the circulation, vWF prevails in veins, and TM in blood vessels of every caliber in all organs apart from the brain.[14–16] These data suggest that ECs originating from different sites of the vascular tree employ site-specific formulas of procoagulants and anticoagulants to balance local hemostasis.[11]

Hepatic Venoocclusive Disease

VOD is the most common and important regimen-related toxicity experienced after allogeneic and autologous SCT and is characterized by painful hepatomegaly, jaundice, ascites, fluid retention, and weight gain,[17,18,19,20] with onset typically prior to day 35 after stem cell reinfusion.[17,18,19,20] VOD develops in 1% to 22% of patients after SCT and ranges in severity from a mild, reversible disease to a severe syndrome associated with multiorgan failure and death.[18] The incidence and severity of VOD can be influenced by differences in patient characteristics, conditioning regimens, and source of graft, with allogeneic transplantation conferring a threefold increase in risk compared to autologous transplantation.[21,22]

Pathogenesis

Hepatic sinusoidal ECs, which comprise 50% of hepatic nonparenchymal cells, are discontinuous. They possess large (100 to 200 nm) membrane-bound, nondiaphragmed cytoplasmic holes or fenestrae, occupying 6% to 8% of the endothelial surface,[23] and also display gaps and lack an organized basement membrane. The lack of basement membrane generates a space between sinusoidal ECs and hepatocytes called the space of Disse. The sinusoidal endothelium functions as a selective sieve. The fenestrae act as a dynamic filter for fluids, solutes, and particles, allowing

Table 103.1

Etiology and treatment of thrombotic and hemorrhagic complications in SCT

	VOD	TMA	DAH	Hemorrhagic Cystitis
Etiology	Chemotherapy (alkylating agents)/irradiation in SCT[21,22]	Extensive prior chemotherapy[73–75]	Chemotherapy/irradiation in SCT[107]	Chemotherapy (alkylating agents)/ irradiation in SCT[119,121]
	Allogeneic > autologous[21,22]	Reactivation of CMV[73–75]	WBC recovery[99,100]	GVHD[119,121]
		Drugs (cyclosporine-A, tacrolimus)[73–75]	Lung infection[97]	Viral infection (BK, adenovirus, herpes simplex)[119,122,123]
		Allogeneic > autologous[10,69,70]	Acute GVHD[2,102]	Allogeneic > autologous[118,119]
			Renal insufficiency[99,100]	
			DMSO[95,104]	
			Granulocyte colony-stimulating factor (G-CSF)[105,106]	
			Endotoxin[107]	
			Allogeneic = autologous[92–96]	
Treatment	[a]Ursodeoxycholic acid[41–44]	[a]Plasma exchange[71,74,88]	[a]Steroids[93,95,107,112]	[a]Hydration[125]
	[a]Heparin[45–49]	Defibrotide[89]	[a]Tranexamic acid, aprotinin[117]	[a]Alum[125]
	t-PA[51,52]	Immunoglobulin G[90]	rFVIIa[116]	Hyperbaric oxygen[127]
	Defibrotide[54–56,64,65,66–68]			Antiviral antibiotic[123]
				Estrogen[126]
				rFVIIa[115,129]
				Silver nitrate[125]
				Formalin[125]
				Selective embolization[128]

[a]Commonly used treatment.

passage of small particles (up to medium-sized chylomicrons) from blood to hepatocytes via the space of Disse. Although the fenestrae are not large enough to accommodate leukocyte transmigration, they do allow cytoplasmic extensions from such cells to penetrate and "touch" the underlying matrix, stellate cells, and hepatocytes. Blood flow velocity in the sinusoids has been estimated to be 400 to 450 μm/s, compared with 500 to 1,000 μm/s in "true capillaries," thus prolonging interactions between blood and sinusoidal ECs and promoting filtration.[23]

Sinusoidal obstructive syndrome, also known as hepatic VOD, occurs most commonly in response to conditioning regimens (chemotherapy with or without irradiation) used in bone marrow transplantation. The first lesions appear in the sinusoids. Experimental models suggest that exposure to toxins results in swelling and rounding up of sinusoidal ECs, especially in the centrilobular area that has the poorest oxygenation, and is relatively more sensitive to ischemic injury, after which red blood cells enter the space of Disse.[24] Here, blood flow essentially dissects the sinusoidal endothelial lining away from the underlying parenchymal cells, resulting in embolization of sinusoidal ECs and occlusion of the downstream venous vessel (FIGURE 103.1A–C).[24] Subsequent changes include deposition of fibrin within sinusoids and veins, fibroblast cell proliferation, and collagen accumulation in the extracellular matrix (FIGURE 103.1D).[25,26,27] As the process of venous microthrombosis, ischemia, and fibrosis advances, widespread zonal disruption leads to portal hypertension, hepatorenal syndrome, multiorgan failure, and death.[28]

Injury to sinusoidal ECs caused by high-dose alkylating agents appears to be the primary event in the pathogenesis of VOD.[29–31] Detoxification of these chemotherapy agents in the liver is mainly mediated by cytochrome P450 and glutathione-*S*-transferase enzymes, which are present at high concentrations within the centrilobular area.[32] Depletion of glutathione stores predisposes hepatocytes to necrosis, whereas addition of glutathione mono-8-diester protects hepatocytes from high-dose alkylator injury.[33,34]

Several endothelial injury markers and adhesion molecules are upregulated in patients with VOD, including plasma TM, P- and E-selectins, TFPI, soluble tissue factor, and PAI-1.[9,35] *In vitro* studies show that endothelial PAI-1 production is triggered by cytokine transforming growth factor beta (TGF-β) released from activated platelets.[36] Elevated pretransplant plasma levels of TGF-β have been associated with the development of hepatic VOD.[37] Additionally, proinflammatory cytokines could also contribute to the initial endothelial injury, and increased tumor necrosis factor (TNF-α), interleukin (IL)-6, IL-8, and IL-1β are correlated with the jaundice, renal dysfunction, and pulmonary disease of SCT.[4,38]

The role of thrombophilic factors in the pathogenesis of thrombotic complications in SCT is unclear. In a small retrospective study, the prothrombin gene 20210 G-A mutation was found to be a predisposing factor for VOD,[39] and a prospective study in children revealed a strong association between factor V Leiden mutation and VOD.[40] More studies are warranted to confirm the association between thrombophilia and VOD.

Therapy

The most established practice in VOD prevention has been the use of pharmacokinetics to monitor chemotherapeutic drug levels to minimize hepatic injury. Prophylactic administration of ursodeoxycholic acid, a hydrophilic water-soluble bile acid, has been studied in randomized placebo-controlled trials, some showing a statistically significant benefit in patients predicted to be at a high risk of VOD.[41–43] However, a large phase III study failed to demonstrate significant benefit of this approach.[44] The role of heparin anticoagulation, alone or in combination with other agents, in VOD prophylaxis has been assessed in only one randomized study, which reported a beneficial effect of low-dose continuous heparin infusion.[45] Low molecular weight heparin is relatively safe and may have some impact on VOD prevention,[46–49] but well-designed studies are needed to confirm these results.

There is no approved therapy for established hepatic VOD. Current approaches focus on supportive care and on anticoagulant or fibrinolytic drugs,[45,46,50–52] based on pathologic findings of hepatic venules or sinusoids obliteration by fibrin.[18,26,27] The major disadvantage of exposure to heparin or recombinant t-PA is the high risk of life-threatening bleeding in patients with concurrent thrombocytopenia. The lack of response to platelet transfusions due to portal hypertension, splenic sequestration, and antiplatelet antibodies hampers effective management.[51,53]

Defibrotide, a single-stranded polydeoxyribonucleotide that exerts antithrombotic activity by binding to vascular endothelium, has shown promising results in VOD management.[54–56] Defibrotide upregulates the endothelial release of prostacyclin (PG I_2), prostaglandin E_2, TM, and t-PA,[57–59] and decreases thrombin generation, tissue factor expression, PAI-1 release, and endothelin activity.[57,60] Defibrotide exerts no significant effect on systemic coagulation,[61] but it has antiangiogenic potential in ECs and in an animal model.[62] Preclinical studies have demonstrated profibrinolytic effects and inhibition of fibrin deposition, with selective activity in

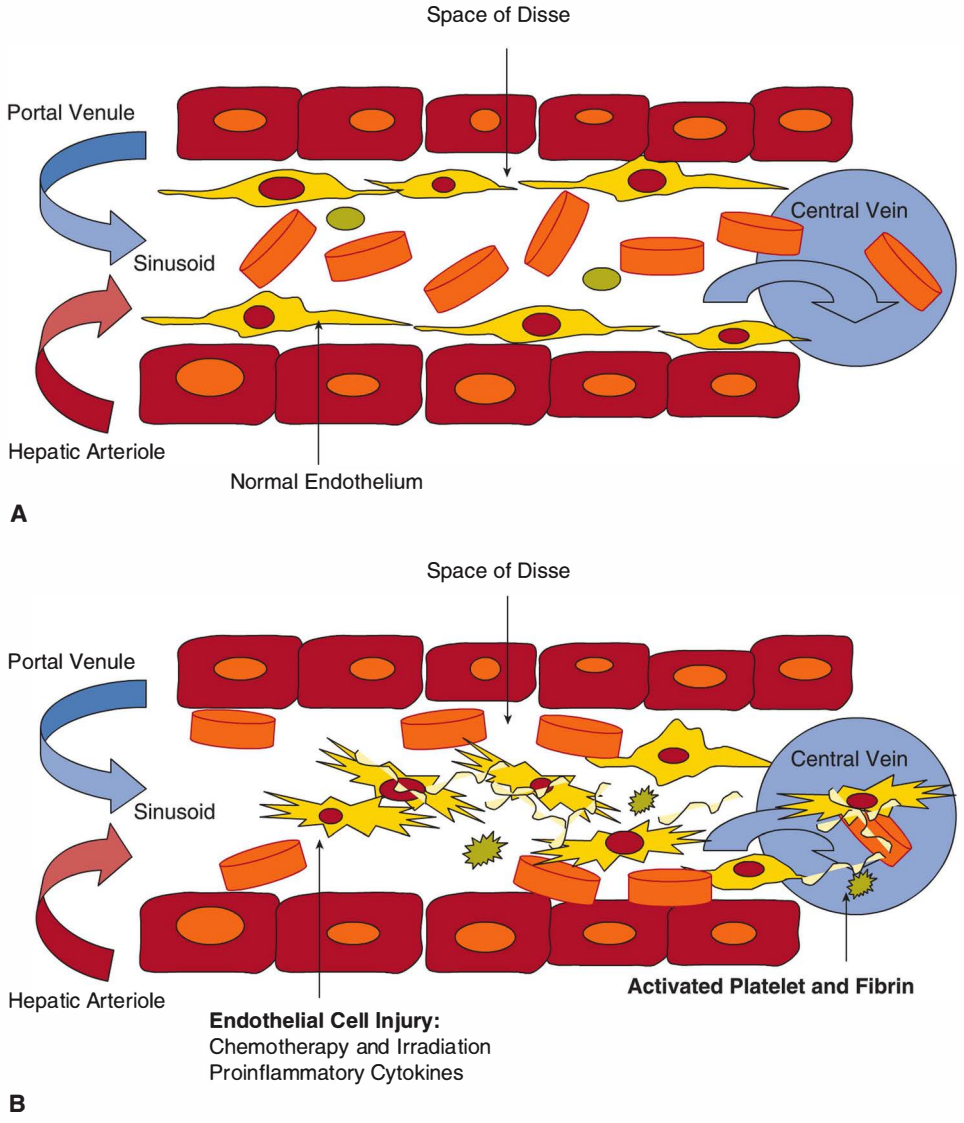

FIGURE 103.1 Mechanism of VOD. **A:** Normal blood flow in hepatic sinusoid. No basement membrane is present. **B:** Damaged ECs torn away from the sinusoid. Red blood cells enter the potential space of Disse and aggravate EC separation. Damaged ECs and the exposure of the subendothelial space activate platelets and the coagulation system to form fibrin.

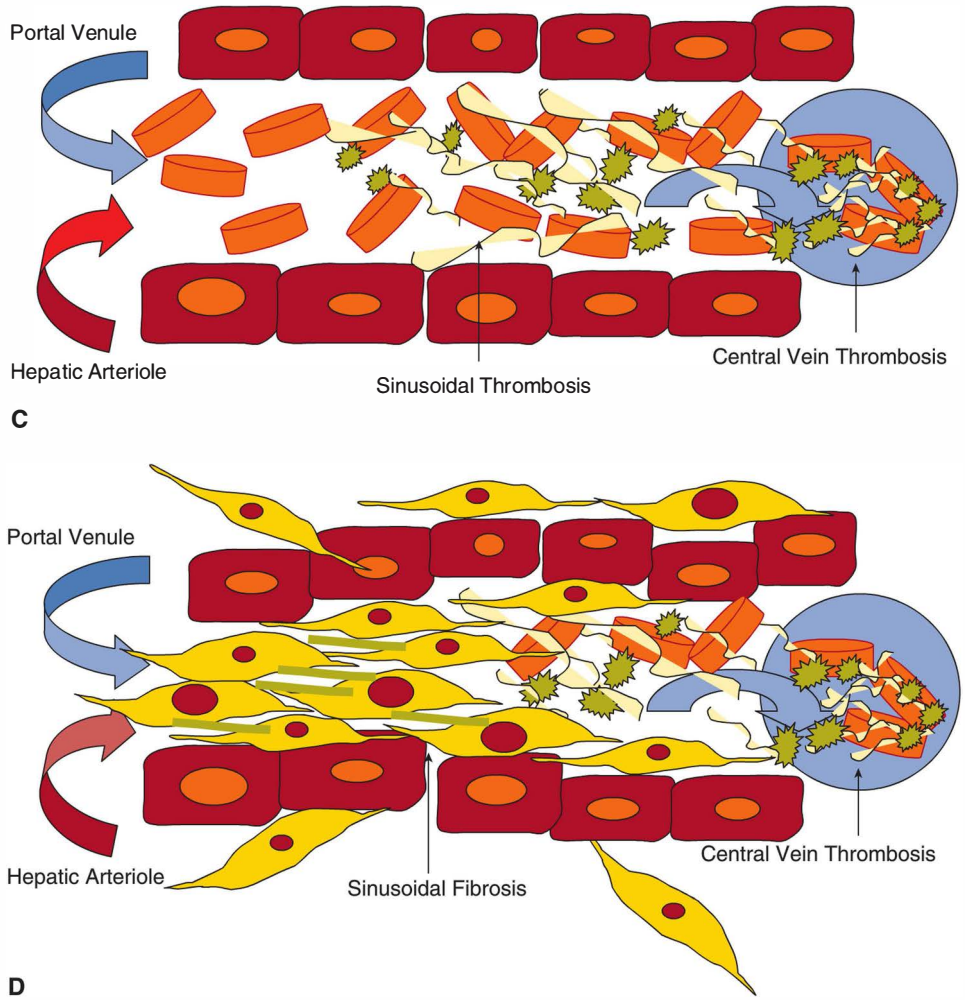

FIGURE 103.1 *(Continued)* **C:** The formed thrombus obstructs the sinusoid and central vein. **D:** Secondary changes of fibroblasts invasion and collagen deposition resulting in sinusoidal fibrosis.

small vessels.[63] Initial clinical reports of defibrotide for severe VOD recorded complete resolution in 36% to 42% of patients, with most individuals surviving beyond day 100.[54,55,64] In a recent randomized phase II dose-finding trial in adult and pediatric patients, defibrotide 25 mg/kg/d was selected for forthcoming phase III trials.[65] Preemptive antithrombin replacement and combined antithrombin/defibrotide therapy allowed excellent remission and survival rates in a prospective case series study of pediatric SCT patients.[66] In another report on 58 adults undergoing SCT, no patient developed VOD following the use of defibrotide prophylaxis (without concurrent heparin).[67] Cappelli et al.[68] reported on 57 children with beta thalassemia at a very high risk for developing VOD (liver fibrosis, iron overload, hepatitis C virus infections, busulfan-based conditioning), and only one patient developed VOD, after discontinuation of defibrotide 6 days prior to VOD onset. To date, no phase III randomized studies have been published on the use of defibrotide as prophylaxis or treatment of VOD.

Transplantation-related Thrombotic Microangiopathy

Transplantation-related TMA is a devastating complication occurring following SCT. The incidence of TMA after allogeneic and autologous transplantation is 0.5% to 15% and 0.1% to 0.25%, respectively.[10,69,70] Diagnosis is based principally on the presence of thrombocytopenia and microangiopathic hemolytic anemia in the absence of an alternative clinically apparent etiology.[71,72] Similar to idiopathic thrombotic thrombocytopenia purpura (TTP), transplantation-related TMA is associated with renal dysfunction and neurologic complications.[72] However, since both thrombocytopenia and fragmentation of red blood cells are extremely common after SCT, and renal dysfunction and neurologic complications can occur secondary to a diverse range of etiologies, definitive diagnosis of transplantation-related TMA is often uncertain.[73,74] Transplantation-related TMA can be attributed to extensive prior therapy, GVHD, reactivation of cytomegalovirus (CMV), and drugs (cyclosporine-A, tacrolimus).[70,75,76] Cyclosporine A is widely used as an immunosuppressive agent and is the most commonly reported cause of drug-induced TMA,[77] not only in bone marrow transplantation, but also in 3% to 5% of renal transplant recipients.[78] Chemotherapy-induced TMA is rare, reported in association with mitomycin-C,[79] cisplatin, alone or with bleomycin,[80] and gemcitabine.[81] These drugs are not routinely used in SCT chemotherapy protocols. Although transplantation-related TMA is associated with a shorter survival due to multifactorial causes, the exact prognosis is difficult to assess.[82]

Pathogenesis

Posttransplant TMA is not associated with severe vWF-cleaving protease deficiency, and the elevated vWF antigen found in these patients is more likely the result of diffuse endothelial injury (FIGURE 103.2A–C).[70,83] Cyclosporine-A is cytotoxic to cultured ECs at concentrations similar to the peak plasma levels achieved *in vivo*.[84] During episodes of cyclosporine-A–induced microangiopathy, plasma levels of vWF and endothelin are elevated,[85] as well as those of prostacyclin and thromboxane A_2 released by ECs.[84]

In patients with transplant-related TMA who received tacrolimus, a rise in serum levels of endothelin and several cytokines (IL-10, IL-12, TNF-α, interferon-γ) occurs, consistent with EC injury and cytokine-induced expression of adhesion molecules.[86] In addition, cyclosporine-A enhances platelet aggregation and platelet thromboxane A_2 release. Platelet hyperaggregability in renal allograft patients on long-term cyclosporine-A therapy reverts toward normal following the replacement of the drug with azathioprine.[87]

Therapy

Optimal management of transplantation-related TMA is currently not clear. Certainly, any suspected offending drug should be discontinued. Because of clinical similarity to idiopathic TTP, treatment-related TMA has been managed with plasma exchange, albeit with limited efficacy and relatively low response rates of <20% to 50% compared with idiopathic TTP (80%).[71,74,88] Other modalities of treatment, such as defibrotide or immunoglobulin G, have been attempted with variable success.[89,90]

HEMORRHAGIC COMPLICATIONS

Diffuse Alveolar Hemorrhage

Respiratory insufficiency associated with lung injury, both infectious and noninfectious, is a major cause of death in SCT patients. In a retrospective cohort study, medical and autopsy records of 71 autologous or allogeneic SCT recipients with

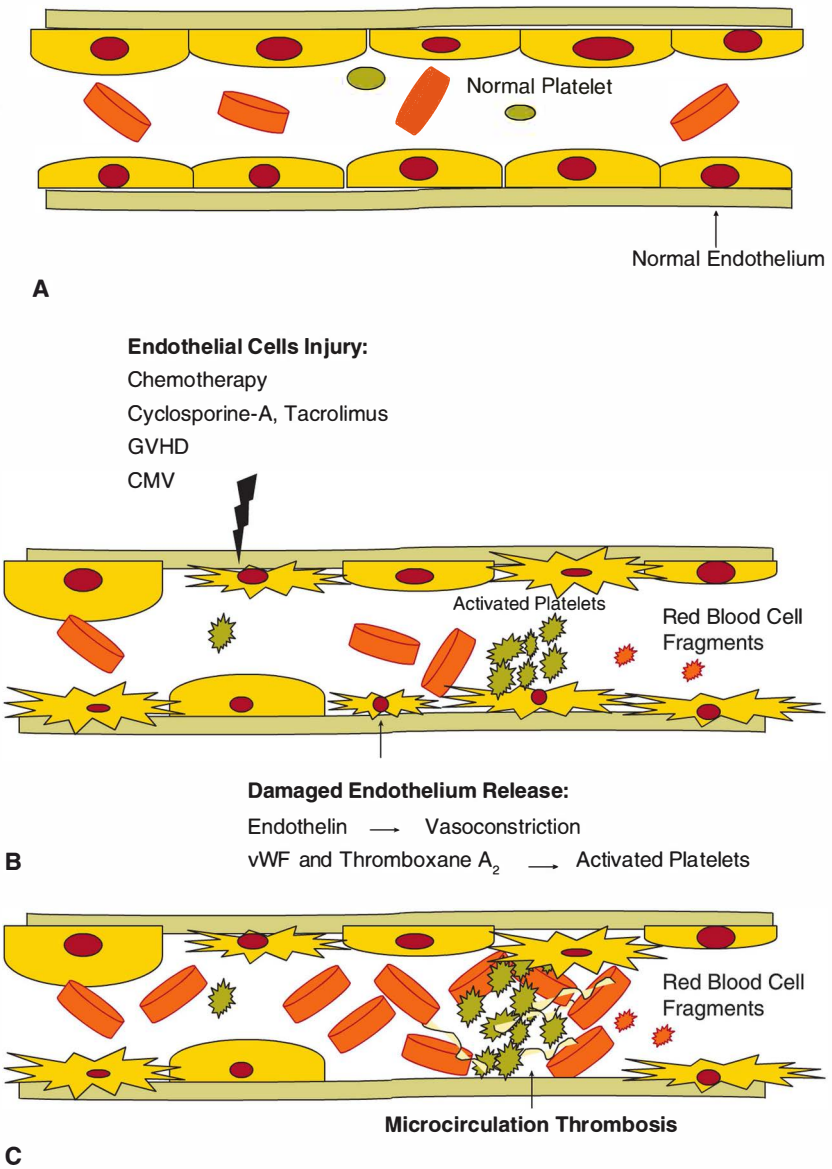

FIGURE 103.2 Endothelial damage in transplantation-related TMA. **A:** Normal blood flow in a capillary. **B:** Mechanism of endothelial damage involves drugs, GVHD, and CMV infection. Damaged ECs release endothelin, vWF, and thromboxane A_2, resulting in vasoconstriction and platelet activation. **C:** Capillary occlusion with a thrombus.

pulmonary complications found an infectious cause of death in 27 (37%) patients.[91] Noninfectious causes included diffuse alveolar damage, amyloidosis, leukemia/lymphoma, bronchitis obliterans, bronchiolitis obliterans, pulmonary alveolar proteinosis, acute organizing pneumonia, and aspiration pneumonia. Hemostatic problems were identified in 21% of the patients, including alveolar hemorrhage in 14% and pulmonary embolism in 7%.[91]

The reported incidence of diffuse alveolar hemorrhage (DAH) varies from 1% to 21% in autologous and from 2% to 17% in allogeneic SCT patients, suggesting that the type of transplantation does not play a major role in this pathology.[92–96] DAH can present with hypoxemia, pulmonary infiltrates, and progressive bloody alveolar lavage. A documented associated infectious cause occurred in 50% to 60% of the patients.[97] The probability of a 60-day survival from the onset of hemorrhage was 16% for the DAH and 32% for the infection-associated alveolar hemorrhage group.[97] Allogeneic SCT using reduced-intensity conditioning (RIC) has lower morbidity and mortality compared to transplantation using myeloablative conditioning (MAC), although alveolar hemorrhage occurs in 18/206 (8%) and 85/1112 (7%, $P = 0.56$), respectively, indicating that reduction in the intensity of a preparative regimen does not prevent transplantation-related DAH.[98] White blood cell (WBC) recovery and renal insufficiency, but not prolonged prothrombin time, partial thromboplastin time, or low platelets counts, are associated with DAH;[99,100] although most patients have thrombocytopenia, the condition is not corrected with platelet transfusion.[100]

Pathogenesis

Autopsies of SCT recipients with acute hemorrhagic pulmonary edema show intraalveolar and, to a lesser extent, interstitial collections of edema, fibrin, and erythrocytes, without hyaline membranes, pneumocyte changes, interstitial mononuclear cell infiltration, and fibrosis.[101] Thus, although the clinical presentation is hemorrhagic, autopsy findings imply a thrombotic etiology (FIGURE 103.3A,B). The incidence of DAH is high in SCT recipients with acute GVHD.[102] In addition to toxicity of the GVHD therapy, antigen-specific injury of endothelium may be a contributing factor to the development of DAH.[103] Mice receiving bone marrow T lymphocytes develop alveolitis, characterized by alveolar hemorrhage, increased alveolar leukocytes, platelet microthrombi, and damage to endothelial and epithelial cells during the acute phase of the graft-versus-host reaction.[2] The use of dimethyl sulfoxide (DMSO)

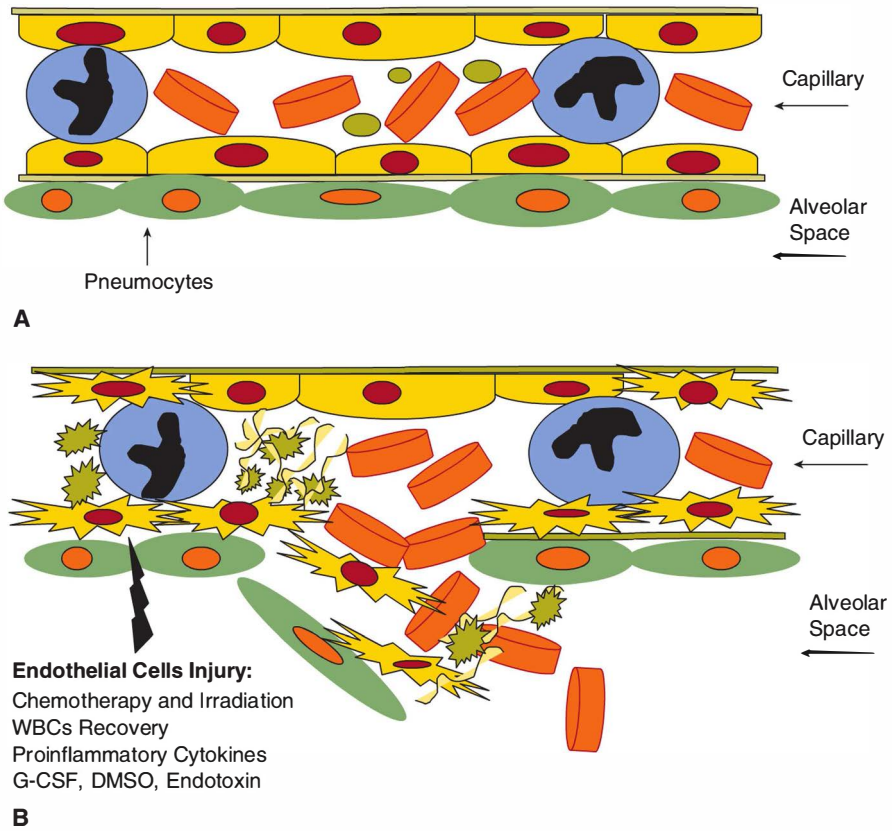

FIGURE 103.3 Proposed mechanism of DAH. **A:** Normal capillary and alveolar space. Pulmonary capillary bed has small diameter, hardly enabling WBC passage. **B:** Alveolar capillary EC injury is the primary insult. WBC recovery exacerbates the thrombotic potential due to the large diameter of neutrophils and tissue factor exposure during inflammation, infection, and G-CSF treatment. Injured endothelium activates platelets and coagulation factors to form fibrin. The thrombotic capillary rupture and red blood cells enter the alveolar space resulting in alveolar hemorrhage.

for cryopreservation of blood stem cells has been implicated in causing damage to the alveolar endothelial lining, thereby leading to the development of DAH,[95,104] and hematopoietic growth factors may also play a role in worsening the alveolar damage and capillary leakage by increasing neutrophil infiltration into the lungs.[105,106] It is speculated that damage to alveolar capillary endothelial membranes begins during preparative chemotherapy or total body irradiation, resulting in release of inflammatory mediators,[107] a response that may be amplified after the release of endotoxin into circulation from an inflamed gastrointestinal tract.[107]

Although alveolar capillary ECs do not express vWF,[108] they produce and secrete the clotting factor VIII that may enhance local coagulation.[109] Alveolar capillary blood contains an increased concentration of neutrophils and other leukocytes compared with large vessels, due to the structure of the pulmonary capillary bed, the diameter of spherical leukocytes, and their poor deformability compared with erythrocytes.[110] While capillary diameter in most organs is about 10 μm, it is about 8 μm in the pulmonary bed, enforcing deformability of granulocytes and monocytes which are 10 to 17 μm in diameter.[110] DAH typically occurs in parallel to WBC recovery,[99,100] and most contributing factors involve an inflammatory process. Impaired ability of WBCs to transmigrate lung ECs, as was shown in the mice model of DAH,[2] together with endothelial damage, may engender local alveolar capillary endothelial thrombosis resulting in endothelial damage and hemorrhage (FIGURE 103.3B). Although no supporting clinical data are available, this suggested obstructive and thrombotic damage may be potentially treated with regular preventive inhalation of heparin. Heparins are capable of transmigrating thin alveolar cells and reaching the alveolar capillary ECs. The ability of heparins to interfere with WBC rolling and adhesion to ECs[111] may prevent the alveolar endothelial sequestration.

Therapy

There are no prospective, randomized trials that address treatment of DAH in SCT recipients. Given that the pathogenesis of DAH is considered to be an inflammatory response and based on anecdotal experience and retrospective studies, SCT recipients with DAH are treated with systemic corticosteroids.[93,95,107,112] Fresh frozen plasma transfusion and plasmapheresis have been tried in one study with inconclusive results.[113] Paucity of reports on the off-label use of recombinant factor VIIa (rFVIIa) in SCT recipients with DAH precludes drawing definitive conclusions regarding the efficacy of this therapy.[114,115] In a phase II multicenter trial in SCT patients with major bleeding, a total dose of 280 to 1,120 μg/kg rFVIIa was used,[116] with regimens 40, 80, or 160 μg/d administered in seven successive doses every 6 hours for 36 hours. In this study, 7 patients out of 100 suffered from DAH, 6 thromboembolic events were observed in the treated group, and the trial did not show a significant benefit of rFVIIa in the setting of SCT-related severe bleeding.[116] The use of antifibrinolytic agents, such as tranexamic acid and aprotinin, is not well documented. Solomonov et al.[117] reported on six patients with significant hemoptysis, two of whom bled during bronchoscopy biopsy and four with spontaneous bleeding, treated successfully with local tranexamic acid without adverse events.

Hemorrhagic Cystitis

Hemorrhagic cystitis occurs as microscopic hematuria (grade I), or as gross hematuria, with clots and urinary tract obstruction (grades II to IV) in 5% to 40% of SCT recipients and causes significant morbidity and mortality. It occurs during or following SCT. Its incidence is twofold to threefold higher in allogeneic than autologous transplantation.[118,119]

Pathogenesis

Histologic analyses reveal mucosal bladder alterations such as edema, necrosis, and hemorrhage (FIGURE 103.4A,B).[120] Acute GVHD, the use of alkylating agents, and irradiation as part of conditioning regimen as well as viral reactivation have been implicated in the pathogenesis of hemorrhagic cystitis.[119,121] Reactivation of viral infection (BK virus, adenovirus, and herpes simplex viruses) has been associated with late-onset (>2 weeks posttransplant) hemorrhagic cystitis.[119,122,123] A recent study demonstrated that hemorrhagic cystitis was less common in patients with related donors than in those with unrelated donors and was less frequent in patients receiving a RIC regimen, compared to those exposed to MAC.[124]

Therapy

Various palliative treatments, including hydration, hyperbaric oxygen, antiviral antibiotic, estrogen, selective embolization of the internal iliac arteries, intravesicle instillation of materials as alum, silver nitrate, and formalin, showed variable success in alleviating symptoms.[123,125–128] A multicenter randomized trial in SCT patients with major bleeding[116] included 26 individuals with intractable hemorrhagic cystitis, which was not controlled with rFVIIa treatment.[116] A trial of four patients with SCT-related severe hemorrhagic cystitis treated with rFVIIa (150 to 1,200 μg/kg) was effective without thrombotic events.[129]

CONCLUSIONS

Hemostatic complications are common in allogeneic as well as autologous SCT patients, and there is an overlap in contributing factors. Due to the possibility that DAH could also be a thrombotic event, the three typical hemostatic complications in SCT, namely, VOD, TMA, and DAH, may be manifestations of thrombosis in the microcirculation. Hemorrhagic cystitis is likely to be a primary epithelial injury. ECs line the vascular bed, and each vascular bed has unique structural and functional properties, and therefore, understanding of these properties may hold important clues to site-specific diagnostics and therapeutics. Better understanding of the unique endothelial changes in each thrombotic condition would promote advanced treatment strategies. Hemostatic complications are known to cause high morbidity and mortality in SCT patients. The use of more intensive

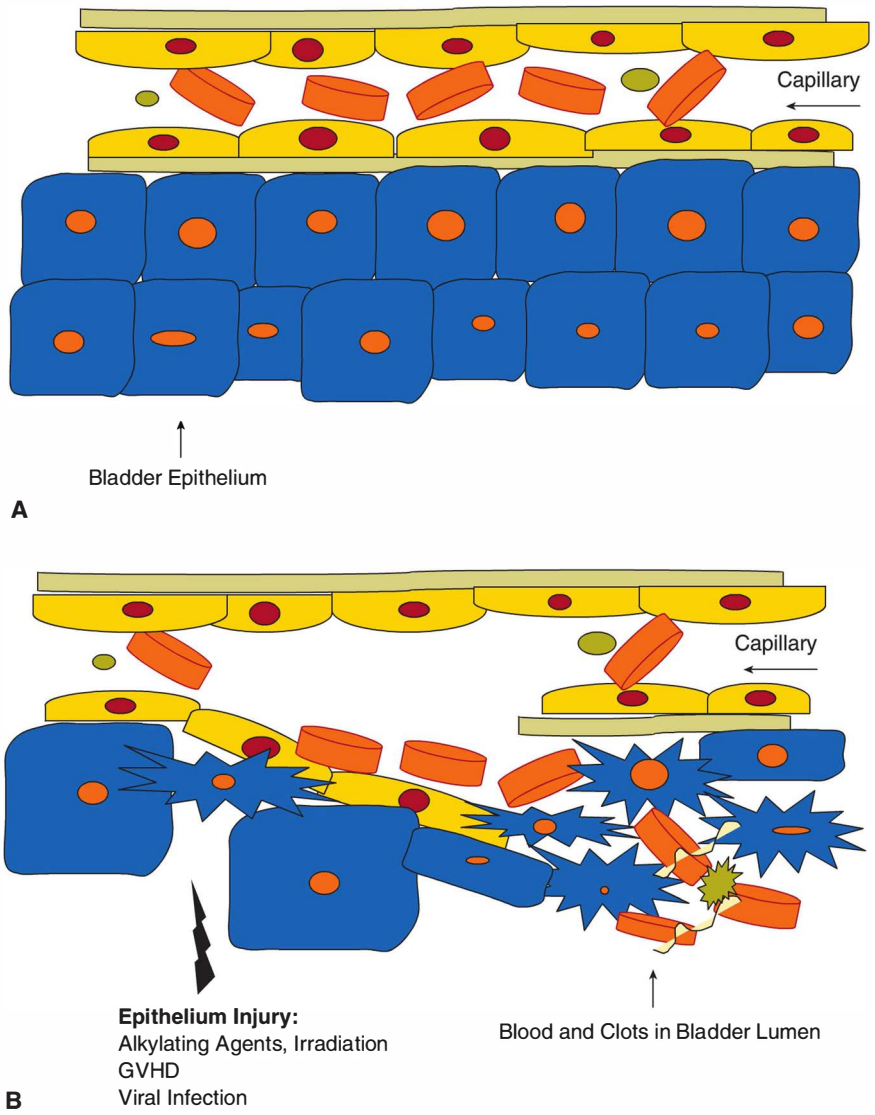

FIGURE 103.4 Epithelial damage in hemorrhagic cystitis. **A:** Normal bladder epithelium. **B:** Epithelial injury resulting in mucosal bladder alterations such as edema, necrosis, and hemorrhage.

conditioning regimens and mismatched donors increase the incidence of bleeding and thrombosis. Given that the ability of treating patients with severe thrombocytopenia, frequently refractory to platelets transfusions, is currently limited, the search for hemostatic agents that do not significantly interfere with coagulation system, such as defibrotide, is essential.

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CHAPTER 104

Overview of Therapeutic Agents Used in Thrombotic Disorders

SAM SCHULMAN AND VICTOR J. MARDER

In this section, the many recent developments in drug and device therapy for venous or arterial thromboembolism are presented together with reviews of the biochemistry, pharmacology, mechanism of action, and side effects of the various agents. Thrombolytic therapy has gone through many modifications regarding both agents and regimens over the past 40 years from the initial infusions of streptokinase or urokinase. An important reason for the many attempts to improve this kind of therapy has been the wish to reduce the risk of bleeding and to simplify the treatment. Conversely, the drive to improve treatment with antiplatelet agents during the past 30 years has mainly been related to a desire to improve efficacy. Among the anticoagulants, vitamin K antagonists (VKAs) and unfractionated or low molecular weight heparins have dominated the clinical stage until the last decade. Recently, an unprecedented number of new anticoagulants have been taken to phase II and III clinical trials, and the standard therapy is about to undergo a shift in paradigm. Finally, vena cava filters have been refined to enhance retrieval after they have served their purpose, and sophisticated catheters for local injection of thrombolytic agents in combination with mechanical clot disruption are for the first time investigated in randomized control trials.

This introduction to the section focuses on some therapeutic dilemmas that will continue to haunt the clinicians for the foreseeable time, despite improvements of the therapeutic agents.

ANTICOAGULANTS AND COMPLIANCE

The VKAs are very effective—as long as they are taken as ordered and the proportional time in therapeutic range (TTR) is high. Even in well-conducted randomized clinical trials, about 30% of the time is spent outside of the therapeutic range.[1,2] Many countries have difficulties keeping an average TTR above 50% due to their therapeutic traditions. The new orally available anticoagulant agents may improve the efficacy by reducing the risk of nontherapeutic levels related to drug–drug or drug–food interactions. Poor compliance from the patients will, however, continue to result in treatment failures. An attempt to solve this problem was seen with the development of a very long-acting, more negatively charged derivative of the pentasaccharide fondaparinux. Idraparinux, with a half-life of 80 hours, was designed to be injected once a week, but with the actual experiences from phase III clinical trials and a half-life after multiple injections of 60 days,[3] it might have been suitable for even less frequent administration. This could have been a helpful solution for the poorly compliant patients employing relatively inexpensive support from medical staff.

The favorable results from treatment of deep vein thrombosis (DVT) in phase II,[4] were replicated with the selected dose of 2.5 mg idraparinux also in phase III in patients with DVT.[5] Nevertheless, the same dose was inferior regarding efficacy in the treatment of pulmonary embolism (PE),[5] and, conversely, it caused an increased risk of bleeding, particularly intracranial bleeding, compared to warfarin in patients with atrial fibrillation[6] (Table 104.1). The trial program of idraparinux has been discontinued, but the dilemma of how to best manage poor compliance will be highlighted with the introduction of new oral anticoagulants without the need for routine laboratory monitoring.

Furthermore, this lesson has taught us that results from trials in DVT cannot be directly extrapolated to other types of patients not only to those with atrial fibrillation but also to the very closely related condition PE. Although often thought of as one and the same disease, PE and DVT are probably best described as different expressions of a disease—with discrepancies regarding clinical picture, short-term prognosis in case of missed diagnosis,[7] location of recurrence,[8] and case fatality of recurrences.[9]

With respect to the issue of compliance, the use of the new, nonmonitored anticoagulants will require imaginative steps to ensure adherence. Otherwise, the promising results from clinical trials[2,10] (EINSTEIN) will not be replicated in the real-world setting. The solutions may include clinical follow-up with unannounced point-of-care screening test, for example with activated partial thromboplastin time for oral thrombin inhibitors. It would be of interest to develop a test that identifies activation of coagulation over prolonged periods, corresponding to the HbA1c for diabetes. Another option is the use of "intelligent" drug packaging that records removal of tablets and alarms when a dose is missed.[11]

Finally, in terms of new development of glycosaminoglycans—our oldest clinically used anticoagulant—the most recent agent currently in phase III trials is a hemisynthetic heparin with a molecular weight of only 2,000 to 3,000 Da (semuloparin, AVE5026), which makes it almost a pure factor Xa inhibitor.[12] Whether it can be adapted for oral administration, in view of its low molecular weight, is unclear, but previous attempts with longer glycosaminoglycans have only had limited success.[13,14] The glycosaminoglycans are reviewed in

Table 104.1

Example of discrepant results with the same drugs (warfarin with therapeutic range, INR 2–3, vs. idraparinux 2.5 mg daily) for different indications

	Idraparinux (%)	Warfarin (%)	Hazard Ratio (95% CI)	Interpretation
Efficacy				
DVT: Recurrence	3.7	3.7	1.01 (0.66–1.55)	Noninferior
PE: Recurrence	4.0	2.0	2.09 (1.22–3.57)	Inferior
Atrial fibrillation: Stroke, systemic embolism	0.9	1.3	0.71 (0.39–1.30)	Noninferior
Bleeding				
DVT: Major	0.8	1.2	NA	Noninferior, $P = 0.35$
PE: Major	1.1	2.1	NA	Borderline superior, $P = 0.05$
Atrial fibrillation: Intracranial	1.1	0.4	2.58 (1.18–5.63)	Inferior
Death				
DVT	2.3	2.0	NA	Noninferior, $P = 0.61$
PE	5.1	2.9	NA	Inferior, $P = 0.006$
Atrial fibrillation	3.2	2.9	NA	Noninferior, $P = 0.49$

CI, confidence interval; NA, not available.

detail regarding their pharmacology and mechanism of action in Chapter 105.

DURATION OF ANTICOAGULATION AFTER VENOUS THROMBOEMBOLISM

For most of the indications for anticoagulant treatment, until now mainly with VKAs, the duration is minimally disputed. Thus, 1 to 3 months is considered sufficient after cardioversion for atrial fibrillation, 3 months after anterior myocardial infarction and/or left-ventricle thrombus, ablation therapy for atrial fibrillation with low risk for stroke, and (if at all) after bioprosthetic valve replacement or postoperative transient atrial fibrillation. Indefinite treatment duration is chosen for patients with mechanical heart valves or atrial fibrillation with risk factors for stroke. The situation is different for venous thromboembolism (VTE) for the following reasons: (a) the risk of recurrence seems to remain indefinitely, albeit with some decrease over time, (b) the balance between recurrence without treatment and bleeding with treatment is tight for many patients with suboptimal control of the anticoagulation, (c) the individual risk of recurrence varies widely depending on the presence or absence of a large number of known risk factors, and (d) despite more randomized trials than for any other indication no clear answer has emerged.

For a minority of patients—those with a distal DVT provoked by temporary risk factors such as surgery or trauma—6 weeks of therapy seems sufficient.[15,16] At the other end of the spectrum are the patients with unprovoked and recurrent VTE, or thromboembolism associated with noncurable cancer or with serious thrombophilia (deficiency of antithrombin or combinations of defects). The vast majority of patients with VTE fall between these groups. Since several studies on the duration of treatment showed that the risk of recurrence after unprovoked VTE was 10% during the 1st year after stopping anticoagulation, irrespective of the treatment duration being 3, 6, 12, or 24 months, it has been suggested that the choice should be between 3 months and indefinite duration.[17] This dichotomous decision should be influenced by the risk of bleeding and the quality of anticoagulant management provided.

Common risk factors for recurrence are male sex,[18] cancer,[19] proximal location of the DVT,[20] and elevated level of factor VIII[21] or of D-dimers.[22] Absence of most of these risk factors as well as of the relatively common mutations, factor V Leiden, and prothrombin G20210A is not very helpful in the prediction of thrombosis-free survival,[23] but a negative D-dimer has a negative predictive value of 93% and a likelihood ratio associated with the absence of pos D-dimer, LR(–) was 0.58. Therefore, if an additional and objective decision support is needed, the use of this risk factor is probably the most cost-effective. A negative D-dimer after anticoagulant treatment for 3 to 6 months with subsequent discontinuation and a repeat negative D-dimer 1 month later predicts an annual risk of recurrence of 3.5%.[24] This risk is for most patients and physicians acceptably low in comparison with an annual risk of major bleeding on anticoagulation of about 2% per year and the inconvenience

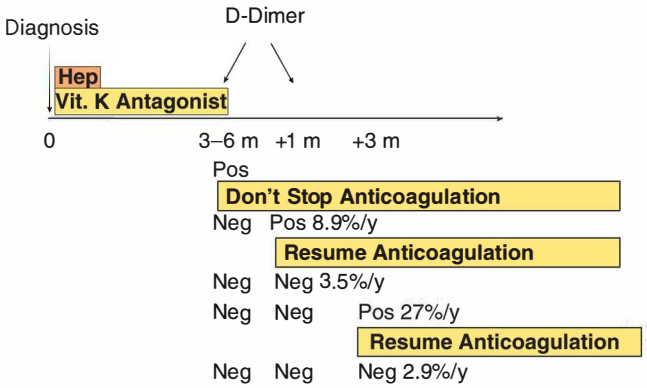

FIGURE 104.1 The use of D-dimer to predict risk of recurrent VTE. Rates (in %) show risk of recurrence during 1 year.

of treatment. With a third negative D-dimer, 3 months after stopping anticoagulation, some additional narrowing of the risk for recurrence is possible[25] (FIGURE 104.1).

A somewhat more sophisticated decision algorithm includes the sex of the patient and the location of the VTE, as presented in the Vienna model.[26] A cumulative recurrence rate for 12 months and for 60 months is obtained, and then the physician must decide what risk is acceptable. The data regarding the influence of the location of the VTE are, however, controversial since a large Canadian study demonstrated higher risk for recurrence after DVT than after PE,[20] which is in contrast to the Vienna model rule.[26]

Finally, the decision can be individualized according to a combination of risk factors for thrombosis and for bleeding and by also taking into account patient preferences (FIGURE 104.2).[27] This requires the physician to spend some time with the patient to obtain a good medical history and to discuss the options and understand the preferences of the patient.

With the introduction of new oral anticoagulants, such as dabigatran, rivaroxaban, or apixaban, the decision process may

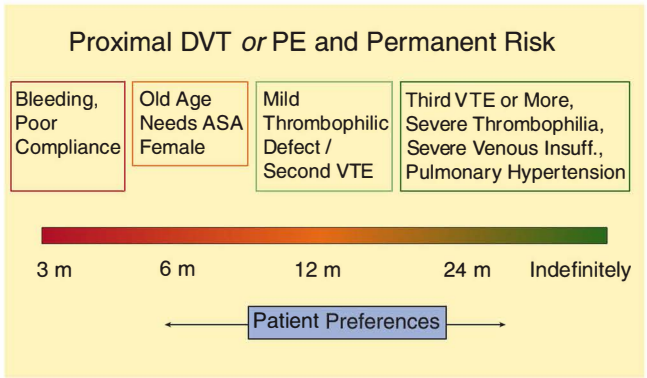

FIGURE 104.2 Individual decision tool for duration of anticoagulation after unprovoked VTE. ASA, acetylsalicylic acid; PE, pulmonary embolism; DVT, deep vein thrombosis. (From Schulman S, Ogren M. New concepts in optimal management of anticoagulant therapy for extended treatment of venous thromboembolism. *Thromb Haemost* 2006;96:258–266.)

change with a preference for long-term treatment. This prediction is based on the eliminated need for frequent laboratory monitoring, dose adjustments, and limitations regarding diet and use of interacting drugs as well as possibly lower risk of bleeding compared to warfarin.[10]

The biochemistry and pharmacology of VKAs are described in Chapter 106, whereas the duration of secondary prophylaxis after VTE is discussed further in Chapter 87. The above-mentioned new anticoagulants are presented in more detail in Chapter 110.

COMBINATIONS OF ANTIPLATELET AGENTS AND VITAMIN K ANTAGONISTS—"TRIPLE THERAPY"

Whereas some thromboembolic conditions are more effectively prevented or treated with anticoagulants, others require antiplatelet agent(s). The former include VTE, stroke prophylaxis in atrial fibrillation or mechanical heart valves. Conversely, for patients with coronary stents, stroke prophylaxis in carotid stenosis, or secondary prophylaxis after acute coronary syndromes (ACSs), platelet inhibition seems to be the most effective strategy. The situation becomes complex when a patient has concomitant conditions from both of these groups. Typical scenarios are (a) a patient with atrial fibrillation, already receiving stroke prophylaxis with a VKA, and then due to ACS managed with percutaneous coronary intervention (PCI) and stent insertion or (b) anterior myocardial infarction with development of left-ventricle aneurysm and thrombus but also treated with primary PCI and insertion of a stent.

Combinations with an increasing number of antithrombotic agents augment inevitably the risk of bleeding. In a registry study on more than 400,000 patients, the risks for bleeding with different agents and combinations were compared (Table 104.2).[28] The risk of bleeding requiring hospitalization in patients with triple therapy was 12.0% per year, which reasonably corresponds to the 1st year since hardly any patients receive this combination longer than that. The risk of bleeding is usually highest initially, partly due to proximity to invasive procedures and partly because patients with a high risk of bleeding will manifest the complication early and are thereafter weeded out. A meta-analysis of observational studies on triple therapy demonstrated a pooled bleeding rate of 2.2% during the 1st month.[29] With these two studies, it is plausible that the risk of major bleeding on triple therapy is 2% the 1st month and thereafter 1% per month. With aspirin + clopidogrel, the annual rate of bleeding is one-third of that on triple therapy.

The annual risk of stroke risk in patients with atrial fibrillation and a mean $CHADS_2$ score[30] of 2.0 is 5.5% for patients on aspirin + clopidogrel versus 3.7% for those continuing previous therapy with warfarin (relative risk 1.50), as shown in the ACTIVE W trial.[1] The annual risk of stroke without prophylaxis according to the $CHADS_2$ risk stratification is 1.9 for score 0, 2.8 for 1, 4.0 for 2, 5.9 for 3, 8.5 for 4, 12.5 for 5, and 18.2 for 6.[30] Warfarin reduces this risk by 67%. The bleeding risk increases also with the $CHADS_2$ score but not as much as the stroke risk. In an Italian study, the high $CHADS_2$ scores were a risk factor, independent of age and associated with approximately a doubling of the bleeding rate.[31] From this information, the excess risk of bleeding with triple therapy versus aspirin + clopidogrel and the excess risk of ischemic stroke with aspirin + clopidogrel versus triple therapy can be calculated for different $CHADS_2$ scores (FIGURES 104.3 and 104.4). The excess risk of

Table 104.2

Annual risks and adjusted hazard ratios for bleeding requiring hospitalization on antithrombotic therapy in patients with myocardial infarction

	Annual Incidence (%)	Adjusted Hazard Ratio	95% Confidence Interval	Number Needed to Harm
One Drug				
Aspirin	2.6	Reference		Reference
Clopidogrel	4.6	1.33	1.11–1.59	115.7
VKA	4.3	1.23	0.94–1.61	165.9
Two Drugs				
Aspirin + clopidogrel	3.7	1.47	1.28–1.69	81.2
Aspirin + VKA	5.7	1.84	1.51–2.23	45.4
Clopidogrel + VKA	12.3	3.52	2.42–5.11	15.2
Three Drugs				
Aspirin + clopidogrel + VKA	12.0	4.05	3.08–5.33	12.5

Adapted from Sørensen R, Hansen ML, Abildstrom SZ, et al. Risk of bleeding in patients with acute myocardial infarction treated with different combinations of aspirin, clopidogrel, and vitamin K antagonists in Denmark: a retrospective analysis of nationwide registry data. *Lancet* 2009;374:1967–1974.

bleeding is always higher than the excess risk of ischemic stroke, for any duration of therapy and for any $CHADS_2$ score. The excess bleeding risk with triple therapy is so much higher than the excess stroke risk with aspirin + clopidogrel for the low $CHADS_2$ scores that it seems unreasonable to recommend triple therapy. This is the case even if only 20% to 25% of the major bleedings are intracranial. However, for the high $CHADS_2$ scores and with drug-eluting stents, requiring 6 to 12 months of aspirin + clopidogrel, it may be justified to add warfarin, if most of the excess bleedings are gastrointestinal.

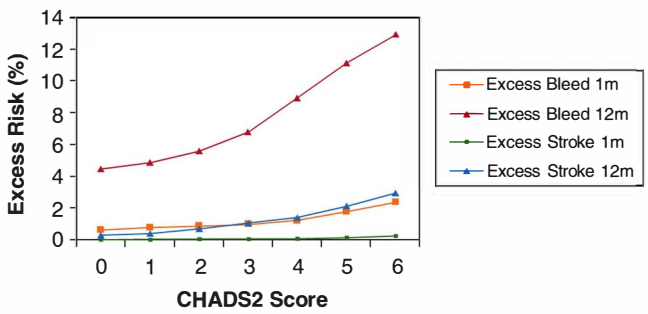

FIGURE 104.3 Excess risk of bleeding with triple therapy (aspirin + clopidogrel + warfarin) versus aspirin + clopidogrel compared to excess risk of stroke with aspirin +clopidogrel versus triple therapy for patients with atrial fibrillation and different risk levels for stroke according to $CHADS_2$ score. (Adapted from Gage BF, Waterman AD, Shannon W, et al. Validation of clinical classification schemes for predicting stroke: results from the National Registry of Atrial Fibrillation. *JAMA* 2001;285:2864–2870.) Results are shown for 1 month of therapy (*bare metal stent*) and for 12 months (*drug-eluting stent*). See text for further explanation.

Aspirin and clopidogrel have drawbacks with interindividual differences in efficacy and lacking a reliable laboratory method to predict the clinical response. New antiplatelet agents such as the direct-acting ADP-receptor $P2Y_{12}$-inhibitor ticagrelor seem to be more predictable than clopidogrel and may change the scenario for complex patients.[32] Bleeding complications on anticoagulant treatment are discussed in more detail in Chapter 107 and the old and new antiplatelet drugs are described in Chapter 111.

Several new drugs for humans have their origin in the animal kingdom or sometimes from the most primitive organisms (streptokinase from streptococci). Refined derivatives of animal proteins may prove very useful with improved risk: benefit ratio. One example is bivalirudin,[33] developed from the salivary protein hirudin from the medicinal leech. Procoagulant proteins from animals are useful as components in coagulation assays. These topics are dealt with in Chapter 113.

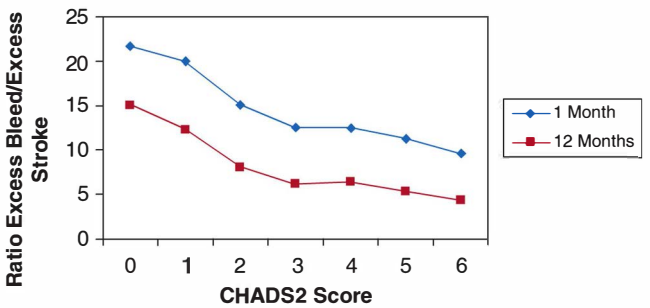

FIGURE 104.4 Ratio of risk of excess bleeding (with triple therapy) to risk of excess stroke (with aspirin + clopidogrel), based on results in FIGURE 104.3.

Despite all advances in drug therapy, there are sometimes important contraindications to the use of anticoagulants, treatment failures, or large limb- or life-threatening thrombi. In those situations, mechanical devices or mechanical thrombus removal may be required, as described in Chapter 114.

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CHAPTER

105 Pharmacology and Mode of Action of Heparin and Glycosaminoglycans

MEYER MICHEL SAMAMA, JEANINE M. WALENGA, EVI KALODIKI, AND JAWED FAREED

Heparin belongs to a class of linear acidic polysaccharides known as glycosaminoglycans (GAGs). Unfractionated heparin (UFH) came into clinical use in the 1930s, making extracorporeal circulation and cardiovascular surgery possible. Despite its discovery almost 100 years ago, heparin is still a life-saving compound, crucial for procedures such as treatment of deep venous thrombosis (DVT), pulmonary embolism (PE), and acute coronary syndrome (ACS), as well as for anticoagulation in surgery, interventional cardiology, and hemodialysis.

Low molecular weight heparins (LMWHs) are depolymerized heparin derivatives developed in the 1980s, with pharmacologic profiles distinct from UFH.[1] Other heparin-related GAGs (heparinoids) of clinical interest include heparan sulfate, dermatan sulfate, chondroitin sulfate, and heparinoids.

There have been major developments recently for new anticoagulant drugs including advancements in new oral anticoagulants. However, heparins continue to be widely used as a clinical anticoagulant.

UNFRACTIONATED HEPARIN

History of the Discovery of Heparin

Jay McLean[2] discovered heparin in 1916 by serendipity. He was at that time a medical student in the physiology laboratory of William Henry Howell at Johns Hopkins University Hospital in Baltimore, working on dog liver in search of a procoagulant substance, not an anticoagulant.[3,4,5]

The first publication was authored by McLean[2] without the name of Howell, who was skeptical that a real anticoagulant had been discovered. Yet Howell made McLean fight the rest of his life for recognition that the great discovery of heparin was in fact McLean's.

Howell[6] gave the term heparin (Greek for liver "hepar") to the anticoagulant material discovered by McLean. Finally in 1963, 6 years after McLean's death, the New York Academy of Medicine presented to Johns Hopkins a plaque in recognition of McLean's work.[4,7]

Several researchers worked on the purification of heparin including Charles Best.[8] The story of heparin also includes a history of the discovery of its primary mechanism of anticoagulant activity through binding to the endogenous protease inhibitor antithrombin (AT),[9,10] as outlined (see also Chapter 18):

- 1895: Discovery of thrombin inhibition by AT[11]
- 1916: Discovery of heparin (McLean)[2]
- 1935: Heparin active only in the presence of heparin cofactor (Brinkhous)[9]
- 1950: Confirmation of AT as heparin cofactor (Seegers' group and others)[12,13]
- 1968: Isolation of AT (Abildgaard)[10]
- 1973: Procedure to obtain large amounts of AT (Rosenberg)[14]
- 1976: AT-binding site separation of active and inactive heparin[15]
- 1980: Heparin oligosaccharides with only anti–factor Xa (anti-FXa) activity (Lindahl; Choay)[13,16]

The ultimate proof of AT as a cofactor for the anticoagulant activity of heparin was provided by a specific pentasaccharide sequence[17] and its subsequent chemical synthesis,[16,18] which became the pharmaceutical compound fondaparinux.

Chemistry of Heparin

Heparin is a heterogeneous natural polysaccharide (FIGURE 105.1), the complexity of which makes its study of pharmacologic characteristics difficult.[19,20]UFH is a biologic product isolated from mammalian tissues, most commonly porcine intestinal mucosa or bovine lung. Heparin is synthesized in these tissues by mast cells as part of a high molecular weight (MW) proteoglycan (MW 750,000–1,000,000 Dalton [Da]), which consists of a center core protein composed of 20 to 25 residues each of glycine and serine.[21] There are 15 carbohydrate chains[22] (MW 40,000 to 300,000 Da) attached to the core peptide via a galactosyl–galactosyl–xylosyl trisaccharide sequence.[23–25] These chains are polymers of disaccharide repeats that are highly sulfated and therefore negative in charge. Polysaccharide chain elongation and a series of modification reactions of the polymer occur in the final synthesis step.[26,27]

The GAGs attached to the proteoglycan central core protein include chondroitin sulfate, heparan sulfate, and heparin.[28] Chondroitin sulfate is composed of repeats of glucuronic acid and D-acetyl galactosamine with sulfate groups in the 4 or 6 position of the galactosamine. Its sulfate/carboxylate ratio is equal to 1.0; its sulfur content is ≥5%. Dermatan sulfate is a copolymer composed of repeated iduronic acid and D-galactosamine. It has anti–factor IIa anticoagulant activity.

Heparan sulfate consists of repeats of glucuronic or iduronic acid and *N*-acetylglucosamine. Total sulfate content and sulfate/carbohydrate ratios differ between GAG types, which play a role in the biologic processes of tissue physiology,[29] particularly cell adhesion, cell migration, and cell differentiation, as well as in coagulation, fibrinolysis, and inflammation.[28]

Four enzymatic modifications of the polysaccharide backbone occur following its synthesis. The majority of the *N*-acetyl groups on the glucosamine residues are removed,[30] and the *N*-deacetylated glucosamines are subsequently sulfated. In the next step, D-glucuronic acid residues are epimerized to L-iduronic acid units by uronosyl C-5 epimerase.[31,32] During the epimerization process, most iduronic acids are 2-O sulfated.

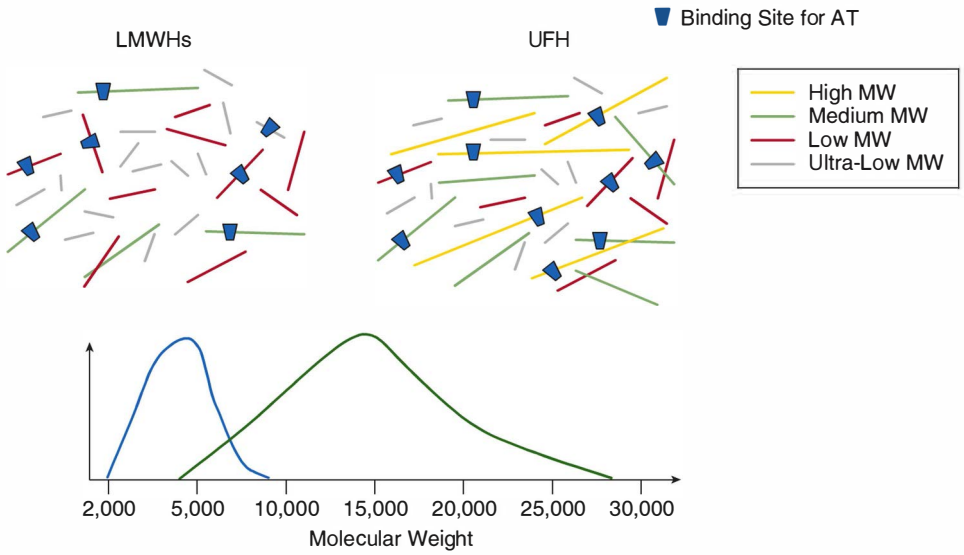

FIGURE 105.1 Characteristics of UFH and LMWHs (Source Material = Porcine Intestinal Mucosa Heparin).

Finally, 3-O and 6-O sulfate groups are added onto the glucosamine units. *N*-sulfation allows more efficient O-sulfation to occur.[22]

Heparin consists of a mixture of GAG chains that are made of disaccharide subunits and possess a reducing end and a nonreducing end (FIGURE 105.2). The heparin backbone is composed of alternating sugar units of α-D-glucosamine (*N*-sulfated, or *N*-acetylated, O-sulfated) and O-sulfated uronic acids (α-L-iduronic acid or β-D-glucuronic acid).[33] It is the linear pattern of the disaccharide units and the placement of specific disaccharide units within a certain chain length that impart the various and heterogeneous biologic activities of heparin. The most notable illustration of the heterogeneous sulfation pattern is the presence of a particular 3-O sulfate group required for specific interaction of heparin with AT.[34] This 3-O sulfate group is present on only a small percentage of heparin chains.

The mean MW of UFH is around 12,000 to 15,000 Da, with individual polysaccharide heparin chains varying in MW from 3,000 to 30,000 Da (FIGURE 105.1).[19,35]

Models for Evaluation of Anticoagulant Activity of Heparin

The Wessler model of human serum-induced thrombosis (1959), based on the teachings of Virchow,[36] was a commonly used model to determine whether heparin fractions and LMWHs possessed an anticoagulant effect. In this model, an intravenous (IV) serum injection is followed by ligation of an isolated segment of the jugular vein.[37] After a 10- or 20-minute period, a full red cell-fibrin thrombus forms in controls, but clot formation is inhibited in the presence of an anticoagulant. Variations of this thrombosis model utilize thromboplastin, activated prothrombin complex concentrate,

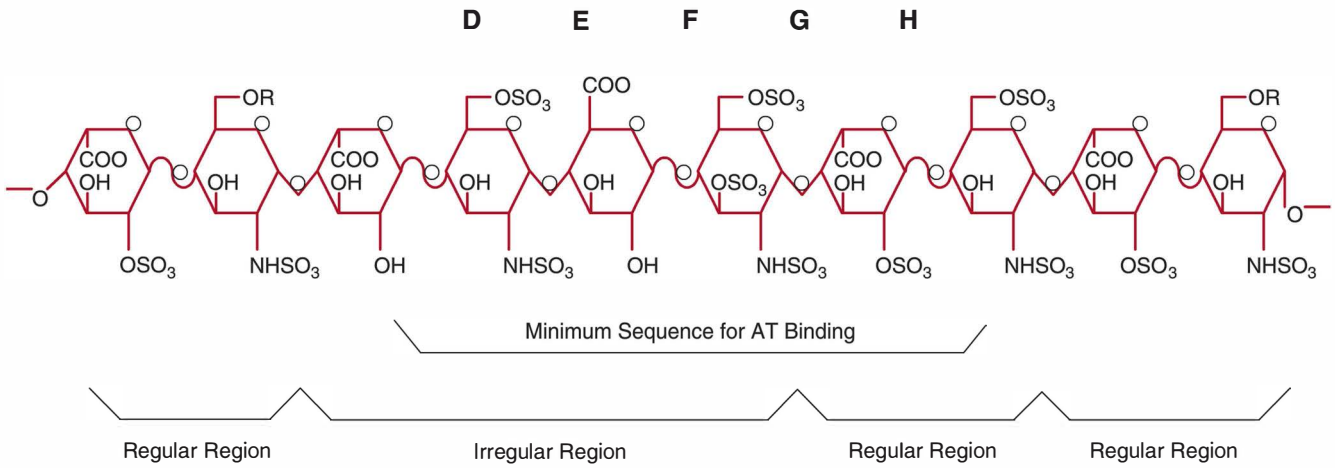

FIGURE 105.2 Heparin chains are a mixture of heterogeneous saccharide unit sequences. An example of the linkage of the disaccharide subunits of heparin is shown. The specific pentasaccharide with high affinity binding to AT is represented by the DEFGH sequence.

or factor Xa as the thrombogenic stimulus.[38] Vena cava ligation using a steel coil, collagen, or kaolin has also been used.[39] These methods are reproducible and allow for the evaluation of the antithrombotic activity of pharmaceutical preparations.[40,41]

Investigations of the anticoagulant activity of heparin and LMWHs in the arterial system were largely performed in a model of vessel wall damage-induced thrombosis using chemical damage ($FeCl_3$), mechanical crushing, or electrical stimulation.[42,43] The Folts model using stenosis and compression injury of the right common carotid artery in the rabbit was another popular model.[44,45]

Mechanisms of the Anticoagulant Action of Heparin

The anticoagulant effect of heparin is primarily attributed to inhibition of the serine protease coagulation factors mediated through heparin binding to these plasma proteins.[46] The most important anticoagulant activities are the inhibition of the coagulation factors thrombin (factor IIa) and factor Xa by heparin bound to AT.

Heparin has a polycomponent anticoagulant mechanism inhibiting numerous coagulation factors, inhibiting platelet function, and enhancing the antithrombotic functions of vascular endothelium as well as fibrinolysis. In addition, by inhibiting thrombin, heparin affects thrombin-mediated coagulation mechanisms involving factor V, factor VIII, protein C, and thrombin activatable fibrinolytic inhibitor (TAFI). It also releases tissue factor pathway inhibitor (TFPI),[1,47] inhibits the release of P-selectin (an adhesion molecule that in the presence of tissue factor and microparticles modulates the interaction between thrombosis and inflammation),[48,49] impairs vascular barrier properties, and attenuates nitric oxide-mediated vasodilatation during dynamic changes in blood flow.[50]

Antithrombin

The primary mechanism of heparin is through association with AT, a 55,000-Da single-chain glycoprotein, with a plasma concentration of 2 to 3 μM.[51–53,54] Heparin binding produces an allosteric modification of AT, which promotes stable complexes, exposing the arginine-385 residue of AT, allowing for interaction with the serine-active site of coagulation enzymes.[14,54,55,56]

In the presence of heparin, inhibition of coagulation is accelerated 300 to 2,000-fold more than by AT alone.[14,57–59] Some of the higher MW heparin chains possess two binding sites for AT (FIGURE 105.3).[60] The longer heparin chains (i.e., chains of >18 saccharide units; >5,400 Da) bind both AT and the activated coagulation factor, whereas the shorter heparin chains (e.g., pentasaccharide; 1,727 Da) bind only AT.[61–63] Single-site binding of short heparin chains to AT is sufficient to inhibit factor Xa (anti-FXa activity), whereas a second binding site on the higher MW heparin chains is required for the binding and inhibition of thrombin (anti-FIIa activity), FXIIa, FIXa, kallikrein, etc. Thrombin is 10-fold more sensitive to inhibition than factor Xa.

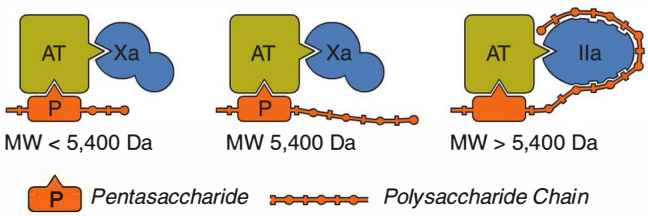

FIGURE 105.3 Schematic representation of the interaction of heparin, AT, FXa, and thrombin.

Structure-activity studies have identified a unique minimum saccharide sequence that binds with high affinity to AT (FIGURE 105.2), causing inhibition of FXa but not thrombin.[41] Heparin chains that lack this particular pentasaccharide sequence have minimal anticoagulant activity.[34]

Heparin-bound AT weakly inhibits coagulation factors IXa, XIa, and XIIa, as well as the enzymes trypsin, plasmin, and kallikrein,[61,64] and requires high concentrations of AT (~5 units). A weak inhibitory activity of this complex toward factor VIIa has been described, but the mechanism is unknown.[65]

Heparin Cofactor II

Heparin cofactor II (HCII) is a second plasma inhibitor (MW 60,000 Da) that binds heparin and inhibits thrombin by forming a 1:1 stoichiometric complex with the enzyme. Thrombin interacts with the active site of HCII on the C-terminus and forms a covalent bond.[64] The reaction of HCII and heparin is charge and chain length dependent, but independent of the pentasaccharide sequence.[18]

Whereas the activation of AT is dependent upon a specific sequence in the heparin chain, HCII can be activated by a wide variety of agents, including heparin, heparan sulfate, dermatan sulfate, pentosan polysulfate, and dextran sulfate.[66–68] Agents with relatively little sulfation such as chondroitin 4-O- or 6-O-sulfate, keratan sulfate, and hyaluronic acid do not activate HCII.[69] High and low AT affinity fractions of heparin equally activate HCII if charge density is equal.[70]

Tissue Factor Pathway Inhibitor

TFPI (MW 42,000 Da) is a coagulation protease inhibitor synthesized in the vascular endothelium,[71,72] the largest pool of which is bound to the endothelial cell surface.[47,73,74] Also found in plasma and platelets,[75] TFPI has three Kunitz domains linked in tandem between a negatively charged amino terminus and a positively charged carboxy terminus.[76] TFPI simultaneously binds to and inhibits the factor VIIa–tissue factor complex and FXa via two of its Kunitz domains.

Heparin displaces TFPI bound to endogenous GAGs on the surface of the endothelium.[77–79,80,81] Repeated heparin administration releases TFPI showing no diminishing releasability with TFPI levels reaching 2- to 10-fold over baseline.[77] LMWHs have a variable effect on TFPI release.[82] Neutralization of heparin by protamine sulfate results in a dramatic decrease in the plasma TFPI level.[83,84] The anticoagulant activity of TFPI can be detected by the prothrombin time (PT), the activated partial thromboplastin time (aPTT), and clot-based anti-FXa assays.[85–87]

Other Anticoagulation Properties of Heparin

Platelets

Heparin is a weak platelet agonist, but is also a potent inhibitor of platelet activation induced by thrombin, the LMWHs less so.[88] A weak or no response occurs with very low MW heparins (<3,000 Da), having almost no reactivity on ADP, collagen, epinephrine, and thrombin-induced platelet aggregation. Heparin also binds to and inhibits von Willebrand factor

(vWF), the clinical implication being a reduction of thrombotic risk in general and control of platelet–endothelium interactions in specific.[89] The weaker antiplatelet effect of LMWHs also is associated with a lower bleeding risk, along with a lesser antithrombotic effect than for UFH. The role of low affinity material which does not contain the binding site of AT is not well documented.[90–93]

Fibrinolysis

A weak profibrinolytic effect has been described,[94] but this was not clearly related to the MW of heparin.[95,96] Heparin regulates tissue plasminogen activator and plasminogen activator inhibitor release from endothelial cells, and the anti–FIIa activity of heparin induces a reduced activation of TAFI that facilitates fibrinolysis.[97,98] An increase in the porosity of the fibrin network has been demonstrated.[99] At comparable anti-FXa *in vitro* concentrations, LMWHs exhibit different profibrinolytic activities.[100] The clinical significance of these results remains to be established.

Nonanticoagulant Properties of Heparin

In addition to the heterogeneous mixture of saccharide chains, heparin also contains three functional groups that occupy multiple sites on its individual saccharide units: $-OSO_3^-$, $-NHSO_3^-$, and $-COO^-$.[19,28] This structural diversity induces a diverse range of nonanticoagulant properties. Only 20% to 30% of GAG components produce anticoagulant activity by binding AT, the other 70% to 80% exhibit multiple biologic actions that are only partially understood.[29,101]

Heparin and heparan sulfate are found on cell surfaces, in the extracellular matrix, and in intracellular granules. These GAGs can interact with a large number of proteins and biologic activities of cell proliferation, inflammation, angiogenesis, viral infectivity,[102] and cell function.[103]

Heparin-binding Proteins

Heparin-binding proteins can be divided into 1) proteins that enhance the anticoagulant activity such as AT and HCII found in blood and released by heparin such as TFPI and lipase and 2) proteins that inhibit the anticoagulant activity of heparin such as PF4, histidine-rich glycoprotein (HRG), protamine, and vitronectin.[28,104–106,107] Some of these interactions are dependent on the MW of the heparin molecules, while others require calcium ions.[106]

There are different types of heparin binding.[22,107,108] The first type relates to a specific saccharide sequence of heparin, present on only one-third of the heparin chains, the second is covalent bonding, and the third depends on charge density and chain length.

Histidine-rich Glycoprotein

HRG belongs to the group of proteins that neutralize heparin anticoagulant activity.[109,110] It has an MW of 75,000 Da, has a plasma concentration of approximately 100 mg/L, and binds to heparin and LMWHs. However, only the long-chain fragments of heparin have both anti-FXa and anti–FIIa activities completely neutralized.[106,111] The antiheparin action of HRG requires calcium.[109]

Vitronectin

Vitronectin, also called complement S protein, is present in the plasma (about 400 mg/L) and in the extracellular matrix. Binding of vitronectin to UFH or LMWHs is associated with neutralization of the anticoagulant activity, but this antiheparin effect is of minor importance.[112]

Vascular Cells

The direct interaction of endothelial cells with heparin depends on the MW of the heparin.[113] Once attached, heparin molecules lose their anticoagulant effect and are internalized. Heparin also binds to smooth muscle cells, which could play a role in atherosclerosis.[114,115] LMWHs and pentasaccharide have the same beneficial activity against atherosclerosis.[116] Heparin interacts with endothelial cell growth factors, which results in an enhanced mitogenic activity at the vascular cell lining.[117]

Platelet Factor 4

Heparin bound to PF4 produces an immunogenic response that can lead to the potential life-threatening disorder of heparin-induced thrombocytopenia (HIT)[118–121] (Chapter 108).

Lipases

Lipoprotein lipase and hepatic lipase are two critical enzymes in lipid and lipoprotein metabolism.[122,123] They are released after an IV injection of heparin or LMWH. Repeated injections of heparin or LMWH do not exhaust the release.[124] Elevated levels cause a lipid-clearing lipolytic effect on the blood that is less for LMWHs than for heparin.[125] Lipase release is associated with an anti-FXa activity that is not neutralized by protamine or PF4.[126,127]

Anti-inflammatory Effect

Heparin has anti-inflammatory actions, for example, in inflammatory bowel disease[128,129] and ulcerative colitis.[130] Removal of the 2-0, 3-0 sulfate groups of heparin reduces its anticoagulant activity without impacting its anti-inflammatory effect.[131] Heparin inhibits the release of P-selectin from platelets and endothelial cells and also binds to P-selectin and L-selectin and CD11b/CD18 expressed on leukocytes.[132] Heparin also binds to components of the complement cascade, reducing activation, a process that may account for the reduced early phase of the asthmatic reaction with inhaled heparin, and for protection of the heart from ischemic reperfusion injury.[133–135] Heparin binding to cytokines inhibits the recruitment of neutrophils and reduces proinflammatory cytokine levels, thereby producing immunomodulating and anti-inflammatory actions.[134–136]

Antineoplastic Effect

A link between improved survival and heparin treatment was suggested by Lebeau[137] in cancer patients receiving heparin or LMWH for the prevention or treatment of venous thromboembolism (VTE),[137] an observation that launched fundamental research and clinical trials.[138–141] In both animal studies and clinical trials, heparin has an antiproliferative property, whereby it inhibits cell growth, cell adhesion, cellular microparticle formation, smooth muscle cell proliferation, and tumor growth.[133,142–145,146,147] Fondaparinux is inactive in these respects.[138,139] Heparin has been postulated to have an antimetastatic effect due to a decrease in neoangiogenesis, possibly mediated by binding to vascular endothelial growth factors, cytokines, and adhesion molecules. Heparin could inhibit metastasis by interference with P- and L-selectin–moderated cell–cell interactions. Other mechanisms have been attributed to the modulation of the angiogenic activity of fibroblast growth factor by heparin[140–145,146,147,148] or by nonanticoagulant components that inhibit cancer growth.[149]

Pharmacokinetics of Heparin

Heparin (sodium or calcium salt) is administered either by IV bolus injection followed by continuous infusion or by subcutaneous (SC) injection. Upon entering the blood stream, heparin binds to a variety of plasma proteins, thereby lowering its bioavailability and producing a variable anticoagulant response.[150] Major binding proteins include HRG, PF4, fibronectin, vitronectin, and vWF.[106,111,151–153] Heparin chains of varying MW exhibit different binding capacities reflected in varying pharmacokinetic behavior, with shorter heparin chains exhibiting a higher and more reliable bioavailability and slower clearance than larger chains.

Heparin exhibits complex pharmacokinetics, and its anticoagulant effect is not linearly related to the dose.[35,154–156] The half-life of heparin's anticoagulant activity is approximately 1 hour at therapeutic doses;[157] it increases from 30 minutes following an IV bolus dose of 25 U/kg to 150 minutes following a dose of 400 U/kg.[158]

Heparin is cleared by two mechanisms. The rapid, saturable phase of elimination is due to receptor-mediated internalization of heparin by endothelial cells and macrophages,[158–160] while a slower, nonsaturable renal mechanism is also operative.[161–163] Accumulation of UFH is modest in severe renal deficiency in contrast to LMWH.

Alternative Routes of Heparin Administration

Attempts have been made to give heparin via oral, sublingual, rectal, nasal, pulmonary, topical, and transdermal routes of administration.

Intrapulmonary or Intranasal

Animal studies demonstrated that heparin is efficiently, rapidly, and reproducibly delivered through the lungs.[164,165] In a limited study, 16 VTE patients were treated with intrapulmonary heparin without adverse effects.[166] The intrapulmonary dose of LMWH needed to produce the same anti-FXa level and TFPI release as obtained with SC administration is about 10-fold higher as shown in healthy volunteers.[167]

Topical

Heparin and other GAGs have been shown to enhance the healing process when applied to the surface of an ulcer.[168,169] The mechanism may be through proteinases, which digest necrotic tissue, and the release of growth factors that accelerate granulation and epithelialization.

Oral

Oral heparin formulations have been developed, the prototype being a diamine salt that showed the importance of charge neutralization for transport across the gastrointestinal epithelium.[170,171] Heparin carriers with the highest bioavailability were chitosan/peg-alginate microspheres of heparin with 70% release,[172] nanoparticles with a blend of polyester and polycationic polymethacrylate with 51% to 59% release,[171] and sodium *N*-(8[2-hydroxybenzoyl]amino) caprylate (SNAC) with 38% release.[173] Other absorption enhancers are abrasol, gelucire, and thiolated polymers in combination with reduced glutathione, biodegradable (poly-epsilon-caprolactone and poly-lactic-co-glycolic acid) and nonbiodegradable polycationic polymers. While preclinical and clinical studies suggested that the heparin–SNAC combination facilitated higher absorption of heparin, the clinical development has stalled.[174,175]

Contamination of Heparin

In early 2008, the US Federal Drug Administration recalled several batches of heparin due to the increased incidence of adverse reactions and death.[176] Fatal hypotension from anaphylactoid-type reactions following heparin administration was responsible for over 149 deaths worldwide.[177] Researchers identified a heparin-like semisynthetic contaminant, which was oversulfated chondroitin sulfate (OSCS), in significant amounts (5% to 30%) that appeared to be an intentional adulteration of therapeutic heparin.[177] Varying degrees of high MW dermatan sulfate and heparan sulfate were also detected.[176,178,179] Because LMWHs are produced from UFH, OSCS was also found in various batches of LMWHs.[177,180]

Potency Designation and Standardization of Heparin

The heparin contamination issue that occurred in 2007 to 2008 led the United States Pharmacopeia (USP) to rewrite the heparin monograph in 2009. Simultaneous with the changes to the monograph, the USP instituted a new heparin reference standard, with the intent to harmonize the US heparin unit with the European Pharmacopeial Commission heparin unit based on International Standard for heparin issued by the World Health Organization.

LOW MOLECULAR WEIGHT HEPARINS

Differences Between LMWHs and UFH

The LMWHs are prepared by controlled depolymerization from porcine mucosa-derived heparin (FIGURE 105.1). The LMWHs have approximately one-third the MW of the parent material (MW 3,000 to 10,000 Da) with a mean MW around 5,000 Da (Table 105.1).[181] The different depolymerization processes also cause partial desulfation, reduction in charge density, and other changes in the heparin saccharide chains (Table 105.2).

Table 105.1

Biochemical and pharmacologic characteristics of UFH and LMWH

	UFH	LMWHs
Mean MW (Da)	15,000	5,000
Anti-FXa/anti–FIIa ratio	1	2–7
Bioavailability at small doses	Poor	Very good
Binding to endothelium	Yes	Low
Dose-dependent clearance	Yes	No
Affinity for plasma protein binding	High	Low
Neutralization by protamine	Yes	Partial
Heparin-induced thrombocytopenia	Rare	Very rare
Osteoporosis	Rare	Very rare

Table 105.2

Characteristics of LMWHs

Depolymerization Method	MW (Da)	Anti-FXa (U/mg)	Anti-FIIa (U/mg)	Ratio
Alkaline depolymerization				
• Enoxaparin	4,034	105	27	3.9
Heparinase depolymerization				
• Tinzaparin	4,500	83	45	1.8
Nitrous acid				
• Nadroparin	4,279	95	27	3.5
• Reviparin	4,395	130	40	3.3
• Dalteparin	5,663	130	58	2.2
• Certoparin	4,959	88	32	2.8
Peroxidative cleavage				
• Parnaparin	3,190	88	30	2.9
• Ardeparin	6,000	100	30	2.0

Impact of manufacturing processes.
Different manufacturing processes produce different LMWH products.

The LMWHs are similar to UFH in their biologic properties yet, because of their lower MW, certain of their biologic properties differ.[181,182] Only heparin molecules that contain at least 18 saccharides (corresponding to a MW of ~5,400 Da) simultaneously bind to AT and thrombin, which is required for the inactivation of thrombin by the heparin–AT complex. Smaller heparin molecules are only able to bind to AT and catalyze the inactivation of FXa.[182] By definition, the ratio of anti-FXa activity to anti–FIIa activity for UFH is 1, whereas the ratio for the originally developed LMWHs ranges from two to four.[181] Newer LMWHs have ratios up to 7.

The LMWHs in experimental models of venous thrombosis are active antithrombotic agents.[183] The increased specificity for anti-FXa activity with LMWHs seems to result in the inhibition of thrombosis with a reduced risk of bleeding.[158,181] Reduced molecular size also decreases nonspecific binding of LMWHs to plasma proteins.[184] Specifically, LMWHs exhibit less interaction with PF4, resulting in a reduced risk of HIT.[185,186]

The pharmacologic differences between LMWHs and UFH result in several practical advantages for LMWHs. First, the bioavailability following SC administration is much higher with LMWHs.[35,187] As a consequence of reduced binding to cells and plasma proteins, LMWHs have a longer half-life, 3 to 6 hours, with more predictable anticoagulation.[187] These properties permit convenient once-daily or twice-daily SC administration without the need for routine monitoring or dose adjustments[187] (Tables 105.3 and 105.4). After SC administration, circulating anti-FXa activity is not detectable in samples collected 12 hours after the administration of prophylactic doses of LMWHs.[182]

Distinct Biochemical and Biologic Properties of Different LMWHs

Commercial LMWH preparations are derived from heparin using a number of different depolymerization processes to cleave the longer saccharide chains (Table 105.2). Each manufacturer uses a distinct chemical (β-elimination, nitrous acid, oxidative cleavage), enzymatic (β elimination), or physicochemical (γ irradiation) process; thus specific LMWH products differ substantially from one another in their physical structure, biologic properties, and pharmacologic properties.[181,184,187,188]

Although the average MW of LMWHs is similar, the proportion of the different saccharide chain lengths differs within each LMWH, and the end-residue alterations of the heparin fragments are specific to the depolymerization method.[189] Some LMWHs are characterized by the presence of a sulfated pentose at the reducing end of the molecule or double bonds at the reducing terminus.[181,188] Although enoxaparin and tinzaparin are derived via β-elimination reactions, only enoxaparin contains specific 1,6-anhydromanno and 1,6-anhydrogluco groups at the reducing end.[190]

Studies comparing different LMWHs using standard analytical methods have demonstrated considerable biochemical differences between the different products.[181,191–193] The anti-FXa

Table 105.3

Clinical Indications for UFH

1. Prophylaxis of VTE[251,252]
2. Treatment of VTE[251,252]
3. Prophylaxis and treatment of atrial fibrillation with systemic embolization
4. Treatment of acute and chronic consumptive coagulopathies (disseminated intravascular coagulation)
5. Prevention of clotting in cardiac surgery
6. Prevention of clotting in hemodialysis procedures
7. As an anticoagulant in:
   i. blood transfusions
   ii. blood samples for laboratory purposes

Table 105.4

Clinical Indications for LMWHs

1. For VTE prophylaxis:
   After total hip and knee replacement surgery[253]
   After abdominal surgery[254]
   In high-risk medical patients[255]
2. For the treatment of VTE
3. For long-term prophylaxis against VTE in pregnant women[256]
4. For long-term secondary prophylaxis after VTE in cancer[146]
5. For treatment of unstable angina[257]
6. In thrombolytic therapy
7. For treatment of superficial thrombophlebitis[258]

activities ranged from 80 to 160 international units (IU)/mg, and the anti-FXa:anti–FIIa ratios ranged from 2:1 to 8:1.[193] The HCII affinity, thrombin generation inhibition, PF4 and protamine neutralization, cellular interactions, and release of mediators from the vascular endothelium also vary from product to product.[184,194]

The clearance half-life is not dose dependent in contrast to UFH.[195] The LMWHs are cleared by the kidney, and their biologic half-life is prolonged in patients with renal failure.[196,197] Bioaccumulation of LMWHs at prophylactic doses can be observed in patients with various degrees of impaired renal function and weight extremes, more pronounced for some LMWHs.[198–200] Pharmacologic studies have demonstrated significant differences among the various LMWHs.[184,201,202,203] Differences in efficacy and hemorrhagic risk have also been demonstrated with different LMWHs,[35] suggesting that LMWHs are not clinically interchangeable.

Generic/Biosimilar LMWHs

Several biosimilar LMWHs have been developed and are available worldwide. The biosimilar LMWHs have a similar average MW and anti-FXa potency as their branded counterpart, but biologic and pharmacologic behaviors have not been proven to be the same.[204–209]

SYNTHETIC HEPARIN PENTASACCHARIDE AND ITS DERIVATIVES

Fondaparinux

Fondaparinux (MW 1,727 Da) was the first of a new class of antithrombotic agents distinct from LMWHs and UFH.[210] It is a chemically synthetic pentasaccharide mimicking the active site of heparin that binds to AT, exhibiting only factor Xa inhibitory activity leading to an inhibition of thrombin generation.[18] There is nearly complete bioavailability by the SC route, rapid onset of action, a prolonged half-life in both IV and SC dosing regimens, and it is renally excreted.[211]

While fondaparinux was more effective than enoxaparin in reducing asymptomatic DVT in orthopedic surgery patients, hemorrhagic complications were either comparable to or more frequent than those for the LMWH.[211] Fondaparinux has been used in a limited number of cases with HIT, since it has a low cross-reactivity with heparin antibodies.[212] However, there have also been very rare cases of HIT reported where fondaparinux was implicated.[213]

Idraparinux and Idrabiotaparinux

A long half-life of fondaparinux derivative, idraparinux (1,729 Da), has been obtained by incorporating an additional 3-O-sulfate group in the glucose moiety at the reducing end of the fondaparinux molecule and additional methyl groups.[190,214] This alteration in the chemical structure causes a significantly stronger binding to AT, increased potency to inactivate factor Xa via AT, and increased half-life of elimination (120 to 200 hours), allowing for a weekly rather than a daily SC administration. The complete pharmacokinetic profile is complex and not well understood. After weekly SC administration of 2.5 mg idraparinux over 12 weeks, the elimination half-life increased to about 600 hours, and in clinical trials the elimination half-life was about 60 days after reaching steady state.[215] In clinical application, idraparinux showed a higher risk of PE recurrence compared to warfarin.[216,217] In patients with atrial fibrillation, idraparinux was equivalent to warfarin in terms of efficacy but caused more bleeding.[218] A biotinylated form of idraparinux/idrabiotaparinux (1,853 Da) can be specifically neutralized by the IV administration of avidin.[219] The biotin arm does not interfere with the AT-dependent inhibition of FXa.

DANAPAROID AND RELATED HEPARINOIDS

Danaparoid has been in clinical use for more than 20 years for the prevention and treatment of thrombosis, during pregnancy (does not cross the placenta and is not excreted in breast milk) and hemodialysis.[220] Danaparoid is a mixture of low MW GAG components that result from the manufacture of UFH, mainly comprising heparan sulfate (84%), dermatan sulfate (12%), and chondroitin sulfate (4%), obtained without further depolymerization. Danaparoid has a mean MW of 5,500 Da, half-life approximately 18 hours, and biologic and pharmacokinetic profiles distinct from UFH and LMWH.

Danaparoid has a weaker anti-FXa activity than LMWH, but multiple other biologic targets of danaparoid exert profound effects on cellular responses (anti-inflammatory[221] and antiulcer) and protease regulation by mechanisms other than those mediated by AT and HCII. Despite the sustained anticoagulant activity, the risk of bleeding with danaparoid is low (except in cardiopulmonary bypass where major bleeding can occur).

Due to low cross-reactivity with heparin antibodies, danaparoid has been used to treat patients with HIT.[220] Potential other clinical uses include anticoagulation for percutaneous coronary intervention and treatment of ACS, leg ulcers, cancer, sepsis, and acute ischemic stroke.[222]

Other Heparinoids

Dermatan sulfate has been developed for the prophylaxis of venous thrombosis. Other heparinoids include heparan sulfate and sulfaminoheparosan derivatives derived from bacterial cell wall, but none of these drugs are in clinical use.[223]

LABORATORY MONITORING OF HEPARIN ANTICOAGULANTS

Heparin

The laboratory monitoring of heparin reduces the risk of bleeding and decreases the rate of thrombotic recurrences,[224,225] the therapeutic range of aPTT in patients receiving IV infusion being 1.5 to 2.5 times control.[226] The variability of aPTT to heparin is dependent on the reagent,[227,228] which can be selected with more sensitivity to heparin based on their lipid composition and the concentration of phospholipids.[229]

In an effort to standardize heparin monitoring, the therapeutic range of 1.5 to 2.5 times aPTT control was considered by the American College of Chest Physician (ACCP) as equivalent to 0.2 to 0.4 U/mL heparin by protamine titration or 0.35 to 0.70 U/mL by anti-FXa assay.[20,229] However, the concentration of heparin associated with an aPTT ratio of 1.5 to 2.5 also varies significantly with different reagents.[20,230,231] Since the aPTT ratio and the anti-FXa activity may be discrepant in a given patient, it is recommended by the ACCP that the dose in patients with heparin resistance, who require unusually high doses of heparin to achieve a therapeutic aPTT ratio, should be adjusted on the basis of anti-FXa levels rather than on the aPTT results.[20,232,233] In some groups of patients, often in intensive care units, an unusually low dose of heparin is sufficient to achieve a therapeutic aPTT ratio. The increased response to heparin could be due to congenital or acquired contact factor deficiency.[234] In these patients, monitoring heparin with a chromogenic anti-FXa assay would be a useful alternative.

Low Molecular Weight Heparins

Routine monitoring of LMWHs given at prophylactic doses is not required, but may be used in special clinical situations such as obesity, renal insufficiency, long-term treatment in pregnant women, and pediatric patients.[20] At prophylactic doses, the PT and aPTT are not significantly prolonged by LMWHs. Therapeutic doses prolong the aPTT, although to variable degree with different reagents.[227,228] The test recommended for laboratory monitoring of LMWHs is a chromogenic assay measuring anti-FXa activity,[225,235] the results being expressed in IU of anti-FXa, using a LMWH reference preparation for assay calibration.[225,236,237]

Fondaparinux

Routine monitoring is not required due to predictable pharmacokinetics. A chromogenic anti-FXa measurement can be used, with fondaparinux as the assay calibrator, the results then reported as fondaparinux concentration (μg/mL) and not in IU as for heparin.[238] The aPTT is not affected by fondaparinux.

Danaparoid

Routine monitoring is not required; if desired, the chromogenic anti-FXa measurement can be used with danaparoid as the calibrator.[239] The aPTT is not affected by danaparoid.

SIDE EFFECTS OF HEPARIN THERAPY

The most common side effect of heparin therapy is hemorrhage, which can range from minor to life threatening and is related to the total administered dose and the degree of prolongation of the aPTT rather than the route of administration. Fondaparinux and LMWHs have a lower bleeding risk. Although bleeding can be attributed to thrombin inhibition, the effect of these drugs on vascular endothelium and platelets also contribute to the bleeding. HIT occurs in 0.1% to 5% of patients treated with heparin[118] (see Chapter 108). A rare but potentially lethal acute "anaphylactic" reaction to heparin can also occur.[182] Heparin therapy is also associated with transient elevations in serum transaminase levels, which is likely not of clinical importance. Prolonged exposure to heparin can result in osteoporosis, and skin reactions, eosinophilia, alopecia, and hyperkalemia are rare complications.[182] Multiple anticoagulants, antiplatelet drugs, and thrombolytic drugs have an additive, even synergistic effect, and nonanticoagulant agents can affect the anticoagulant effects of heparin.

NEUTRALIZATION OF HEPARINS

The anticoagulant and bleeding effects of heparin are reversed with IV protamine sulfate and protamine chloride. Protamine is associated with hemodynamic effects, can cause severe hypotension if administered too rapidly,[240] and may also activate the complement system causing pulmonary vasoconstriction, pulmonary hypertension, and peripheral vascular collapse.[241] While protamine effectively neutralizes heparin, it has limited value in the neutralization of LMWHs,[242] only partially reversing the anti-FXa activity.[243] Protamine does not reverse the anticoagulant effect of fondaparinux. Several approaches have been unsuccessful as an antagonist for heparin and LMWHs, including recombinant PF4 and heparinase-1; a salicylamide derivative, PMX-60056, is under development.[244]

FUTURE HEPARIN-DERIVED DRUGS

UltraLow Molecular Weight Heparins

Recently, several newer depolymerized heparin-based agents have been developed, which mimic the anticoagulant and antithrombotic effects of heparin. These include the ultra-LMWHs such as AVE-5026 (semuloparin; enriched in AT-binding oligosaccharides) and bemiparin.[245,246] Several approaches to modify heparin have been used to obtain selective therapeutic agents, including hypersulfation, desulfation, oxidation; agents that have been clinically tested include Astenose, Vasoflux, and M-118, but none is available for clinical use.[247–249]

Heparin Mimicking Oligosaccharides

The elucidation of the mechanism of heparin's anticoagulant action led to the development of synthetic drugs, beginning with the development of the heparin pentasaccharide obtained by *de novo* chemical synthesis[250] followed by idraparinux.[214] Other additions to this class of agents are compounds constructed of a pentasaccharide analogue conjugated via a spacer to a specific thrombin inhibitor group derivative that provides thrombin inhibitory activity.[16,190] EP-42675 uses a specific thrombin

inhibitor that can inhibit both fibrin-bound and fluid-phase thrombin. A biotinylated form, EP-217609, which can be neutralized by avidin, has also been prepared with the same biologic activities.[190]

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CHAPTER

106

Vitamin K Antagonists: Biochemistry, Pharmacology, and Management

NIRMISH SHAH, THOMAS L. ORTEL, AND SAM SCHULMAN

INTRODUCTION/HISTORY

Vitamin K antagonists (VKAs) are currently the most common oral anticoagulants used worldwide. The discovery of VKAs began in 1921 to 1922, with initial investigations into bleeding cattle that had eaten improperly cured hay from the sweet clovers *Melilotus alba* and *Melilotus officinalis*. These cattle were given the diagnosis of sweet clover disease and found to have an increased prothrombin time (PT).[1] In 1939, crystals of the anticoagulant were isolated and the structure was subsequently identified as 3,3′-methylenebis(4-hydroxycoumarin), a conjugation of two molecules of 4-hydroxycoumarin with a methylene bridge. In addition, this dicumarol anticoagulant was found to be counteracted by vitamin K.[2] Although the anticoagulant effect was not understood until the 1970s, efforts then focused on development of coumarin-type compounds.[3] The development and synthesis of these compounds eventually led to patents for both dicumarol and WARF 42, which were held by the Wisconsin Alumni Research Foundation (WARF). WARF 42 was later renamed as warfarin and was initially used as a rodenticide due to its relative nontoxic effects to humans and domestic animals.[4] All coumarin derivatives have a central 4-hydroxycoumarin with a substituent in the 3-position and an asymmetric carbon atom resulting in enantiomers. The anticoagulant effect of both dicoumarol and warfarin quickly became a significant weapon in the arsenal of the cardiac specialist and has remained so to this day. VKA are now used worldwide to treat patients with thrombosis or an increased risk for thrombosis. This chapter reviews the biochemistry and pharmacology of VKA, as well as issues related to the management of patients treated with VKA.

BIOCHEMISTRY

Mechanism of Action

Effect on Coagulation Factor Synthesis/Degradation

Vitamin K is a cofactor for the posttranslational γ-carboxylation of glutamate residues to γ-carboxyglutamates on the *N*-terminal regions of vitamin K–dependent proteins. The anticoagulant effect of VKA is due to interference of the cyclic conversion of vitamin K and its 2,3-epoxide, which blocks the regeneration of the reduced form of vitamin K (FIGURE 106.1A). The VKAs interfere by inhibiting the enzyme vitamin K epoxide reductase, subunit 1 (VKOR*C1*). The reduced form of vitamin K (KH_2) is responsible for the carboxylation of glutamic residues on the procoagulant forms of factors II, VII, IX, and X. By inhibiting vitamin K conversion, coumarins cause hepatic production of partially carboxylated and decarboxylated proteins with reduced procoagulant activity, resulting in their observed anticoagulant effect.[5,6]

Conformational changes in coagulation proteins occur as a result of carboxylation and in the presence of calcium. These conformational changes are important to promoting binding to cofactors on phospholipid surfaces and procoagulant activity.[7,8] It should be noted that VKAs also inhibit carboxylation of the regulatory anticoagulant proteins C, S, and Z, which can lead to an increased procoagulant risk in certain clinical settings.

The oxidation of vitamin KH_2 to vitamin K epoxide also requires molecular oxygen and carbon dioxide. Vitamin K epoxide is subsequently recycled to vitamin KH_2 through two reductase steps. The first step reduces vitamin K epoxide to vitamin K_1 (common in natural food) and is sensitive to VKAs.[9–11] The second step reduces vitamin K_1 to vitamin KH_2 and is relatively insensitive to VKA (FIGURE 106.1A).

The use of VKAs reduces availability of vitamin KH_2, which subsequently leads to decreased γ-carboxylation of the vitamin K–dependent coagulant proteins. Whereas normally about 10 glutamic acid residues on the vitamin K–dependent factors are carboxylated, only approximately seven residues undergo this posttranslational change at a therapeutic level of VKAs.[12,13] The effect of VKAs can be counteracted by vitamin K_1 administered therapeutically or from ingestion in food. The counteraction is effectively due to the second reductase step, which is relatively insensitive to VKAs. In fact, the use of large doses of vitamin K_1 will lead to warfarin resistance for up to a week or longer, due to its accumulation in the liver.

Procoagulant Effect

VKAs also have the potential to have a procoagulant effect in addition to their anticoagulant effect. This is due to inhibition of carboxylation of anticoagulant proteins C and S. This rare occurrence leads to complications such as skin necrosis and is discussed in detail below.

PHARMACOLOGY

Pharmacodynamics/Pharmacokinetics

Although there are several oral VKAs, warfarin is currently the most common form in clinical use. It has a high bioavailability due to its water solubility and is rapidly absorbed from the stomach and duodenum.[14,15] In addition, it has maximal blood concentrations in healthy volunteers within 90 minutes of oral administration.[16] Warfarin will circulate in plasma bound to 98% to 99% to proteins, primarily to albumin, and accordingly only a minor fraction of the drug is biologically active. The drug

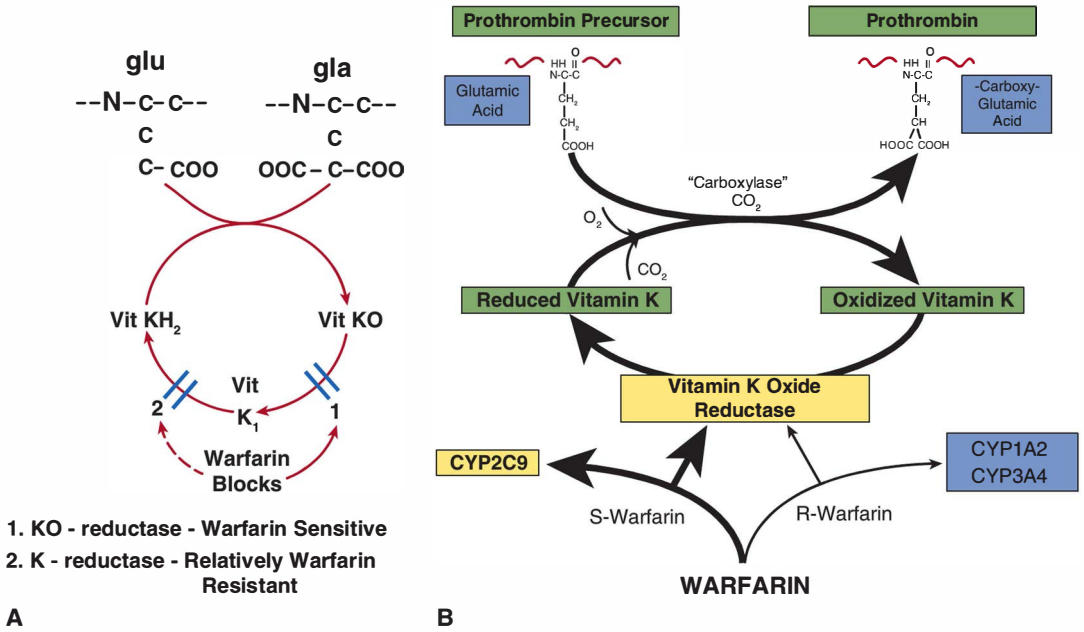

FIGURE 106.1 **A,B**: Metabolism of vitamin K and warfarin. (From Hirsh J, Dalen J, Guyatt G. The sixth [2000] ACCP guidelines for antithrombotic therapy for prevention and treatment of thrombosis. American College of Chest Physicians. *Chest* 2001;119:1S–2S; Ansell J, Hirsh J, Hylek E, et al. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008;133:160S–198S.)

will then accumulate in the liver, specifically in the microsomes.[17] Warfarin is a racemic mixture of S and R enantiomers, which are present in almost equal proportions. Warfarin has a half-life of 36 to 42 hours, which is the result of different metabolism rates of the two enantiomers. The R enantiomer is less potent, is metabolized primarily by two microsomal cytochrome enzymes (CYP1A2 and CYP3A4) to 6- and 8-hydroxywarfarin, and has a half-life of 45 hours. The S enantiomer is 2.7 to 3.8 times more potent than the R enantiomer, is metabolized primarily by the CYP2C9 enzyme of the P450 system to 7-hydroxywarfarin, and has a half-life of 29 hours (FIGURE 106.1B).[18] The inactive hydroxywarfarins are excreted renally.

Although warfarin is the most common VKA used, acenocoumarol, phenprocoumon, indanedione derivatives (phenindione), and rodenticides are occasionally encountered. Similar to warfarin, acenocoumarol and phenprocoumon are both composed of a racemic mixture of R- and S-isomers with different stereochemical characteristics. Although R-acenocoumarol has an elimination half-life of 9 hours, the less potent S-acenocoumarol has an elimination half-life of 0.5 hours.[19] The longer acting phenprocoumon has an elimination half-life of 5.5 days, with the S-phenprocoumon isomer 1.5 to 2.5 times more potent than R-phenprocoumon.[20] The most commonly used superwarfarin rodenticide is brodifacoum, a 4-hydroxycoumarin, which has higher lipid solubility and an elimination half-life of 16 to 36 days.[21] Patients with accidental ingestion or overdoses of brodifacoum should be administered vitamin K daily for an extended period due to the long half-life.[22]

Alterations in Pharmacodynamics/ Pharmacokinetics

Drugs, diet, and various disease states influence the pharmacokinetic properties of warfarin. Therefore, monitoring should occur more frequently in patients with any changes to medications or illness. The response to warfarin is also affected by multiple drugs, which lead to alterations in absorption or metabolic clearance. In addition, recently identified genetic polymorphisms can also influence the response to warfarin and should be considered in treatment protocols. Finally, anticoagulant effect may also vary based on the result of inaccurate laboratory testing, patient noncompliance, and miscommunication between patient and physician.

Pharmacokinetics of Interactions

Displacement of Warfarin from Protein-Binding Sites

Warfarin is bound to albumin at binding site I and may theoretically be displaced by compounds that are primarily albumin bound (phenylbutazone, azapropazone). Displacement may then acutely increase the effects of warfarin.[23] This effect, however, seems to be transient since the rate of elimination also increases, and it is rarely of clinical importance.[24]

Induction/Inhibition of Metabolism

Multiple drugs reduce gastrointestinal absorption of warfarin and influence pharmacokinetics. Other drugs potentiate warfarin effect by decreasing warfarin clearance through stereoselective or nonselective pathways.[25–27] Either the S-isomer or R-isomer is selectively influenced by stereoselective interactions. The influence on the S-isomer of warfarin metabolism is clinically important.[26,27] Examples of stereoselective inhibition of the S-isomer include phenylbutazone, sulfinpyrazone, metronidazole, and trimethoprim-sulfamethoxazole.[28]

Hepatic dysfunction impairs synthesis of vitamin K–dependent factors and potentiates the response to warfarin.[29] Fever or hyperthyroidism may also increase warfarin response by inducing a hypermetabolic state and increasing catabolism of vitamin K–dependent coagulation factors.[30,31]

Diet

The intake of dietary vitamin K will change daily and is an important cause of intraindividual variability over time. Vitamin K is primarily found in high amounts in foods such as green leafy vegetables and certain vegetable oils. Foods low in content can also become clinically important when ingested in large amounts. Patients need to be advised which foods contain vitamin K, as well as the variations in the international normalized ratio (INR) that can occur with warfarin use in the setting of vitamin K deficiency. The information should aim at avoiding very large amounts of these foods but make sure the patient understands that a moderate intake of vitamin K is beneficial. Reduced dietary vitamin K may arise in poor nutritional states, fat malabsorption, and sick patients treated with antibiotics and intravenous fluids without vitamin K supplementation (Table 106.1).

Genetics

Allelic variants in either CYP2C9 or VKOR*C1* are found in more than two-thirds of the White population and up to 90% of East Asians[32,33] (see Table 106.2). Individuals with certain polymorphisms of these genes will usually require a lower dose of warfarin to maintain a therapeutic INR, as well as need a longer time to achieve a stable dose.[33,34]

The first gene identified to influence the effects of warfarin was CYP2C9, which encodes for a cytochrome P450 2C9 hepatic microsomal enzyme. Certain polymorphisms in this gene lead to an alteration in the pharmacokinetics of warfarin. The most common CYP2C9 variant alleles are designated 2C9*2 and 2C9*3 and lead to decreased metabolism of the S-warfarin, especially in those individuals who are homozygous for these mutations.[35] More recently, mutations in genes encoding for the enzyme VKOR*C1* have been identified to lead to variable sensitivity to inhibition by warfarin, affecting the pharmacokinetics and dosing.

Rarely, patients may have a mutation affecting the factor IX propeptide that leads to a selective reduction in factor IX. This leads to a decreased factor IX level without prolonging the PT and may increase risk of bleeding.[36] These mutations are estimated to affect <1.5% of the population and are phenotypically silent except when the patient is receiving warfarin therapy.

Carriers of these variant alleles are at increased risk of bleeding complications, especially at the initiation of therapy. Several studies have investigated the utility of pharmacogenetic-guided strategies for the dosing of warfarin, reviewed under *Genetic Guidance* below.

Drug Interactions

There are numerous drugs that alter the pharmacodynamics of warfarin by either inhibiting synthesis or increasing clearance of vitamin K–dependent factors (see Table 106.1). For example, second- and third-generation cephalosporins,[37] erythromycin,[38] and some anabolic steroids[39] augment the effects of warfarin; thyroxine increases metabolism of coagulation factors[40]; sulfonamides as well as broad-spectrum antibiotics augment the warfarin effect by eliminating bacterial flora and aggravating vitamin K deficiency[41]; and amiodarone inhibits the metabolic clearance of both the S and R enantiomer of warfarin and potentiates the warfarin effect. Following medication changes for a patient, increased monitoring is usually required to determine the potential effect on the INR and warfarin dosing.

Dietary Supplement Interactions

A common difficulty of health care providers is the use of nutritional supplements and herbal products by patients on warfarin therapy. Most physicians do not ask about the use of these products, and patients rarely divulge this information. However, survey data would suggest that as many as 12% of the general public have used a herbal preparation or supplement within the prior year.[42] Difficulty arises from little to no standardization of the contents of these products, and interactions are not well studied. Ginkgo, ginger, and coenzyme Q10 were not shown to have an effect on warfarin.[24,43] Ginseng, however, was shown to reduce the effect of warfarin.[44] As expected, products containing vitamin K, such as green tea, also reduce warfarin effect. Table 106.1 details dietary supplement interactions with warfarin by level of supporting evidence.

MANAGEMENT

Warfarin Initiation

Commercially available warfarin has a half-life of approximately 36 hours; however, treatment for 4 to 5 days is usually required to stably reduce the vitamin K–dependent coagulation proteins to achieve a therapeutic INR. This is due to the >2 day half-life of factor X and prothrombin, despite the shorter half-lives of factors VII and IX (FIGURE 106.2). In addition, the therapeutic effects of warfarin seem to be primarily mitigated by decreased levels of factors X and II.[45] This has been the rationale for using heparin or low molecular weight heparin (LMWH) in the initial treatment period to provide an effective anticoagulant until a therapeutic warfarin effect can be achieved.[46] Treatment with warfarin should be overlapped with heparin or LMWH for at least 2 days following a therapeutic INR on two consecutive measurements 24 hours apart. This allows further reduction of factors X and II.[45]

Studies have evaluated the effect of an initial "loading" dose (e.g., ≥10 mg) of warfarin, which shortens the time to reach the therapeutic range (TTR). This may be of benefit for outpatients, who have to overlap with heparin/LMWH, and was evaluated as safe in two large case series.[47,48] Loading doses lead to a more rapid decrease in factor VII and protein C levels, as well as theoretically an increased risk of warfarin skin necrosis, and should be avoided in generally ill, hospitalized patients.[49,50]

Further attempts have also been made to supplement with oral vitamin K, at a dose corresponding to usual dietary intake, to improve stability of control of anticoagulation with warfarin. Supplementation seemed to decrease the variability of and increase the time of INR within the TTR.[51–53] This has so far not translated into a lower risk of clinical events, and larger studies will be needed to determine any clinical benefit.

It is usually recommended to begin warfarin therapy with an average maintenance dose of 5 mg, due to this dose resulting in an INR of >2.0 in 4 to 5 days with less excessive anticoagulation.[45] There are situations in which this standard dose may not be appropriate. A lower dose may be appropriate in elderly patients; in patients with impaired nutrition, liver disease, or congestive heart failure; and in patients at high risk for bleeding. For example, a patient who is undergoing heart valve replacement may begin with an initial dose of 2 to 3 mg[54] and in elderly a nomogram starting with 4 mg daily and using the INR directly as predictor led to a correct decision on the maintenance dose in 88% of the patients.[55]

Table 106.1

Level of evidence for drug, food, and dietary supplement interactions with warfarin

Level of Causation	Anti-Infectives	Cardiovascular	Analgesics, Anti-Inflammatories, and Immunologics	CNS Drugs	GI Drugs and Food	Herbal Supplements	Other Drugs
Potentiation Highly probable	Ciprofloxacin Cotrimoxazole Erythromycin Fluconazole Isoniazid Metronidazole Miconazole oral gel Miconazole vag supp Voriconazole	Amiodarone Clofibrate Diltiazem Fenofibrate Propafenone Propranolol Sulfinpyrazone (biphasic with later inhibition)	Phenylbutazone Piroxicam	Alcohol (if concomitant liver disease) Citalopram Entacapone Sertraline	Cimetidine Fish oil Mango Omeprazole	Boldo-fenugreek Quilinggao	Anabolic steroids Zileuton
Probable	Amoxicillin/clavulanate Azithromycin Clarithromycin Itraconazole Levofloxacin Ritonavir Tetracycline	Aspirin Fluvastatin Quinidine Ropinirole Simvastatin	Acetaminophen Aspirin Celecoxib Dextropropoxyphene Interferon Tramadol	Disulfiram Chloral hydrate Fluvoxamine Phenytoin (biphasic with later inhibition)	Grapefruit	Danshen Don quai Lycium barbarum L PC-SPES	Fluorouracil Gemcitabine Levamisole/fluorouracil Paclitaxel Tamoxifen Tolterodine
Possible	Amoxicillin Chloramphenicol Gatifloxacin Miconazole Nalidixic acid Norfloxacin Ofloxacin Saquinavir Terbinafine	Amiodarone-induced toxicosis Disopyramide Gemfibrozil Metolazone	Celecoxib Indomethacin Leflunomide Propoxyphene Rofecoxib Sulindac Tolmetin Topical salicylates	Felbamate	Orlistat	Danshen/methyl salicylates	Acarbose Cyclophosphamide/methotrexate/ fluorouracil Curbicin Danazol Ifosfamide Trastuzumab
Highly improbable	Cefamandole Cefazolin Sulfisoxazole	Bezafibrate Heparin	Levamisole Methylprednisolone Nabumetone	Fluoxetine Diazepam Quetiapine			Etoposide/carboplatin Levonorgestrel

Inhibition Highly probable	Griseofulvin Nafcillin Ribavirin Rifampin	Cholestyramine	Mesalamine	Barbiturates Carbamazepine	High vitamin K content foods/ enteral feeds Avocado (lg amts)		Mercaptopurine
Probable	Dicloxacillin Ritonavir	Bosentan	Azathioprine	Chlordiazepoxide	Soy milk Sucralfate	Ginseng	Chelation therapy Influenza vaccine Multivitamin supplement Raloxifene HCl
Possible	Terbinafine	Telmisartan	Sulfasalazine		Sushi containing seaweed		Cyclosporine Etretinate Ubidecarenone
Highly improbable	Cloxacillin Nafcillin/ dicloxacillin Teicoplanin	Furosemide		Propofol		Green tea	

From Ansell J, Hirsh J, Hylek E, et al. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008;133:160S–198S.

Table 106.2

Distribution of CYP2C9 and VKOR polymorphisms in different racial populations*

	CYP2C9 Poylmorphisms†					VKOR Polymorphisms	
	*1	*2	*3	*4	*5	–1639 G/A	1173 C/T
Caucasians	0.743	0.008–0.143	0.109	0	0	0.366–0.384	0.422
African-Americans	0.953	0	0.005–0.023	0	0.008	0.101–0.103	0.086
Asians	0.92	0–0.006	0.021–0.045	–	–	0.759–0.92	–
Japanese	0.984	0	0.011–0.068	0	0	–	0.891

*Compiled from references (Xie 2002; Takahashi 2006; Xiong Y, Wang M, Fang K, et al. A systematic genetic polymorphism analysis of the CYP2C9 gene in four different geographical Han populations in mainland China. *Genomics* 2011; 97: 277–281; Limdi NA, Wadelius M, Cavallari L, et al. Warfarin pharmacogenetics: a single VKORC1 polymorphism is predictive of dose across 3 racial groups. *Blood* 2010; 115: 3827–3834)
†CYP2C9 polymorphisms include *1, wild type; *2, Arg/Cys144; *3, Ile/Leu359; *4, Ile/Thr359; *5, Asp/Glu360

Warfarin Discontinuation

Discontinuation of warfarin will remove inhibition of vitamin K–dependent coagulation proteins and lead to a gradual normalization of INR. The rate of decrease of an INR is influenced by factors including liver dysfunction and age, with average time to normalization of 96 hours.[56] For patients on chronic anticoagulation with warfarin, elective procedures require either discontinuation of warfarin or bridging therapy (see below). Current guidelines by the American College of Chest Physicians (ACCP) suggest that if the annual risk of thromboembolism is low, warfarin therapy may be held for 4 to 5 days prior to the procedure and reinitiated shortly thereafter.[45]

Temporary discontinuation of warfarin is also recommended for nonbleeding patients with a supratherapeutic INR. Once the INR is at the therapeutic level, warfarin can be resumed with more frequent monitoring. Bleeding patients with supratherapeutic INRs require additional interventions in addition to discontinuation of warfarin (see below). Discontinuation of warfarin at the end of planned therapy can be abrupt, since several trials have failed to show any benefit of a tapered regimen.[57–61]

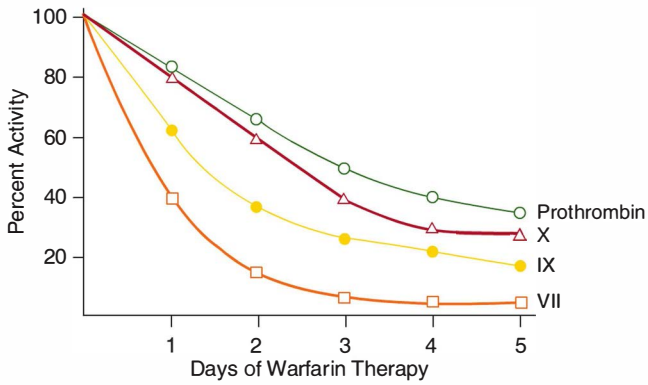

FIGURE 106.2 Effects of warfarin therapy on the plasma vitamin K–dependent procoagulant proteins. Administration of 5 to 10 mg daily of warfarin results in inhibition of synthesis of functional vitamin K–dependent proteins. The coagulant activity of these proteins in plasma declines as a function of their half-life. Half-lives of factors VII, IX, and X and prothrombin are 6, 24, and 40 and 60 hours, respectively. Although 1 to 2 days of warfarin prolongs the PT assay (because of the rapid decrease in factor VII concentration), therapeutic anticoagulation requires at least 4 to 5 days. (Data from O'Reilly RA. The pharmacodynamics of the oral anticoagulant drugs. *Prog Hemost Thromb* 1974;2:175–213; reproduced from Greenberg PL, Negrin R, Rodgers GM. Hematologic disorders. In: Melmon KL, Morrelli HF, Hoffman BB, et al., eds. *Clinical pharmacology: basic principles in therapeutics*, 3rd ed. New York: McGraw-Hill, 1992:524–599, with permission; from Wintrobe MM, Greer JP. *Wintrobe's clinical hematology*, 12th ed. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2009:1488, Ref. 178.)

Monitoring

The most useful assay to monitor therapy with warfarin is the PT assay, which measures three vitamin K–dependent coagulation proteins (factors VII, X, and prothrombin). Factor VII has a half-life of 4 to 6 hours; therefore, factor VII levels often decrease significantly following only 1 day of warfarin therapy. This may lead to a prolonged PT value, but, due to the longer half-lives of the other vitamin K–dependent coagulation proteins, therapeutic anticoagulation usually takes 4 to 5 days.[62]

The PT is performed by adding calcium and thromboplastin to citrated plasma. Thromboplastins vary in their response to reduced levels of the vitamin K–dependent coagulation factors. PT monitoring of warfarin treatment is not standardized when expressed in seconds, as a simple ratio of the patient's plasma value to that of plasma of a healthy control subject, or as a percentage of diluted normal plasma.[45] To standardize PT assays among different laboratories, an international reference thromboplastin preparation and calibration model was adopted by the World Health Organization (WHO) in 1982. To determine the responsiveness of each thromboplastin reagent, all commercial thromboplastin reagents are calibrated against the primary WHO reference preparation. These results are used to calculate the relative sensitivity of the unknown preparation compared to the WHO standard providing an international sensitivity index (ISI).[63] Once a particular thromboplastin is adjusted for the ISI, an INR is defined as the PT ratio that would be obtained if the WHO standard thromboplastin had been used. The calculation of the INR is determined by $INR = (PT\ ratio)^{ISI}$

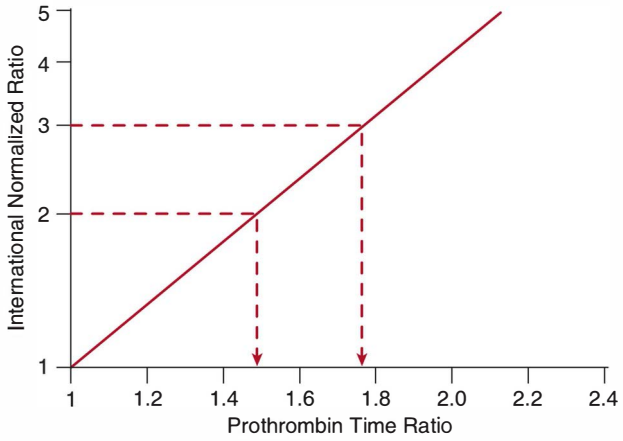

FIGURE 106.3 Relationship between PT ratio on warfarin therapy and the corresponding INR. The slope of the line represents the ISI value of the particular thromboplastin preparation used in the laboratory's PT assay. In this example, for low-intensity warfarin therapy (INR 2.0 to 3.0), a PT ratio between 1.50 and 1.75 would be required. Thromboplastins with higher ISI values would have slopes greater than that shown and would be less sensitive reagents for the PT assay, whereas thromboplastins with lower ISI values would have slopes less than that shown (more sensitive reagents). (From Greenberg PL, Negrin R, Rodgers GM. Hematologic disorders. In: Melmon KL, Morrelli HF, Hoffman BB, et al., eds. *Clinical pharmacology: basic principles in therapeutics*, 3rd ed. New York: McGraw-Hill, 1992:524–599, with permission; from Wintrobe MM, Greer JP. *Wintrobe's clinical hematology*, 12th ed. Philadelphia, PA: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2009:1490.)

(FIGURE 106.3).[64] With the wide use of the standardized INR, several of its limitations have become apparent (Table 106.3). These include decreased precision when insensitive thromboplastins are used; there is an incorrect assignment of an ISI value by the manufacturer; a different reagent–instrument combination is used than that recommended by the manufacturer; and if an incorrect control PT value is used.[45]

Recommendations by the ACCP are for standard-intensity warfarin therapy (INR 2.0 to 3.0) for all indications except prosthetic mechanical heart valves and prophylaxis against recurrent myocardial infarction (MI) for which a higher intensity warfarin therapy (INR 2.5 to 3.5) is suggested.[45]

Frequency of Monitoring

Many factors influence the response to warfarin, and as a result, the dose required to achieve a therapeutic dose will vary over time. Therefore, warfarin dosing is adjusted in response to ongoing PT measurements to maximize the proportion of time in the TTR. Appropriate anticoagulation is important because (a) subtherapeutic anticoagulation (particularly INR < 1.5) increases the risk of thromboembolism; (b) supratherapeutic anticoagulation (particularly INR > 5.0) increases the risk of bleeding; and (c) poor anticoagulation control leads to an increased burden of anticoagulation therapy and discourages patients and health care providers from continuing warfarin therapy when indicated.[65] In a systematic review, the TTR was 56.7% in community practices compared to 66.4% in randomized trials.[66]

For hospitalized patients, although INR monitoring is usually daily, frequency is often guided by the attempt to keep patients therapeutic. Once a therapeutic INR has been obtained for two consecutive days, it is often recommended to subsequently monitor two to three times weekly for 1 to 2 weeks, then less often depending on the stability of the INR.[45]

As an outpatient, initial therapy is often monitored every few days until a stable dose is achieved and then frequency is often monitored at reduced intervals. It is suggested, however, that more frequent monitoring may lead to an increased TTR.[67] For example, patient self-testing, in which patients have unlimited access to testing, seems to increase TTR,[68] although the benefit of more frequent testing begins to decrease once testing occurs more frequently than several times per week.

The optimal frequency will ultimately be influenced by patient compliance, changes in diet, interactions with medications, as well as fluctuations in comorbid conditions. In addition, physician dose adjustment decisions will also lead to variability in obtaining a therapeutic effect. To assist health care providers in decisions of warfarin dosing adjustments, there are several predictive models being used to reduce the frequency of testing while keeping patients therapeutic. The current ACCP guidelines recommend that patients receiving a stable dose of oral anticoagulation should be monitored no less than every 4 weeks.[45] A randomized trial comparing 4 versus 12 weeks interval for very stable patients indicated that the frequency can be decreased without compromising the TTR.[69]

Management of Nontherapeutic INR

The management of patients with nontherapeutic INRs is variable due to few studies comparing the various options. Changes in INR may occur due to multiple causes including (a) inaccurate INR testing; (b) inconsistent vitamin K intake; (c) changes in vitamin K or warfarin absorption; (d) changes in warfarin metabolism; (e) changes in vitamin K–dependent coagulation factor synthesis or metabolism; (f) influence of additional medications being taken; or (g) noncompliance.[45]

Patients with nontherapeutic INRs will require omitting or adjustment of the warfarin dose. If the INR is near the TTR, then adjustments of 5% to 20% based on the cumulative weekly dose and increases monitoring is usually accepted as an appropriate management plan. Due to a low absolute daily bleeding risk when the INR is minimally elevated (e.g., INR between 2.5 and 5.0), many health care providers will make no change to dose and initiate more frequent monitoring. In otherwise stable patients with an occasional INR minimally above or below the TTR, no change at all versus a single-day dose adjustment resulted in a similar proportion back in the range after 2 weeks.[70]

When the INR is elevated between 5.0 and 9.0, it is recommended to hold warfarin for 1 day or more, reduce the weekly dose, and monitor more frequently. Patients at increased risk of bleeding (intermediate to high risk) may receive vitamin K (1 to 2.5 mg) orally in addition to discontinuation of therapy.[71] Patients requiring urgent procedures and more rapid reversal may consider receiving vitamin K (≤5 mg) orally and expect a reduction with 24 hours. If the INR is still elevated, then an additional dose of oral vitamin K (1 to 2 mg) is indicated. More recent studies have revealed that vitamin K may not be as beneficial, with recent evidence that vitamin K supplementation for an INR 4.5 to 10.0 did not reduce bleeding events in

Table 106.3

Causes of incorrect INR results

Problem	Description
1. Incorrect PT ratio from erroneous PT determination due to: pretest variables (sampling and blood collection problems) Incorrect normal value	Trisodium-citrate concentration, storage time, storage temperature, evacuated tube effects, inadequate sample, variations in manual technique From nonuse of MNPT, error in MNPT due to (a) unrepresentative selection; (b) technical faults (see above); (c) nonuse of geometric mean
2. Incorrect ISI of local thromboplastin reagent/test system from lack of reliability of the ISI result provided by the manufacturer	Incorrect choice of international reference preparation; poor distribution of coumarin test samples across treatment range; inadequate numbers of test samples in ISI calibration; incorrect transformation of PT ratio of test plasmas to INR
3. Drift of ISI since original calibration	
4. Instrument (coagulometer) effects on INR at local site	
5. Lupus anticoagulant effects on some thromboplastin reagents	
6. Lack of reliability of the INR system when used at the onset of warfarin therapy and for screening for a coagulopathy in patients with liver disease	
7. Relative lack of reliability of INR > 4.5 as these values excluded from ISI calibrations	
8. Sample carryover in INR determination[181]	

MNPT, mean normal prothrombin time; ISI, international sensitivity index.
Modified from Ansell J, Hirsh J, Hylek E, et al. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008;133:160S–198S.

these patients.[72] The use of vitamin K in nonbleeding patients, therefore, should be considered but is not required.

For bleeding patients, rapid correction of the INR requires fresh-frozen plasma (FFP) or prothrombin complex concentrates (PCCs), containing FII, IX, X with or without FVII, as well as protein C and protein S. Although FFP will replace vitamin K–dependent factors and reverse an elevated INR, it is not the optimal product to use in this situation. Due to the amount of FFP needed for rapid reversal, PCCs should be considered.[73] In addition, although recombinant factor VIIa (rFVIIa) is not approved, there is evidence supporting its use to reverse supratherapeutic INR results to normal.[74–76] Table 106.4 outlines recommendations by the ACCP for management of an elevated INR or of bleeding patients receiving warfarin. See also under *Hemorrhage*, below.

Periprocedure Management or "Bridging"

Bridging is the use of shorter acting anticoagulation for elective procedures and surgery. The risk of thromboembolism, risk of bleeding, and the type of procedure should all be considered when evaluating patients on chronic VKA therapy for bridge therapy. Patients are initially classified as low, intermediate, or high risk for thromboembolism. For patients at low risk of thromboembolism, simply discontinuing warfarin prior to surgery without a bridge can be considered.

Patients requiring long-term warfarin treatment, who have an intermediate to high risk of thromboembolism, will require bridging therapy for procedures or elective surgery. It is recommended to discontinue warfarin 4 to 5 days prior to surgery and begin heparin or LMWH prior to the procedure. LMWH has the advantage of being administered safely in an outpatient setting,[77] and this approach is more cost-effective than the use of heparin as an inpatient.[78] Twice-daily dosing of LMWH should be used, and the final preoperative dose should be administered 24 hours prior to the procedure. This should decrease the incidence of bleeding during the procedure; however, there is a residual anticoagulant effect at the time of surgery that may be important in cases at high risk of bleeding.[79] Postoperatively, LMWH and warfarin may be reinitiated when the risk of bleeding is judged to be minimal.

There are several procedures that are not high-risk procedures for thromboembolism, but may have increased risk for serious bleeding with aggressive anticoagulation. Procedures such as prostate and kidney biopsy, pacemaker or defibrillator implantation, and resection of colonic polyps, especially sessile polyps and polyps larger than 2 cm in diameter, should also be taken into consideration when developing a bridge therapy plan.[80]

ACCP Guidelines

The most current ACCP guidelines review consensus recommendations for the treatment and monitoring of VKAs. Although these guidelines are referenced in this chapter, for full

Table 106.4

Management of excessive INRs or of bleeding in patients on VKAs

Condition[a]	Intervention
INR > TTR but <5.0; no significant bleeding	Lower dose or omit dose; monitor more frequently, and resume at lower dose when INR therapeutic; if only minimally above TTR, no dose reduction may be required
INR ≥ 5.0, but <10.0; no significant bleeding	Omit next 1–2 doses, monitor more frequently, and resume at lower dose when INR in TTR In addition, give vitamin K (1–2.5 mg orally), if at increased risk of bleeding. If more rapid reversal is required because the patient requires urgent surgery, vitamin K (2–5 mg) can be given intravenously with the expectation that a reduction of the INR will occur in 6–12 h. If the INR is still high, additional vitamin K_1 (1–2 mg orally) can be given
INR > 10.0; no significant bleeding	Hold warfarin therapy and give vitamin K_1 (2.5–5 mg orally) with the expectation that the INR will be reduced substantially in 24–48 h. Monitor more frequently and use additional vitamin K if necessary. Resume therapy at lower dose when INR therapeutic
Serious bleeding at any elevation of INR	Hold warfarin therapy and give vitamin K (10 mg by slow IV infusion), supplemented with PCC, FFP, or rFVIIa depending on the urgency of the situation; vitamin K can be repeated every 12 h
Life-threatening bleeding	Hold warfarin therapy and give PCC or rFVIIa supplemented with vitamin K (10 mg by slow IV infusion); repeat if necessary depending on INR
Administration of vitamin K	In patients with mild to moderately elevated INRs without major bleeding, we recommend that when vitamin K is to be given, it be administered orally rather than SC

[a]If continuing warfarin therapy is indicated after high doses of vitamin K_1, then heparin or LMWH can be given until the effects of vitamin K_1 have been reversed and the patient becomes responsive to warfarin therapy. It should be noted that INR values >4.5 are less reliable than values in or near the therapeutic range. Thus, these guidelines represent an approximate guide for high INRs.
Modified from Ansell J, Hirsh J, Hylek E, ET AL. Pharmacology and management of the vitamin K antagonists: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008;133:160S–198S.

detailed recommendations the guidelines should be reviewed[45] or any future edition replacing this.

IMPROVING CONTROL

Due to the increased risk of complication when patients are not in the therapeutic INR range, it is necessary to closely monitor treatment with VKAs. To improve anticoagulation control, monitoring has commonly involved anticoagulation clinics, self-testing strategies, and computer guidance for dose adjustments. More recently, the use of algorithms have been used following genetic testing to guide initiation of warfarin, but these have not been shown to be cost-effective for the typical patient with nonvalvular atrial fibrillation.[81]

Education

The amount of education that a patient and family receive has been reported to correlate with the results in patient knowledge tests. However, there is no solid evidence for a reduction of clinically important adverse events. Nevertheless, patients should be involved in warfarin education programs, which has recently been recommended to be a part of oral anticoagulation management plan.[45] Education includes both written and verbal information, as well as potential interactions with food and drugs.

Anticoagulation Clinics

History

The narrow therapeutic index of VKAs, numerous medication and dietary interactions, and genetic and physiologic variations in metabolism have led to the development of specialized health care services dedicated to maximizing the effectiveness and safety of oral anticoagulation. The first organized effort to use a coordinated network of services specifically dedicated to managing oral anticoagulant therapy was developed in the Netherlands, using a model from Utrecht in 1949.[82] The underlying philosophy was that only adequately maintained therapy is of value to the patient, which requires considerable effort and dedication and cannot be offered if the staff is continually changing. This fundamental concept for the management of anticoagulant therapy is still critical for optimal efficacy and safety, even as new anticoagulants are being introduced for patient management.

Nonrandomized, observational studies of either routine medical care or an anticoagulation management service (AMS) beginning in the 1980s demonstrated lower observed rates

of hemorrhage (7.6% vs. 2.8% per patient-year of therapy, respectively) and thrombosis (8.1% vs. 2.6% per patient-year of therapy) for patients followed in an AMS.[64] These observations were subsequently confirmed in several studies that used a before-and-after trial design.[83–86] Four prospective, randomized trials comparing routine medical care to an AMS have been reported.[87,88,89,90] None of the studies demonstrated a significant difference in major hemorrhage or thromboembolism, although the study by Wilson et al.[88] did show an increased frequency of INR results within the target TTR (82% vs. 76%, $P = 0.034$) and a decrease in high-risk INR values, defined as being <1.5 or >5.0 (30% vs. 40%, $P = 0.005$), favoring the AMS model.

Although the literature comparing routine medical care with the AMS model is not as robust as would be preferred, the results are almost always consistent, indicating that care provided through an AMS is associated with better outcomes and more stable therapy than by routine medical care. The current recommendation from the ACCP concerning this issue is that health care providers who manage oral anticoagulation therapy "…do so in a systematic and coordinated fashion, incorporating patient education, systematic INR testing, tracking, follow-up, and good patient communication of results and dosing decisions as occurs in an AMS (Grade 1B)."[45]

Personnel and Infrastructure

A variety of different structures exist for anticoagulation services. Clinics may be primarily composed of pharmacists,[83,85,91] integrated provider groups,[92,93] telephone-based services,[94,95] or, more recently, internet-based connections.[96,97] These providers benefit from specialized training, and national certification for anticoagulation providers is available through the Anticoagulation Forum, a multidisciplinary nonprofit organization of health care professionals who manage patients on antithrombotic therapy (http://www.acforum.org).

A point-of-care (POC) instrument for determination of the INR with capillary blood obtained by a finger stick can enhance the interaction with the patient by providing immediate feedback with the laboratory result. These monitors measure a thromboplastin-mediated clotting time from capillary whole blood or from a nonanticoagulated venous whole blood sample, converting the result to a PT and/or an INR.[98] Numerous studies have reported on the accuracy and precision of these instruments and on their suitability for monitoring anticoagulant therapy.[98–101] Limitations of these instruments have been described, however, including greater differences compared to a standard, plasma-based methodology as the INR results increase above the TTR,[102] incorrect calibration of the ISI, inability to calculate a mean normal PT, and inaccuracies in certain patients with antiphospholipid antibodies.[103]

Optimal management of oral anticoagulant therapy also depends on a robust data management software package to track patients' INR results, dosing adjustments, nonanticoagulant medication changes, and any complications encountered. In addition to simple data management, a variety of computer programs have been described that provide recommendations concerning anticoagulant dosage and timing for follow-up testing.[104–106] Recently, two of these computer-assisted oral anticoagulant dosage programs were evaluated in large, multicenter, randomized clinical trials comparing management by the software program with manual dosage by experienced medical staff.[107,108] Importantly, these two studies were the first to compare clinical outcomes (hemorrhagic and thromboembolic events) in addition to therapeutic control of the INR. One study, using the DAWN AC program, found that clinical events were almost identical between the computer-assisted dosing group and the experienced manual dosage group.[108] The second study, using the PARMA 5 program, found a nonsignificant reduction in clinical events in the computer-assisted group, and this reduction was significant in the subset of patients with deep vein thrombosis/pulmonary embolism ($P = 0.005$).[107] A subsequent cost-effectiveness analysis of both studies revealed that the computer-assisted dosage strategy was less expensive than manual dosage, indicating that investment in this technology was cost-effective.[109]

The Future of Anticoagulation Management Services

With the development of novel oral anticoagulant agents that do not need routine laboratory monitoring, there has been some concern about the future of these effective management services. More likely, there will be an evolution in their services to more broadly assist in the management of patients who may be on a variety of antithrombotic agents. When AMSs can take a leadership role, patient and provider education concerning antithrombotic therapy will continue to be critical. Certain patients will not be eligible for new antithrombotic agents for various medical reasons (e.g., patients with prosthetic cardiac valves, patients with renal failure, etc.) and will likely be continued on warfarin therapy. The cost of the newer therapies may also be prohibitive for many patients. Although routine laboratory management is not required for the new therapies, expertise in evaluating and managing patients with thrombotic complications or hemorrhagic events can be provided through the AMS, particularly those with an integrated group of providers (including physicians, physician extenders, and pharmacists). Perioperative management and management of patients on combined anticoagulant and antiplatelet regimens would also benefit from an AMS model. Consequently, the AMS will evolve from providing supervision of a single therapeutic agent to one that provides essential expertise for managing patients on a variety of antithrombotic therapies.

Self-Testing/Management

Several studies have demonstrated that more frequent testing of the INR, up to once or twice a week, can lead to an increased time in the TTR.[110] Patient self-testing using a POC monitor to measure the PT from a finger stick sample of capillary whole blood makes this frequency of testing feasible, and several meters are currently available for patients to use. Patient self-testing refers to the patient performing their own PT, but decision making about adjustments to the warfarin dose and time of next INR test requires interaction with a health care provider. In contrast, patient self-management refers to the patient performing their own PT as well as making adjustments in their warfarin dose and determining when to recheck their PT. These two strategies are described in further detail below.

The first reports of patients performing their own PT testing using a POC meter appeared in 1989.[111,112] In the study from White et al.,[111] patients who were randomized to using a portable PT monitor and calling in their results to their provider for management were subtherapeutic significantly less frequently than patients randomized to follow-up in the anticoagulation clinic ($P < 0.001$). In the study from Ansell et al.,[112] a total of

16 patients were given a portable PT instrument and structured guidelines concerning how to adjust their warfarin dose based on the test result. No clinical complications were encountered with either study, but both enrolled a small number of patients.

Since these initial reports, several randomized, controlled clinical trials have confirmed that patient self-testing can be reliably used to manage a patient's oral anticoagulant therapy.[113] In addition, novel approaches have been developed to simplify the therapeutic adjustments of patients using home INR monitors. Ryan et al.[96] demonstrated that an internet-based, direct-to-patient expert system could be used to effectively manage patients using INR self-testing with home monitors. More recently, Matchar et al.[114] described the results of The Home INR Study (THINRS) trial, which used an interactive voice-response reporting system across the telephone with Web-based local monitoring of INR results. For the THINRS study, it was found that approximately four out of five patients who underwent training to perform self-testing demonstrated competency with the home INR device. Factors associated with failure to perform self-testing included age, prior stroke, poor cognition, and poor manual dexterity.[115]

Following the initial report from Ansell et al. in 1989, self-management of warfarin using a home INR monitor and an algorithm to self-adjust oral anticoagulant dosing has been shown to be safe and efficacious in several prospective, randomized clinical trials.[116–118] A recent Cochrane review summarized results from 18 clinical trials investigating self-monitoring of oral anticoagulant therapy, of which 12 investigated patients performing self-management in addition to self-testing. They found that patients performing self-management had significant reductions in thromboembolic events (relative risk [RR] 0.47, 95% confidence interval [CI] 0.31 to 0.70) and all-cause mortality (RR 0.55, 95% CI 0.36 to 0.84), but no reduction was observed with major hemorrhage (RR 1.12, 95% CI 0.78 to 1.61).[119] Although most studies also report an improved quality of INR control, defined by the TTR, this has not been observed in all studies of patient self-management.[116]

The adoption of patient self-testing and/or self-management as a strategy to manage chronic oral anticoagulant therapy with VKAs is extremely variable in different countries. In the United States, this approach is rarely used, with common barriers to adoption including the cost of the instrument and/or test strips, concern that self-testing might lead to self-management, and loss of clinic revenue.[120] In contrast, self-management of oral anticoagulant therapy using a structured training approach is widely used in Germany and other European countries, particularly in patients receiving mechanical heart valves.[118,121,122]

Genetic Guidance

The use of pharmacogenetic guidance for the dosing of warfarin has been promising, but it has had few studies to support recommendations for testing in all patients. The presence of polymorphisms of the genes encoding CYP2C9 and VKOR*C1* has been found to need lower doses of warfarin at initiation and required a longer time to achieving a stable dose.[123] Based on these findings, the Federal Drug Administration (FDA) recommended a label change to describe the reported effects of CYP2C9 and VKOR*C1* on dose adjustment (FDA press release 2007). The increased risk of hemorrhage associated with overanticoagulation has led to recent investigations of individualized treatment algorithms for determining the dosage of warfarin at initiation.

Millican et al. recently attempted to develop an algorithm based on evaluation of warfarin treatment and genotyping in patients undergoing knee or hip surgery. They suggested warfarin initiation as per previous recommendations, however also suggested that genotype be determined. Further refinement of the dose would then occur with the third dose of warfarin to allow time for results of an offsite genotyping.[124] The group has subsequently developed an interactive and free Web site that is accessible to clinicians.

Current efforts are focused on definitively proving that genotyping will lead to improved safety and efficacy. Four randomized controlled trials with a total of 544 patients have been performed to compare the advantages of genotyping.[125–128] They have not been able to demonstrate any advantage regarding clinical outcomes.[34] There are currently no recommendations, however, for the use of genotyping and algorithms utilizing evaluation of genotyping to guide therapy with warfarin. Table 106.5 gives an overview of the arguments for and against genotype-guided dosing of VKAs.

The Future of Vitamin K Antagonists

The past decade has brought forward in clinical trials a dozen new oral anticoagulants without need for frequent laboratory monitoring or dose adjustments (Chapter 110). Some of these

Table 106.5

Advantages and drawbacks with pharmacogenetic-guided dosing of VKAs

Pros	Cons
Identifies patients who need ≤21 mg warfarin per week and may have high risk of bleeding	Systematic review of randomized controlled trials failed to show a reduction of risk of bleeding[34]
Identifies patients who need ≥49 mg warfarin per week	All trials were underpowered
Genotype predicts up to 50% of interindividual dose variability	Intervention arm differed in each trial
Possibly more rapid achievement of stable dose	Algorithm including sex, age, and body weight also has predictive value
May reduce cost by shortening concomitant parenteral therapy	Incurs additional analysis costs

agents have already achieved registration in many countries for the indication "prophylaxis against venous thromboembolism after major orthopedic surgery" and for "stroke prophylaxis in atrial fibrillation." At the time of writing, the oral thrombin inhibitor dabigatran is gaining part of the market from warfarin. Many patients starting on oral anticoagulant therapy prefer a more convenient drug to a drug that initially will require intensive monitoring. There is, however, a substantial proportion of patients on warfarin with very stable INR levels and monitoring only once per month, which is not perceived as an inconvenience. Studies indicate that they might be safely managed with even less frequent monitoring.[69,129] The management of major bleeding on VKAs is well defined, whereas at this point there is no available antidote against dabigatran. Even though the risk of intracranial bleeding is reduced by 60% with dabigatran,[130] some patients and physicians prefer the VKA with its antidote (PCC and vitamin K). Disturbing gastrointestinal adverse effects occur in 5% to 10% of the patients on dabigatran, whereas intolerable side effects from VKAs are very rare and some patients have asked to be switched back to warfarin. Patients with mechanical valves have not been studied yet on the new agents, and severe renal dysfunction seems to be a contraindication for several of these anticoagulants. The most complex issue is the reimbursement. Warfarin is one of the most inexpensive drugs. Conversely, its management incurs significant direct and indirect costs,[131] which correspond to the drug cost of dabigatran but involve different sources of funding.

It therefore seems likely that VKAs will continue to be used for at least a decade ahead (a) by patients with very stable INRs, (b) by patients with mechanical heart valves and possibly other less common indications, and (c) wherever there is restricted funding for drugs.

WARFARIN COMPLICATIONS

The situation with an asymptomatic high INR has been described above under "management on nontherapeutic INR."

Hemorrhage

Hemorrhagic complications to anticoagulant therapy are discussed in detail in Chapter 107. The risk of bleeding increases sharply with the INR (FIGURE 106.4).[132] Patients who have hemorrhage during treatment with VKAs will need rapid reversal following discontinuation of the VKAs. Patients should receive intravenous vitamin K and replacement of coagulation factors. There have been several attempts to determine ideal dosing of oral vitamin K for warfarin-associated coagulopathy. Several randomized controlled trials as well as a recent meta-analysis have found that the use of low-dose oral vitamin K leads to a reduction in an excessively elevated INR without lowering it into a subtherapeutic range.[71,133,134] The intravenous form of vitamin K has effects more rapidly than oral or subcutaneous forms and causes a progressive reduction of the INR starting from 2 hours.[135,136] In addition, normalization of the INR usually occurs within 24 hours following a sufficiently large dose of vitamin K with normal hepatic function.[137] There are reports that intravenous vitamin K causes anaphylactoid reactions in 3:100,000,[138] and therefore it is recommended to inject slowly. Subcutaneous injection of vitamin K leads to a slower and less predictable reduction of INR than intravenous injection[139] and should be avoided.

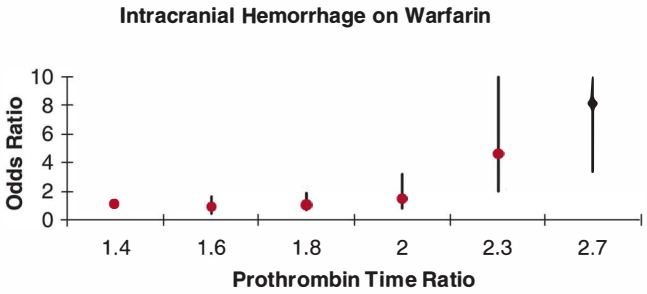

FIGURE 106.4 Relationship between PT and risk for intracranial hemorrhage. (Adapted from Hylek EM, Singer DE. Risk factors for intracranial hemorrhage in outpatients taking warfarin. *Ann Intern Med* 1994;120:897–902.)

The use of coagulation factor replacement products are additionally needed to rapidly reverse the coagulopathy. FFP is commonly given at a dose of 15 mL/kg, although a standard dose has not been determined. Limitations to the use of FFP include (a) administration of a large volume, which may be an issue for patients with volume overload; (b) the usual dose of FFP is insufficient to normalize factor IX levels; (iii) therapy is delayed because being a frozen product it requires a thawing process; and (iv) FFP has a small risk of transfusion-transmitted infections.[137]

Alternative products available for hemorrhage or rapid reversal of VKA-associated coagulopathy include PCCs and rFVIIa. PCCs include factors II, IX, X, various levels of VII, as well as anticoagulant proteins C and S. PCCs seem to be more effective at rapidly correcting a supratherapeutic INR. Makris et al.[140] found that a complete correction of the INR to normal was achieved within 15 minutes in nearly all patients treated with PCCs (25 to 50 U/kg) versus no patients achieving correction of their INR when treated with FFP. Although there are several different PCCs, no direct comparison has been made between them, and currently there is no standard dose. Increased usage of PCCs has been limited by the fear of a risk of thrombotic complications such as stroke, MI, and deep-vein thrombosis. A recent literature review revealed seven patients with evidence of thrombosis among 460 patients treated with PCCs in 14 separate studies.[141] There are, however, also thrombotic complications when VKAs are interrupted without PCCs, for example, for elective surgery.

rFVIIa appeared to be effective in rapidly correcting supratherapeutic INRs in patients with central nervous system bleeds, as shown in a study with 13 patients.[75] However, due to its risk of thrombosis and lack of clinical evidence for its use, there have been limited recommendations for its use. Should rFVIIa be used, close monitoring of coagulopathy and bleeding should occur due to its short half-life of approximately 60 minutes.

Skin Necrosis/Limb Gangrene

The use of VKAs may uncommonly cause nonhemorrhagic complications such as skin necrosis or limb gangrene. Although not well understood, reports of potential mechanisms include thrombosis, hypersensitivity, hemorrhage, factor

VII deficiency, protein C and S deficiency, and direct toxic effects of warfarin.[142] The effect seems to involve the venules and capillaries of subcutaneous fat or massive venous outflow obstruction, leading to skin necrosis and limb gangrene respectively.[45] These complications usually occur between the 3rd and 8th day of VKA therapy.

Warfarin-induced skin necrosis usually presents with pain, edema, and erythematous changes to the skin. Over the initial 24 hours, petechiae develop and eventually hemorrhagic bullae signal significant necrosis of the skin.[143] Once skin necrosis develops, there is no specific treatment that has been shown to affect outcome,[144] and nearly half of the patients will require surgical intervention.[143] The discontinuation of warfarin and administration of vitamin K, however, usually limits further development. The development of skin necrosis also does not preclude further use of VKA; however, most recommendations include a slow initiation and additional treatment with LMWH.[145] In a small study, the use of prostacyclin did seem to be beneficial in treating patients with skin necrosis.[146] Warfarin-induced skin necrosis is discussed in detail in Chapter 109.

Cutaneous Manifestations—Purple Toe Syndrome

Another rare complication of VKAs is "purple toe syndrome" and may develop several weeks after initiation. Initial reports described patients with bilateral purple discoloration of the feet on the plantar surface and postulated to be due to the direct toxic effect of warfarin on capillaries and venules.[147] More recently, histopathologic examination seems to indicate cholesterol embolization as a possible etiology.[148] No specific recommendations beyond discontinuation of warfarin have been recommended for this uncommon complication.

Other Cutaneous Manifestations

Rashes that are very itchy and are maculopapular,[149] vesicular, or urticarial[150] have been described with onset weeks to months after the start of anticoagulation with coumarin derivatives. The reactions seem to be at least as often a result from the coloring of the tablet as from the drug itself, and the first action should be to try another strength or white warfarin tablets. If that does not alleviate the problem, a switch to another VKA may be attempted. Phenindione occasionally causes severe hypersensitivity reactions.[151]

Other Complications of Warfarin

Warfarin has been reported to cause eosinophilic pleurisy[152] or vasculitis.[153] Toxic hepatitis[154] and intrahepatic jaundice[155] are very rare complications from warfarin therapy.

Alopecia occurs in <1% of patients on warfarin[156] and may be alleviated by switching to another VKA. There is also a report of two patients, whose alopecia was treated successfully with ubidecarenone 30 mg/day.[157]

Warfarin also has an effect on bone mineral density possibly via a reduction of the vitamin K–dependent osteocalcin.[158] In a retrospective study of hospital records, it was found that males on long-term warfarin had an increased risk of osteoporotic fractures (OR 1.63, 95% CI 1.26 to 2.10).[159] Conversely, warfarin can cause unwanted valvular and vascular calcification via hypocarboxylation of the vitamin K–dependent matrix Gla protein, which seems to play a role for inhibition of calcification.[160]

Yet another vitamin K–dependent protein, growth arrest-specific gene 6 protein stimulates mesangial proliferation in glomerular kidney disease.[161] However, in this situation, warfarin—and actually at lower than therapeutic doses—may provide a benefit.[162]

Pregnancy and Lactation

VKAs readily cross the placenta and produce characteristic embryopathy, central nervous system abnormalities, and fetal bleeding. The first reported case of multiple congenital abnormalities in a mother ingesting coumarin was published in 1966.[163] Subsequently, Shaul and Hall summarized the abnormalities in 11 patients exposed to warfarin during gestation. The abnormalities most typically seen included nasal hypoplasia, stippled epiphyses, eye anomalies, and developmental delay.[164] Retrospective reviews initially noted the incidence of warfarin embryopathy to be approximately 8% in infants exposed between week 6 and 9 of gestation.[165] A prospective study of 63 women with prosthetic valves, however, reported that 28.6% of all infants exposed to warfarin between week 6 and 12 of gestation developed congenital abnormalities.[166] Importantly, warfarin embryopathy was not found in the 19 patients in whom heparin was substituted for warfarin between weeks 6 and 12.[166] Neurologic abnormalities have been reported in 3% of liveborn infants exposed to coumarin derivatives. Abnormalities often seen include mental retardation, blindness, spasticity, and seizures.[165] Finally, an expected high incidence of fetal hemorrhage and death is seen in pregnant women on warfarin. Animal studies suggest significant numbers of stillborn fetuses and widespread hemorrhage, although a prospective analysis by Hirsh et al.[167] reported no fetal or neonatal complications when VKAs were discontinued after 37 weeks of gestation and replaced with heparin.

As described above, the literature suggests a strong association between maternal warfarin use and adverse effects to the fetus. Women who conceive while taking VKAs should be advised to discontinue their use prior to 6 weeks of gestation, if feasible. The use of unfractionated heparin (UFH) or LMWH would be useful alternatives. For patients with mechanical valves or at high risk for thrombosis, it is suggested to use either adjusted-dose LMWH or UFH twice daily throughout pregnancy or until the 13th week with substitution to warfarin thereafter. Warfarin would then be continued only until 2 to 4 weeks prior to delivery to avoid an anticoagulated fetus at birth.[168,169] Finally, patients with high-risk mechanical valves (e.g., older generation valves, history of thromboembolism) are suggested to continue using VKA rather than heparin.

There are few studies evaluating the safety of VKAs in lactating women. Warfarin is polar, nonlipophilic, and highly protein bound.[170] There have been two relevant studies, which reveal no evidence of warfarin within breast milk and that it does not induce an anticoagulant effect in the breastfed infant.[171,172] Therefore, the use of warfarin in lactating mothers is regarded as safe.

Warfarin Resistance

Requirement for extremely high doses of VKAs to achieve a therapeutic INR is a rare phenomenon (<0.1%).[173] There are several possible mechanisms for this. An absorption defect has been

described for cases with resistance to warfarin or dicoumarol.[174] Another explanation has been identified with mutations in the target protein VKOR*C1*,[175] which also seems to be behind the selection of warfarin-resistant rodents.[176]

Increased elimination via duplication of the CYP2C9 gene has also been described.[177] Before hereditary defects are investigated, it is crucial to review acquired causes such as poor compliance, high intake of vitamin K in any form, other dietary habits, and concomitant medication with rifampicin, carbamazepine, etc., as well as malabsorption disorders. An algorithm for investigation of patients with a daily requirement of >15 mg of warfarin has been published.[177]

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CHAPTER

107 Hemorrhagic Complications of Anticoagulation

SAM SCHULMAN AND MARK A. CROWTHER

Bleeding is the most common complication of anticoagulant therapy. The spectrum is from seemingly harmless subcutaneous ecchymoses, albeit cosmetically disturbing for some individuals, to severely disabling intracerebral hemorrhages and fatal bleeding. Parenteral anticoagulants, for example, unfractionated heparin (UFH) or low molecular weight heparin (LMWH), may cause serious hemorrhage despite careful monitoring or predictable plasma levels, respectively, because of the underlying illness and invasive procedures performed in the hospital. Vitamin K antagonist (VKA) therapy is in many patients difficult to control, and excessive anticoagulation increases dramatically the risk of bleeding. New anticoagulants with more predictable pharmacokinetics and a minimum of drug–drug and drug–food interactions may have a lower risk of bleeding. However, a substantial number of bleeding events occur without provoking risk factors and while patients are within the therapeutic range—these events are impossible to avoid during anticoagulant therapy. It is therefore also crucial to be familiar with the reversal of anticoagulants and management of hemorrhage.

DEFINITIONS

Comparison of incidences of bleeding between clinical studies is hampered by the plethora of definitions for serious or major bleeding. Some of the most used or well-reasoned classifications and their criteria are provided in Table 107.1.[1–5] Many studies have, however, used their own definition or a "modified" version of any of those in Table 107.1.[1]

The International Society on Thrombosis and Haemostasis (ISTH) definition of major bleeding for nonsurgical patients[2] is supported by the European Medicines Agency,[3] and many contemporary clinical trials have chosen this definition. A criterion for major bleeding that has been used in several clinical trials on prophylaxis against venous thromboembolism (VTE) after major orthopedic surgery is the bleeding index of at least 2. This index is the sum of the number of red cell and whole blood units transfused plus the difference between pre- and postbleeding hemoglobin (in g/dL).

The number of major bleedings is often compared with the number of thromboembolic events to evaluate the net benefit of a treatment. It is then necessary to analyze the proportion of different types of major bleeding events, since an intracranial hemorrhage has about the same clinical burden as an ischemic stroke, whereas a gastrointestinal bleeding usually has far less severe consequences. In many large trials, the greatest proportion of major hemorrhages were indeed gastrointestinal.[8]

INCIDENCE OF ANTICOAGULATION-ASSOCIATED BLEEDING

Vitamin K Antagonists

The risk of bleeding complications in randomized control trials (RCTs) is lower than that seen in routine clinical practice since the study population is selected and patients with risk of bleeding are usually excluded. Paradoxically, the risk of bleeding reported in observational studies is often lower than in RCTs. This could be due to recall bias in retrospective studies, missed events in registry studies, or a result of studying a VKA-experienced cohort. Conversely, RCTs may demonstrate a high incidence of bleeding if they are pivotal registration trials, where all events are carefully captured, and if a large proportion of patients are VKA naïve.[4]

The largest RCTs on VKA therapy have been performed in patients with *atrial fibrillation*. In Table 107.2, the annualized incidence of fatal, intracranial, and major bleeding on VKA is shown for RCTs versus placebo, aspirin + clopidogrel, and dabigatran as well as for observational studies of an inception cohort and of VKA-experienced patients. The annual incidences of intracranial bleeding and of major hemorrhage range from 0.3% to 1.7% and from 1.3% to 4.7%, respectively.[4–9]. Detailed information on incidence of bleeding for each RCT can be found in the guidelines of the American College of Chest Physician.[10]

In a pooled analysis of five studies on VKA versus placebo the attributable risk of major bleeding or of intracranial hemorrhage for VKA was 0.3% and 0.2% per year, respectively.[6]

In a meta-analysis of 33 RCTs and prospective cohort studies with 10,757 patients treated with VKAs for VTE for an average of 5 months, the annualized risk of fatal, intracranial, and major bleeding was 1.3%, 1.15%, and 7.2%, respectively.[11] It should be noted that these patients are all VKA naïve and the risk of bleeding is highest shortly after start of therapy. For the subpopulation with duration of anticoagulation longer than 3 months, the annualized incidence of fatal, intracranial, and major bleeding for the period after 3 months was 0.6%, 0.6%, and 2.7%, respectively.[11]

In nine RCTs on VKA versus combined VKA–antiplatelet therapy for patients with *mechanical prosthetic heart valves*, there were 59 major bleeding events in the VKA-alone arm during a total of 1,926 patient-years, corresponding to an annualized risk of 3.1%.[12] Three of these studies aimed for a therapeutic international normalized ratio (INR) of 3.0 to 4.5, and the risk includes nonfatal intra- and extracranial major bleeding.

Whenever VKA is combined with other antithrombotic agents, the risk of bleeding increases. The annual risk of bleeding requiring hospitalization was in a large registry study 4.3% on VKA, 5.7% with aspirin added, and 12% with aspirin and clopidogrel added[13] (Table 104.2, Chapter 104).

Table 107.1

Commonly used and/or well-reasoned classifications for major or severe bleeding in nonsurgical and surgical patients

Nonsurgical Patients

TIMI Major Bleeding[149]	GUSTO Severe Bleeding[150]	ISTH Major Bleeding[2]
Intracranial bleeding ≥5 g/dL decrease in Hgb ≥15% decrease in Hct	*At least one of*: Intracranial bleeding Bleeding causing hemodynamic compromise	*At least one of*: Fatal bleeding Bleeding in critical organ Bleeding with either of (a) ≥2 g/dL decrease in Hgb or (b) transfusion of ≥2 units of whole blood or red cells

Surgical Patients

ISTF—Adjudicated Serious Bleeding[151]	ISTH Major Bleeding (Surgical)[152]
At least one of:	*At least one of*:
Fatal bleeding	Fatal bleeding
Intracranial bleeding	Bleeding in critical organ
Retroperitoneal bleeding	Extrasurgical site bleeding with either of:
Bleeding causing treatment cessation	(a) ≥2 g/dL decrease in Hgb or (b) transfusion of ≥2 U of whole blood or red cells
Bleeding causing reoperation	Complicated hemarthrosis Surgical site bleeding requiring second intervention Surgical site bleeding with (a) ≥2 g/dL decrease in Hgb or (b) transfusion of ≥2 U of whole blood or red cells *AND* that, as assessed by the surgeon, is either (c) unexpected and prolonged or (d) causing hemodynamic instability

TIMI, thrombolysis in myocardial infarction; GUSTO, global utilization of streptokinase and tPA for occluded coronary arteries; ISTH, International Society on Thrombosis and Haemostasis; ISTF, International Surgical Thrombosis Forum.

Unfractionated Heparin

The administration of UFH should be by continuous infusion or subcutaneous injections, since the risk of bleeding is increased with intermittent intravenous injections.[10] The risk of bleeding depends very much on the indication for treatment. In patients with acute myocardial infarction, there is a high risk due to invasive procedures, frontloaded thrombolysis, and concomitant (frequently multiagent) antiplatelet therapy. The frequent use of revascularization procedures in non–ST-elevation acute coronary syndromes probably explains the high risk of major bleeding seen in this setting (Table 107.3).[14] Patients with ischemic stroke have a risk of hemorrhagic stroke transformation,[15] whereas those treated for VTE are less susceptible unless they have underlying cancer.

Low Molecular Weight Heparin

Whereas UFH is almost exclusively given for short durations, LMWH is, due to the possibility of once-daily administration without monitoring, sometimes used for many months, particularly during pregnancy or in patients with VTE and cancer. The risk of bleeding for different indications and durations of treatment is shown in Table 107.4.[14,15,16,17,18] In comparison with UFH, LMWH resulted in more bleeding in acute coronary syndromes, and the difference was statistically significant in ST-elevation myocardial infarction, although this was outweighed by better efficacy for LMWH.[14] In acute stroke and in pulmonary embolism, there was a nonsignificant decrease of risk of major hemorrhage with LMWH compared to UFH,[15] whereas in short-term treatment of deep vein thrombosis LMWH resulted in significantly fewer major bleedings.[17]

Fondaparinux

The pentasaccharide fondaparinux is almost exclusively used for durations up to approximately 10 days, both in prevention and treatment of thromboembolism. The risks of major bleeding during treatment for cardiac or for venous indications are presented in Table 107.5.[19–22]

Dabigatran

The orally available direct thrombin inhibitor, dabigatran etexilate, was evaluated in a study with 18,000 patients versus warfarin and was given as either 110 mg b.i.d. or 150 mg b.i.d. for a mean of 2 years.[7] The annualized risk for major bleeding was 2.7% and 3.1%, respectively, and for intracranial bleeding it was 0.2% and 0.3%, respectively. The risk of intracranial

Table 107.2

Annual risk (in %) for different types of hemorrhages with VKA therapy in studies with various designs

Study/Year	Design	Fatal	Intracranial	Major Bleed
Atrial Fibrillation				
Atrial fibrillation Investigators/1994[6]	Pooled analysis of RCTs; warfarin vs. control	NA	0.3	1.3
SPAF III/1996[5]	RCT; vs. low, fixed dose VKA plus aspirin	NA	0.5	2.1
ACTIVE W/2006[4]	RCT; vs. aspirin plus clopidogrel; 23% of patients VKA naïve	0.26	0.36	2.21
RE-LY/2009[7]	RCT; vs. dabigatran; 50% of patients VKA naïve	NA	0.74	3.36
Hylek/2007[9]	Inception cohort; elderly	0.8	1.7	4.7
Go/2003[8]	ATRIA cohort, retro and prospective	NA	0.5	1.5
VTE				
Linkins/2003[11]	Meta-analysis, RCTs, and prospective cohorts	1.3	1.15	7.2
Linkins/2003[11]	Same, but 1st 3 mo excluded	0.6	0.6	2.7
Akl/2008[16]	Meta-analysis, four RCTs, VTE and cancer, 3–6 mo	NA	NA	1.9
Mechanical Heart Valves				
Little/2003[12]	Meta-analysis of RCTs	NA	NA	3.1

bleeding was for both dose levels significantly lower than for patients randomized to warfarin (0.7%).

Parenteral Direct Thrombin Inhibitors

Several parenteral, direct thrombin inhibitors (argatroban, bivalirudin, and lepirudin) are used for limited indications and often with small populations studied.[10]

Table 107.3

Risk for major hemorrhage with UFH according to indication

Study/Year	Design; Duration	Intracranial	Major Bleed
Acute Coronary Syndromes, ST-Elevation			
Murphy/2007[14]	Meta-analysis, six RCTs; 30 d	NA	1.8%[a]
Acute Coronary Syndromes, Non–ST-Elevation			
Murphy/2007[14]	Meta-analysis, six RCTs; 30 d	NA	5.4%[a]
Acute Ischemic Stroke			
Sandercock/2008[15]	Meta-analysis, eight RCTs; 10 d	1.1%[b]	0.1%[c]
Acute VTE			
Quinlan/2004[18]	Meta-analysis, 12 RCTs, pulmonary embolism; 7 d	NA	2.3%
Erkens/2010[17]	Meta-analysis, 8 RCTs, deep vein thrombosis; 7 d	NA	2.1%

[a]Includes intracranial bleeding but not fatal bleeding.
[b]Symptomatic bleeding; for any—including asymptomatic hemorrhagic transformation—the risk was 2.6%.
[c]Extracranial, nonfatal bleeding.

Table 107.4

Risk for major hemorrhage with LMWH according to indication

Study/Year	Design; Duration	Intracranial	Major Bleed
Acute Coronary Syndromes, ST-Elevation			
Murphy/2007[14]	Meta-analysis, six RCTs; 30 d	NA	2.6%[a]
Acute Coronary Syndromes, Non–ST-Elevation			
Murphy/2007[14]	Meta-analysis, six RCTs; 30 d	NA	6.3%[a]
Acute Ischemic Stroke			
Sandercock/2008[15]	Meta-analysis, four RCTs; 10 d	0.7%[b]	0.6%[c]
Acute VTE, Short-Term			
Quinlan/2004[18]	Meta-analysis, 12 RCTs, pulmonary embolism; 7 d	NA	1.4%
Erkens/2010[17]	Meta-analysis, eight RCTs, deep vein thrombosis; 7 d	NA	1.0%
VTE, Long-Term			
Akl/2008[16]	Meta-analysis, four RCTs, patients with cancer, 3–6 mo	NA	6.4%[d]

[a]Includes intracranial bleeding but not fatal bleeding.
[b]Symptomatic bleeding; for any—including asymptomatic hemorrhagic transformation—the risk was 1.7%.
[c]Extracranial, nonfatal bleeding.
[d]Annualized risk was 2.0%.

Argatroban has been studied in treatment after stroke, and the incidence of intracranial bleeding was 3.4% or 5.1% with a bolus dose of 100 μg/kg followed by continuous infusion at 1 or 3 mg/kg/min, respectively, compared to 0% with placebo.[23] In three trials including patients with myocardial infarction after initial thrombolytic therapy, the risk of bleeding was similar[24] or lower[25,26] with argatroban compared to UFH. In a pooled analysis of two cohort studies with argatroban for patients with heparin-induced thrombocytopenia (HIT), the incidence of major bleeding was 6% compared to 7% in historical controls for whom heparin was stopped and sometimes replaced with VKA.[27] Argatroban is eliminated by hepatic metabolism, and the dose has to be reduced in case of liver impairment or a drug with alternative route of elimination chosen.

Bivalirudin was compared with UFH in patients with ST-elevation myocardial infarction in the HERO-2 trial and was associated with a trend to more severe bleeding and intracranial bleeding events.[28] Conversely, after percutaneous coronary interventions, bivalirudin has consistently resulted in only about half the number of major bleeding events as with UFH (~3% vs. 6%).[29,30]

Lepirudin as anticoagulant after thrombolysis in myocardial infarction resulted in a similar risk of major or intracranial bleeding as UFH.[31] In patients with acute coronary syndrome

Table 107.5

Risk for major hemorrhage with fondaparinux according to indication

Study/Year	Design	Fatal	Intracranial	Major Bleed
Acute Coronary Syndromes, ST-Elevation				
OASIS 6/2006[21]	RCT, 9 d	0.6%	0.2%	1.8%[a]
Acute Coronary Syndromes, Non–ST-Elevation				
OASIS 5/2006[22]	RCT, 9 d	NA	NA	2.2%
	Same, 30 d	NA	NA	3.1%
Acute VTE, Short-Term				
MATISSE PE/2003[20]	RCTs, pulmonary embolism, 7 d	0.1%	NA	1.3%
MATISSE DVT/2004[19]	RCTs, deep vein thrombosis, 7 d	0.2%	NA	1.1%

[a]Fatal and intracranial hemorrhages are included in this result. Severe bleeding according to modified TIMI definition occurred in 1.0%.

without thrombolysis, lepirudin was, however, associated with a higher incidence of major bleeding than UFH.[32] In patients with HIT treatment, lepirudin appears in indirect or historical comparisons to give more major bleeding (10% to 14%)[33,34] than with argatroban (6%),[27] danaparoid (2.5%),[33] or controls (8.5%).[34] Both bivalirudin and lepirudin are eliminated renally and are contraindicated in severe kidney disease.

Rivaroxaban

Several direct and orally available factor Xa inhibitors are under development. Rivaroxaban was, in a phase III RCT for patients with deep vein thrombosis, associated with a similar risk of major bleeding and fatal bleeding as LMWH and VKA (0.8% vs. 1.2% and 0.1% vs. 0.3%, respectively).[35] The same was true in a study on 14,000 patients with atrial fibrillation with major hemorrhage in 3.6% per 100 patient-years with rivaroxaban versus 3.45% with warfarin.[36]

RISK FACTORS FOR BLEEDING

The risk factors for bleeding can be classified as drug related and patient related. Drug-related risk factors may result in excessive drug levels—every known antithrombotic agent has a dose–bleeding relationship, although this curve may be more or less steep for different drugs.

Drug-Related Risk Factors

The risk of bleeding with overly intensive treatment has been best documented with VKAs. The risk of intracranial bleeding doubles with each increase of approximately 1 in the INR.[37] The risk for major bleeding on warfarin doubles when the treatment range is above INR 3.0 compared to when it is INR 2.0 to 3.0.[38–41] Patients with myocardial infarction, treated with UFH in a large trial, had an increase of the risk of bleeding of 7% for every 10-second additional prolongation of the activated partial thromboplastin time (APTT).[42] Increased bleeding with higher doses have also been reported for lepirudin,[43] argatroban,[25] and dabigatran.[7]

High variability in the drug level will inevitably result in frequent periods of excessive anticoagulation. The VKAs are most susceptible to such variations due to a multitude of interactions with other drugs and with food and a narrow therapeutic range. The variability is a risk factor that is independent of the mean INR.[5,44,45] Furthermore, "time in therapeutic range" (TTR) is closely related to hemorrhagic as well as thromboembolic complications.[46] Nevertheless, a patient that has INR results consistently a few decimal points above the upper limit of the therapeutic range and accordingly a very low TTR has probably a lower risk of bleeding than another patient with alternating low and high INR results and a modest TTR. Therefore, a method that estimates both the variability of INR and the time spent in range probably provides the best estimate of risk for complications.[47]

The management of anticoagulation with VKA needs to be meticulous and delivered by experienced staff to minimize the complication rate. A systematic review of 67 studies with more than 50,000 patients monitored by specialized anticoagulation clinics (68%), community practices (24%), or in clinical trials (7%) demonstrated that the study setting had the greatest influence on TTR, which was lowest in community practices.[48] Dosing decision support with manual algorithms[49,50] or computer software[51] can effectively improve the quality of anticoagulant management.

Concomitant medications can result in increased risk of bleeding with VKAs either via direct interacting effects on the pharmacokinetics of VKA and resulting high INRs (see Chapter 106) or by exerting inhibition on other components in the hemostatic system, typically on the platelets (nonsteroid anti-inflammatory drugs, serotonin reuptake inhibitors, aspirin, glycoprotein IIb/IIIa, or $P2Y_{12}$ receptor inhibitors) (Table 104.2, Chapter 104; Table 106.1, Chapter 106). Multiple inhibitions of the hemostatic system or concomitant thrombolysis and increased bleeding have also been described for other drugs, for example, UFH.[52–55]

A frequently described interaction is between VKA and paracetamol (acetaminophen), resulting in increased INR,[56–58] which has been associated with increased risk for gastrointestinal bleeding requiring hospitalization.[59] Nonsteroid anti-inflammatory drugs increase the risk of upper gastrointestinal bleeding 3.6 times compared to a matched nontreated population and with VKA added the risk is 8 to 11 times the expected.[59,60]

Patient-Related Risk Factors

Age is an independent risk factor for bleeding on treatment with VKA[61–64] and UFH,[65,66] but it is more controversial whether female sex is such a risk factor for either of these drugs.[66–68] In recent large trials with new anticoagulants in atrial fibrillation, there was no apparent difference in risk of bleeding between males and females,[36,69,70] supporting the notion of play of small numbers in the above-mentioned older studies.

Genetic variants that influence the effect of VKAs and may cause increased risk of bleeding can involve the metabolism (microsomal enzyme CYP2C9*2 or *3) and the target protein (C1173T polymorphism of vitamin K epoxide reductase complex 1 [VKOR*C1*])[71] or result in dysfunction of hemostatic components such as platelets (A1A1 variant of glycoprotein IIb/IIIa)[72] or factor IX (drastic decrease on VKA, unnoticed with prothrombin time).[73] However, the influence of genetic polymorphisms on warfarin response, expressed as bleeding complications, remains controversial. Whereas on one hand observational studies have indicated strong associations between polymorphisms in CYP2C9 or VKOR*C1* and dose requirements and also an association between a low maintenance dose and the risk of bleeding,[74,75] randomized trials on pharmacogenetic dosing have not demonstrated a reduction of clinical adverse events[76] (see also Chapter 106).

A history of previous bleeding may be a risk factor for recurrent bleeding on VKAs.[77,78,79] Comorbid diseases are frequently reported as risk factors for bleeding on VKAs[10] and include hypertension, cerebrovascular disease, ischemic stroke, serious heart disease, diabetes, alcoholism, liver disease, malignancy, and renal insufficiency. Chronic kidney disease is obviously also a risk factor for bleeding with drugs that are dependent on renal elimination (LMWH,[80] pentasaccharides,[81] lepirudin,[43] and dabigatran[82]), and a glomerular filtration rate of <30 mL/min is usually considered a contraindication for their use. However, increased risk of bleeding in chronic kidney disease has been observed with UFH[83] as well. A body weight of <50 kg was in a registry study associated with increased risk of bleeding with an

odds ratio of 2.2 (95% CI 1.2 to 4.0) compared to 50 to 100 kg.[84] This was partly, but not entirely, explained by the use of excessive doses of anticoagulants or by other comorbidities.

Finally, temporal proximity to trauma or invasive procedures has been reported as a determinant of bleeding for several anticoagulants.[22,85–89]

CLINICAL PREDICTION RULES FOR BLEEDING

A logical step after identification of risk factors for bleeding is the derivation of the proportional importance of independent risk factors into a formula that subsequently is used prospectively in another cohort for validation of the predictive value. Several clinical prediction rules have been published,[77,90–94] and they are summarized in Table 107.6. These tools are for patients starting treatment with VKAs, but some of these are difficult to memorize due to many components and complicated point systems. Furthermore, it is challenging enough for a clinician to stratify the patient according to risk for stroke, and to simultaneously stratify each patient for risk of thromboembolism and for bleeding complications is unrealistic. A systematic review of the bleeding risk rules has been performed,[95] and the outpatient bleeding risk index (OBRI)[77] had the best level of evidence in view of prospective validation by different investigators and in different populations. None of the tools would, however, have a "strong" ability to predict whether a major bleeding will occur or not during treatment with VKAs. Nevertheless, for patients where the assessment of thromboembolic risk leaves the physician uncertain about the optimal choice of prophylaxis, a subsequent analysis of the bleeding risk may be decisive.

PROGNOSIS AFTER BLEEDING

In an analysis of patients with acute coronary syndromes, recruited for two large RCTs, Eikelboom et al.[96] found that a major bleeding was associated with a high risk of death within 30 days but to a much lesser extent after that time period (hazard ratio 5.37; 95% CI 3.97 to 7.26 and 1.54; 95% CI 1.01 to 2.36, respectively). The risk was higher if the bleeding had been major rather than minor. The same association was identified in populations treated with fondaparinux to prevent VTE with 8.6% risk of death by 30 days compared to 1.7% when no major bleeding had occurred.[97]

There are obviously several plausible and perhaps all of them contributing reasons for this association, including that major bleeding is more likely to occur in patients with comorbidities (see above), the fact that antithrombotic treatment usually is stopped after a hemorrhage with subsequent increase in the risk of thromboembolism, the potential for hypovolemia and hypoxemia causing myocardial damage, and possible harmful effects of blood transfusions.[98]

MANAGEMENT OF BLEEDING COMPLICATIONS IN ANTICOAGULATED PATIENTS

Localization of Bleeding

As discussed, bleeding is the most frequent and important complication of anticoagulant therapy. The clinical burden of bleeding is generally underestimated—in a systematic review the case fatality rate for bleeding was 9.7% in patients enrolled in large RCTs.[11] Although minor bleeding is inconvenient and potentially disruptive for patients, it generally does not have an adverse outcome. Thus, ecchymoses, epistaxis, and menorrhagia may be very bothersome for patients, albeit rarely resulting in clinically important outcomes. Chronic blood loss will, however, eventually cause iron deficiency anemia. Major bleeding is generally gastrointestinal, genitourinary, or intracranial. Gastrointestinal bleeding may occur from any location within the gastrointestinal tract and may be an indicator of underlying pathology "unmasked" by the anticoagulant. Thus, 50% to 75% of patients presenting with evidence of gastrointestinal bleeding will have a detectable underlying lesion on endoscopic evaluation.[99] Similarly, genitourinary bleeding is generally an indicator of underlying pathology, irrespective of the patient being anticoagulated or not and requiring investigation if renal tract infection has been ruled out. In a prospective study, 81% of individuals with microscopic hematuria had identifiable genitourinary disease.[100] Women treated with VKAs reported menorrhagia in 70.8% compared to 44.2% before therapy.[101] VKAs did, however, not increase the incidence of hematuria in those studies.[100,101]

Intracerebral bleeding may be subdural (usually occurring as a result of traumatic injury), subarachnoid (rarely a complication of anticoagulant therapy), or parenchymal. Subdural hematoma will usually present with chronic and progressive symptomatology associated with slow expansion and compression. Rarely, rapid enlargement may cause a drastically deteriorating neurologic course due to tentorial herniation. Intracerebral hematoma will usually present with a sudden, oftentimes catastrophic, deterioration in neurologic function associated with focal neurologic findings, which may progress rapidly to unconsciousness and death due to raised intracranial pressure.

General Approach to Management

In all cases, management of bleeding requires an organized approach to reduce morbidity and potential mortality.[102] For all major bleedings, the anticoagulant medication should be stopped. Intubation, ventilation, and administration of inotropic drugs and volume substitution may be required during the initial stabilization or while investigations are carried out. Acute resuscitation of a critically ill patient with anticoagulant-associated bleeding will require prompt and thorough evaluation by an interventional or surgical expert. The source of bleeding should be identified and interventions proposed to halt the bleeding. This will require either a surgical, radiologic, or endoscopic intervention. Clinically important bleeding is rarely rectified by medical therapy alone. Local therapies to treat bleeding (such as cauterization of a gastrointestinal lesion or surgical resection of bleeding lesions) can result in immediate cessation of bleeding. More aggressive treatments such as embolization of briskly bleeding vessels may be lifesaving in selected circumstances. Prompt administration of an antidote (if available) should occur in all patients with major or life-threatening hemorrhage. In many patients, empiric therapy with an antidote may antecede imaging. For example, acute neurologic deterioration in a patient with previously normal neurologic function in the setting of administration of a thrombolytic agent is likely to be due to hemorrhage; in this setting, it is more appropriate

Table 107.6

Published bleeding risk prediction tools for patients starting on VKAs

Acronym or First Author	Indication for VKA	N (Derivation/ Validation)	Components and Scoring	Scores and Bleeding Risk		
				Low	Intermediate	High
OBRI[77]	Mixed	556/264	Age ≥ 65, previous stroke, GI bleed <2 wk ago, ≥1 of (recent myocardial infarction, hematocrit <30%, creatinine >1.5 mg/dL, diabetes mellitus) (1 p each)	0	1–2	3–4
Kuijer[90] (simplified score)	VTE	241/780	Age ≥ 60 (1.6 p), female (1.3 p), malignancy (2.2 p)	0	1–3	>3
HEMORR^{2}HAGES[91]	Atrial fibrillation	3,138 patient-y	Hepatic or renal disease, ethanol abuse, malignancy, age > 75, ↓ platelet count or function, hypertension, anemia, genetic factors, fall risk, previous stroke/TIA (1 p each), previous bleed (2 p)	0–1	2–3	4
Shireman[92]	Atrial fibrillation	19,875/6,470	Age ≥ 70 (0.49 p), female (0.32 p), remote bleed (0.58 p), recent bleed (0.62 p), alcohol/drug abuse (0.71 p), diabetes (0.27 p), anemia (0.86 p), antiplatelet Rx (0.32 p)	≤1.07	1.08–2.18	≥2.19
RIETE[93]	VTE	13,057/6,572	Recent bleeding (2 p); creatinine >1.2 mg/dL, anemia (1.5 p each), cancer, symptomatic PE, age > 75 (1 p each)	0	1–4	>4
HAS-BLED[94]	Atrial fibrillation	3.978/not done	Hypertension, abnormal liver function, abnormal kidney function, stroke, previous bleeding, labile INRs, age > 65, alcohol abuse, drugs affecting platelet function (1 p each)	NA	NA	NA

to provide agents to reverse the thrombolytic agent than it is to send the patient for imaging since the delay that such imaging implies will probably result in worsening outcomes or death.

Transfusion Therapy

Transfusion of blood components should be undertaken to address identified coagulation factor defects or to replace red blood cells, platelets, or coagulation factors reduced due to dilutional coagulopathy. Transfusions should not be ordered without adequate clinical basis; they may cause an unrealistic expectation of efficacy, carry the potential for harm, and will delay needed treatments. Thus, for example, the administration of fresh frozen plasma (FFP) or prothrombin complex concentrates (PCCs) to patients with UFH- or LMWH-associated bleeding is inappropriate as antidote, and FFP is potentially dangerous for volume overload.[103,104] However, administration of FFP or PCCs to a patient who has received VKAs is very appropriate. Red blood cells should be administered based on assessments of the hemoglobin value, interpreted in light of the amount of bleeding. FFP and platelets should be administered based on laboratory evaluations of coagulation parameters except in the setting of profuse bleeding where FFP may be administered at an initial dose of 10 to 15 mL/kg.[105]

Nonspecific Agents

A number of nonspecific prohemostatic agents are available. These include deamino-D-arginine vasopressin (DDAVP), antifibrinolytics, and estrogens. These agents should be considered in patients with major or life-threatening bleeding, although their efficacy in patients with antithrombotic therapy–induced bleeding has generally not been studied and they may be associated with complications including thrombosis. Recombinant activated factor VII (rfVIIa) has been widely promulgated as an effective agent for the reversal of a variety of anticoagulants,[106,107] and it is indeed able to generate a thrombin burst on the surface of platelets even in the setting of severe coagulopathy.[108] However, use of rfVIIa is largely based on animal models, impact on surrogate markers in healthy volunteers, and occasional case reports in patients who are receiving multiple interventions in the setting of acute bleeding. Given its cost and potential for thromboembolic complications,[109] it should only be entertained in patients with life-threatening bleeding who have not responded to other interventions.

MANAGEMENT OF BLEEDING ON SPECIFIC AGENTS

The principles are summarized in Table 107.7.

Vitamin K Antagonists

Warfarin, acenocoumarol, and phenprocoumon are the three commonly used oral VKAs (although others are available in some jurisdictions). The initial management of a patient with major or life-threatening bleeding receiving VKAs should adhere to the first principles outlined previously. In critical situations, rapid reversal of the INR is of the utmost importance; this should precede imaging to determine the source of bleeding. Traditionally, FFP was the agent of choice for reversal of VKAs. FFP is slow to obtain, is associated with the potential for fluid overload,[110] may not be virally inactivated, and requires a group and screen unless AB plasma is used. Furthermore, the volume of FFP required to normalize the INR is very large and, in many settings, impractical. Usual FFP transfusions (e.g., 4 to 6 U) will not normalize the INR in the bulk of patients.[104] Estimates of the volume of FFP required to restore vitamin K–dependent coagulation factors and normalize the INR are as high as 35 mL/kg,[111] a dose far in excess of what is practical to administer to a bleeding patient. PCCs are now available in many jurisdictions. Generally, the so-called three-factor PCCs have adequate levels of coagulation factors II, IX, and X and reduced levels of VII. As a result, when administered to patients who have received warfarin, they may not normalize the INR.[112] However, the coadministration of a three factor PCCs with a small dose of FFP or rfVIIa can rapidly correct the INR.[112] The four-factor PCCs have adequate levels of coagulation factors II, VII, IX, and X. As a result, when administered in appropriate doses, they can rapidly correct the INR to normal.[113] Advantages of PCCs include the ability to rapidly reconstitute them from a lyophilized powder, viral inactivation, a small volume of administration allowing rapid infusion, and a lack of a need for cross-match. In general, they are somewhat more expensive than FFP (300 U resource costs for PCCs, 500 IU, vs. 176.5 U resource costs for one bag of FFP), but their ability to rapidly correct the INR results in better cost-effectiveness for PCCs.[114] Currently, there are a number of three- and four-factor PCCs available or under evaluation in many jurisdictions. Their composition regarding contents of the four coagulation factors—protein C, protein S, antithrombin, and heparin—differ somewhat, but there are no comparisons to demonstrate whether this has clinical implications.[115] Clinicians are advised to contact their transfusion medicine service to better understand what is available in their area. The required dose of PCCs can be calculated from the current and the desired INR and the body weight[116] or appreciated according to a simplified algorithm (Table 107.8).

When reversing oral VKAs, it is critically important to remember to administer supplemental vitamin K. In the setting of major or life-threatening bleeding, this is best administered in doses of 5 to 10 mg given by slow intravenous infusion. Vitamin K has been reported to cause anaphylaxis at an incidence of 3 per 100,000

Table 107.7

Efficacy of different antidotes for the antithrombotic agents[a]

	Vitamin K	Plasma	PCC	rfVIIa	APCC	Protamine	DDAVP	Platelets
VKA	+++ very slow	+	+++	++				
UFH				+		+++		
Low molecular weight heparin				++		++		
Direct thrombin inhibitors					?			
Fondaparinux				++				
Direct factor Xa inhibitors			++					
Aspirin							++	++
Clopidogrel, Prasugrel							++	+++
Ticagrelor								–

[a]+++ indicates very good effect; ++ indicates effect shown in human volunteers or in clinical situations under certain conditions; + indicates some effect, other alternatives preferred; ? indicates effect in animal model or single case reports; – indicates no expected effect.
PCC, prothrombin complex concentrate; rfVIIa, recombinant-activated factor VII; APCC, activated prothrombin complex concentrate; DDAVP, deamino-D-arginine vasopressin.

Table 107.8

A simplified nomogram for estimation of dose of PCC for reversal of VKAs

Dosing for Target INR:	~1.5	<1.2
Current INR	Dose	Dose
2–3	20 IU/kg	30 IU/kg
3–6	30 IU/kg	40 IU/kg
>6	40 IU/kg	50 IU/kg

intravenous doses (95% CI, 0.04 to 11).[117,118] Failure to administer vitamin K may result in "rebound" anticoagulation on the day following admission due to diminution of the activity of transfused coagulation factors in the setting of a persistent effect of the VKAs (particularly with warfarin and phenprocoumon that have longer half-life than acenocoumarol). Vitamin K at doses of 1 to 2 mg, administered orally, are effective treatments for excessive anticoagulation without bleeding, or in the setting of minor bleeding where a short period of reduction or normalization of the INR is deemed necessary. However, low-dose oral vitamin K has been shown to not reduce bleeding in asymptomatic patients with excessively prolonged INR values.[119] If the INR is markedly prolonged (e.g., >10.0), the administration of a small oral dose of vitamin K (e.g., 2.5 mg) is generally recommended.[46,120]

Unfractionated and Low Molecular Weight Heparin

Heparin is a complex macromolecular biologic agent that produces the majority of its anticoagulant effect through an antithrombin-dependent catalytic action on serine proteases that are active in coagulation, including factors IIa, Xa, VIIa, VIIIa, Xia, and XIIa. Chemical or physical depolymerization of heparin produces LMWH, which (due to reduced nonspecific binding) has more predictable pharmacokinetics. UFH and LMWH can be given in prophylactic doses (for the prevention of thrombosis) or therapeutic doses (for its treatment). Although bleeding may complicate lower dose heparin therapy, it is predominately a complication of higher doses. Very large doses of UFH, associated with a high risk of major bleeding, are used in procedures such as cardiopulmonary bypass, extracorporeal membrane oxygenation, and in other settings with intense prothrombotic stimuli or where a surface is in contact with the blood.

UFH can be completely reversed with protamine sulphate, which inhibits heparin through a covalent interaction. One mg of protamine reverses about 100 U of heparin activity. When judging the dose of protamine sulphate, the amount of heparin in the circulation should be estimated, assuming a half-life of infused drug of 30 to 60 minutes. The effectiveness of protamine can be assessed using an APTT, activated clotting time, or thrombin clotting time (TCT); full neutralization should result in normalization of these tests. When UFH is given by subcutaneous injection or in very large doses, its half-life may exceed that of protamine; in such cases "rebound" anticoagulation may be seen and additional doses of protamine may be required.[121] Care should be taken with protamine; it is a fish-derived protein and may cause allergic reactions in selected groups of patients, including those with an allergy to iodine. Rapid administration is associated with hypotension and a systemic inflammatory state—thus the infusion should be at a moderate rate, and slowed or stopped if these complications are seen.

Protamine partially reverses the effect of LMWHs—it reverses all of their anti-IIa effect and a variable proportion of their anti-Xa effect.[122,123] The degree of reversal appears to correlate *in vivo* with the average chain length and charge of the LMWH with products such as tinzaparin being more reversible than enoxaparin.[123] However, the clinical impact of these differences is unknown.

In addition to the administration of protamine, those measures discussed in the introduction should be instituted. As the half-life of heparin is short (UFH 0.5 to 2 hours, LMWH ~5 hours), its effect will wane rapidly—thus with mild or moderate bleeding simple heparin withdrawal may be all that is required.

A variety of heparinases[124] and synthetic heparin-binding agents such as concatemeric peptides[125] and platelet factor 4[126] have been evaluated in clinical trials—none are approved or commercially available.

There is no evidence that fibrinolytic inhibitors of PCCs, are effective for the reversal of heparin-associated bleeding.

The amount of LMWH present may be determined using an anti-Xa heparin level. Although there are concerns about the reliability of this test, any detectable level indicates the presence of drug. Serial anti-Xa levels may be useful in the case of intentional overdose or to monitor for residual or excessive anticoagulant effect in patients with renal insufficiency.

Thrombin Inhibitors

Until recently, direct thrombin inhibitors were exclusively parenteral and used in limited clinical circumstances. Thrombin inhibitors available include argatroban, the hirudins, and hirudin derivatives such as bivalirudin. Recently, dabigatran was approved for the prevention of cerebrovascular events in patients with atrial fibrillation. Other approved or sought indications include orthopedic prophylaxis and the prevention of recurrent thrombosis in patients with acute deep vein thrombosis or pulmonary embolism. With the advent of these medications, thrombin inhibitors are likely to become commonly used.

There are no antidotes for the anticoagulant effect of the thrombin inhibitors. Management of major or life-threatening hemorrhage is contingent on the basic principles previously outlined: withdrawal of the agent, investigations to identify and treat the bleeding source, lifesaving therapy, and transfusion to treat identified cytopenias or coagulopathies.

Argatroban has a half-life of <1 hour after intravenous administration, and thus its effect wanes quickly with withdrawal. Argatroban is not renally excreted; thus, it can be used in patients with severe renal insufficiency. Argatroban is very dependent on normal hepatic function, and its half-life will be markedly prolonged in patients with hepatic dysfunction. The INR is markedly prolonged during treatment with argatroban. Concomitant treatment with warfarin should start at the expected daily dose without any loading dose. The argatroban infusion at doses up to 2 μg/kg/min is discontinued when INR is >4, and it should then be checked again after 4 to 6 hours. If the repeat INR is below 2.0, the argatroban infusion should be resumed.

Hirudins are renally excreted, in part. Their half-lives are markedly prolonged in the setting of renal failure. Bivalirudin is cleared in part by autocatalysis. As a result, it is less affected than the other parenteral direct thrombin inhibitors by organ dysfunction. It has a very short circulating half-life of about 25 minutes.

Dabigatran is 80% excreted by the kidneys.[127] After oral administration, it has a half-life of approximately 15 hours, becoming prolonged in patients with renal insufficiency. Dabigatran is only 25% to 35% protein bound[128] and thus may be removed by dialysis. Unabsorbed drug may be cleared by oral charcoal administration and gastric lavage.[129] Dialysis, oral charcoal administration, and gastric lavage may be particularly useful in the case of intentional overdose where dabigatran may be present in the gastrointestinal tract and circulation in much higher than normal levels.[129]

It is unclear whether "prohemostatic" agents such as PCC, rfVIIa, fibrinolytic inhibitors, or DDAVP are able to mitigate bleeding complications due to thrombin inhibitors. Small studies in animals and in normal volunteers suggest that *ex vivo* markers of activity (such as coagulation tests) may be normalized with PCCs or rfVIIa.[129] Conversely, rfVIIa failed to reverse the effect of another oral thrombin inhibitor, melagatran, in volunteers.[130] Activated prothrombin complex concentrate (APCC) at a dose of 50 U/kg effectively stopped bleeding in an animal model with melagatran.[131] There is no evidence of the efficacy of these agents in bleeding patients. In the case of major or life-threatening bleeding, any of these agents may be considered, keeping in mind the lack of evidence for their efficacy and their potential to cause side effects including thrombosis.

There are no commonly available quantitative assays for any of the direct thrombin inhibitors. These agents variably prolong the INR, the APTT, and other coagulation tests such as the ecarin clotting time.[129] TCT using 2 U thrombin/mL of reagent is very sensitive to the presence of direct thrombin inhibitors. As a result, a normal TCT rules out the presence of any of these agents.

Indirect Factor Xa Inhibitors

Fondaparinux is a parenteral antithrombin-dependent inhibitor of coagulation factor Xa. After subcutaneous administration, it has a half-life of 17 to 21 hours. It is dependent on renal function for clearance, and its half-life is markedly prolonged in patients with severe renal insufficiency. Protamine is ineffective for the reversal of fondaparinux. When bleeding occurs, standard management (previously outlined) should be invoked. There is no evidence that transfusion therapy effectively reduces the activity of fondaparinux. Small studies in animals and healthy volunteers suggest that rfVIIa can ameliorate the anticoagulant effect of fondaparinux, and there are case reports supporting the efficacy of 30 to 90 μg/kg rfVIIa to control bleeding associated with fondaparinux or the long-acting pentasaccharides idraparinux.[132–135]

Fondaparinux levels can be determined using an anti-Xa heparin level. For quantification, the assay should use a fondaparinux standard, although in emergency situations an LMWH standard could be used realizing that the resultant levels will inaccurately convey the quantity of fondaparinux but should accurately reflect the presence or absence of the medication.

Direct Factor Xa Inhibitors

Rivaroxaban is available in many countries, and apixaban has been approved for prophylaxis against VTE after orthopedic surgery in the European Union at the time of writing. Additional agents (including edoxaban and betrixaban) are currently in the late stage of clinical evaluation. The direct inhibitors of factor Xa are oral agents. They are dependent to a greater or lesser extent on normal renal function for excretion. After oral administration, they are rapidly absorbed and have relatively short half-lives (<12 hours).

With major bleeding, patients should receive standard management (previously outlined). If a dose has been taken recently or in the case of intentional overdose, elimination from the gastrointestinal tract with gastric lavage and oral administration of charcoal might be considered. Rivaroxaban is highly protein bound and thus is not amenable to dialysis.[136] It might be removed by hemoperfusion, but this has not been studied. The use of rfVIIa is recommended for bleeding; however, there is no clinical evidence to support its effectiveness.[137] *Ex vivo* studies suggest that PCCs may be able to reverse the effective rivaroxaban. This effect, if it actually exists, is probably due to the fact that rivaroxaban is a stoichiometric inhibitor of factor Xa—administering PCCs presumably "floods" the circulation with factor X that binds to and inactivates rivaroxaban.[138]

Prothrombin time and anti-Xa show a linear response to rivaroxaban levels, but the tests are assay dependant. Standard calibrators for rivaroxaban are available and should be used to develop assay-specific calibration curves.

Antiplatelet Drugs

Bleeding complications commonly occur in patients taking antiplatelet agents. The risk of hemorrhage is increased by the use of multiple agents particularly when in concert with an anticoagulant such as warfarin.[139]

In general terms, there are no specific antidotes for the antithrombotic effect of any of the antiplatelet agents. Treatment is further complicated by the observation that most agents produced sustained inhibition of platelet activity, with the degree of inhibition depending on the type of agent. Recently, a number of short-acting agents and/or reversible agents have been evaluated,[140,141] although it is likely that their use will be limited given the widespread acceptance and relative inexpensiveness of agents such as aspirin and clopidogrel.

There are no easily available assays, which accurately reflect the degree of inhibition of platelet activity. As a result, treatment of bleeding should be undertaken with the assumption that an antiplatelet effect is present if, based on an estimation of half-life, it is likely that drug effect will still be present. It is important to remember that the platelet inhibitory half-life of aspirin and clopidogrel considerably exceeds their pharmacokinetic half-life due to their irreversible impact on platelet function (in the case of aspirin) or the presence of a metabolite with an extended half-life (clopidogrel).

In the setting of major or life-threatening bleeding, the general approach to managing hemorrhage (previously outlined) should be implemented. Use of nonspecific prohemostatic agents (such as DDAVP) has been evaluated in small experiments in normal volunteers or in animals, but there is little evidence to guide practice in patients with hemorrhage.[142] In

truly life-threatening hemorrhage or in patients requiring time-limited interventions, transfusion of platelets may provide enhanced prothrombotic activity particularly if the drug has been removed from the circulation while the patient's native platelets continue to be inhibited.[143] For example, the antithrombotic effect of aspirin persists well beyond its pharmacologic half-life due to irreversible inhibition of the cyclooxygenase enzyme. Transfusion of normally functional platelets should reconstitute some degree of platelet function if administered after the clearance of all of the aspirin from the circulation. Ticagrelor binds reversibly to the ADP receptor and can therefore inhibit freshly transfused platelets to the same extent. Further details on the management of antiplatelet agents are provided in Chapter 111.

MANAGEMENT OF ANTICOAGULATION AFTER MAJOR BLEEDING

Patient with a VKA-associated major bleeding have to be evaluated carefully for (a) resumption of anticoagulation and (b) the optimal timing, in case resumption is deemed having a greater benefit than risk. The most difficult constellation is the patient with a clear indication for anticoagulation (mechanical valve or atrial fibrillation with a previous ischemic stroke) and an intracranial bleeding. In a decision analysis using a Markov model, stratified for the location of an intracerebral hemorrhage being lobar or deep hemispheric, Eckman et al.[144] found that for lobar bleeding withholding future anticoagulation resulted in improved quality-adjusted life expectancy. Conversely, for deep hemispheric hemorrhage and a high risk of stroke, resumption of anticoagulation was preferred. Majeed et al.[145] performed a retrospective study of 234 patients with warfarin-associated intracranial hemorrhage, out of whom 59 resumed anticoagulation. By estimation of the rates of the two main adverse events—recurrent intracranial bleeding and ischemic stroke—in relation to different time points of resumed anticoagulation, it was demonstrated that the optimal resumption point for VKAs is 10 to 30 weeks after the index bleeding. Prophylaxis against VTE should, however, be started already after 2 to 4 days with low-dose subcutaneous heparin or LMWH.[146,147]

The most common major hemorrhagic complication on VKA, gastrointestinal bleeding, is more tolerable, even in case of recurrence. Half of these bleeding are from the upper part of the gastrointestinal tract, usually caused by an ulcer and the early mortality is about 4%.[148] Recurrent bleeding, including life-threatening events, are not uncommon.[78] It is probably optimal to delay resumption of anticoagulation until healing of an ulcer has been demonstrated by repeat endoscopy, although this has not been proven in any appropriately designed study.

SUMMARY

Antithrombotic agents are highly effective for the prevention and treatment of a variety of prothrombotic disorders. Complications of their use largely consist of bleeding. As novel agents are introduced, management becomes more complex. The lack of antidotes and a proliferation of agents with variable half-lives, pharmacologic effects, and dosing increases the likelihood of errors leading to excessive anticoagulant effect. Management of hemorrhage should consist predominantly of a withdrawal of the agent, evaluation of the source of bleeding, disruption of the source of bleeding, administration of an antidote (if one exists), administration of nonspecific prohemostatic agents, and heroic interventions (when required) including the use of dialysis or hemoperfusion. In most cases, symptomatic management and the passage of time will lead to elimination of the anticoagulant effect of these agents and cessation of bleeding.

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CHAPTER

108 Heparin-Induced Thrombocytopenia

THEODORE E. WARKENTIN AND ANDREAS GREINACHER

Heparin-induced thrombocytopenia (HIT) is an immune-mediated adverse drug reaction caused by heparin-dependent, platelet-activating immunoglobulin (Ig) G antibodies that recognize complexes of platelet factor 4 (PF4) bound to heparin.[1–3] HIT represents a strong, independent risk factor for venous and arterial thrombosis.[4]

HIT typically causes thrombosis in large veins and arteries; platelet-rich, intra-arterial "white thrombi" were first described in 1958.[5,6,7] However, HIT is also a risk factor for microvascular thrombosis in certain situations, such as in coumarin treatment of deep vein thrombosis (DVT) (i.e., coumarin-associated venous limb gangrene [see Chapter 109]) or severe HIT-associated disseminated intravascular coagulation (DIC).[8,9,10]

Thrombosis typically occurs in anti-PF4/heparin antibody-positive patients who develop significant declines in platelet count,[11,12,13,14] pointing to clinically relevant consequences of *in vivo* platelet activation induced by these antibodies. A central role for *in vivo* thrombin generation in HIT[8] provides a rationale for the use of anticoagulants that inhibit thrombin or its generation.[15]

DEFINITION AND TERMINOLOGY

HIT can be defined as any clinical event best explained by platelet-activating anti-PF4/heparin antibodies ("HIT antibodies") in a patient who is receiving, or who has recently received, heparin (or another polyanion implicated in this syndrome).[1] Thrombocytopenia is the most common event in HIT and occurs in at least 90% of the patients, depending on the definition of thrombocytopenia. A high proportion (50% to 75%) of patients with HIT develop thrombosis.

A consensus report[15] recommended that the term *heparin-induced thrombocytopenia* should be used to refer to the antibody-mediated disorder. Therefore, HIT can be considered a *clinicopathologic* syndrome in which the diagnosis can be made accurately when one or more *clinical* events (e.g., thrombocytopenia, thrombosis, necrotizing skin lesions at heparin injection sites, acute systemic [anaphylactoid] reactions, decompensated DIC) occur together with laboratory detection of the *pathologic* HIT antibodies. In contrast, it is recommended that the term *nonimmune heparin-associated thrombocytopenia* (HAT) should be used to describe thrombocytopenia occurring during heparin use that is not associated with HIT antibodies. Often, nonimmune HAT is due to a comorbid disorder (e.g., perioperative hemodilution), although a direct platelet-activating effect of heparin that is not mediated by antibody may occur in some patients.[16] Unlike the situation in HIT, the causative role of heparin in nonimmune HAT in a given patient cannot readily be ascertained.

PATHOGENESIS

FIGURE 108.1 summarizes the pathogenesis of HIT.[17] The central concept is the formation of heparin-dependent IgG that activates platelets via their FcγIIa receptors.[18] It remains controversial—although in our view unlikely—whether IgA and IgM antibodies evince pathogenicity.[19–21,22] The target antigen is a complex of heparin and the positively charged platelet α-granule protein, PF4,[23,24–26] a member of the CXC subfamily of chemokines. HIT antibodies are directed against one or more sites on PF4 that have been conformationally altered by binding to negatively charged heparin.[27–29,30] This model is consistent with reports that other highly sulfated, nonheparin carbohydrates such as pentosan polysulfate[31] and polysulfated chondroitin sulfate[32] can trigger HIT. This possibly triggered additional adverse effects by oversulfated chondroitin sulfate within "contaminated" heparin.[33] It may also explain why low molecular weight heparin (LMWH) is less likely to trigger HIT,[11,12] as only heparin molecules of at least 12 saccharides in length bind PF4 in such a way as to induce a large number of neoepitopes required for clinical breakthrough of HIT.[34] Rarely, a disorder mimicking HIT on clinical and serologic grounds can occur in the absence of preceding heparin exposure ("spontaneous HIT"); reported cases have featured preceding inflammatory events such as surgery or infection.[35–37] This indicates that the immune reaction to PF4/polyanion complexes can also be induced by endogeneous factors, a feature relevant to understanding the temporal features of antibody formation. Moreover, many HIT-positive sera[38,39]—but especially those obtained from patients whose HIT begins[40] or worsens after stopping heparin—are able to activate platelets *in vitro* even in the absence of pharmacologic heparin, a phenomenon consistent with "autoimmune-like" features.

Anti-PF4/heparin seroconversion has certain atypical features[41,42]: Antibodies are formed relatively quickly—approximately 4 days (median) after even a first-time immunizing heparin exposure, and without evidence for more rapid formation with previous heparin exposure or even with previous HIT.[43] Anti-PF4/heparin antibodies of any of the three major Ig classes can develop and in any combination, in the frequency IgG > IgA > IgM.[41,42] Moreover, when IgM antibodies are formed, these are detectable simultaneously with IgG and/or IgA, that is, there is no IgM precedence (as would be expected in a "classic" immune response). This indicates that the immune response induced by heparin is a secondary, rather than a primary, immune response; PF4 complexed to polyanions on bacterial surfaces might be the "preimmunizing" trigger.[44]

Several factors contribute to the prothrombotic nature of HIT (FIGURE 108.1): (a) the potent platelet-activating

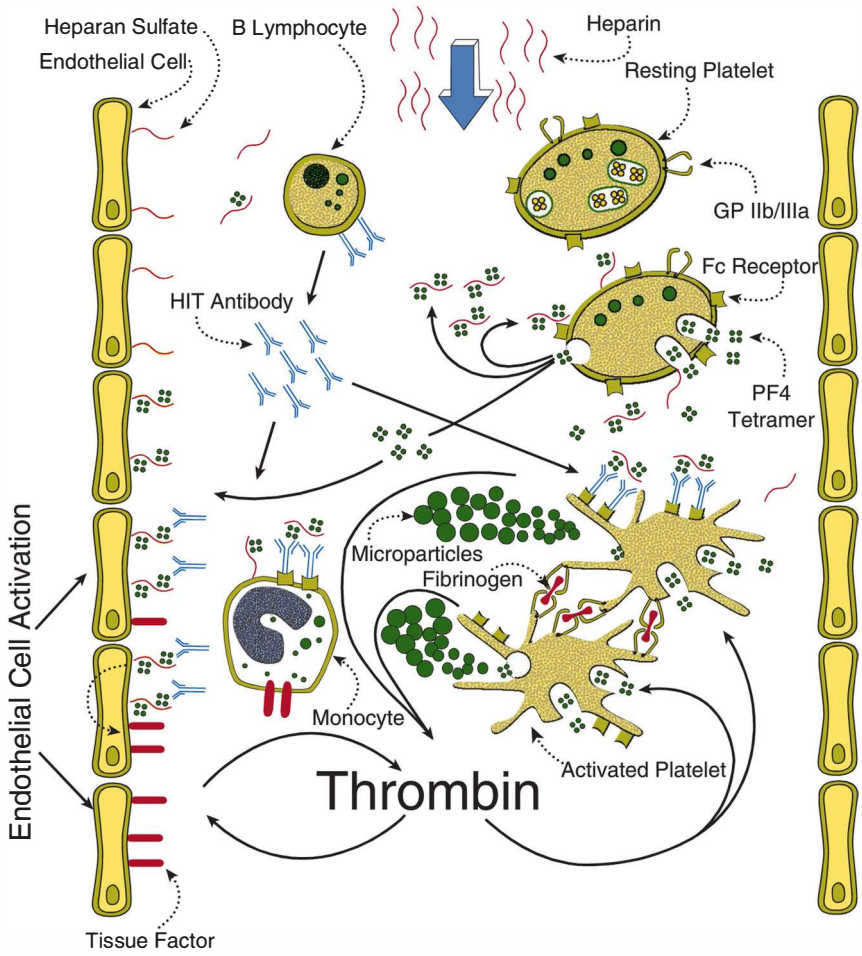

FIGURE 108.1 Pathogenesis of HIT. Heparin produces mild platelet activation, resulting in the release of PF4 from platelet α-granules and in the formation of immunogenic PF4/heparin complexes. B lymphocytes generate IgG that recognize the PF4/heparin complexes; the Fc "*tails*" of the IgG bind to platelet FcγII receptors, resulting in Fc receptor clustering and consequent strong platelet activation. Platelet aggregation is mediated by glycoprotein (GP) IIb/IIIa. Platelet-derived microparticles that accelerate thrombin generation are produced. The HIT antibodies also recognize PF4 bound to endothelial heparan sulfate, leading to tissue factor expression on endothelium. HIT antibodies also can activate monocytes. In all, increased thrombin generation results can explain some of the unusual clinical manifestations of HIT (e.g., venous limb gangrene and DIC) and provide a rationale for treatment that reduces thrombin generation. (From Greinacher A, Warkentin TE. Treatment of heparin-induced thrombocytopenia: an overview. In: Warkentin TE, Greinacher A, eds. *Heparin-induced thrombocytopenia*, 4th ed. New York: Informa Healthcare USA, 2007:283–317.)

properties of the HIT antibodies, which also generate procoagulant, platelet-derived microparticles[45,46]; (b) pancellular activation (either directly or indirectly by HIT antibodies),[47,48] manifesting as tissue factor expression on endothelium[25,48,49] and monocytes,[50,51] and possibly platelet–leukocyte complex formation[52]; and (c) neutralization of the heparin anticoagulant effects by PF4. Collectively, these factors result in increased thrombin generation in HIT.[8,53,54]

Women are more likely than men to develop HIT (~ twofold increase by odds ratio).[55] However, no HLA association or other genetic risk factor has been identified.[56] Although the platelets bearing FcγIIa receptors of the His131 phenotype (~70% of the population) are more readily activated by HIT antibodies of IgG1 subclass (the predominant subclass in HIT),[57,58] no clear association between HIT and Fc receptor genotype has been established.[59,60] A strong, albeit indirect, argument against a genetic risk factor is the surprising observation that even patients with a previous history of HIT do not usually regenerate HIT antibodies upon repeat heparin exposure.[43,61]

RISK FACTORS FOR HIT

The frequency of HIT is variable and is influenced by the type of patient population (major surgery or trauma > minor surgery > medical > obstetrical), sex (female > male), the type of heparin used (bovine unfractionated heparin [UFH] > porcine UFH > porcine LMWH > fondaparinux), duration of heparin therapy (the risk increases until ~10 to 14 days of heparin administration), and, possibly, the timing of the first

dose of anticoagulant in relation to surgery (postoperative > preoperative) and body mass index (BMI) (high BMI > low BMI).[55,62–64] A stoichiometric model of optimally immunizing PF4:heparin ratios has been proposed to explain some of these observations.[64,65]

A relatively high frequency (~5%) of HIT occurs in postoperative orthopedic surgery patients receiving prophylactic-dose UFH for up to 14 days.[11,12,66] The frequency is even higher (8% to 10%) in postcardiac surgery patients who receive therapeutic-dose UFH after implantation of a ventricular assist device.[67–69] In contrast, HIT was not observed in several large studies in which LMWH was administered for many weeks during pregnancy.[70–72] The frequency of HIT occurrence is estimated to be about 1% in patients receiving UFH for treatment of venous thromboembolism[62] and, perhaps, for prevention of thrombosis in medical patients,[73] and approximately 0.1% to 0.5% in postoperative patients receiving LMWH[62]; case reports suggest a small risk of HIT (probably <0.01%) with fondaparinux thromboprophylaxis after surgery.[74–76] However, it might be that in these cases the antibodies (albeit triggered by fondaparinux) show the autoantibody-like behavior described above. Given the decreasing use of UFH after orthopedic surgery, one common scenario for HIT today is antithrombotic prophylaxis with UFH following cardiac surgery (risk: ~1% if UFH is given for >1 week).[77]

Patient-dependent risk factors influence the type of HIT-associated thrombotic event. For example, venous thrombosis occurs in at least half of postorthopedic surgery patients who develop HIT, with relatively few arterial thrombi observed.[11,12,66] In contrast, arterial thrombosis occurs at least as often as venous thrombosis in patients with HIT after cardiac surgery, suggesting a predisposing role for atherosclerosis.[62,78,79] Risk of limb amputation is greater in patients who develop HIT in the setting of peripheral vascular surgery.[80] Patients with a central venous catheter who develop HIT have about a 10% chance of developing symptomatic upper limb DVT at the catheter site, reflecting the interaction of localized vessel injury with the systemic hypercoagulability of HIT.[81]

CLINICAL PRESENTATION

HIT is a distinct immunohematologic syndrome that usually can be distinguished on clinical grounds from IgG-mediated thrombocytopenia caused by drugs such as quinine/quinidine or sulfa antibiotics.[82–84]

Thrombocytopenia

The thrombocytopenia is usually mild to moderate in severity (median platelet count nadir, ~50 to 60 × 10^9/L)[1,9,82–84] (see FIGURE 108.2). The platelet count is <20 × 10^9/L in only 5% to 10% of patients with HIT; in contrast, severe thrombocytopenia typically occurs in patients with immune thrombocytopenia caused by other drugs.[84–86] Petechiae and ecchymoses are usually absent even in patients with HIT who have severe thrombocytopenia. Thrombosis is common in HIT, irrespective of the severity of the thrombocytopenia, and can occur in patients with a decreased platelet count that may not necessarily be lower than 150 × 10^9/L[1,12] (see FIGURE 108.3), although the risk and severity of thrombosis increase with the degree of thrombocytopenia, whether expressed in absolute or proportional terms.[12,13,14,80,87,88] In postoperative patients, a proportional fall in platelet count (≥50%) between postoperative days 4 and 14 is more sensitive (and similarly specific) for HIT than the conventional definition of thrombocytopenia (platelet count fall to <150 × 10^9/L). Identifying the postoperative platelet count peak as the appropriate "baseline," together with regular platelet count monitoring (particularly in patients receiving UFH after a major surgery), is important for being able to recognize a HIT-associated decrease in platelet count when a thrombotic event occurs (FIGURE 108.3).[12,83,87,88]

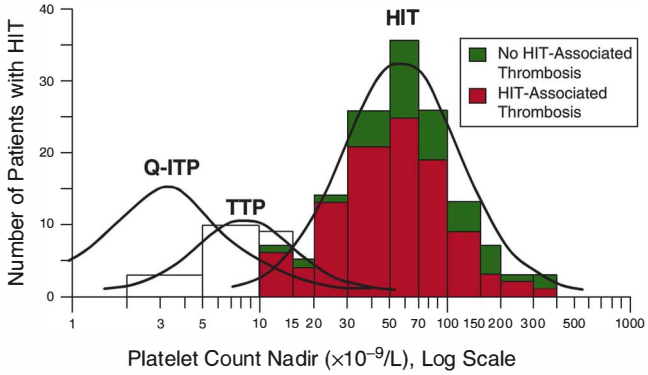

FIGURE 108.2 Platelet count nadirs in HIT, quinine-induced immune thrombocytopenic purpura (Q-ITP), and thrombotic thrombocytopenic purpura (TTP) with absent ADAMTS-13 activity. The majority of patients with HIT have HIT-associated thrombosis, and the risk of thrombosis is substantial irrespective of the severity of the thrombocytopenia. Data on TTP were kindly provided by James N. George, MD. (From Warkentin TE. Think of HIT. *Hematology Am Soc Hematol Educ Program* 2006:404–414, with permission.)

Timing

Patients with HIT typically develop a platelet count fall that begins between days 5 and 10 of heparin treatment (1st day of heparin use [especially intraoperative or early postoperative heparin] = day 0).[9,43,89] Sometimes, thrombocytopenia can begin earlier in patients who have been exposed previously to heparin. In general, this is observed only in patients whose previous heparin exposure is recent (within the past 3 months), in which case an immediate recurrence of thrombocytopenia can occur on reexposure to heparin.[43,89] A true anamnestic immune response, in which previous heparin exposure predisposes to a greater probability of forming more avid (higher-titer, higher-affinity) antibodies, is *not* a feature of HIT. Sometimes, HIT can begin several days after all heparin has been stopped (delayed-onset HIT) or progress despite stopping heparin.[40,90–92,93] This clinical picture is characterized by high-titer HIT antibodies that can activate platelets even in the absence of heparin.[40]

Prospective studies indicate a characteristic "timeline" of anti-PF4/heparin seroconversion and development of clinically evident HIT: Antibodies first become detectable at day 4, the platelet count begins to fall at day 6, thrombocytopenia (≥50% platelet count fall) is manifest at day 8, and thrombosis occurs at day 10 (days all expressed as median values). However, while the timing of the immune response and the associated platelet

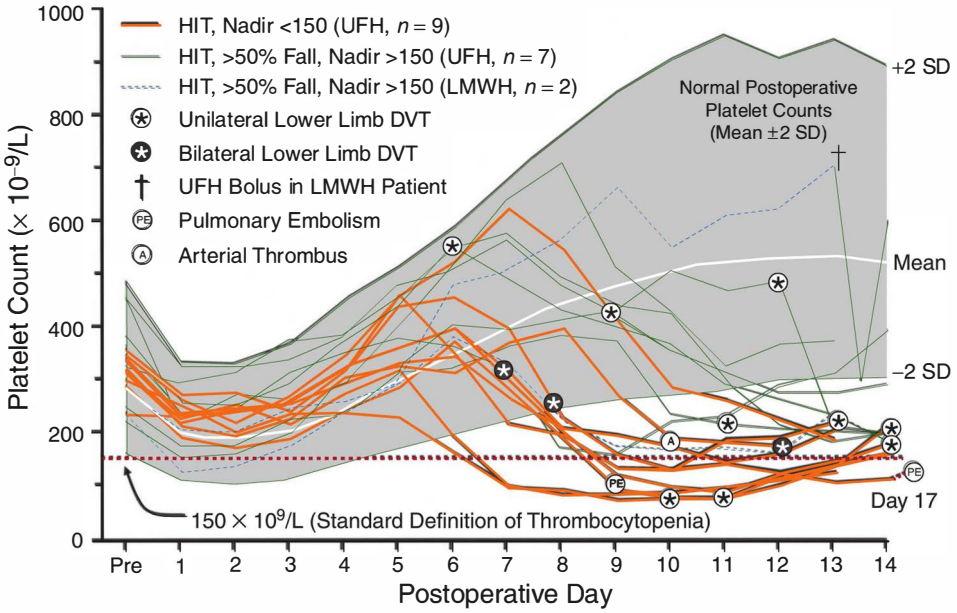

FIGURE 108.3 Characteristic timing, severity, and prothrombotic nature of HIT. The *shaded area* indicates the mean (±2 standard deviation [SD]) platelet count range in the reference population without HIT. There is an immediate postoperative decrease in the platelet count (maximal, days 1 to 3), followed by a rise to levels greater than baseline (maximal, days 11 to 14). Antithrombotic prophylaxis with subcutaneous UFH (7,500 U twice daily) or the LMWH, enoxaparin (30 mg twice daily), was begun usually on postoperative day 1. Eighteen patients developed HIT (50% or greater fall in the platelet count from the postoperative peak), with nine evincing a platelet count nadir <150 × 10^9/L and nine a platelet count nadir >150 × 10^9/L. The *symbols* indicate thrombotic events, 18 of which occurred in 13 of the 18 patients with HIT. One patient who received enoxaparin developed an abrupt platelet count fall when given a 5,000 U bolus of UFH (†). (Created from figures in Warkentin TE, Levine MN, Hirsh J, et al. Heparin-induced thrombocytopenia in patients treated with low-molecular-weight heparin or unfractionated heparin. *N Engl J Med* 1995;332:1330–1335; Warkentin TE, Roberts RS, Hirsh J, et al. An improved definition of immune heparin-induced thrombocytopenia in postoperative orthopedic patients. *Arch Intern Med* 2003;163:2518–2524.)

count fall is rather uniform, for thrombotic events, the onset ranges widely, sometimes occurring even before the fall in platelet count is readily apparent, or up to several weeks later.[14,83,88]

Complications

Table 108.1 lists various complications of HIT by frequency. The strong association between HIT and thrombosis means that this diagnosis should be considered when a patient develops thrombosis or other potential HIT-associated sequelae in association with a falling platelet count during, or shortly after stopping, heparin therapy (FIGURE 108.3).

Venous thrombosis is the most common complication of HIT and includes DVT and pulmonary embolism.[1,11,12,13,94] Abdominal or flank pain suggests adrenal hemorrhagic necrosis, which can lead to shock secondary to acute adrenal failure if bilateral. This complication results from adrenal vein thrombosis.[9] Headache and acute neurologic sequelae suggest dural sinus (cerebral venous) thrombosis.[91,92] Splanchnic vein thrombosis (e.g., mesenteric vein thrombosis) is a rare complication of HIT.

Arterial thrombosis is relatively common in HIT and most often involves large limb arteries, resulting in acute arterial occlusion with absent pulses.[8,94,95] Thrombotic stroke, myocardial infarction, or mesenteric artery thrombosis occurs less often.[9,94]

Limb ischemia or gangrene can be categorized as limb ischemia with and without palpable or Doppler-identifiable pulses (see FIGURE 108.4).[5,8,95] Absent pulses suggests that the acute limb ischemia is caused by large artery occlusion by platelet-rich "white clots." In contrast, severe limb ischemia with pulses present indicates microvascular thrombosis in HIT and is most commonly caused by treatment of DVT using warfarin (coumarin) (see Chapter 109).[8,96–98] The laboratory hallmark of coumarin-associated venous limb ischemia is a supratherapeutic international normalized ratio (INR) (usually >3.5), which is a surrogate marker for severe protein C depletion (in parallel with a severe reduction of factor VII) complicating coumarin therapy for HIT-associated DVT (see Chapter 109). Rarely, venous limb ischemia or necrosis is associated with decompensated DIC in HIT in the absence of coumarin therapy, with or without associated DVT,[9,10] possibly because of acquired natural anticoagulant depletion.

Classic (nonacral) coumarin-induced skin necrosis has been reported in patients with HIT,[8,98,99] but this is less common than venous limb gangrene (see Chapter 109).

Table 108.1

Complications of HIT

Venous Thrombosis	Arterial Thrombosis	Miscellaneous
DVT (50%)	Aortoiliac thrombosis (acute limb ischemia) (5%–15%)	Heparin-induced necrotizing skin lesions (small minority of sensitized patients receiving subcutaneous heparin injections)
Pulmonary embolism (25%)	Acute thrombotic cerebrovascular accident (5%–10%)	Acute systemic reactions postintravenous heparin bolus (≈25% of sensitized patients who receive a heparin bolus)
Warfarin-induced venous limb gangrene (≈10% of HIT patients with DVT treated with warfarin)	Myocardial infarction (3%–5%)	Adrenal hemorrhagic infarction (1%–3%)—manifestation of adrenal vein thrombosis
Rare: cerebral sinus thrombosis (<3%); splanchnic vein thrombosis (e.g., mesenteric or portal vein thrombosis) (<3%)	Rare: thrombosis of upper limb, renal, mesenteric, spinal, other arteries (<3%)	Rare: DIC with hypofibrinogenemia and microvascular thrombosis (<3%)

Estimated frequencies of the various complications of HIT are taken from several series of patients with serologic support of the diagnosis of HIT.
HIT, heparin-induced thrombocytopenia; DVT, deep vein thrombosis; DIC, disseminated intravascular coagulation.

Heparin-induced skin lesions are dermal reactions noted within 24 hours of subcutaneous heparin injection that begin 5 or more days after heparin initiation.[100,101] These lesions are associated with HIT antibodies. Interestingly, only about one-fourth of patients who develop skin lesions together with HIT antibodies develop thrombocytopenia, but those with heparin-induced skin lesions and thrombocytopenia are at particularly high risk for arterial limb thrombosis.[102] The antibodies found in patients with necrotizing skin lesions are indistinguishable from those associated with other manifestations of HIT.

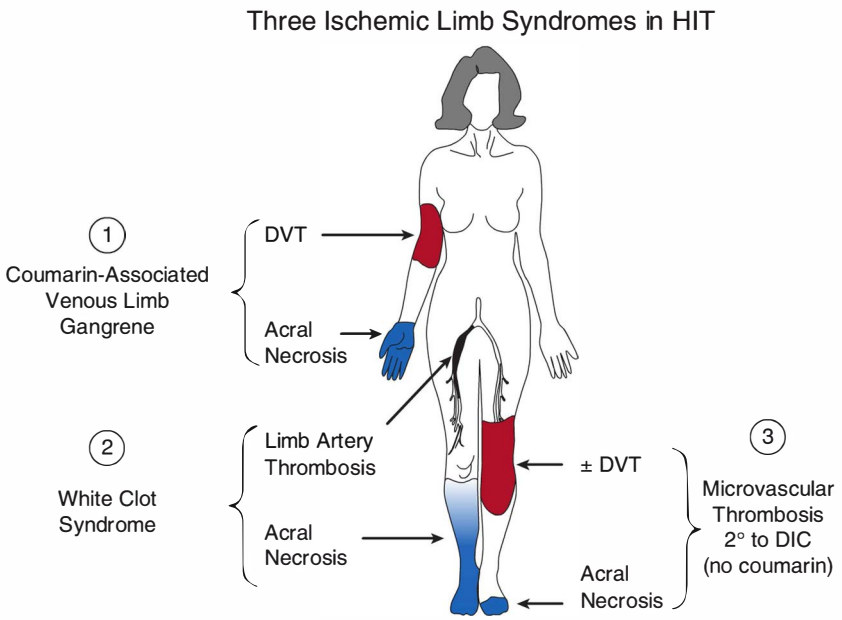

FIGURE 108.4 Three ischemic limb syndromes in HIT. The two most common causes of limb loss in HIT are coumarin-induced venous limb gangrene (microvascular thrombosis in a limb affected by DVT) and white clot syndrome (occlusion of large limb arteries by platelet-rich white clots). Rarely, microvascular thrombosis (with or without associated DVT) is associated with limb ischemia in the absence of coumarin use. DIC, disseminated intravascular coagulation. (Modified from Warkentin TE. Heparin-induced thrombocytopenia: IgG-mediated platelet activation, platelet microparticle generation, and altered procoagulant/anticoagulant balance in the pathogenesis of thrombosis and venous limb gangrene complicating heparin-induced thrombocytopenia. *Transfus Med Rev* 1996;10:249–258.)

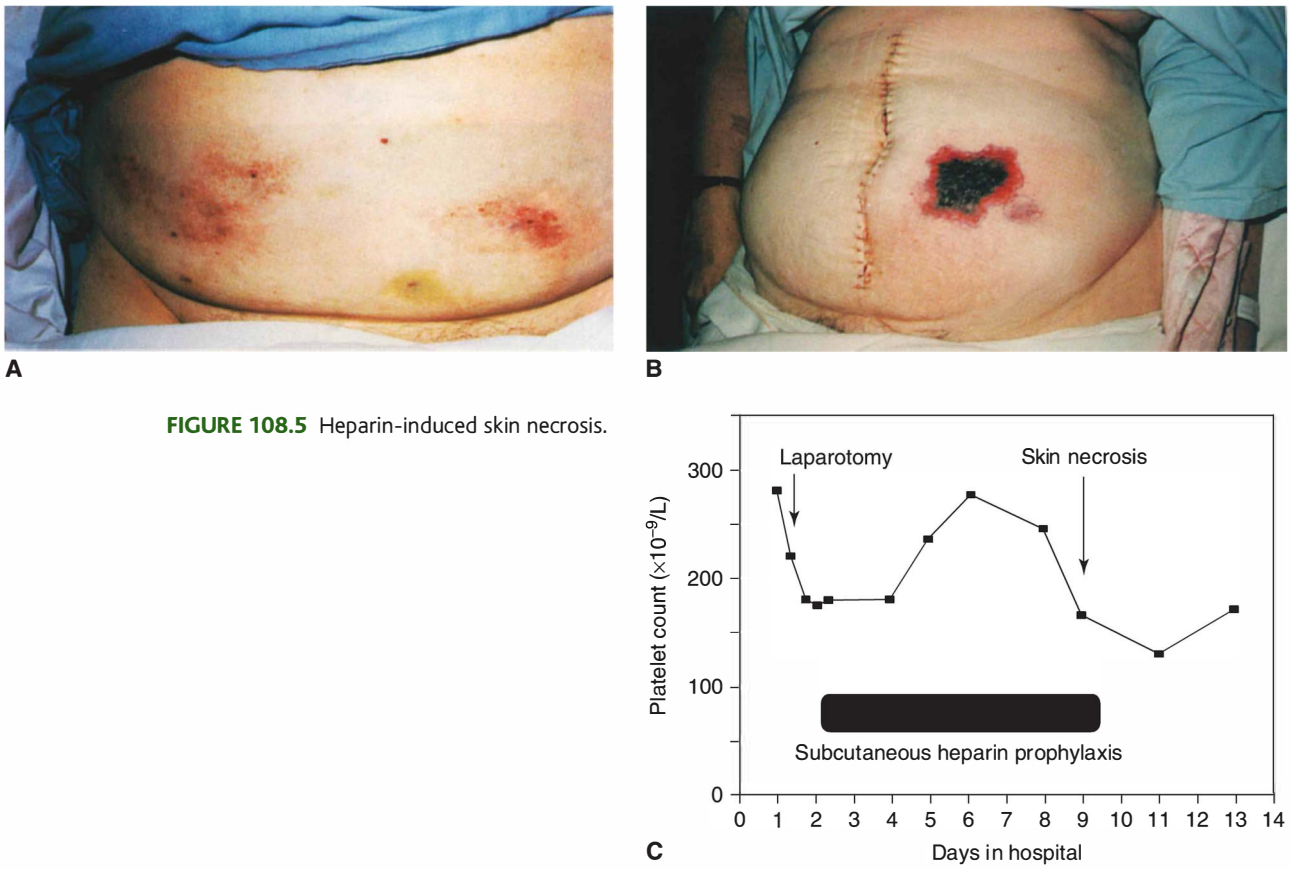

FIGURE 108.5 Heparin-induced skin necrosis.

Two types of skin lesions are described: painful, red plaques and frank skin necrosis (see FIGURE 108.5). Whereas the former can represent delayed-type hypersensitivity reactions with coincidental anti-PF4/heparin seroconversion,[103,104] the latter (necrotizing) reactions appear to be specific for HIT. Close clinical observation and monitoring of platelet count should be performed for several days after stopping heparin injections in patients with skin lesions because thrombocytopenia and thrombosis can begin several days later.[102]

Acute anaphylactoid reactions (*acute systemic reactions*) refer to a variety of systemic symptoms and signs that begin within 5 to 30 minutes of receiving an intravenous heparin bolus—or less often, following subcutaneous injection—and are accompanied by an abrupt decrease in platelet count.[9,105–107] Clinical features are inflammatory (i.e., fever, chills, and flushing), cardiopulmonary (i.e., hypertension, tachycardia, dyspnea or tachypnea, and cardiac or respiratory arrest), neurologic (i.e., transient global amnesia and pounding headache), and gastrointestinal (i.e., watery diarrhea). These differ from the anaphylactic reactions induced by heparin contaminants such as oversulfated chondroitin sulfate.[33,107]

Decompensated DIC refers to an elevated INR and/or activated partial thromboplastin time (aPTT); reduced concentration of fibrinogen; presence of red cell fragments and/or circulating normoblasts; or other laboratory features of severe DIC. Although increased thrombin generation is a universal feature of HIT (as shown by increased levels of thrombin–antithrombin complexes),[8] only 10% to 15% of the patients evince one or more of these features of decompensated DIC. These patients are at an unusually high risk for developing multiple thrombi and/or microvascular thrombosis.

SCORING SYSTEMS

Scoring systems have been developed for use in diagnosis of HIT, to evaluate new laboratory tests for HIT antibodies, or to estimate the pretest probability of HIT.[108–110,111,112,113] Table 108.2 shows the 4Ts scoring system; depending on the clinical setting in which the 4Ts is used, the likelihood of HIT ranges from <2% (low score), to 10% to 30% (intermediate score), to 50% to 70% (high score).[111] Thus, the main utility of the 4Ts is in predicting a low likelihood of HIT when the score is low (≤3 points), that is, a high negative predictive value.[111] A recent scoring system developed using broad expert opinion, the HIT Expert Probability Score, exhibited favorable operating characteristics but requires prospective validation.[113]

LABORATORY TESTING

Both platelet activation and PF4-dependent antigen assays are useful to support the diagnosis of HIT.[114–116] In general, the more abnormal the test result, for example, higher optical density (OD) in the enzyme (-linked) immuno(sorbent) assay (EIA or ELISA), the greater the likelihood that the patient has HIT.[117–119] HIT antibodies are usually detectable in patient serum or plasma only for a few weeks or months,[43] and therefore, acute serum or plasma should be tested.

Table 108.2

Estimating the pretest probability of HIT: 4Ts scoring system

	Points (0, 1, or 2 for Each of 4 Categories: Maximum Possible Score = 8)[a]		
	2	**1**	**0**
*T*hrombocytopenia (acute)	>50% platelet fall (nadir ≥ 20 × 10^9/L)	30%–50% platelet fall (or >50% fall due to surgery) or nadir 10–19 × 10^9/L	<30% platelet fall or nadir <10 × 10^9/L
*T*iming[b] of platelet count fall, thrombosis, or other sequelae (1st day of immunizing heparin course = day 0)	Clear onset between days 5 and 10 or ≤1 d (if heparin exposure within the past 30 d)	Consistent with day 5–10 fall, but not clear (e.g., missing platelet counts) or ≤1 d (heparin exposure within the past 31–100 d) or platelet fall after day 10	Platelet count fall ≤4 d without recent preceding heparin exposure
*T*hrombosis or other sequelae (e.g., skin lesions, ASR)	New thrombosis; skin necrosis[c]; ASR after IV heparin bolus	Progressive or recurrent thrombosis; suspected thrombosis (not yet proven); asymptomatic upper limb DVT)	None
o*T*her causes of thrombocytopenia not evident	No explanation (besides HIT) for platelet count fall is evident.	Possible other cause is evident.	Definite other cause is present.

Note: The score can increase or decrease as additional information is obtained and as the thrombocytopenia evolves.
[a]Pretest probability score: 6–8 = high; 4–5 = intermediate; 0–3 = low.
[b]First day of immunizing heparin exposure is considered day 0; the day the platelet count *begins* to fall for any given episode of thrombocytopenia is considered the day of onset of thrombocytopenia (it generally takes 1–3 more days until an arbitrary threshold that defines thrombocytopenia is passed). In general, giving heparin during or soon after surgery is most likely to induce immunization, and this scenario (when applicable) should be primarily evaluated.
[c]At site(s) of heparin injections.
ASR, acute systemic reaction (i.e., anaphylactoid reaction); DVT, deep vein thrombosis; HIT, heparin-induced thrombocytopenia; IV, intravenous.
Modified from Warkentin TE. Clinical picture of heparin-induced thrombocytopenia. In: Warkentin TE, Greinacher A, eds. *Heparin-induced thrombocytopenia*. New York: Informa Healthcare USA, 2007:21–66.

Activation Assays

Activation assays exploit the platelet-activating properties of the HIT–IgG antibodies. The characteristic profile of HIT sera is the activation of normal donor platelets using therapeutic heparin concentrations (optimal at 0.1 to 0.3 U/mL) and the inhibition of activation of normal donor platelets at supratherapeutic heparin concentrations (10 to 100 U/mL).[120,121] As noted earlier, many HIT sera also induce considerable platelet activation in the absence of heparin (i.e., buffer control), a feature associated with delayed-onset or worsening of HIT despite stopping heparin.[40] Washed platelets that have been resuspended in divalent cation-containing buffer—with "washing" performed in the presence of apyrase (an enzyme that preserves platelet reactivity to adenosine diphosphate—an important potentiator of HIT antibody-induced platelet activation[122])—respond optimally to HIT antibodies. These features have been incorporated into two "gold standard" assays, the platelet serotonin release assay (SRA)[120,123] and the heparin-induced platelet activation (HIPA) test.[124] In one study, a positive SRA was strongly associated with thrombocytopenia that began on day 5 or later of heparin treatment (odds ratio 78, 95% CI 12 to 819, $P < 0.001$).[11]

Methods to improve quality control in the detection of HIT antibodies include using an Fcγ receptor (FcγRIIa)-blocking monoclonal antibody to ascertain inhibition of platelet activation by HIT serum[18] and testing a panel of negative, weak, and strong HIT control sera to confirm that the test platelets selected are adequate to detect HIT antibodies of varying reactivity.[123] When performed by experienced laboratories, the sensitivity and specificity of washed platelet assays for HIT are >95%[114]; in approximately 2% of sera, however, the serum induces platelet activation at all concentrations of heparin, and the result is "indeterminate" for presence of HIT antibodies.[125]

Conventional platelet aggregation assays using normal donor platelets suspended in citrated plasma have a relatively low sensitivity for HIT (50% to 80%)[126] and are no longer widely used. Specificity also is lower because heparin can produce nonspecific activation of platelets under these test conditions.

Antigen Assays

Table 108.3 summarizes commercially available antigen assays.[21,23,34,108,116,126–137] These can be divided into PF4-dependent EIAs (polyspecific and IgG-specific) and various particle-based immunoassays.

Antigen assays using the EIA technique have been developed using the PF4/heparin (or PF4/polyanion) target antigen.[21,23,34,116,126,127] Potentially, false-negative EIA results could arise if HIT antibodies recognize chemokines other than PF4 (e.g., interleukin-8 and neutrophil-activating peptide-2).[138,139] However, these antigens are rarely implicated (<1% of HIT patients); indeed, for >99% of sera that cause a clear positive result in a platelet activation assay, anti-PF4/heparin antibodies are readily detectable. Although the PF4/heparin EIA is more sensitive than the SRA or HIPA for detecting PF4-/heparin-dependent antibodies, this may not be an advantage because more clinically insignificant antibodies are detected,[127,140]

Table 108.3

Commercially available PF4-dependent immunoassays

Assay	PF4 Source	Polyanion	Ig Classes Detected
Enzyme Immunoassays			
Asserachrom HPIA	Recombinant	Heparin	Polyspecific (IgG/A/M)
PF4 enhanced	Platelets	PVS	Polyspecific (IgG/A/M)
PF4 IgG	Platelets	PVS	IgG
Zymutest HIA	Platelet lysate	Heparin (bound to protamine)	Polyspecific (IgG/A/M) IgG or IgA or IgM (individually)
Particle-Based Immunoassays			
PaGIA	Platelets	Heparin	Polyspecific (IgG/A/M)
PIFA	Platelets	Heparin	Polyspecific (IgG/A/M)
HemosIL HIT-$Ab_{(PF4-H)}$	Platelets	PVS	Polyspecific (IgG/A/M)
Chemiluminesent-Based Immunoassays			
HemosIL AcuStar HIT-$IgG_{(PF4-H)}$	Platelets	PVS	IgG
HemosIL AcuStar HIT-$Ab_{(PF4-H)}$	Platelets	PVS	Polyspecific (IgG/A/M)

resulting in a lower specificity for clinical HIT.[117] The nonspecific nature of the negatively charged coantigen of HIT is illustrated by the development of an antigen assay using PF4 complexed to polyvinylsulfonate (PVS), a negatively charged material devoid of both carbohydrate and sulfate groups.[34] Despite the absence of heparin, this assay has high sensitivity for detecting HIT antibodies. A high heparin "confirmatory" step—inhibition of reactivity by >50% at high heparin concentrations—has been suggested to increase diagnostic specificity; this maneuver has some utility for weakly reacting sera (positive but OD < 1.0 U), but for stronger reacting sera (OD > 1.0), lack of inhibition does not rule out the presence of platelet-activating antibodies.[141–144]

There are two types of rapid, particle-based assays. The "particle gel immunoassay" (PaGIA),[132–134] which utilizes gel centrifugation technology (used in blood banks), has operating characteristics (sensitivity–specificity trade-offs) intermediate between the commercial EIA and a washed platelet activation assay[127,128,133]; one study[134] suggested that the utility of this test is enhanced by interpreting the assay results quantitatively (rather than simply as positive or negative). Unlike the very high sensitivity (~99%) of the EIAs, approximately 5% of HIT sera test negative in the PaGIA (i.e., ~95% sensitivity).[127,128] The second assay, particle immunofiltration assay (PIFA), however, showed very poor operating characteristics in two laboratories and cannot be recommended for the diagnosis of HIT.[135]

Two automated assays have recently been introduced in Europe.[136,137] One assay is based on the agglutination of latex particles coated with a monoclonal antibody specific for PF4/polyanion complexes. In the presence of anti-PF4/heparin antibodies in the patient plasma and added PF4/PVS complexes, agglutination by the monoclonal antibody is inhibited. The degree of agglutination is inversely proportional to the concentration of antibodies in the sample and is determined by measuring the decrease of transmitted light caused by the aggregates. The other assay is based on chemiluminescence. A potential advantage of this latter assay is the much wider range of reactivities, which may yield additional information, for example, potential association of very high antibody titers and thrombotic complications. These automated assays could allow for greater standardization of interlaboratory results, compared with other assays.

Iceberg Model and Interpretation of HIT Assays

The "iceberg" model (FIGURE 108.6) provides a framework for interpreting HIT antibody test results.[7,62,145] Among the totality of the anti-PF4/heparin immune response, only the "tip" of the iceberg extending above the waterline—constituting platelet-activating antibodies of IgG class—is potentially capable of causing HIT. Thus, whereas washed platelet activation assays and PF4-dependent EIAs (whether IgG-specific or not) have similar high sensitivity for HIT, diagnostic specificity increases as: EIA-IgG/A/M < EIA-IgG < washed platelet activation assay.

Results of HIT antibody tests must be interpreted in the appropriate clinical context of pretest probability.[117,146] Relying on a positive EIA alone to diagnose HIT risks overdiagnosis: Only approximately half of the patients who have a positive EIA test result actually have HIT.[147] The magnitude of the positive EIA test result provides useful diagnostic information. For example, predictivity of a positive polyspecific EIA for the presence of platelet-activating antibodies ranges from approximately 5% (for weakly positive results with OD 0.40 to 1.0 U) to 90% for strong-positive results (>2.0 U).[118,119] However, simply using a higher cutoff (e.g., 1.0 OD units) will result in some false-negative results. Laboratories should report the results of antigen tests at least semiquantitatively (negative, weak, intermediate, strong) to allow an estimate of the likelihood of platelet-activating antibodies being present. In the case of an intermediate pretest score of HIT (e.g., 4Ts score of 4 to 5),

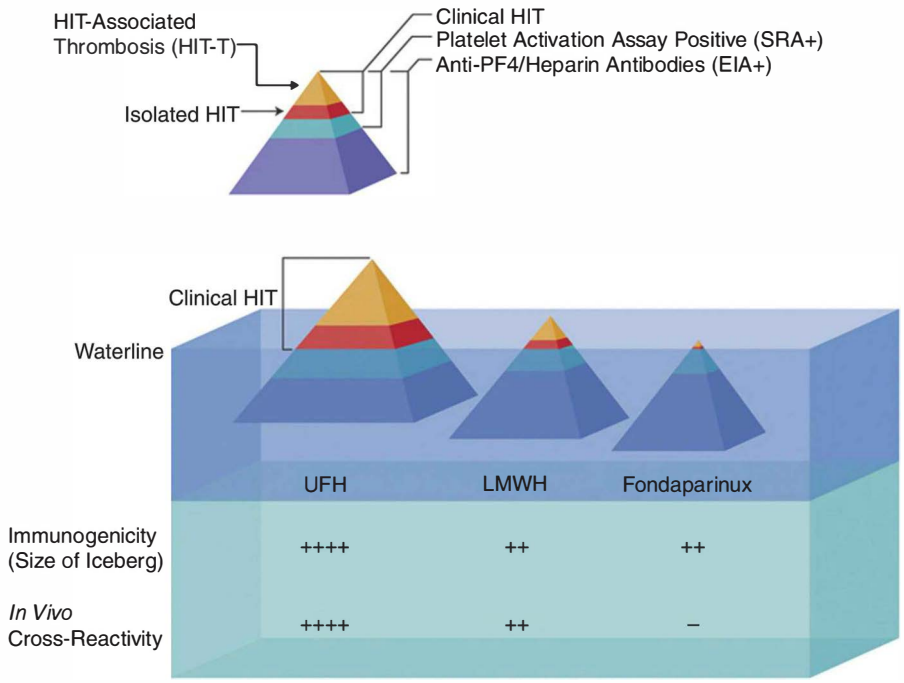

FIGURE 108.6 The relationship among the clinical expression of HIT (thrombocytopenia with or without thrombosis), the type of heparin used, and the antibodies that cause HIT. This can be conceptualized as an "iceberg." The visible component of the iceberg (*the portion above the waterline*) represents clinically evident features of HIT, such as thrombocytopenia and/or thrombosis. The mass of the iceberg corresponds to the entire spectrum of anti-PF4/heparin antibodies generated. Some of these antibodies will be biologically active (platelet-activating), and others will be non–platelet-activating, which may make them unlikely to cause clinical consequences. The type of heparin given to the patient determines the overall size of the iceberg, with UFH being the largest (most immunogenic) and LMWH and fondaparinux having less immunogenicity. Also illustrated in this figure is the *in vivo* cross-reactivity. UFH forms antibodies that are readily recognized by HIT antibodies. In contrast, fondaparinux forms poorly recognized antigens. LMWH is intermediate. (From Kelton JG, Warkentin TE. Heparin-induced thrombocytopenia: a historical perspective. *Blood* 2008;112:2607–2616.)

it makes a great difference, whether the test is weakly positive (0.5 to 1.0 OD units) or very strong (OD >2.0 U), with only the latter positive result representing a likely HIT scenario (~5% vs. >90%, respectively).

The presence of anti-PF4/heparin antibodies but without platelet-activating properties (i.e., negative washed platelet activation assay) does not appear to indicate the presence of HIT, based upon clinical comparisons with antibody-negative and activation assay–positive controls.[147] Adverse prognosis associated with anti-PF4/heparin antibodies, but in the absence of clinical HIT, has been observed in some[148–155] but not all studies.[156,157] One possibility for the potential association of nonactivating anti-PF4/heparin antibodies with adverse outcomes is that inflammation could potentiate generation of these antibodies.[158] In so, inflammation itself could be the key factor for adverse prognosis.

TREATMENT

Table 108.4 lists consensus conference treatment recommendations for HIT.[159] Although it is recommended that heparin be discontinued when a patient is strongly suspected (or confirmed) as having HIT, the risk for subsequent thrombosis postheparin discontinuation ranges from 25% to 50% in patients with serologically confirmed HIT.[1,79,94,160] Further, the risk of sudden thrombotic death is about 5%.[1,94,160] The lack of benefit of stopping heparin is further illustrated by the nature of "delayed-onset HIT": In such patients, thrombocytopenia begins and intensifies, with the development of thrombosis, while no heparin is being given.[40,90–92,93] Furthermore, microvascular thrombotic complications of HIT typically occur only *after* all heparin has been stopped.[8] One explanation for this is the anticoagulant activity of heparin, which protects against part of the prothrombotic effects of HIT. However, the half-life of the anticoagulant activity of heparin is shorter (1 to 2 hours) than that of the non–antithrombin-binding heparin molecules (up to 24 hours),[161] which can still form antigenic complexes with PF4. Therefore, when HIT is strongly suspected or confirmed, heparin should be substituted by a rapidly acting, alternative anticoagulant, such as a direct thrombin inhibitor (DTI) (e.g., lepirudin, argatroban, or bivalirudin) or factor Xa inhibitor (e.g., danaparoid or fondaparinux), generally in therapeutic doses, until the platelet count has recovered.[159,162,163] In addition, to rule out DVT, duplex ultrasound examination of the lower limbs should be performed prior to discharge in patients strongly suspected or confirmed as having HIT.[159]

Table 108.4

Treatment of HIT: recommendations[a] of the eighth ACCP conference on antithrombotic and thrombolytic therapy: evidence-based guidelines, 2008

Treatment Strategy	Recommendation
Nonheparin anticoagulants for HIT	For patients with strongly suspected (or confirmed) HIT, whether or not complicated by thrombosis, we recommend the use of an alternative, nonheparin anticoagulant (danaparoid [grade 1B], lepirudin [grade 1C], argatroban [grade 1C], fondaparinux [grade 2C], bivalirudin [grade 2C]) over the further use of UFH or LMWH therapy or initiation/continuation of a VKA (grade 1B).
Screening for DVT	For patients with strongly suspected or confirmed HIT, whether or not there is clinical evidence of lower limb DVT, we recommend routine ultrasonography of the lower limb veins for investigation of DVT over not performing routine ultrasonography (grade 1C).
Management of nonheparin anticoagulant–VKA overlap	For patients with strongly suspected or confirmed HIT, we recommend *against* the use of VKA (coumarin) therapy until after the platelet count has substantially recovered (i.e., usually to at least 150×10^9/L) over starting VKA therapy at a lower platelet count (grade 1B); that VKA therapy be started only with low, maintenance doses (maximum, 5 mg of warfarin or 6 mg of phenprocoumon) rather than with higher initial doses (grade 1B); and that the nonheparin anticoagulant (e.g., lepirudin, argatroban, danaparoid) be continued until the platelet count has reached a stable plateau, the INR has reached the intended target, and after a minimum overlap of at least 5 days between nonheparin anticoagulation and VKA therapy rather than a shorter overlap (grade 1B).
Reversal of VKA anticoagulation	For patients receiving a VKA at the time of diagnosis of HIT, we recommend the use of vitamin K (10 mg PO or 5 to 10 mg IV) (grade 1C).
LMWH for HIT	For patients with strongly suspected HIT, whether or not complicated by thrombosis, we recommend against the use of LMWH (grade 1B).
Prophylactic platelet transfusions for HIT	For patients with strongly suspected or confirmed HIT who do not have active bleeding, we suggest that prophylactic platelet transfusions should not be given (grade 2C).

[a]Grading of recommendations: Recommendations are ranked as grade 1 (strong recommendation) and grade 2 (weak recommendation). Methodologic strengths of supporting evidence are ranked as grade 1B (strong recommendation, moderate-quality evidence); grade 1C (strong recommendation, low or very low-quality evidence); and grade 2C (weak recommendation, low or very low-quality evidence).
HIT, heparin-induced thrombocytopenia; DVT, deep vein thrombosis; INR, international normalized ratio; IV, intravenous; UFH, unfractionated heparin; LMWH, low molecular weight heparin; VKA, vitamin K antagonist (e.g., warfarin).
From Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines [8th edition]. *Chest* 2008;133(Suppl):340S–380S.

Two DTIs, lepirudin and argatroban, are approved for management of HIT in the United States. Both agents have relative advantages and disadvantages. Careful drug selection, dosing, and monitoring are important because neither agent has a specific antidote.

Recombinant Hirudin

Lepirudin (Refludan) is a recombinant hirudin (r-hirudin) derivative that inhibits thrombin via a high-affinity, irreversible, noncovalent interaction.[10,164–167] The *C*-terminal region of this 65-amino acid polypeptide (6,980 Da) binds to thrombin's fibrinogen-binding site (exosite 1), and a highly packed hydrophobic region near the *N*-terminus (produced by three disulfide bridges) binds to the apolar region of thrombin, thereby blocking the active (catalytic) site of thrombin. Its anticoagulant effect is usually monitored with the aPTT. Lepirudin is renally eliminated, and greatly reduced dosing is required in patients with renal failure. Antihirudin antibodies are commonly generated in patients treated with lepirudin; in a minority of patients, this can lead paradoxically to a greater anticoagulant effect of lepirudin[168] due to an immune complex–mediated prolonged half-life of active agent. It is advisable to avoid the initial intravenous bolus[159] to minimize the risk of postbolus anaphylaxis[169] and to reduce the risk of overdosing.[170–173] Lepirudin is approved in the United States and in the European Union to treat HIT complicated by thrombosis, but the potential for bleeding has prevented approval for other indications, such as acute coronary syndrome. Table 108.5 lists consensus conference dosing recommendations (which are lower than the manufacturer's recommendations given in the package insert).

Desirudin (US trademark, Iprivask; EU trademark, Revasc) is another r-hirudin, which is effective for thrombosis prophylaxis after hip replacement surgery[174] and is approved for this (non-HIT) indication in the United States and the European Union (and additionally approved for knee replacement surgery in the European Union); the dose (normal renal function) is 15 mg every 12 hours by subcutaneous injection. It has the potential to be used for thrombosis prophylaxis in patients who cannot receive heparin.[175] Its pharmacologic characteristics are very similar to lepirudin, including risk of rapid accumulation in renal insufficiency and induction of antibodies.[176]

Table 108.5

Lepirudin for treatment of HIT

Standard protocol: *HIT with thrombosis*: ± initial bolus, 0.2 to 0.4 mg/kg IV; followed by IV infusion at 0.05 to 0.10 mg/kg/h (target aPTT, 1.5–2.0 × baseline), aPTT adjusted 4 h later and at 4-h intervals until steady state is reached and 4 h after any dose adjustment; increase rate by 20% and decrease by 50% if aPTT below or above target range, respectively. *HIT without thrombosis*: no initial bolus; initial infusion rate, 0.05 to 0.10 mg/kg/h, adjusted by aPTT as in the preceding text.

Other Dosing Considerations	Rationale
Laboratory tests to determine standard curve of plasma lepirudin (using normal plasma "spiked" with lepirudin) to aPTT, including low- and high-therapeutic lepirudin levels (0.6–1.4 μg/mL)[83]	Because different aPTT reagents show flattening of the lepirudin–aPTT curve at different lepirudin concentrations, the standard curve is used to determine the lepirudin level beyond which accurate assessment by aPTT is no longer possible.
Avoid the initial bolus dose, beginning with infusion alone (unless severe thrombosis is present)	Safety enhanced by minimizing risk of postbolus anaphylaxis, with only minor delay (~6 h) in achieving therapeutic anticoagulation
In elderly patients (>60 y), even with normal creatinine levels, begin with lower infusion rate (0.05 mg/kg/h)	Renal function declines with age and may be underestimated by serum creatinine levels; overdosing can occur at 0.15 mg/kg/h (with or without bolus).
Repeat aPTT monitoring at 4-h intervals until steady state is obtained.	Steady state may not be reached at 4 h, especially if any renal dysfunction is present.
Monitor aPTT daily while on lepirudin	Antihirudin antibodies can affect lepirudin clearance (either increase or decrease aPTT).

Dose Adjustments in Renal Insufficiency

Serum Creatinine mg/dL (μmol/L)	Initial IV Infusion Rate (then Adjust by aPTT)
1.0–1.6 (90–140)	0.05 mg/kg/h
1.6–4.5 (141–400)	0.01 mg/kg/h
>4.5 (>400)	0.005 mg/kg/h

HIT, heparin-induced thrombocytopenia; IV, intravenous; aPTT, activated partial thromboplastin time.
Modified from, with dosing guidelines from, Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines [8th Edition]. *Chest* 2008;133(6 Suppl):340S–380S.

Argatroban

Argatroban (non-US trademark, Novastan) is a synthetic, small-molecule DTI (527 Da) derivative of arginine.[177,178] Argatroban inhibits thrombin at therapeutically relevant concentrations. The anticoagulant effect is monitored using the aPTT (usual target: 1.5 to 3.0 times baseline, maximum 100 seconds). Unlike lepirudin, however, argatroban is predominantly metabolized by the liver and, therefore, may be safer in patients with renal failure. Argatroban prolongs the INR to a greater extent than does bivalirudin or lepirudin,[179] complicating the monitoring of overlapping warfarin anticoagulation.

Argatroban is approved in the United States, Canada, and several countries within Europe for treatment of HIT with or without associated thrombosis[177] and also for anticoagulation during percutaneous coronary intervention (PCI) in patients with, or at risk for, HIT.[177,180]

Bivalirudin

Bivalirudin (Angiomax) is a 20-amino acid (2,180 Da) analog of hirudin comprising two subunits: the *C*-terminal dodecapeptide of hirudin and a thrombin active site–binding *N*-terminal tetrapeptide (D-Phe1–Pro2–Arg3–Pro4) bridged by four glycine "spacers."[164,181–184] As with hirudin, the *C*-terminus moiety binds to the fibrinogen recognition site of thrombin (exosite 1). This "designer molecule" has several theoretical advantages over hirudin,[183] including (a) predominant nonrenal elimination (80% of its degradation is by plasma proteases), (b) a shorter half-life (25 vs. >80 minutes), (c) reduced or absent immunogenicity, and (d) minimal prolongation of the INR (greater than lepirudin, but less than argatroban). Unlike r-hirudin, thrombin inhibition is reversible. Favorable results of "off-label" use of bivalirudin for HIT have been reported in uncontrolled studies,[183,185] with initial dosing of 0.15 mg/kg/h and aPTT monitoring (target aPTT 1.5- to 2.5-fold greater than baseline). Bivalirudin has undergone evaluation for both "on-pump" and "off-pump" cardiac surgery in patients with HIT or with anti-PF4/heparin antibodies.[77,183,186–188] It is widely used in the United States for PCIs and is the anticoagulant of choice in patients with HIT, or a history of HIT, requiring coronary interventions.

Direct Thrombin Inhibitor–Coumarin Overlap

The risk of warfarin-associated venous limb gangrene (discussed in Chapter 109) remains when warfarin is prematurely started and/or when the DTI is prematurely discontinued in a

patient with HIT-associated venous thrombosis and ongoing thrombocytopenia.[97,98] Therefore, it is prudent to delay commencing warfarin until the platelet count has substantially recovered (preferably, to >150 × 10^9/L) and any clinically manifest thrombosis is improving. Further, there should be at least a 5-day overlap between the DTI and warfarin (with target INR levels achieved for at least the last 2 days), and the DTI should not be stopped until the platelet count has reached a stable plateau. If warfarin has already been initiated when HIT is recognized, vitamin K should be given for two reasons: (a) to reduce the risk of warfarin-induced microvascular thrombosis and (b) to avoid underdosing of DTI because warfarin also prolongs the aPTT.[159,189]

Confounding of aPTT Monitoring in HIT-Associated Coagulopathy

The observation that aPTT prolongation by warfarin confounds subsequent dosing and aPTT-based monitoring of DTI therapy raises the issue whether HIT-associated coagulopathy itself can confound DTI therapy. Indeed, there are reports of frequent dose interruptions and reductions—associated with progressive limb necrosis—in patients who had baseline (pre-DTI) aPTT prolongation due to HIT-associated consumptive coagulopathy.[10,93] In theory, the use of drug levels could avoid this problem,[190] but these assays are not widely available.

AT-Dependent, Factor Xa Inhibition

Table 108.6 summarizes advantages and disadvantages of indirect (AT-dependent) factor Xa inhibitors (danaparoid, fondaparinux) with DTIs.[93]

Danaparoid sodium (Orgaran) is a mixture of anticoagulant glycosaminoglycans (e.g., low-sulfated heparan sulfate, dermatan sulfate) with predominant anti–factor Xa activity; the anti-Xa/anti-IIa ratio is 22:1.[191,192] Approximately 10% to 40% of HIT sera will show some degree of *in vitro* cross-reactivity for danaparoid. However, *in vitro* cross-reactivity is usually not of clinical significance,[193] and treatment should not be delayed to perform *in vitro* cross-reactivity studies. Indeed, a unique property of danaparoid is that high therapeutic concentrations *inhibit* HIT antibody–induced platelet activation.[194] Anticoagulant monitoring of danaparoid is performed by measuring anti–factor Xa activity. However, monitoring is not usually required because the anticoagulant effect is largely predictable when administered on a weight-adjusted basis, and the likelihood of obtaining therapeutic anticoagulant levels is high using an empirically derived standard treatment protocol that begins with intravenous bolus administration. Danaparoid can accumulate in renal failure; therefore, the maintenance dose should be reduced in these cases by approximately 30%, and anti–factor Xa activity should be closely monitored. No antidote for danaparoid exists. Danaparoid's long half-life and lack of effect on the INR generally make for a smooth transition to warfarin monotherapy, suggesting that the risk of iatrogenic venous limb gangrene

Table 108.6

A comparison of two classes of anticoagulant used to treat HIT

	Indirect AT-Dependent Factor Xa Inhibitors: Danaparoid, Fondaparinux	Direct AT-Independent Thrombin Inhibitors: r-Hirudin, Argatroban, Bivalirudin
Half-life	√ Long (> 16 h): avoids potential for rebound hypercoagulability	SHORT (<2 h): potential for rebound hypercoagulability. Half-life of lepirudin and bivalirudin depends on renal function; half-life of argatroban depends on liver perfusion/function.
Dosing	√ Both prophylactic- and therapeutic-dose regimens[a]	Prophylactic-dose regimens are not well established.
Monitoring	√ Direct (anti-Xa levels): accurate drug levels obtained	Indirect (aPTT): risk for DTI underdosing due to aPTT elevation for non-DTI factors
Effect on INR	√ No significant effect: thus, simplifies overlap with warfarin	Increases INR: argatroban > bivalirudin > r-hirudin; complicates warfarin overlap
Protein C pathway	√ No significant effect	Thrombin inhibition could impair activation of protein C pathway.
Reversibility of action	√ Irreversible inhibition (AT forms covalent bond with factor Xa)	Irreversible inhibition only with r-hirudin
Platelet activation	√ Danaparoid inhibits platelet activation by HIT antibodies	No effect
Drug clearance	Predominantly renal	Variable (see half-life [above] and text)

Legend: "√", that is, check mark, indicates positive feature in comparison of drug classes.
[a]Although therapeutic dosing is recommended for HIT, availability of prophylactic-dose regimens increases flexibility when managing potential non-HIT situations.
Modified from Warkentin TE. Agents for the treatment of heparin-induced thrombocytopenia. *Hematol Oncol Clin North Am* 2010;24:755–775.

during overlapping therapy with danaparoid and coumarin is very low (in contrast to DTI–warfarin overlap). Danaparoid is the only therapy for HIT evaluated in a randomized clinical trial (vs. the antiplatelet agent, dextran-70).[195] A retrospective comparison with ancrod (defibrinogenating snake venom) and/or coumarin therapy showed significantly fewer thrombotic events with danaparoid, and with less bleeding.[196] Danaparoid is not available in the United States.

Fondaparinux (Arixtra) is a synthetic, sulfated pentasaccharide that catalyzes antithrombin-mediated inactivation of factor Xa. It only rarely cross-reacts with HIT antibodies[197,198] and, theoretically, should be effective in patients with HIT. Indeed, recent experience[199–203] supports the thesis[93,204] that fondaparinux is an effective anticoagulant for treatment of HIT (Table 108.7). Its long half-life and renal elimination, however, require careful dosing and monitoring if used in critically ill patients.

New Oral Anticoagulants

The new oral anticoagulants, dabigatran (thrombin inhibitor) and rivaroxaban and apixaban (FXa inhibitors), cannot induce HIT, as they have a different molecular structure than heparin.[205] Although no experience with these agents in acute HIT exists, they are an option for patients with a history of HIT who require anticoagulation.

PROGNOSIS

Many patients with HIT present with life- or limb-threatening thrombosis,[79,94] and thus even "effective" anticoagulation may not prevent long-term sequelae (e.g., disabling stroke, limb amputation). Despite stopping heparin, as many as 40% to 50% of the patients with "isolated HIT" develop thrombosis.[1,79,94] Limb amputation occurred in approximately 5% of patients with HIT-associated thrombosis treated with danaparoid, fondaparinux, or lepirudin, and 10% to 15% of patients treated with argatroban.[159,160] The mortality rate is 15% to 25%, but deaths often reflect comorbidities rather than thrombotic death.[159,160]

Table 108.8 lists treatment outcomes of lepirudin and argatroban, assessed in prospective cohort studies (with historic controls), as well as that of danaparoid, which was compared against dextran-70 in a randomized controlled trial.[160,206,207,208–210] All three agents appeared to prevent thrombosis in HIT, although an impact on overall mortality and limb loss is less clear.

PREVENTION OF HEPARIN-INDUCED THROMBOCYTOPENIA AND ITS COMPLICATIONS

HIT is a potentially preventable adverse drug reaction: The frequency of HIT with LMWH thromboprophylaxis is approximately one-tenth that observed with UFH[55,211] and is likely lower still with fondaparinux.[76,212]

The recent emphasis on substituting heparin with an alternative anticoagulant even in patients with isolated HIT suggests an important role for platelet count monitoring for HIT. Consensus conferences[114,159] have adopted the view that the most frequent monitoring (at least every other day) should be performed in situations in which HIT is common (>1%), such as in postoperative patients receiving antithrombotic prophylaxis with UFH or treatment of thrombosis with UFH. Less frequent monitoring (two or three times per week) is appropriate if HIT occurs at a frequency of 1/100 to 1/1,000, such as in medical and surgical patients receiving prophylaxis with UFH and LMWH, respectively. Routine monitoring is not recommended in circumstances in which HIT is rare (<1/1,000 in

Table 108.7

Fondaparinux for treatment of acute HIT: studies of five or more patients

Study	N	HIT-T	Median Platelet Count Nadir, × 10^9/L (Range)	EIA Results (OD Units)	Dose (mg)[a]	Thrombosis Rate	Amputation Rate	Major Bleeding Rate
Ref. 199	5	5/5	43 (5, 79)	>1.0 in all 5	7.5	0/5	0/5	0/5
Ref. 200	7	6/7	66 (11, 139)	0.7 (median)	7.5[b]	0/7	1/7[c]	0/7
Ref. 201	24	14/24[d]	66[e] (17, 600)	1.4 (mean)[f]	7.5	0/24	1/24[g]	0/24
Ref. 202	16	9/16	79 (10, 169)	2.53 (mean)[h]	7.5[i]	0/16	1/16[j]	1/16
Ref. 203	8	6/8	56 (23, 99)	2.10 (mean)[k]	7.5	0/8	0/8	0/8
Pooled	60	40/60 (66.7%)	66 (5, 600)[e]	Positive in all	7.5[i]	0/60	3/60 (5.0%)	1/60 (1.7%)

[a]Dose per day administered by subcutaneous injection (10 and 5 mg if the patient weighted >100 and <50 kg, respectively).
[b]The single patient without thrombosis received 2.5mg daily by subcutaneous injection.
[c]One patient with popliteal artery thrombosis and failed thrombectomy required amputation; however, the ischemic limb necrosis was judged to have been present before fondaparinux therapy.
[d]All 10 patients without HIT-T nevertheless had preceding thrombosis that led to the heparin therapy that triggered HIT.
[e]The mean platelet count nadir excludes two patients with myeloproliferative disease whose HIT-associated platelet count declines resulted in relative, rather than absolute, thrombocytopenia.
[f]The mean OD was significantly greater (2.0 U) in the 14 patients with HIT-T.
[g]One patient had limb amputation before commencing fondaparinux.
[h]An IgG-specific EIA was used; all 16 patients also tested strongly positive in the serotonin-release assay (mean percent serotonin-release, 91%).
[i]7.5 mg was the median initial dose; however, some patients received initial dosing of 2.5, 5, 10 or 15 mg/d.
[j]One patient had limb amputation because of ischemic limb necrosis established prior to initiation of fondaparinux.
[k]Mean OD value excludes one result with very high OD value (>7.50 U); the 3 patients with OD values <2.00 tested positive in the serotonin-release assay.
EIA, enzyme-immunoassay; HIT-T, HIT-associated thrombosis; N.A., not available; OD, optical density.

Table 108.8

HIT treatment outcomes

Study, Year (Reference)	Regimen	n	Mean Days of Alternative Anticoagulant	% HIT Antibody Positive	New Thrombosis Rate (RRR)[a]	Amputation Rate (RRR)[a]	Composite Endpoint (RRR)[a]	Major Bleed (% per Day Alternative Anticoagulant Given)[b]
HIT-Associated Thrombosis								
HAT-1/2, 2000 (206)	Lep 0.4 mg/kg bolus + 0.15 mg/kg/h	113	13.3	100%	10.1 (63%)	6.5% (38%)	21.3% (55%)	1.4%
HAT-3, 2004 ([207])	Lep bolus + 0.15 mg/kg/h	98	14.0	100%	6.1% (78%)	5.1% (51%)	21.5% (55%)	1.5%
DMP, 2003 (208)	Lep bolus + 0.15 mg/kg/h	496	12.1	77%	5.2% (NA)	5.8% (NA)	21.9%	0.45%
Arg911, 2001 (160)	Arg 2 μg/kg/min	144	5.9	65%	19.4% (35%)	11.8% (–8%)	43.8% (22%)	1.9%
Arg915, 2003 (209)	Arg 2 μg/kg/min	229	7.1	NA	13.1% (62%)	14.8% (–36%)	41.5% (27%)	0.9%
RCT versus dextran, 2001 (195)	Danap bolus + inf 200 U/h	24	6.4[c]	~92%[d]	12.5% (70%)	4.2% (76%)	25.0% (66%)	0%
Isolated HIT								
HAT1-3, 2002 (210)	Lep: 0.10 mg/kg/h	111	13.5	100%	2.7% (NA)[e]	2.7% (NA)[e]	9.0% (NA)[e]	1.1%[e]
DMP, 2002 (208)	Lep: 0.10 mg/kg/h	612	11	66%	2.1% (NA)[f]	1.3% (NA)[f]	≥15.7%	0.5%[f]
Arg911, 2001 (160)	Arg: 2 μg/kg/min	160	5.9	65%	8.1% (64%)	1.9% (5%)	25.6% (34%)	0.6%
Arg915, 2003 (209)	Arg: 2 μg/kg/min	189	5.1	NA	5.8% (75%)	4.2% (–45%)	28.0 (28%)	1.0%

[a]RRR (relative risk reduction, expressed as percent) compared with historical controls (not shown) or, for danaparoid vs. dextran study,[195] RCT controls (not shown).
[b]Calculated by dividing major bleed rate by the number of mean days of alternative anticoagulant given.
[c]Median (data provided by Dr. Harry Magnani, Organon NV).
[d]34/37 (91.9%) patients tested were positive for HIT antibodies (4 patients did not undergo testing).
[e]Data limited to on-treatment observation period.
[f]Data limited to on-treatment observation period + 1 day.
Arg, argatroban; DMP, drug monitoring program (postmarketing study); Danap, danaparoid; HAT, heparin-associated thrombocytopenia (prospective lepirudin study); Lep, lepirudin; NA, not available; RCT, randomized controlled trial; RRR, relative risk reduction.
Modified from Warkentin TE, Poncz M, Cines DB. Heparin-induced thrombocytopenia. In: Young NS, Gerson SL, High KA, eds. *Clinical hematology*. Philadelphia, PA: Elsevier/Mosby, 2006:887--899. Some data pertaining to a published trial[195] obtained from Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines [8th edition]. *Chest* 2008;133(Suppl):340S–380S.

medical or obstetrical patients receiving LMWH). Nevertheless, HIT is frequently recognized only after a new thrombosis has occurred, even when frequent platelet count monitoring is performed,[1,11,12] and there is no proof that frequent platelet count monitoring will prevent HIT-associated thrombosis.

Physicians should suspect HIT in any patient who develops new or progressive thrombosis during or within several days after stopping heparin therapy. In 10% to 15% of the patients with HIT, the platelet count nadir is >150 × 10^9/L (FIGURE 108.2), although usually a proportional platelet count decline of at least 50% had occurred (FIGURE 108.3). Heparin should be discontinued and an alternative management strategy instituted in any patient in whom HIT is the most likely diagnosis, even prior to obtaining diagnostic laboratory results.

Although the frequency of HIT, HIT-associated thrombosis, and HIT–IgG seroconversion is lower in patients treated with LMWH than in those treated with UFH (e.g., ~90% lower in patients receiving heparin >5 days after a major orthopedic surgery),[11,12,62,211] LMWH should not be used to treat HIT,[159] because HIT antibodies frequently activate platelets strongly in the presence of LMWH.[11,213]

HEPARIN REEXPOSURE

Table 108.9 lists treatment recommendations of the American College of Chest Physicians (ACCP) in special situations such as heparin reexposure in patients with previous HIT and PCI.[159] For patients with a previous history of HIT who have

Table 108.9

Treatment of HIT: special patient populations; recommendations of the eighth ACCP conference on antithrombotic and thrombolytic therapy: evidence-based guidelines, 2008

Clinical Situation	Recommendation (Grade of Recommendations[a])
Patients with previous HIT undergoing cardiac or vascular surgery	For patients with a history of HIT who are HIT antibody negative and require cardiac surgery, we recommend the use of UFH over a nonheparin anticoagulant (grade 1B). For patients with a history of HIT who are antibody positive by PF4-dependent EIA but antibody negative by washed platelet activation assay, we recommend the use of UFH over a nonheparin anticoagulant. Remark: Preoperative and postoperative anticoagulation, if indicated, should be given with a nonheparin anticoagulant.
Patients with acute HIT undergoing cardiac surgery	For patients with acute HIT (thrombocytopenic, HIT antibody positive) who require cardiac surgery, we recommend one of the following alternative anticoagulant approaches (in descending order of preference): delaying surgery (if possible) until HIT has resolved and antibodies are negative or weakly positive (grade 1B); using bivalirudin for intraoperative anticoagulation during cardiopulmonary bypass (if techniques of cardiac surgery and anesthesiology have been adapted to the unique features of bivalirudin pharmacology) (grade 1B) or during "off-pump" cardiac surgery (grade 1B); using lepirudin for intraoperative anticoagulation (if ECT is available and the patient has normal renal function and is judged to be at low risk for postcardiac surgery renal dysfunction) (grade 2C); using UFH plus the antiplatelet agent epoprostenol (if ECT monitoring is not available or renal insufficiency precludes lepirudin use) (grade 2C); using UFH plus the antiplatelet agent, tirofiban (grade 2C); or using danaparoid for intraoperative anticoagulation for off-pump coronary artery bypass surgery (grade 2C) over performing the surgery with UFH when platelet-activating anti-PF4/heparin antibodies are known to be present in a patient with acute or recent HIT (grade 2C).
Patients with subacute HIT undergoing cardiac surgery	For patients with subacute HIT (platelet count recovery, but continuing HIT antibody positive), we recommend delaying surgery (if possible) until HIT antibodies (washed platelet activation assay) are negative and then using heparin over using a nonheparin anticoagulant (grade 1C). If surgery cannot be delayed, we suggest the use of a nonheparin anticoagulant over the use of UFH (grade 2C).
PCI	For patients with strongly suspected (or confirmed) acute HIT who require cardiac catheterization or PCI, we recommend a nonheparin anticoagulant bivalirudin (grade 1B), argatroban (grade 1C), lepirudin (grade 1C), or danaparoid (grade 1C) over UFH or LMWH (grade 1B). For patients with preious HIT (who are antibody negative) who require cardiac catheterization or PCI, we suggest the use of a nonheparin anticoagulant over UFH or LMWH (grade 2C).

[a]See footnote of Table 114.3 for grading of recommendations.
HIT, heparin-induced thrombocytopenia; LMWH, low molecular weight heparin; UFH, unfractionated heparin; ECT, ecarin clotting time; EIA, enzyme immunoassay; PCI, percutaneous coronary interventions; PF4, platelet factor 4.
From Warkentin TE, Greinacher A, Koster A, et al. Treatment and prevention of heparin-induced thrombocytopenia. American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th edition). *Chest* 2008;133(Suppl):340S–380S.

an important indication for heparin, such as cardiac or vascular surgery, the recommended approach is to use heparin, provided that HIT antibodies are undetectable.[159] This is because HIT antibodies are transient[43] and usually decline to nondetectable levels (by SRA) within a few weeks following an episode of HIT, although the antigen assay may persist at low-positive levels for a somewhat longer period. In patients who require urgent surgery, for example, heart transplantation, it is acceptable to wait until platelet-activating antibodies become undetectable and to reexpose the patient to heparin even while the antigen assay is still positive.[214] Furthermore, HIT does not exhibit features of a typical anamnestic response: upon heparin reexposure, HIT antibodies are usually not regenerated,[61] or if regenerated do not form more rapidly than *de novo* HIT (i.e., minimum of 5 days),[43] thereby safely permitting at least a brief intraoperative reexposure to heparin. Usually, alternative nonheparin anticoagulants are used for preoperative and postoperative anticoagulation in this setting. Patients with recent HIT, whose platelet count has recovered but who still have detectable platelet-activating antibodies (subacute HIT), should be considered to be at risk for developing rapid-onset HIT upon heparin reexposure.[159,214] Alternative anticoagulant approaches for patients in whom heparin is not judged appropriately (because of acute or very recent HIT with residual antibodies) are listed in Table 108.9.[159]

Two recent studies[215,216] describing reintroduction of LMWH or UFH for hemodialysis—following disappearance of HIT antibodies—reported successful hemodialysis and lack of HIT recurrence.

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CHAPTER

109 Coumarin-Induced Skin Necrosis and Venous Limb Gangrene

THEODORE E. WARKENTIN

Thrombotic events that result from coumarin administration are incongruous but well-documented complications of anticoagulant therapy. The key role of coumarin (vitamin K antagonist) in causing thrombosis and necrosis is by the depletion of one or both vitamin K–dependent natural anticoagulants, protein C, and protein S. In this chapter, *coumarin-induced necrosis* denotes both (central) skin necrosis and (acral) venous limb gangrene syndromes. *Coumarin-induced skin necrosis* (CISN) was first recognized by Verhagen,[1] who described 13 cases in 1954, noting features such as its female predominance, characteristic localization of lesions, and typical onset within a few days of initiating coumarin. CISN is characterized by infarction and necrosis of the skin and underlying subcutaneous tissues.[2,3] A distinct though related syndrome, *coumarin-induced venous limb gangrene*, is linked to warfarin treatment of acute deep vein thrombosis (DVT) complicating certain hypercoagulability disorders, most notably immune heparin-induced thrombocytopenia (HIT) and adenocarcinoma.[4–6,7–11]

COUMARINS

The *coumarins* are a group of naturally occurring lactones that are produced by a number of plants and microbes. Ironically, the least complex member, *coumarin* (1,2-benzopyrone), has no anticoagulant action despite its synonymous designation for those chemically related molecules that possess anticoagulant activity.[12] Warfarin (Coumadin), a 4-hydroxycoumarin derivative, is the most widely used oral anticoagulant in North America and worldwide.[2] Therefore, most instances of CISN in North America are warfarin induced. However, the syndrome can be caused by other coumarin congeners (e.g., phenprocoumon, acenocoumarol, and nicoumalone), as well as by the rarely used phenindione group of oral anticoagulants.[13,14] Phenprocoumon (Marcumar) is widely used in continental Europe. Because its half-life is longer than that of warfarin (6 vs. 1.5 days), relatively large loading doses are often given; however, whether these differences result in a lesser or greater frequency of CISN, compared with warfarin, is unknown. The coumarins interfere with vitamin K epoxide reductase,[15] producing post-translational modifications of four procoagulant and two anticoagulant vitamin K–dependent factors[16] (see Chapter 106).

PATHOGENESIS

Several observations suggest that the fundamental pathobiology of coumarin necrosis is a transient disturbance in procoagulant/anticoagulant balance that leads to microthrombosis in susceptible tissue sites. First, coumarin necrosis has a characteristic temporal profile, usually occurring within 1 week of initiating oral anticoagulant therapy.[5,6,7,9,17,18,19,20] During this interval, functional levels of protein C decline much more quickly than the major procoagulant factor, prothrombin,[21,22] because of differences in their half-lives[16] (see Table 109.1); given that prothrombin inhibits the ability of activated protein C to inactivate factor Va,[23] this early discrepancy in prothrombin versus protein C concentrations—(low-)normal and very low, respectively—can impair the protein C anticoagulant system. Second, the pathology is that of non-inflammatory, small-vessel thrombosis affecting the dermal and subcutaneous postcapillary venules and small veins.[5,19] This is consistent with the role of the protein C anticoagulant pathway in preventing thrombosis of these small vessels.[24] Third, a relatively high proportion of patients with CISN (although not with venous limb gangrene) have a hereditary abnormality of the protein C anticoagulant pathway, which predisposes such patients to further, potentially dangerous reductions in protein C during the first few days of oral anticoagulant treatment. Fourth, the clinical and pathologic appearance of CISN resembles that of neonatal purpura fulminans caused by severe congenital protein C deficiency.[25] Fifth, skin necrosis can be a feature of acquired deficiency of protein C or protein S due to infection, autoimmunity, or the antiphospholipid syndrome[26–28] or functional inhibition of protein C or protein S by antiphospholipid antibodies.[29,30] Sixth, coumarin necrosis (particularly venous limb gangrene) is associated with markedly increased thrombin generation.[4–6,31]

COUMARIN-INDUCED SKIN NECROSIS

CISN refers to *central* (nonacral) necrosis of skin and subcutaneous tissues (see FIGURE 109.1) (4); it occurs more often in women (3:1 ratio), with mean age being 50 to 55 years, and most commonly involves sites with substantial underlying fatty tissues, such as the breast, anterior abdomen, buttocks, hips, thighs, and calves and less commonly, the flank, back, penis (ischemic priapism, necrosis), legs, arms, and face (e.g., cheeks, nose, eyelids, pinnae).[3,13,17,18,19,20,32–39] Multiple (sometimes symmetric) lesions are observed in about one-third of patients. CISN is rare (estimated frequency, 0.01% to 0.1%).[3,18]

The typical temporal onset between 3 and 6 days of oral anticoagulant therapy occurs in 90% of patients,[13,17,18,19,20,40] although late onset after weeks or months has been reported.[41–43] Localized pain, induration, and erythema progress over hours to central purplish black skin discoloration, usually with blistering, and ultimately evolving to well-demarcated, full-thickness necrosis of the skin and subdermal tissues (see FIGURE 109.2). With or without surgical debridement, there usually is a permanent region of depressed skin that reflects the loss of underlying tissues. The necrotic skin also presents the real possibility of secondary fatal sepsis.[40,42,44]

Table 109.1

Half-lives of the vitamin K–dependent procoagulant and anticoagulant factors

Procoagulant Factors	Half-Life, h	Anticoagulant Factors	Half-Life, h
Factor II (prothrombin)	60	Protein C	9
Factor X	40	Protein S	60
Factor IX	24		
Factor VII	4–6		

There are six vitamin K–dependent hemostatic factors: four with procoagulant activity and two with anticoagulant activity. Treatment with coumarin anticoagulants reduces functional levels of these factors. The paradoxic procoagulant effect of warfarin can be explained by the different half-lives[16] of these factors following the onset of action of coumarin—the time to a therapeutically significant reduction of the major procoagulant factor, prothrombin (half-life, ~60 h), is much longer than the time to a clinically important reduction in the major anticoagulant factor, protein C (half-life, ~9 h). Thus, within the first several days of coumarin anticoagulation, and under certain clinical circumstances (see text), there can arise a transient, but clinically important, disturbance in procoagulant—anticoagulant balance.

The earliest stages of CISN can resemble subcutaneous hemorrhage. Incipient skin necrosis is suggested by (a) recent initiation of coumarin, (b) typical location of lesions, (c) absence of trauma, and (d) an international normalized ratio (INR) that is within (in 33% to 60% of patients) or above the therapeutic range.[17,18] CISN that occurs when the INR is within the therapeutic range suggests that the patient has a preexisting abnormality of the protein C natural anticoagulant pathway, as noted in four family members with hereditary protein S deficiency.[45] In contrast, when warfarin-induced venous limb gangrene complicates disorders associated with disseminated intravascular coagulation (DIC), such as HIT or adenocarcinoma, there typically is a *supra*therapeutic INR (>3.5). This reflects the special susceptibility of factor VII and protein C to severe depletion during coumarin therapy in DIC. In such patients, congenital abnormalities of the protein C natural anticoagulant pathway are usually not present.[5,6,10,44]

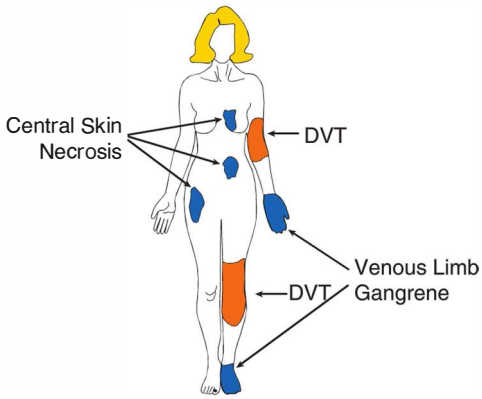

FIGURE 109.1 Two syndromes of coumarin-induced necrosis. (a) CISN and (b) coumarin-induced venous limb gangrene. CISN most often affects the breast, abdomen, thigh, buttock, and calf and often is symmetrical; it is usually associated with congenital or acquired abnormalities in the protein C natural anticoagulant pathway. In contrast, venous limb gangrene occurs in patients with DIC associated with immune HIT or adenocarcinoma. This syndrome is characterized by microvascular thrombosis in a limb affected by DVT that leads to acral ischemic necrosis. (Reprinted with modifications from Warkentin TE. Heparin-induced thrombocytopenia: IgG-mediated platelet activation, platelet microparticle generation, and altered procoagulant/anticoagulant balance in the pathogenesis of thrombosis and venous limb gangrene complicating heparin-induced thrombocytopenia. *Transfus Med Rev* 1996;10:249–258, with permission.)

FIGURE 109.2 Coumarin-induced skin necrosis. Breast necrosis necessitating bilateral mastectomies occurred during the use of warfarin to treat pulmonary embolism complicating HIT. Follow-up studies showed no congenital deficiency in protein C, protein S, or antithrombin. Factor V_{Leiden} and the prothrombin G→A20210 variant were not present. (Reprinted from Warkentin TE, Sikov WM, Lillicrap DP. Multicentric warfarin-induced skin necrosis complicating heparin-induced thrombocytopenia. *Am J Hematol* 1999;62:44–48, with permission.)

Often, multiple risk factors can be identified in individual patients (see Table 109.2).[5,6,7–10,31,32–34,40,44,45,46–64,65,66,67,68,69,70,71,72–76] An example is the concurrence of postpartum DVT, antiphospholipid syndrome, "loading doses" of warfarin, and premature discontinuation of heparin in one patient[50]; another is the concurrence of pulmonary embolism, factor V_{Leiden}, "loading doses" of warfarin, and premature discontinuation of heparin in another.[46]

Abnormalities of the protein C natural anticoagulant pathway are a relatively common finding in patients with CISN. Indeed, congenital deficiency of protein C was the first hemostatic abnormality identified in some patients with CISN (although reports do not always distinguish hereditary deficiency from the reversible reduction expected in a patient receiving coumarin).[68,69,70] Congenital deficiency of protein S[45,48,49,51,52,64,71] and of antithrombin[74] also has been observed, as has been the presence of factor V_{Leiden},[46,49,53,63,72,73] a common mutation that renders factor V less susceptible to proteolytic degradation by activated protein C. Most patients with CISN have acute DVT (rather than a nonthrombotic indication such as atrial fibrillation)[55] as their indication for oral anticoagulants,[3] suggesting

Table 109.2

Multifactorial nature of coumarin necrosis

Local Risk Factors
Sites of increased subcutaneous fat[a] (e.g., breast, abdomen, thigh, calf; obesity is common; lesions often are symmetric, e.g., bilateral thighs)[40,44,45,46–51]
DVT[a,b;40,44,47–50,52,53] and/or pulmonary embolism[46]
Peripheral vasospasm, for example, Raynaud phenomenon[54,55]
Coumarin Pharmacokinetics (Often Resulting in Supratherapeutic INR)
"Loading doses" of warfarin[a;40,44,46–51,53,56]
Drugs enhancing pharmacologic effect of warfarin, for example, antibiotics[57–59]
Hepatic dysfunction (including congestive heart failure)[59,60]
Vitamin K deficiency[61,62]
Premature discontinuation of heparin or low molecular weight heparin (or DTI) during coumarin overlap[c;40,44,46,47,50,54,63,64,65]
Cytochrome P-450 mutations (CYP2C9*2, CYP2CP9*3) affecting coumarin pharmacokinetics[d;66,67]
Disorders Associated with DIC[e]
Heparin-induced thrombocytopenia[a;5,31,49,52,54,65]
Adenocarcinoma[a;6,7–10,47,54]
Congenital or Acquired Hypercoagulability Disorders
Abnormalities of the protein C natural anticoagulant pathway
Congenital protein C deficiency[a;32–34,53,68,69,70]
Congenital or acquired protein S deficiency[a;45,48,49,51,52,64,71]
Factor V_{Leiden} (heterozygous or homozygous)[a;46,49,53,63,72,73]
Antiphospholipid syndrome[50]
Miscellaneous
Postpartum state[a;49–52]
Antithrombin deficiency[d;74,75]
Prothrombin gene mutation G20210A[d;76]
Hyperhomocysteinemia (methyltetrahydrofolate reductase C677T polymorphism)[d;48]
HIV infection with active tuberculosis[40]
The multifactorial nature of coumarin necrosis is illustrated by four or more risk factors in many patients.[46–54]

[a]Common association with coumarin necrosis that is likely of pathogenetic significance.
[b]DVT also predisposes to subtending acral limb necrosis, that is, venous limb gangrene.
[c]Early protein C depletion is more likely to be clinically significant if heparin is prematurely discontinued during heparin–coumarin overlap for treatment of thrombosis.
[d]Theoretical risk factor for coumarin necrosis that is not established.
[e]DIC predisposes more strongly to venous limb gangrene than to CISN.
INR, international normalized ratio; DTI, direct thrombin inhibitor; DIC, disseminated intravascular coagulations.

that associated clinical factors (perhaps thrombin generation related to an acute thrombus) are relevant in CISN pathogenesis.

COUMARIN-INDUCED VENOUS LIMB GANGRENE

A form of coumarin necrosis known as *venous limb gangrene* affects acral tissues such as toes/feet/legs or fingers/hands/arms (see FIGURES 109.1 and 109.3A–C)[4,5]; ischemic necrosis starts at the most distal part of the extremity and progresses proximally.[77] Tissue necrosis occurs in the limb with acute DVT, sometimes with a discernible prodrome of phlegmasia cerulea dolens[5,44,77–79] (see FIGURE 109.3D). Manifestations of acral tissue necrosis range from sloughing of skin of the toes or fingers that does not require amputation to extensive gangrene that requires below-the-knee or even above-the-knee amputation. In contrast, necrosis in CISN is limited to skin and subcutaneous tissues.

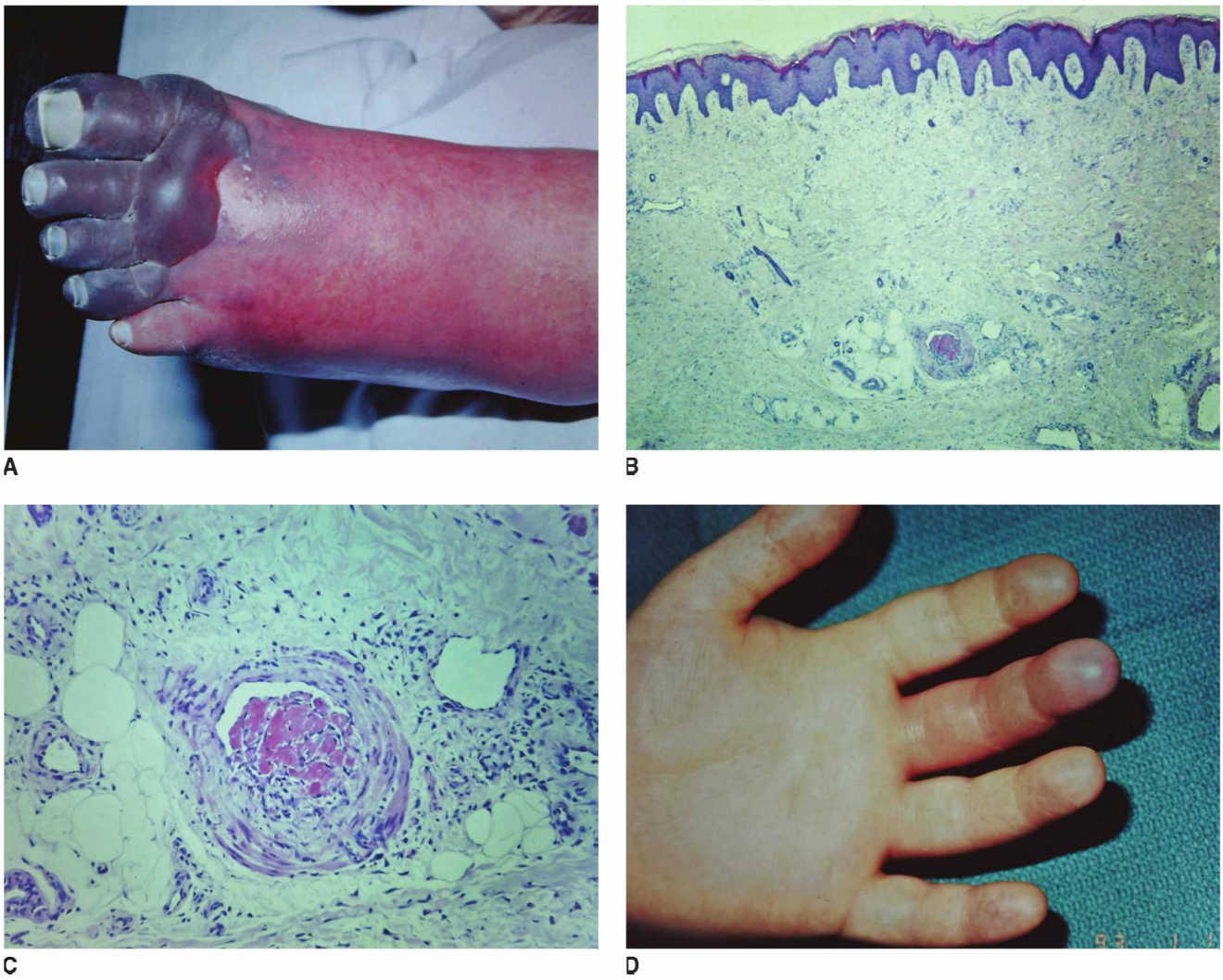

FIGURE 109.3 **A:** Warfarin-induced venous limb gangrene complicating HIT. **B:** An occluding thrombus can be seen in a subcutaneous venule (original magnification, ×40). **C:** High-power view (original magnification, ×100) of the subcutaneous venule from part **(B)**. **D:** Warfarin-associated phlegmasia cerulea dolens complicating HIT. (Photograph from patient 4, reported previously.[5]) (FIGURE 109.3A–C: From Warkentin TE, Elavathil LJ, Hayward CPM, et al. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. *Ann Intern Med* 1997;127:804–812, with permission.)

Two hypercoagulability disorders have been linked to coumarin-induced venous limb gangrene: immune HIT[5,11,31,44] and DIC associated with adenocarcinoma.[6,7–11,47,54,79,80] Occasionally, CISN and venous limb gangrene occur in the same patient.[5,31,78,79]

Clinical and laboratory data support a pathogenic role for coumarin in the pathogenesis of venous limb gangrene complicating HIT. First, a case–control study[5] of patients with HIT observed a higher frequency of warfarin use coinciding with the onset of venous limb gangrene than in control patients with limb ischemia secondary to arterial thrombosis (8 of 8 vs. 3 of 10; $P = 0.004$). Additional analysis showed that patients with HIT, DVT, and venous limb gangrene had a significantly greater median INR (5.8 vs. 3.1; $P < 0.001$) than control patients with HIT who had DVT and who were also treated with warfarin but did not develop limb gangrene.

Laboratory studies corroborated an important role for warfarin in the pathogenesis of venous limb gangrene (see FIGURE 109.4A,B). Compared with controls, plasma from patients with venous limb gangrene had a higher ratio of thrombin–antithrombin complex (a marker of *in vivo* thrombin generation) to protein C activity during warfarin treatment (FIGURE 109.4B, bottom). Analysis of individual cases showed that the onset of venous limb gangrene coincided with a fall in protein C activity to very low levels (FIGURE 109.4A, bottom). No hereditable abnormalities in the protein C anticoagulant pathway were seen in any patient. Thrombosis of subcutaneous venules characteristic of this syndrome is consistent with the role of the protein C anticoagulant pathway to downregulate thrombin generated in the microvasculature.[19,24] The normal INR values before initiation of warfarin therapy, as well as normal (or elevated) levels of factors V and VIII during warfarin treatment, rule out DIC as the sole explanation for the elevated INR values.[6]

Venous limb gangrene usually presents as a characteristic clinical triad[4–6]: (a) an acute DVT involving the limb

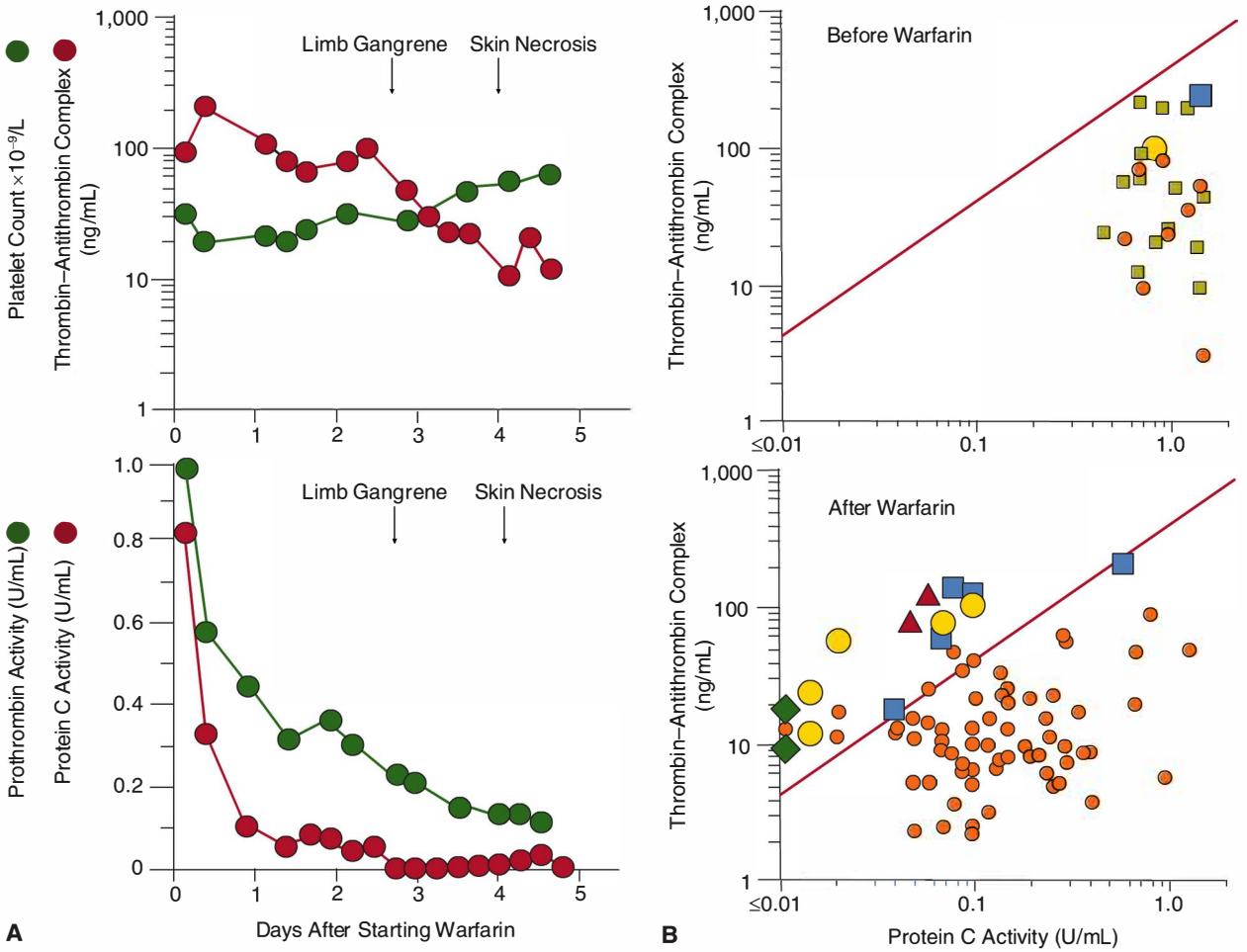

FIGURE 109.4 Coumarin-induced venous limb gangrene: a disturbance in procoagulant–anticoagulant balance. **A:** Hemostatic markers in relation to clinical course in a patient with warfarin-induced venous limb gangrene. Venous limb gangrene and central skin necrosis occurred when warfarin was given during acute HIT; the INR rose to a supratherapeutic value, 7.2 (not shown). **Top:** Platelet counts and thrombin–antithrombin complexes. **Bottom:** Prothrombin activity and protein C activity. **B:** Thrombin–antithrombin complexes compared with protein C activity in patients with HIT. Each data point represents thrombin–antithrombin complexes and protein C activity per single treatment day per patient. In both panels, *large symbols* represent patients who developed venous limb gangrene (n = 3) or cerulea phlegmasia dolens (n = 1). The *diagonal line* indicates an arbitrary ratio of thrombin–antithrombin complex to protein C of 40. **Top:** Results obtained when HIT was first diagnosed and before warfarin therapy began for two patients (*large symbols*) who developed venous limb gangrene or severe venous ischemia and for eight patients (*small orange circles*) who subsequently received warfarin for DVT without developing venous limb gangrene. For comparison, results obtained when HIT was diagnosed in 15 patients (*small olive squares*) without clinically suspected DVT who did not subsequently receive warfarin are shown. **Bottom:** Results in 16 patients who were receiving warfarin for HIT, including four patients (*large symbols*) who developed venous limb gangrene or phlegmasia cerulea dolens and 12 patients (*small orange circles*) (nine of whom had DVT) who received warfarin without developing venous limb gangrene. More than one data point is shown for patients from whom plasma samples were available on more than 1 day of warfarin treatment. (Reprinted with modifications from Warkentin TE, Elavathil LJ, Hayward CPM, et al. The pathogenesis of venous limb gangrene associated with heparin-induced thrombocytopenia. *Ann Intern Med* 1997;127:804–812, with permission.)

subsequently affected by necrosis, (b) a supratherapeutic INR, and (c) thrombocytopenia associated with a procoagulant disorder such as HIT or adenocarcinoma. FIGURE 109.5 summarizes the pathogenesis of warfarin-induced venous limb gangrene in relation to this clinical triad.

Typically, acral necrosis occurs in a limb with a concurrent symptomatic *acute DVT*. In the study of venous limb gangrene complicating HIT,[5] this complication occurred in 10 limbs (among 8 patients). Symptomatic lower limb DVT was present in all 10 limbs within the previous week; necrosis did not occur in upper or contralateral lower limbs unaffected by DVT. These observations suggest a role for large vein thrombosis in predisposing to ipsilateral involvement of small veins and venules, either by direct extension of thrombosis from large to

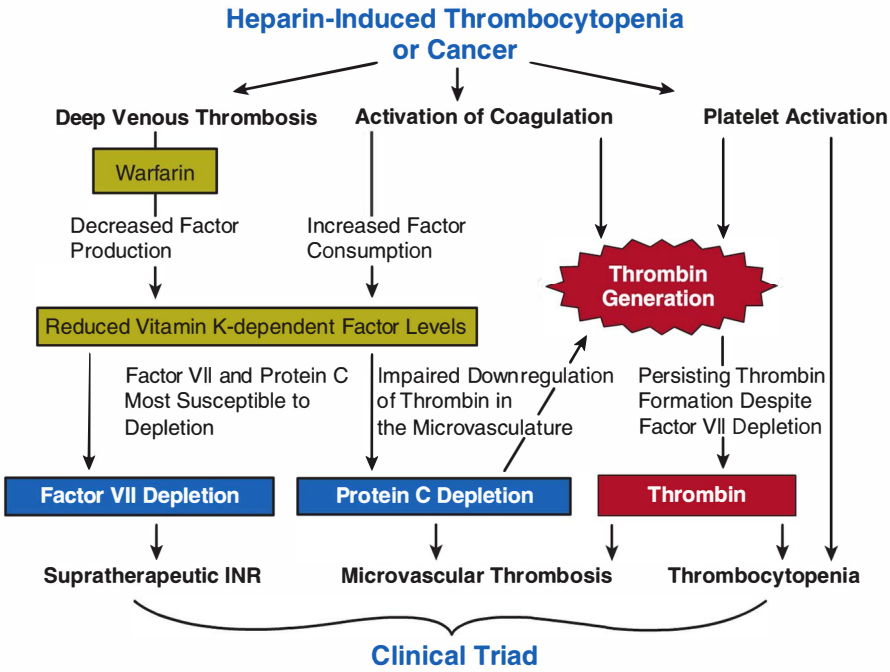

FIGURE 109.5 Proposed pathogenesis of coumarin-induced venous limb gangrene complicating either immune HIT or adenocarcinoma. INR, international normalized ratio. (From Warkentin TE. Venous limb gangrene during warfarin treatment of cancer-associated deep venous thrombosis. *Ann Intern Med* 2001;135:589–593, with permission.)

progressively smaller veins or by venous stasis predisposing to microvascular thrombosis.

An *elevated INR* is the laboratory hallmark of this syndrome. Patients with venous limb gangrene typically have an INR that is at least 3.5.[4–6,7,44,47,49,54,65,80] The high INR is a surrogate marker for severe protein C depletion that reflects parallel depletion in factor VII[6] (see FIGURE 109.6).

Increased thrombin generation and platelet activation, clinically manifesting as thrombocytopenia, are characteristic of coumarin-induced venous limb gangrene.[5,6] Platelet activation by HIT antibodies produces a marked platelet procoagulant response, as shown by *in vitro* and *in vivo* generation of procoagulant, platelet-derived microparticles,[81,82] leading to increased thrombin generation in patients with HIT, with the potential for impaired procoagulant–anticoagulant balance during coumarin treatment.

Table 109.3 lists the reasons why physicians may not recognize the role of coumarin in contributing to venous limb gangrene.[5,77] Clinicians should suspect oral anticoagulation as the explanation for progressive acral ischemia when an otherwise typical DVT worsens in association with a rise in the INR to above 3.5, especially if the patient has thrombocytopenia suggestive of HIT or DIC.[4–6]

Warfarin-induced digital necrosis affecting multiple digits occurred in a patient with adenocarcinoma, HIT, and Raynaud phenomenon; the absence of DVT in three limbs affected by digital ischemia and necrosis suggested that vasospasm predisposed to this unusual presentation of multiple digital gangrene.[54] Raynaud phenomenon was also a feature of warfarin-associated multiple digital necrosis in a patient with protein S deficiency.[55]

TREATMENT

Vitamin K may reverse incipient CISN or phlegmasia cerulea dolens if given in the earliest stages of the disease.[5,44,83] Often, however, the diagnosis is not made until extensive microthrombosis has occurred and necrosis is inevitable. It is not known whether vitamin K, plasma, protein C,[84–86] or activated protein C concentrates alter the natural history at this point. Amputation or skin grafting is required in about half of the patients with coumarin necrosis.

In the original report of CISN by Verhagen,[1] the coumarin was usually continued despite the occurrence of tissue necrosis, and it was the author's view that tissue injury nevertheless did not progress beyond the level at which it was already clinically apparent. An alternative view is that intravenous vitamin K and effective parenteral anticoagulation are important in avoiding progressive macrothrombosis and microthrombosis in a patient with coumarin-associated phlegmasia or venous limb gangrene complicating HIT or cancer. The reasoning is that unlike the transient disturbance of procoagulant–anticoagulant balance seen in some patients with CISN, ongoing consumption of the vitamin K–dependent factors can persist if the underlying hypercoagulability state is uncontrolled. Anticoagulation involves either heparin (or low molecular weight heparin) for cancer-associated DIC, or a nonheparin anticoagulant (e.g., lepirudin, argatroban, danaparoid), if HIT is the diagnosis. A relevant issue is that ongoing vitamin K antagonism tends to prolong the activated partial thromboplastin time (aPTT), thereby confounding the monitoring and dosing of heparin or a direct thrombin inhibitor, underscoring the importance of giving vitamin K, preferably by the intravenous route.[87,88,89]

PREVENTION

A cautious approach to further use of coumarin anticoagulants in acute HIT or DIC secondary to malignancy should be adopted. Warfarin should be started only in patients with acute HIT who are adequately anticoagulated with a nonheparin anticoagulant, and only after the platelet count has recovered

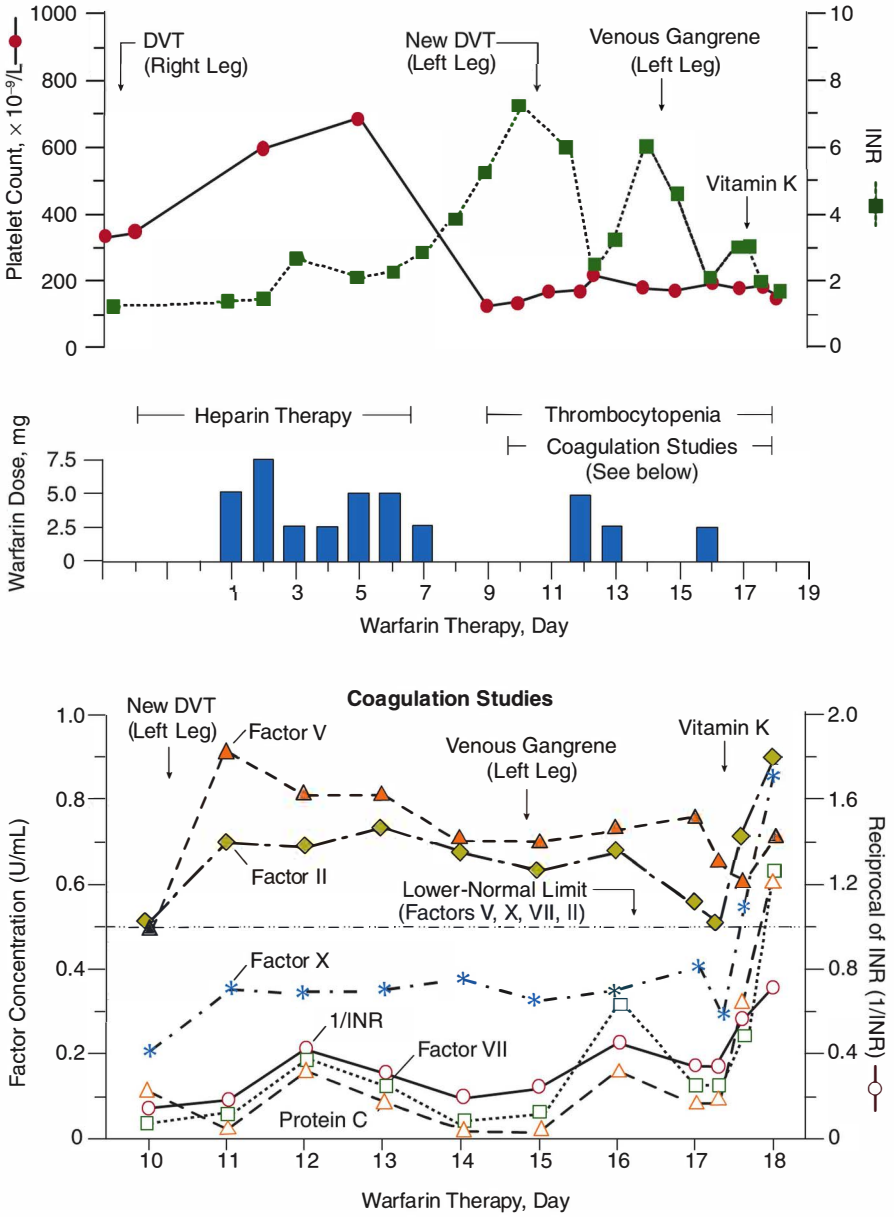

FIGURE 109.6 Top: Cancer-associated venous limb gangrene: serial platelet counts and international normalized ratio (INR) values in relation to heparin and warfarin treatments. The abrupt increase in the INR and decrease in the platelet counts on discontinuation of heparin therapy are consistent with the increase in the consumption of coagulation factors and platelets in this patient with cancer-associated DIC resistant to warfarin. **Bottom:** Plasma factor studies. Eleven plasma samples obtained between days 10 and 18 of warfarin treatment were studied. Vitamin K, 5 mg intravenously, was given where indicated. Of the four coagulation factors that affect the INR (factors II, V, VII, and X), only factor VII levels explained the variability in the reciprocal of the INR: 1/INR = 0.178 + 0.974 (factor VII level) (r^2 = 0.883, P < 0.001). Protein C activity showed a near-identity relationship with factor VII: (protein C level) = −0.01 + 0.94 (factor VII level). The following coagulation factor levels (not shown in the figure) were normal: factor VIII, factor IX, factor XI, factor XII, free protein S, fibrinogen, and antithrombin activity. (From Warkentin TE. Venous limb gangrene during warfarin treatment of cancer-associated deep venous thrombosis. *Ann Intern Med* 2001;135:589–593, with permission.)

to more than 150 × 10^9/L[88,89] (see Chapter 108). FIGURE 109.7 illustrates the danger of initiating warfarin in a patient with DIC and thrombocytopenia associated with HIT; even though concomitant lepirudin was given, when the INR reached 5.5 and lepirudin was stopped, progression to venous limb gangrene rapidly ensued. Low molecular weight heparin rather than warfarin should be considered for patients with cancer-associated DVT[80,90] who have laboratory evidence for DIC.

Routinely avoiding loading doses of oral anticoagulants may reduce the frequency of coumarin necrosis. A maximum of 5 mg for the first dose (even less in case of an elderly patient) is appropriate.[91,92] Harrison et al.[21] performed a randomized controlled trial

Table 109.3

Reasons why venous limb gangrene is often not recognized as being caused by warfarin or other oral anticoagulants

1. Limb necrosis primarily blamed on associated illness alone (e.g., HIT, malignancy) rather than on warfarin
2. DVT usually precedes use of warfarin
3. Classic *clinical* features of CISN are usually lacking (i.e., central skin necrosis involving breast, abdomen, or thigh is usually not present)
4. Classic *laboratory* features of CISN are usually lacking (i.e., congenital abnormality of the protein C anticoagulant pathway usually is not found)

HIT, heparin-induced thrombocytopenia; DVT, deep vein thrombosis; CISN, coumarin-induced skin necrosis.

comparing 5 versus 10 mg warfarin as an initial "loading" dose for typical patients undergoing initial warfarin anticoagulation. The group that received the lower dose of warfarin had significantly higher levels of protein C at 36 and 60 hours after initiation of warfarin and was less likely to have early excess anticoagulation (as measured by the INR). Therefore, there may be an advantage of using the lower dose (avoidance of protein C deficiency and an associated disturbance in procoagulant/anticoagulant balance).[21,93]

The manufacturer of Coumadin advocates avoiding loading doses: "Routine use of loading doses is not recommended as this may increase hemorrhagic and other complications and does not offer more rapid protection against clot formation. .... [T]he initial dose of Coumadin is usually 2 to 5 mg per day. Modify this dose based on consideration of patient-specific clinical factors. Consider lower initiation doses for elderly and/or debilitated patients."[94] An important reason to avoid loading doses of warfarin, although it is safe in most patients, is because it is unsafe in the *minority* of patients who have one or more risk

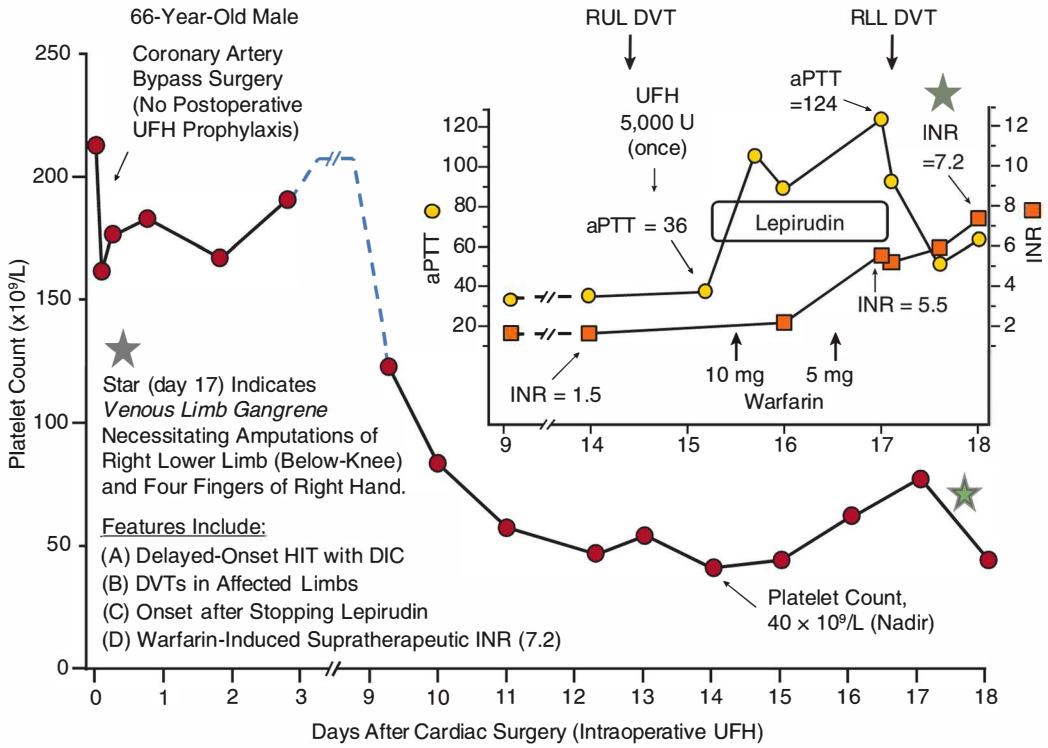

FIGURE 109.7 Patient with delayed-onset HIT, DIC, and venous limb gangrene during overlapping treatment with lepirudin and warfarin. The patient was readmitted to hospital on postoperative day 9 following cardiac surgery because of sternal wound complications. DIC was indicated by elevated INR (1.5) and aPTT (36 s), low fibrinogen (1.5 g/L), and elevated lactate dehydrogenase and reticulocyte counts. On day 14, symptomatic right upper-limb DVT (brachial and basilic veins) occurred, and one subcutaneous dose of UFH was given. An anti-PF4/polyanion enzyme immunoassay was "strongly positive," and thus lepirudin (0.4 mg/kg bolus followed by 0.20 mg/kg/h) was administered. Unfortunately, two doses of warfarin (10 and 5 mg) also were given, in spite of persisting thrombocytopenia (platelet count ~40 × 10^9/L). On day 17, the aPTT of 124 seconds and INR of 5.5 prompted discontinuation of lepirudin; within hours, the patient developed explosive symptoms and signs of right lower-limb DVT (common/superficial femoral and popliteal veins) with rapid progression to venous limb gangrene of the right upper and lower limbs. Other risk factors for venous limb gangrene identified in this patient included heterozygous factor V_{Leiden} status and intravenous ciprofloxacin and amoxicillin (potentiation of warfarin effect). aPTT, activated partial thromboplastin time; DIC, disseminated intravascular coagulation; DVT, deep vein thrombosis; HIT, heparin-induced thrombocytopenia; INR, international normalized ratio; RLL, right lower limb; RUL, right upper limb; U, units; UFH, unfractionated heparin.

factors for coumarin necrosis, the presence of which is generally unknown when warfarin is commenced.[91]

REPEAT USE OF COUMARIN

For patients with a history of CISN or venous limb gangrene, it is acceptable to reintroduce oral anticoagulants with appropriate precautions.[5,50,74] This is because coumarin necrosis is often related to dynamic and transient clinical and biochemical factors that may not exist later. It is prudent to start with low doses of warfarin (e.g., 1 to 2 mg/d for 3 to 4 days), gradually increasing the INR into the target range over 1 to 2 weeks. However, even higher initial doses (2 to 5 mg/d) are reported to be safe.[95] Unless HIT is suspected, combined treatment with therapeutic dose heparin is recommended[96] as thrombin inhibition theoretically reduces the risk of coumarin necrosis. Plasma or protein C concentrates have been given to some patients with known congenital protein C deficiency during initiation of warfarin.[97–99] The risk of recurrence of necrosis despite using any of these precautions is unknown, but likely is low.

COUMARIN-ASSOCIATED "PURPLE TOE" AND CHOLESTEROL EMBOLISM SYNDROMES

Use of oral anticoagulants may contribute to the development of "purple" or "blue" toes (or, rarely, fingers)[100] in patients with severe atherosclerosis. This syndrome was first described by Feder and Auerbach,[101] who reported six patients who developed blue-purple discoloration of the feet, especially the plantar surfaces and sides of the first two toes, that began 3 to 8 weeks after starting a coumarin. Other clinical features included pain and tenderness of the toes, blanching on moderate pressure, and persistence of these features despite stopping oral anticoagulation. Because these patients were male with peripheral vascular disease, it was suggested that "purple toe syndrome" could be explained by cholesterol microembolization,[102,103] a concept that is gaining acceptance.[104,105]

Classically, cholesterol (or atheromatous) embolism syndrome is characterized by a variety of peripheral ischemic signs (livedo reticularis, "blue" toes, focal digital necrosis); renal failure may also occur.[103,106,107] Pedal pulses are usually palpable, consistent with the histopathologic findings of biconvex clefts in small arteries and arterioles indicative of preceding microembolism of cholesterol crystals. Although advanced atherosclerosis is conspicuous, there is often a history of recent vascular injury (e.g., cardiac surgery, arteriography)[103] or of disruption of adherent thrombus (e.g., postthrombolytic therapy).[108]

Only about 20% to 50% of patients who are clinically diagnosed with cholesterol embolism syndrome are receiving anticoagulants.[109,110] Theoretically, coumarins could cause or exacerbate this syndrome by contributing to a gradual loss over weeks of a protective fibrin layer overlying an ulcerated atherosclerotic plaque, facilitating cholesterol microembolization.[111] Fibrin dissolution could also explain the occurrence of this syndrome following thrombolysis.[108] However, any causal role for coumarin or other anticoagulants is speculative. Therefore, the physician must balance the risks of stopping warfarin with the uncertain potential for improvement. Although resolution of cholesterol embolism syndrome might seem improbable, there are well-documented reports of clinical improvement or resolution after stopping warfarin,[107] and even subsequent recurrence of purple toes with resumption of coumarin.[112]

MISCELLANEOUS CUTANEOUS EFFECTS OF ORAL ANTICOAGULANTS

In addition to ecchymotic purpura related to the anticoagulant effects of warfarin and other oral anticoagulants, a few patients may develop macular, papular, vesicular, or urticarial lesions, presumably of an allergic origin.[113,114] Alopecia of varying severity in relation to oral anticoagulation has also been described. Use of a different oral anticoagulant agent may permit further anticoagulation with resolution of these adverse effects.

Calcific uremic arteriopathy (or arteriolopathy), also known as *calciphylaxis*, occurs in patients with chronic renal failure and hyperparathyroidism and is characterized by painful, well-demarcated purpuric plaques and nodules with surrounding livedo reticularis.[115,116,117,118–120] As with CISN, involved skin often overlies adipose tissues, and therefore thigh and abdomen are involved more often than acral locations.[118] Histologic features include medial calcification of small- and medium-sized arteries, with intimal proliferation and endovascular fibrosis.[118] Reduced protein C activity (with normal protein C antigen levels) has been described.[115] Some patients develop this complication while receiving warfarin and may benefit from substituting oral anticoagulation by low molecular weight heparin.[116,119,120]

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CHAPTER

110 New Antithrombotic Drugs

JEFFREY I. WEITZ AND PETER L. GROSS

Currently available antithrombotic drugs include (a) antiplatelet agents, which inhibit platelet activation or aggregation, (b) anticoagulants, which attenuate fibrin formation, and (c) fibrinolytic agents, which degrade the fibrin component of thrombi by catalyzing the generation of plasmin (FIGURE 110.1). Because arterial thrombi consist of platelet aggregates held together by small amounts of fibrin,[1] strategies to prevent or treat arterial thrombosis focus mainly on drugs that block platelet function, but may include anticoagulants to prevent fibrin deposition. Anticoagulants are the treatment of choice for prevention of cardioembolic events in patients with atrial fibrillation or mechanical heart valves. Anticoagulants also are the drugs of choice for their prevention and treatment of venous thromboembolism (VTE) because venous thrombi are composed mainly of fibrin.[1]

Fibrinolytic therapy is used in selected patients with arterial or venous thrombosis. When percutaneous intervention is not feasible, systemic fibrinolytic therapy is often administered to patients with acute myocardial infarction (MI) or selected patients with ischemic stroke to rapidly restore antegrade flow in occluded arteries. Patients with massive pulmonary embolism (PE) may also benefit from systemic or catheter-directed fibrinolytic therapy. Although systemic fibrinolytic therapy is ineffective for treatment of patients with extensive deep vein thrombosis (DVT) involving the iliac and/or femoral veins, catheter-directed fibrinolysis, often in conjunction with mechanical methods of thrombus disruption, may be of benefit in some patients.[2]

Focusing on new antithrombotic agents, this chapter (a) identifies the limitations of current antithrombotic drugs, (b) describes the new antiplatelet and anticoagulant drugs in more advanced stages of development, focusing on their mechanism of action and the results of clinical trials, and (c) provides clinical perspective on the unmet needs in antithrombotic therapy for prevention and treatment of venous and arterial thrombosis. Fibrinolytic drugs are discussed in Chapter 112.

LIMITATIONS OF CURRENT ANTITHROMBOTIC DRUGS

New antithrombotic drugs have been developed to overcome the limitations of existing agents. Most of the advances have been in the area of antiplatelet drugs and anticoagulants. The development of new fibrinolytic agents has lagged behind.

Currently available oral antiplatelet agents include aspirin, clopidogrel, prasugrel, and ticagrelor. The efficacy of aspirin and clopidogrel has established cyclooxygenase-1 (COX-1), a key enzyme in thromboxane A_2 synthesis, and $P2Y_{12}$, the major ADP receptor on platelets, as important targets for antiplatelet drugs. On its own, clopidogrel has been shown to be only marginally more effective than aspirin.[3] The combination of aspirin plus clopidogrel is superior to aspirin alone in patients at high risk for cardiovascular events,[4–7] but combination therapy is associated with a significant risk of bleeding and has yielded disappointing results in patients with stable cardiovascular disease.[8,9] Although the combination of aspirin plus dipyridamole is superior to aspirin alone for secondary prevention in patients with cerebrovascular disease,[10] the efficacy of this combination is similar to that of clopidogrel.[11]

The limitations of existing antiplatelet drugs reflect, at least in part, their capacity to attenuate only a single pathway of platelet activation. Because platelets can be activated via multiple pathways, the potential for bypassing the inhibitory effects of these drugs remains high when there is a potent stimulus for platelet activation. Consequently, it is not surprising that breakthrough cardiovascular events occur, and these should not necessarily be labeled as simple treatment failures.

Another factor that may contribute to breakthrough cardiovascular events is individual variability in the response to antiplatelet drugs. Such variability may reflect poor compliance, pharmacogenetic factors, increased platelet turnover, drug interactions, baseline and residual platelet hyper-reactivity, as well as other factors.[12,13] Decreased responsiveness to aspirin and/or clopidogrel is common in patients with acute coronary syndrome (ACS), particularly those with diabetes.[14] In patients undergoing percutaneous coronary intervention (PCI), a reduced biologic response to aspirin plus clopidogrel has been associated with a poorer outcome. The decreased response may reflect, at least in part, limited metabolic activation of clopidogrel[13] in patients with reduced function variants of the *CYP2C19* gene (see Chapter 111).

Although prasugrel also requires biotransformation to active metabolites, variants of the *CYP2C19* gene do not appear to influence outcomes in prasugrel-treated patients.[15,16] These findings have prompted some experts to recommend tailored antiplatelet therapy based on periprocedural platelet function or genetic testing.[17,18] However, the value of this approach has not been established, and, in one study, doubling the clopidogrel dose in patients with increased platelet reactivity did not improve outcome.[19] Instead, the limitations of existing antiplatelet agents has prompted the development of newer and more potent drugs, some directed against proven targets involved in platelet activation and others against new targets. These agents include novel inhibitors of the thromboxane A_2 receptor, new $P2Y_{12}$ antagonists, and inhibitors of protease activated receptor-1 (PAR-1), the major thrombin receptor on platelets.

On the anticoagulant front, attention has focused on the development of new oral agents to replace vitamin K antagonists (VKA).[20] Rivaroxaban, a direct factor Xa inhibitor, and

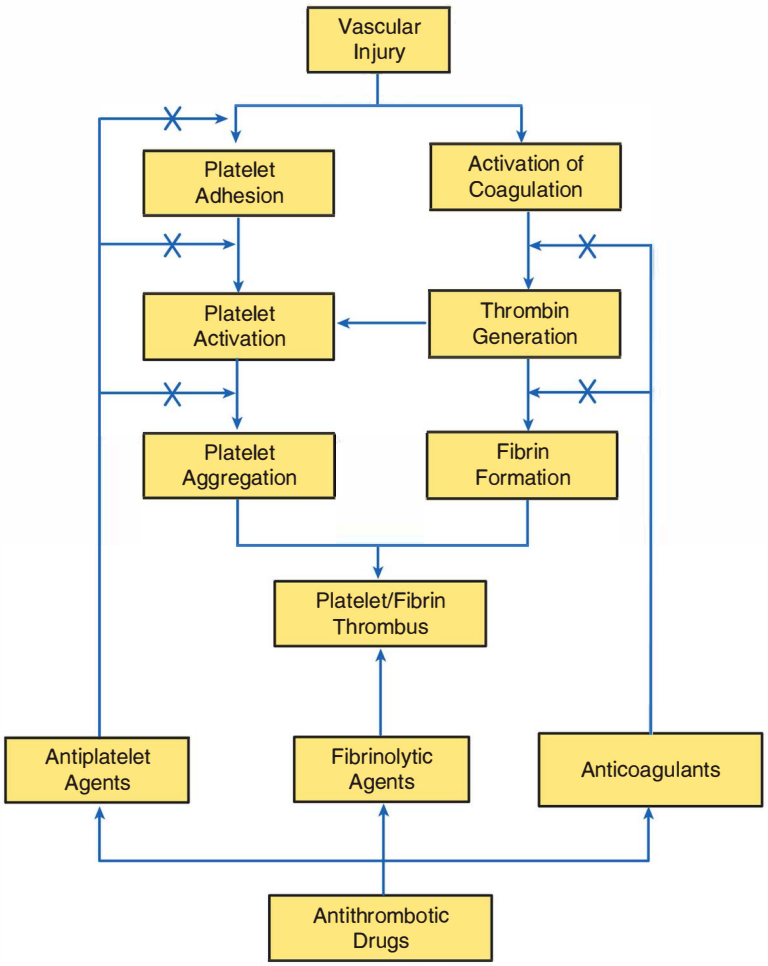

FIGURE 110.1 Targets of new antithrombotic drugs. Thrombosis occurs at sites of vascular injury by coordinated processes that result in platelet activation and activation of the coagulation system. Formation of the platelet plug represents the first step in hemostasis. Subsequent thrombin generation amplifies the platelet response because thrombin is a potent platelet agonist. Thrombin also converts fibrinogen to fibrin, and the fibrin strands stabilize the platelet aggregates, thereby forming a platelet/fibrin thrombus. Given the importance of platelets and fibrin in thrombogenesis, new antithrombotic agents target these components. New antiplatelet strategies focus on blocking platelet adhesion, activation, or aggregation. Novel anticoagulants, which target various coagulation enzymes, are geared at inhibiting thrombin generation or thrombin activity. Finally, fibrinolytic agents digest the fibrin component of the thrombus, thereby causing thrombus dissolution.

dabigatran etexilate, a direct thrombin inhibitor, have been licensed in many countries for short-term thromboprophylaxis after elective hip or knee arthroplasty, and dabigatran etexilate and rivaroxaban have been licensed in the United States, Canada, and Europe for stroke prevention in patients with atrial fibrillation. The pharmacologic properties of these agents are compared in Table 110.1.

PLATELET INHIBITORS

Inhibitors of Platelet Adhesion

The interaction of platelets with subendothelial adhesive proteins exposed at sites of vascular injury promotes platelet signaling pathways that trigger platelet activation and adhesion. Consequently, strategies aimed at blocking platelet adhesion represent a novel antithrombotic approach. The targets for these inhibitors include von Willebrand factor (vWF), glycoprotein (GP) Ib/V/IX, GPVI, GPIa/IIa, collagen, or intracellular signaling molecules that are triggered by platelet adhesion (FIGURE 110.2).

vWF Inhibitors

Because vWF serves as the molecular glue that tethers platelets to the damaged vessel wall, vWF is an attractive target for agents that block platelet adhesion. Strategies to target vWF include (a) aurintricarboxylic acid, a heterogeneous mixture of polycarboxylic and polyaromatic polymers that bind vWF and prevent its interaction with GPIb,[21,22] (b) genetically engineered vWF fragments encompassing the GPIb-binding site, which compete with intact vWF for receptor binding,[23] and (c) monoclonal antibodies or oligonucleotide aptamers directed against the A1 domain of vWF, which block the interaction of vWF with GPIb.[24–27] ARC1779, a DNA aptamer consisting of 40 nucleotides directed against the A1 domain of vWF, is the only one of these agents to be evaluated in humans. Polyethylene glycol was attached to the aptamer to extend its half-life. ARC1779 was well tolerated in a phase I study in healthy volunteers and has shown promise in the treatment of patients with thrombotic thrombocytopenic purpura.[27]

GPIb/V/IX Inhibitors

The interaction of vWF with GPIb/V/IX is an early and pivotal step in platelet adhesion. Thus, thrombus formation is abolished in GPIb-knockout mice.[28] In contrast, when vWF is knocked out, thrombus formation is only modestly attenuated, presumably because other adhesive ligands substitute for vWF and mediate platelet adhesion. Strategies to target GPIb/V/IX include (a) monoclonal antibodies directed against GPIb, (b) GPIb-binding proteins isolated from the venom of *Bothrops jararaca* or *Crotalus atrox*, which compete with vWF for GPIb binding, and (c) R9alpha557, a palmitylated, cell-permeable peptide

Table 110.1

Comparison of the pharmacologic properties of the new oral anticoagulants that are licensed or in the most advanced stages of clinical development

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target	Thrombin	Factor Xa	Factor Xa	Factor Xa
Molecular weight	628	436	460	548
Bioavailability (%)	6	80	50	50
Dose frequency	od/bid	od/bid	bid	od
Tmax (h)	2	3	3	1–2
Half-life (h)	12–17	7–11	9–14	9–11
Protein binding (%)	35	95	87	54
CYP metabolism (%)	None	32	15	<4
P-gp transport	Yes	Yes	Yes	Yes
Renal excretion (%)	80	66[a]	25	35
Drug interactions	Potent P-gp inhibitors and inducers[b]	Potent inhibitors and inducers of P-gp and CYP3A4[b]	Potent inhibitors and inducers of CYP3A4[b]	Potent inhibitors and inducers of P-gp
Antidote[c]	Fab fragment	Factor Xa	Factor Xa	Factor Xa

[a]One-half of renally cleared rivaroxaban is excreted as unchanged drug and one-half as inactive metabolites.
[b]Potent inhibitors of P-gp include quinidine, amiodarone, and verapamil. Potent inhibitors of both P-gp and CYP3A4 include azole antifungals (e.g., ketoconazole), protease inhibitors (e.g., ritonavir), and macrolide antibodies (e.g., clarithromycin). Potent CYP3A4 inhibitors include azole antifungals, macrolide antibiotics, and protease inhibitors. Potent inducer of both P-gp and CYP3A4 is rifampicin.
[c]Neither of these antidotes has been tested in humans.
bid, twice daily; od, once daily; CYP, cytochrome P450; P-gp, P-glycoprotein efflux transporter.

corresponding to the 557 to 569 segment of the cytoplasmic tail of GPIb that attenuates receptor signaling.[29] None of these strategies has been evaluated in humans.

GPVI Inhibitors

As a collagen receptor, GPVI plays an important part in the adhesion of platelets at sites of vascular damage. Mice deficient in GPVI exhibit reduced thrombus formation in response to ferric chloride–induced arterial injury, which exposes collagen.[30] In contrast, thrombus formation in response to laser injury, which does not expose collagen, is minimally affected.[30] Strategies to inhibit GPVI include (a) monoclonal antibodies directed against the receptor,[31,32] (b) soluble GPVI or a chimeric molecule consisting of GPVI linked to the Fc domain of human immunoglobulin,[33–35] and (d) EXP3179, a losartan metabolite that blocks collagen-induced platelet aggregation and platelet adhesion at sites of arterial injury.[36] None of these strategies has been evaluated in humans.

GPIa/IIa Inhibitors

Like GPVI, GPIa/IIa also binds collagen, thereby facilitating platelet adhesion.[37] Although platelets from mice deficient in either of the GPIa/IIa subunits exhibit reduced aggregation in response to collagen, the mice have a normal bleeding time and thrombus formation in response to injury is minimally impaired.[38,39] Strategies to inhibit GPIa/IIa include (a) recombinant protein analogues of the collagen-binding site on the receptor[40] and (b) small molecule receptor inhibitors.[41] Neither strategy has been tested in humans.

Collagen Inhibitors

Anopheline antiplatelet protein, a protein isolated from the saliva of the *Anopheles stephensi* mosquito, binds collagen and inhibits its interaction with GPVI and GPIa/IIa.[42] Additional work is needed to determine the therapeutic potential of this protein or the usefulness of similar antiplatelet strategies.

Inhibitors of Intracellular Signaling

An alternative strategy to targeting platelet receptors or their ligands is to block the downstream signaling pathways that trigger platelet activation. Type Ia phosphoinositide 3-kinase (PI3K) is an intracellular signaling molecule whose activation in platelets is linked to GPIIb/IIIa under high shear conditions. TGX-221, an inhibitor of the β isoform of PI3K, decreases shear-dependent platelet adhesion *in vitro* and in animal models of arterial injury.[43–45] TGX-221 has not been tested in humans.

Inhibitors of Platelet Recruitment

Strategies to block platelet recruitment have targeted the thromboxane A_2 receptor, to which thromboxane A_2 and its endoperoxide precursors bind, $P2Y_{12}$, the most important ADP receptor, or PAR-1, the major thrombin receptor on platelets.

Thromboxane A_2 Receptor Antagonists

The thromboxane A_2 receptor is a G-protein–coupled receptor on platelets that is not only activated by thromboxane A_2, but also by its cyclic endoperoxide precursors. Thromboxane A_2 receptor

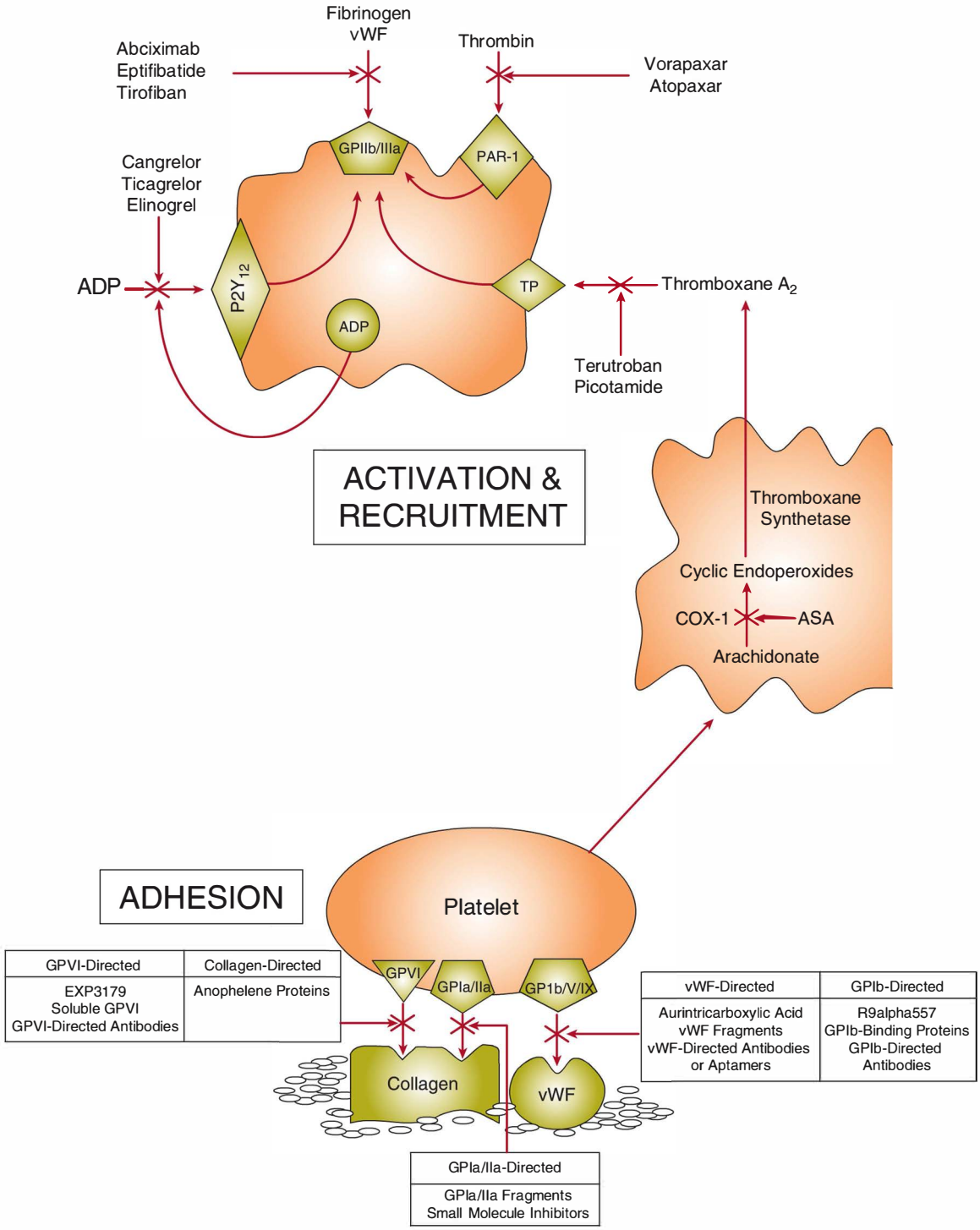

FIGURE 110.2 Sites of action of new antiplatelet drugs. Platelets adhere to subendothelial collagen via glycoprotein (GP) VI and GPIa/IIa, and to von Willebrand factor (vWF) via GPIb/V/IX, respectively (*top panel*). Adherent platelets become activated and release thromboxane A_2 and adenosine diphosphate (ADP), substances that activate adjacent platelets. By irreversibly inhibiting cyclooxygenase-1 (COX-1), aspirin blocks thromboxane A_2 synthesis. Thromboxane A_2 receptor (TP) antagonists attenuate platelet recruitment by preventing thromboxane A_2–mediated platelet activation. ADP induces platelet recruitment by binding to $P2Y_{12}$, the major ADP receptor on platelets, and triggering platelet activation. By binding to $P2Y_{12}$, cangrelor, ticagrelor, and elinogrel block ADP-induced platelet activation. Thrombin, the most potent platelet agonist, binds to, cleaves, and activates protease-activated receptor-1 (PAR-1) on the platelet surface. Vorapaxar and atopaxar inhibit PAR-1 activation, thereby blocking thrombin-induced platelet activation.

antagonists were developed, at least in part, to overcome the variable response to aspirin.[46] Although aspirin blocks thromboxane A_2 synthesis in most individuals, elevated urinary levels of its stable metabolite, thromboxane B_2, have been associated with an increased risk of cardiovascular events.[47,48] Elevated levels of urinary thromboxane B_2 may reflect incomplete blockade of COX-1 in platelets and/or the shuttling of endoperoxide intermediates to platelets from other cells. The thromboxane A_2 receptor antagonists include terutroban and picotamide.

Terutroban

A selective inhibitor of the thromboxane A_2 receptor on platelets, terutroban (previously known as S18886), is orally active.[49] Peak plasma concentrations are achieved within 1 to 2 hours of oral administration, and the drug has a half-life of 6 to 10 hours. Terutroban inhibits thromboxane A_2–induced platelet aggregation in a dose-dependent fashion with maximum inhibition obtained with drug levels above 10 ng/mL—a concentration that can be achieved with daily doses of 10 to 30 mg.[50]

In a phase II study, peripheral arterial disease (PAD) patients were randomized to terutroban, aspirin, or placebo. Terutroban produced dose-dependent inhibition of platelet aggregation in response to thromboxane, ADP, or collagen and was at least as potent as aspirin.[51] The phase III double-blind PERFORM trial compared terutroban (30 mg/day) with aspirin (100 mg/day) for secondary prevention in 19,119 patients with a recent history of ischemic stroke or transient ischemic attacks.[52,53,54] The trial was stopped early because the rate of the primary efficacy endpoint, a composite of fatal or nonfatal ischemic stroke, fatal or nonfatal MI, or other vascular death, was 11% in both groups, and there was a slight increase in the rate of minor bleeding with terutroban compared with aspirin (12% and 11%, respectively).

Picotamide

A derivative of methoxy-isophtalic acid, picotamide not only inhibits the thromboxane A_2 receptor, but also inhibits thromboxane synthetase at equivalent concentrations.[55] In contrast to aspirin, picotamide does not interfere with prostacyclin production. In a double-blind, placebo-controlled study in 2,304 patients with PAD, treatment with picotamide (300 mg bid) or placebo was administered for 18 months.[56] Endpoints of the study included major events (cardiovascular death, MI, stroke, or amputation) and minor events (unstable angina, transient ischemic attacks, hypertension, renal failure, or worsening of PAD symptoms). Although the intention-to-treat analysis revealed an 18.9% reduction in major plus minor events with picotamide, this difference was not statistically significant. However, the on-treatment analysis showed a 22.8% reduction in the same endpoints. Bleeding side effects were similar in the two groups. A *post hoc* subgroup analysis of the data from the 438 diabetic patients included in the study revealed a 45.2% reduction in major and minor endpoints with picotamide compared with placebo.[57] These findings prompted a randomized trial comparing picotamide (600 mg bid) with aspirin (320 mg/day) in 1,209 diabetic patients with PAD.[58] The primary efficacy endpoint was overall mortality, while the secondary endpoint was the composite of death and cardiovascular events. At 2 years, the overall mortality rates with picotamide and aspirin were 3.0% and 5.5%, respectively (relative risk [RR] 0.55, 95% confidence interval [CI] 0.31 to 0.98). Cardiovascular events occurred in 7.1% of patients given picotamide and 8.7% of those treated with aspirin. The difference in the combined endpoint of mortality plus cardiovascular events between the two groups did not reach statistical significance. Bleeding events were infrequent with both picotamide and aspirin (1.3% and 2.0%, respectively). Although these results are promising, additional studies are needed to establish the role of picotamide in diabetics with PAD.

$P2Y_{12}$ Antagonists

ADP receptors on the platelet surface include $P2Y_{12}$, $P2Y_1$, and $P2X_1$. The most important of these is $P2Y_{12}$, which is the target of the thienopyridines: ticlopidine, clopidogrel, and prasugrel. However, $P2Y_1$ also contributes to ADP-induced platelet activation, and maximal ADP-induced platelet activation requires activation of both receptors. The third receptor, $P2X_1$, serves as an adenosine triphosphate (ATP)-gated calcium channel.

Building on the proven benefit of the thienopyridines, $P2Y_{12}$ is the target for several new antiplatelet agents. In contrast to the thienopyridines, these new drugs do not require metabolic activation. Instead, they are active compounds that bind directly and reversibly to $P2Y_{12}$. With no requirement for metabolic activation, the direct $P2Y_{12}$ antagonists have a more rapid onset of action and produce a more potent and predictable level of receptor blockade that is unaffected by the genetic polymorphisms that influence thienopyridine absorption and/or metabolic activation. In addition, because their interaction with $P2Y_{12}$ is reversible, direct $P2Y_{12}$ antagonists have a more rapid offset of action than the thienopyridines, which bind $P2Y_{12}$ in an irreversible fashion. The agents in most advanced stages of development include cangrelor, ticagrelor, and elinogrel (Table 110.2).

Cangrelor

An ATP analogue, cangrelor is a direct competitive inhibitor of $P2Y_{12}$.[58] The drug is only active when administered intravenously, and it produces almost immediate and dose-proportional inhibition of ADP-induced platelet aggregation. Cangrelor is rapidly inactivated by dephosphorylation and has a half-life of 3 to 5 minutes. Upon cessation of therapy, therefore, there is recovery of platelet function within 60 minutes.[58,59]

The interaction of cangrelor with $P2Y_{12}$ prevents the binding of the active metabolites of clopidogrel or prasugrel. This phenomenon complicates the transitioning of patients from cangrelor to clopidogrel or other thienopyridines, which can only exert their inhibitory effects once cangrelor dissociates from $P2Y_{12}$.

Cangrelor has been evaluated in a two-part phase II trial in patients undergoing PCI.[60] For part one, patients were randomized to an 18- to 24-hour infusion of cangrelor or placebo in addition to aspirin plus heparin. In the second part, patients were randomized to intravenous cangrelor or abciximab prior to PCI. In both parts, the primary endpoint, the combination of major and minor bleeding, was not different between cangrelor and the control groups (placebo or abciximab). The 30-day composite of adverse cardiac events (death, MI, or unplanned repeat coronary intervention) was also not significantly different in the two groups.

Building on these phase II data, cangrelor was investigated in two double-blind phase III trials. In the CHAMPION-PCI trial, 8,877 patients scheduled for PCI were randomized to receive intravenous cangrelor (30 μg/kg bolus followed by

Table 110.2

Pharmacologic characteristics of direct-acting reversible $P2Y_{12}$ inhibitors

Characteristic	Cangrelor	Ticagrelor	Elinogrel
Molecular weight	776	523	562
Route of administration	Intravenous	Oral	Intravenous or oral
Site of action	ADP-binding site	Site distinct from ADP-binding site	ADP-binding site
Type of inhibition	Competitive	Noncompetitive	Competitive
Time to peak activity	30 min	2 h	20 min and 12 h for intravenous and oral formulation, respectively
Frequency of oral administration	Inactive orally	Twice daily	Twice daily
Half-life	3–5 min	6–12 h	Not reported
Metabolism	Dephosphorylation	*O*-deethylation and oxidation	None
Elimination	Extrarenal	30% renal; 70% feces	40% renal; 60% feces
Time to recovery of platelet function	60 min	3–5 d	Not reported
Stage of development	Phase III	Completed phase III; approved in United States, Europe and Canada	Phase II

an infusion of 4 μg/kg/min) or placebo starting 30 minutes prior to PCI and continuing for at least 2 hours after completion of the procedure.[61] All patients received aspirin. The primary efficacy endpoint, a composite of all-cause mortality, MI, or ischemia-driven revascularization within 48 hours of PCI, occurred in 7.5% of those randomized to cangrelor and 7.1% of those given placebo (hazard ratio [HR] 1.05, 95% CI 0.88 to 1.24, $P = 0.59$). Rates of major bleeding were similar with cangrelor or clopidogrel (3.6% and 2.9%, respectively; $P = 0.06$), but rates of minor bleeding were higher with cangrelor (17.6% and 15.2%, respectively; $P = 0.003$).

In the CHAMPION PLATFORM trial, 5,362 patients with non–ST-elevation MI or unstable angina with at least one coronary lesion amenable to PCI were randomized to receive cangrelor (in the same regimen used in the CHAMPION-PCI trial) or placebo at the time of PCI.[62] The primary efficacy endpoint, which was the same as that used in CHAMPION-PCI, occurred in 7.0% of those given cangrelor and in 8.0% of patients randomized to placebo (HR 0.87, 95% CI 0.71 to 1.07, $P = 0.17$). Major bleeding was more frequent with cangrelor than with placebo (5.5% and 3.5%, respectively; $P < 0.001$). Based on the negative results of these two trials, more work is needed to determine the role of cangrelor in ACS patients undergoing PCI.

Ticagrelor

An orally active agent belonging to the cyclopentyl-triazolopyrimidine class, ticagrelor acts as a direct inhibitor of $P2Y_{12}$.[63] Ticagrelor binds to the receptor at a location distinct from the ADP-binding site and blocks ADP-mediated receptor activation in a noncompetitive fashion, likely through an allosteric mechanism. Because it does not require metabolic activation, ticagrelor has a rapid onset of action and achieves a level of inhibition of ADP-induced platelet aggregation within 30 minutes that exceeds that obtained with a 300 or 600-mg loading dose of clopidogrel.[64] The peak inhibitory effect of ticagrelor is seen about 2 hours after a loading dose of ticagrelor of 180 mg or a maintenance dose of 90 mg twice daily.

When compared with clopidogrel in 200 aspirin-treated patients with atherosclerosis, ticagrelor, at doses of 100 or 200 mg twice daily, or 400 mg once daily, produced more rapid and more potent inhibition of ADP-induced platelet aggregation.[65] The DISPERSE 2 study compared ticagrelor (90 or 180 mg twice daily) with clopidogrel (75 mg once daily) in 990 patients with non–ST-segment elevation ACS.[66] All patients received aspirin. The primary endpoint, a combination of major and minor bleeding, occurred in 10.2% of patients given either dose of ticagrelor and in 9.2% of those treated with clopidogrel.

In the phase III PLATO trial, ticagrelor (180-mg loading dose followed by 90 mg twice daily) was compared with clopidogrel in 18,624 ACS patients.[67] At 12 months, the primary efficacy endpoint—a composite of cardiovascular death, MI, or stroke—occurred in 9.8% of patients treated with ticagrelor and in 11.7% of those given clopidogrel (HR 0.84, 95% CI 0.77 to 0.92, $P < 0.001$). The rates of the individual efficacy components and of bleeding complications are summarized in Table 110.3.

An invasive strategy was planned for 72% of the 18,624 patients entered in the PLATO trial. In this subset, the primary composite endpoint occurred in 9.0% of patients randomized to ticagrelor and in 10.7% of those given clopidogrel (HR 0.84, 95% CI 0.75 to 0.94, $P = 0.0025$). Rates of major bleeding were similar with ticagrelor and clopidogrel (11.6% and 16.5%, respectively; $P = 0.88$).

PLATO-STEMI focused on the 7,544 patients with ST-elevation MI who were scheduled to undergo primary PCI.[68] Of these, 75% received a stent. In this subset, definite stent thrombosis occurred in 1.6% of patients taking ticagrelor and in 2.4% of those given clopidogrel (HR 0.60, 95% CI 0.45 to 0.95, $P = 0.03$). Other endpoints are summarized in Table 110.3. Outcomes in the 1,899 patients in

Table 110.3

Summary of the results of the PLATO trial comparing ticagrelor with clopidogrel

Endpoint	Ticagrelor (180 mg Then 90 mg Twice Daily)	Clopidogrel (300–600 mg Then 75 mg Daily)	P Value
Primary endpoint (cardiovascular mortality, MI, or stroke)			
PLATO	9.8% (HR 0.84, 95% CI 0.77–0.92)	11.7%	<0.001
PLATO-STEMI	9.4% (HR 0.84, 95% CI 0.75–1.01)	10.8%	0.07
PLATO-CABG	10.6% (HR 0.84, 95% CI 0.60–1.16)	13.1%	0.29
MI			
PLATO	5.8%	6.9%	0.005
PLATO-STEMI	4.7%	5.8%	0.03
Cardiovascular mortality			
PLATO	4.0%	5.1%	0.001
PLATO-STEMI	4.1%	9.7%	<0.01
PLATO-CABG	4.1%	7.9%	<0.01
All-cause mortality			
PLATO	4.5%	5.9%	<0.001
PLATO-STEMI	5.0%	6.1%	0.05
PLATO-CABG	4.7%	9.7%	<0.01
Stroke			
PLATO	1.5%	1.3%	0.22
PLATO-STEMI			
Major bleeding			
PLATO	11.6%	11.2%	0.43
PLATO-STEMI	9.0%	9.2%	0.76
Major bleeding not associated with CABG			
PLATO	4.5%	3.8%	0.03
Fatal intracranial bleeding			
PLATO	0.1%	0.01%	0.02

Overall, PLATO enrolled 18,625 patients with acute coronary syndromes. PLATO-STEMI included those 7,544 patients with ST-elevation MI who were scheduled to undergo primary PCI. PLATO-CABG included those 1,889 patients who underwent coronary artery bypass graft surgery postrandomization.

the PLATO trial who underwent coronary artery bypass grafting (CABG) surgery postrandomization were reported in PLATO-CABG.[69] The rates of cardiovascular death and of all-cause mortality were reduced with ticagrelor (Table 110.3), but there was no difference in MI or stroke and no reduction in CABG-related bleeding.

Side effects of ticagrelor include dyspnea, which is usually mild and dose related,[70] asymptomatic bradycardia with ventricular pauses,[67] and a modest increase in the levels of uric acid. The mechanisms responsible for these side effects are unclear. One possible explanation relates to the capacity of ticagrelor to inhibit adenosine reuptake by erythrocytes, thereby increasing circulating levels of adenosine. In addition to explaining the dyspnea and the bradycardia, the resultant adenosine-induced vasodilatation and increased myocardial perfusion could also endow ticagrelor with beneficial effects that are independent of $P2Y_{12}$ blockade.

Elinogrel

A reversible $P2Y_{12}$ inhibitor available in both intravenous and oral formulations, elinogrel is a competitive inhibitor of $P2Y_{12}$ that blocks ADP binding to the receptor.[71] Consequently, the drug inhibits platelet aggregation in response to low concentrations of ADP, but its inhibitory effects can be overcome with higher ADP concentrations. This phenomenon may endow elinogrel with a more favorable risk–benefit profile if ADP concentrations are higher with hemostatic plug formation than with intravascular thrombosis.

In the phase II INNOVATE-PCI study, 652 patients scheduled for nonurgent PCI were randomized to clopidogrel (300 to 600 mg load followed by 75 mg daily) or elinogrel (80 mg intravenous bolus followed by oral dosing of 50, 100, or 150 mg twice daily).[72] The Data Safety Monitoring Board recommended discontinuation of the 50 mg oral dose and suggested increasing the intravenous dose to 120 mg. The study was not powered for

efficacy, but patients given elinogrel exhibited greater platelet inhibition than those treated with clopidogrel.

Dyspnea occurred more frequently with elinogrel than with clopidogrel. Abnormal liver function tests also were more common with elinogrel, but the abnormalities appeared to resolve over time, even if the drug was continued.[72,73] The plans for phase III evaluation of elinogrel are not available.

PAR-1 Antagonists

PAR-1 belongs to a family of G-protein–coupled receptors that are activated by proteolytic cleavage.[74] Human platelets express PAR-1 and PAR-4, both of which can be activated by thrombin to induce platelet secretion and aggregation. Although activation of either receptor can cause platelet aggregation independently of the other, PAR-1 and PAR-4 act synergistically to induce platelet activation. However, the affinity of PAR-1 for thrombin is 40-fold higher than that of PAR-4. Consequently, PAR-1 is activated by relatively low concentrations of thrombin, whereas PAR-4 activation requires higher thrombin concentrations. Therefore, PAR-1 is considered to be the major thrombin receptor on human platelets.[74]

PAR-1 also is found on endothelial cells, smooth muscle cells, fibroblasts, and cardiac myocytes.[74] Thrombin-mediated activation of PAR-1 on these cells may contribute to the proliferative and proinflammatory effects of thrombin. Therefore, it is possible that PAR-1 antagonism will not only attenuate arterial thrombosis, but may also modulate other thrombin-mediated processes, including restenosis. Two orally active PAR-1 antagonists are under investigation—vorapaxar and atopaxar (Table 110.4).

Vorapaxar

A synthetic tricyclic 3-phenylpyridine analogue of himbacine, an alkaloid isolated from the bark of Australian magnolia trees that is used in several natural products, vorapaxar is a potent and specific competitive inhibitor of PAR-1.[75] The drug has excellent oral bioavailability and produces dose-dependent inhibition of thrombin- or thrombin receptor agonist peptide (TRAP)-induced platelet aggregation. Vorapaxar does not affect platelet aggregation in response to other agonists nor does it affect thrombin-mediated conversion of fibrinogen to fibrin. Vorapaxar has a half-life of 126 to 269 hours and inhibits TRAP-induced platelet aggregation for up to 4 weeks. Although the binding of vorapaxar to PAR-1 is reversible, dissociation of the drug from the receptor is slow, which may explain the long half-life. Vorapaxar is metabolized by CYP3A4.[74]

Table 110.4

PAR-1 antagonists: comparison of the features of vorapaxar and atopaxar

Feature	Vorapaxar	Atopaxar
Molecular weight	591	608
Onset of action (h)	2	3.5
Half-life (h)	250	23
Route of elimination	Feces	Feces
Metabolism	CYP3A4	CYP3A4
Stage of development	Phase III	Phase II

In phase I studies, vorapaxar did not appear to prolong the bleeding time when administered to healthy volunteers.[76] A phase II study in 1,031 patients scheduled for coronary angiography and possible PCI randomized patients to vorapaxar (at loading doses of 10, 20, or 40 mg) or placebo in a 3:1 ratio.[77] A total of 573 patients underwent PCI, all of whom received aspirin, clopidogrel, and an anticoagulant (either heparin or bivalirudin). Those randomized to vorapaxar received maintenance therapy at doses of 0.5, 1.0, or 2.5 mg once daily for 2 months. The primary outcome, a combination of thrombolysis in myocardial infarction (TIMI) major and minor bleeding, occurred in 3.3% of the patients randomized to placebo and in 2.8% of those given vorapaxar. Although underpowered for efficacy, the composite of death, major adverse coronary events, or stroke occurred in 8.6% of patients randomized to placebo and in 6.2% of those given vorapaxar.

The safety of vorapaxar was also evaluated in two small studies in Japanese patients. In the first, 117 patients undergoing PCI were randomized to vorapaxar for 60 days (either 20 or 40-mg loading dose, followed by 1 or 2.5 mg daily thereafter) or placebo in addition to standard-of-care antithrombotic therapy.[78] No differences were observed in the rate of the primary safety endpoint (TIMI major and minor bleeding), but compared with placebo, vorapaxar reduced the rate of nonfatal MI (42.9% and 16.9%, respectively; $P = 0.013$). In the second study, which included 90 patients with a recent history of ischemic stroke,[79] vorapaxar (1 or 2.5 mg daily) or placebo was administered for 60 days. All patients received aspirin. Event rates were low, and vorapaxar appeared to be safe in this setting.

Building on these phase II results, vorapaxar underwent phase III evaluation in two large clinical trials—TRA-CER[80] and TRA2P-TIMI 50.[81] TRA-CER compared vorapaxar with placebo on top of standard-of-care treatment with aspirin and/or clopidogrel in 12,944 patients with non–ST-elevation ACS. Follow-up in the trial was terminated early because of safety concerns. After a median follow-up of 502 days, the rates of the primary efficacy outcome (a composite of cardiovascular death, MI, stroke, hospitalization for recurrent ischemia, or urgent coronary revascularization) in the vorapaxar and placebo groups were 18.5% and 19.9% (HR 0.92, 95% CI 0.85 to 1.01, $P = 0.07$). The composite of cardiovascular death, MI, or stroke was lower with vorapaxar than with placebo occurred in 14.7% and 16.4%, respectively (HR 0.89, 95% CI 0.81 to 0.98, $P = 0.02$). Rates of moderate and severe bleeding with vorapaxar and placebo were 7.2% and 5.2% (HR 1.35, 95% CI 1.16 to 1.58, $P < 0.001$). There was more intracranial bleeding with vorapaxar than with placebo (1.1% and 0.2%). Thus, addition of vorapaxar to standard therapy did not reduce the composite primary efficacy endpoint but significantly increased the risk of bleeding.

TRA2P-TIMI 50 was a randomized double-blind trial that compared vorapaxar (2.5 mg daily) with placebo on top of standard antiplatelet therapy in 26,449 patients with a history of MI, stroke, or PAD. The primary efficacy endpoint was the composite of cardiovascular death, MI, or stroke. After safety review, vorapaxar was discontinued early in patients with a stroke prior to entry or during the course of the trial because of excess bleeding. At 3 years, the primary efficacy endpoint occurred in 9.3% of patients in the vorapaxar group and in 10.5% of those in the placebo group (HR 0.87, 95% CI 0.80 to 0.94). Moderate or severe bleeding was more frequent in the vorapaxar group (4.2% and 2.5%, respectively) and

there was an increase in intracranial hemorrhage with vorapaxar compared with placebo (1.0% and 0.5%, respectively). Therefore, the role of vorapaxar as add-on therapy is uncertain.

Atopaxar

A reversible PAR-1 antagonist, atopaxar is a small molecule that binds PAR-1 with high affinity and blocks thrombin and TRAP-induced platelet aggregation.[74] Because atopaxar inhibits TRAP binding to PAR-1, it is likely that the drug interacts with PAR-1 at or near the tethered ligand-binding site. Atopaxar exhibits good oral bioavailability and is rapidly absorbed. Maximal platelet inhibition is achieved within 3 to 5 hours of dosing and the half-life is about 23 hours. Like vorapaxar, atopaxar does not appear to prolong the bleeding time when administered to healthy volunteers.[74]

In the first of the phase II J-LANCELOT trials, 241 patients with ACS were given atopaxar (loading dose of 400 mg followed by either placebo or atopaxar 50, 100, or 200 mg daily thereafter) for 12 weeks.[82] Over 95% of the patients were also taking aspirin and clopidogrel. In the second trial, 263 patients with coronary artery disease were randomized to placebo or the same doses of atopaxar for 24 weeks.[75] All patients were taking aspirin, and about 40% were also receiving clopidogrel. The primary endpoint in these studies was bleeding events. There was a trend toward more bleeding with increasing doses of atopaxar. The combination of major plus minor bleeding and minimal bleeding requiring medical attention was similar with placebo and atopaxar (6.6% and 5.0%, respectively, in the first study and 1.5% in both groups in the second study). The studies were underpowered for efficacy, but at trough levels, atopaxar produced a mean of over 90% inhibition of TRAP-induced platelet aggregation with the 100 or 200 mg doses and 20% to 60% inhibition with the 50 mg dose.[75]

The clinical development plan for atopaxar is uncertain. Increased transaminase levels were noted in up to 15% of patients receiving atopaxar.[82] It is currently unclear whether these abnormalities are drug related. In addition, prolongation of the QTc interval was seen with higher doses of atopaxar.[82] More studies are needed to determine the clinical significance of these findings.

Inhibitors of Platelet Aggregation

Because the final common pathway of platelet aggregation in response to all agonists involves conformational activation of GPIIb/IIIa, considerable attention focused on development of GPIIb/IIIa antagonists.[83,84] Three parenteral agents, abciximab, eptifibatide, and tirofiban, are of proven efficacy in patients undergoing PCI and in some patients with ACS with maximum benefit seen in those with high-risk features (e.g., elevated troponin levels or diabetes mellitus) and those managed with PCI.[85] However, the use of these parenteral agents has declined with the introduction of more potent oral antiplatelet agents.

Although oral GPIIb/IIIa inhibitors, such as xemilofiban, orbofiban, and sibrafiban, were developed, these agents were associated with an excess of major bleeding in phase III trials, and they produced no significant clinical benefit, even in high-risk patients. Furthermore, a meta-analysis of four trials found an increase in all-cause mortality with their use.[86] Potential mechanisms to explain these findings include suboptimal inhibition of platelet aggregation, partial platelet agonist activity at trough concentrations, lack of responsiveness in patients with certain genetic polymorphisms, and enhancement of cardiac myocyte apoptosis.[87] Based on these disappointing results, further development of this class of drugs has stopped.

NEW ANTICOAGULANTS

Anticoagulants can inhibit the initiation or propagation of coagulation, or by targeting thrombin, they can attenuate fibrin formation (FIGURE 110.3). Drugs that target the tissue factor/factor VIIa complex block the initiation of coagulation, while those that inhibit factor IXa or factor Xa, or their cofactors, factors VIIIa and Va, block the propagation of coagulation. New anticoagulants can be further subclassified as direct or indirect inhibitors. Direct inhibitors bind directly to the target enzyme and block substrate interactions. In contrast, indirect inhibitors exert their anticoagulant effects by binding to naturally occurring plasma cofactors, such as antithrombin or heparin cofactor II, thereby accelerating their interaction with clotting enzymes.

Inhibitors of Initiation of Coagulation

Drugs that target the factor VIIa/tissue factor complex inhibit the initiation of coagulation. Only parenteral agents in this category have reached phase II or III clinical testing. These include

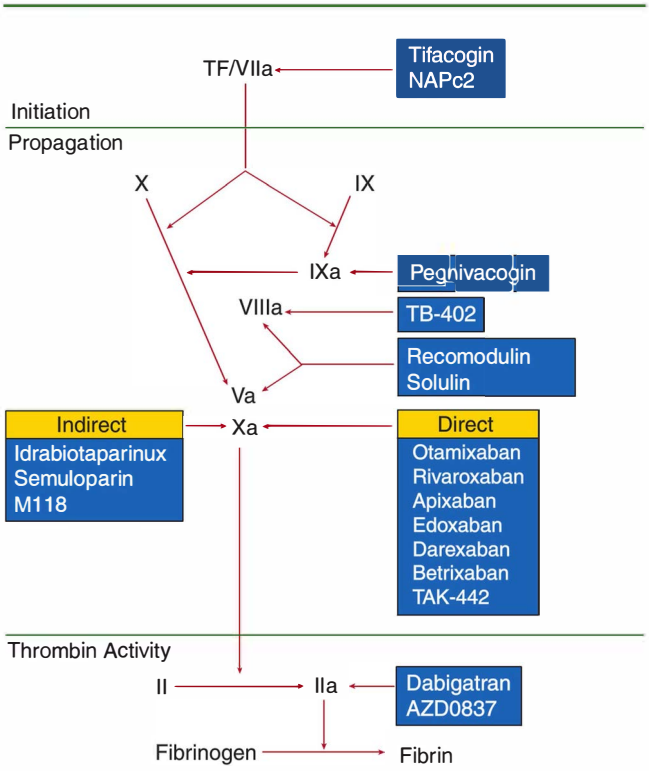

FIGURE 110.3 Targets of the new anticoagulants. Coagulation is initiated by the tissue factor/factor VIIa (TF/VIIa) complex, which activates factors IX and X (IX and X, respectively). The process is propagated by activated factors IX and X (IXa and Xa, respectively) which, together with their cofactors, activated factors VIIIa and Va (VIIIa and Va, respectively), assemble on the surface of activated platelets to form intrinsic tenase and prothrombinase, respectively. Intrinsic tenase activates factor X, whereas prothrombinase converts prothrombin (II) to thrombin (IIa). In the final step, IIa converts fibrinogen to fibrin. The targets for the new anticoagulants are indicated by the *arrows*.

tifacogin, which is a recombinant form of tissue factory pathway inhibitor, recombinant nematode anticoagulant peptide (NAPc2), and active site–inhibited factor VIIa (factor VIIai).

Tifacogin

A recombinant form of tissue factory pathway inhibitor expressed in *Saccharomyces cerevisiae*, tifacogin has been evaluated in patients with sepsis. The drug has a half-life of minutes, which necessitates intravenous infusion, and is cleared by the liver. In a phase II trial,[88] 210 sepsis patients were randomized to receive tifacogin (25 or 50 mg/kg/h) or placebo for 4 days. Tifacogin produced a 20% relative reduction in 28-day mortality. Major bleeding occurred in 9% of patients treated with tifacogin and in 6% of those given placebo, a difference that was not statistically significant. In a phase III trial that compared tifacogin with placebo in 1,754 severe sepsis patients,[89] 28-day mortality was similar with tifacogin and placebo (34.2% and 33.9%, respectively), while the rate of bleeding was significantly higher with tifacogin (6.5% and 4.8%, respectively). A *post hoc* subgroup analysis suggested a benefit of tifacogin in the 780 patients with severe community-acquired pneumonia; in this subset, 28-day mortality was 28% and 33% with tifacogin and placebo, respectively.[90] Differences in mortality were greater in patients with more severe disease who did not receive adjunctive heparin. Based on this analysis, a phase III double-blind trial compared two doses of tifacogin with placebo in about 2,100 patients with severe community-acquired pneumonia (www.clinicaltrials.gov NCT00084071). Although enrollment was completed in 2008, the results of this trial have not been reported.

NAPc2

An 85–amino acid polypeptide originally isolated from the canine hookworm, *Ancylostoma caninum*,[91] recombinant NAPc2 is expressed in yeast. NAPc2 binds to a noncatalytic site on factor X or factor Xa.[92] Once bound to factor Xa, the NAPc2/factor Xa complex inhibits tissue factor–bound factor VIIa. Because it binds factor X with high affinity, NAPc2 has a half-life of approximately 50 hours after subcutaneous injection.[93] Consequently, the drug can be given on alternate days.

Initial clinical trials with NAPc2 focused on venous thromboprophylaxis. In a phase II dose-finding study,[94] 293 patients undergoing elective knee arthroplasty were given subcutaneous NAPc2 on the day of surgery and every 2nd day thereafter to a maximum of four doses. The best results were observed with an NAPc2 dose of 3.0 μg/kg administered 1 hour after surgery. With this dose, the rate of venographically detected DVT in the operated leg was 12%, while the rate of proximal DVT was 1%. Major bleeding occurred in 2% of patients. Compared with historical controls, NAPc2 appears to have efficacy and safety similar to those of low molecular weight heparin (LMWH). However, prospective randomized trials are needed to confirm these findings.

In the next series of phase II studies, NAPc2 was evaluated in patients with ACS and in those undergoing elective PCI. Addition of NAPc2 to usual antithrombotic therapy reduced levels of prothrombin fragment 1.2 (F1.2) in a dose-dependent fashion without increasing the risk of bleeding.[95,96] Despite these promising results, the disease focus then shifted from thrombosis to cancer. A phase II study examining the feasibility of twice-weekly subcutaneous NAPc2 as an adjunct to chemotherapy in patients with metastatic colon cancer was suspended (www.clinicaltrials.gov: NCT0043573), but the results have not been reported and development of NAPc2 has stopped.

Factor VIIai

With its active site irreversibly inhibited, factor VIIai competes with factor VIIa for tissue factor binding, thereby attenuating the initiation of coagulation. Based on promising results in animal models of thrombosis,[97,98] factor VIIai, given in doses of 50 to 400 mg/kg with or without adjunctive heparin, was compared with heparin alone in 491 patients undergoing elective PCI.[99] Factor VIIai produced no significant reduction in the primary endpoint, a composite of death, MI, need for urgent revascularization, abrupt vessel closure, or bailout use of GPIIb/IIIa antagonists. Because of these disappointing results, factor VIIai has not been developed further.

Inhibitors of Propagation of Coagulation

Factor IXa Inhibitors

Both parenteral and oral factor IXa inhibitors have been developed (Table 110.5). The parenteral agents include factor IX–directed monoclonal antibodies and pegnivacogin, a factor IXa–directed aptamer. Development of TTP889, the only oral factor IXa inhibitor, has been halted.

Factor IX–directed antibodies. Several monoclonal antibodies directed against various factor IX epitopes have been developed. Of these, SB 249417, a humanized mouse monoclonal antibody directed against the Gla-domain of factor IX, has undergone the most extensive investigation. The antibody exhibited antithrombotic activity comparable to that of enoxaparin in a rat arterial thrombosis model but produced less prolongation of the activated partial thromboplastin time (aPTT).[100] In a rat model of thromboembolic stroke, the antibody reduced infarct volumes and neurologic deficits to a greater extent than tissue-type plasminogen activator.[101]

SB249417 has undergone limited evaluation in humans. In a phase I study in 26 human volunteers, a 50-minute infusion of antibody prolonged the aPTT in a dose-dependent fashion.[102] Although these early results were promising, development of the antibody was stopped.

Pegnivacogin. A specific factor IXa–directed RNA aptamer, pegnivacogin, which was previously known as RB006, was isolated from a library of 104 nucleic acid species.[103] Pegnivacogin binds to both factor IX and factor IXa with high affinity and not only inhibits factor IXa activity, but also blocks the activation of factor IX by the factor VIIa–tissue factor complex, but not by factor XIa. As such, pegnivacogin prolongs the aPTT in a dose-dependent fashion, but has no effect on the prothrombin time. A unique feature of pegnivacogin is that its anticoagulant activity can be rapidly reversed with a complementary aptamer, anivamersen (previously known as RB007). Anivamersen exerts its inhibitory effect by binding to pegnivacogin and releasing it from factor IX or factor IXa.

In a phase I study, pegnivacogin and anivamersen were evaluated in 85 healthy volunteers.[104] At doses of 30 to 60 mg, pegnivacogin produced dose-dependent prolongation of the aPTT and activated clotting time; both tests were rapidly restored to baseline levels with intravenous anivamersen.

Two early phase II studies evaluated the safety and tolerability of pegnivacogin and anivamersen in patients with coronary artery disease.[105,106] In the first, 50 patients taking aspirin

Table 110.5

Phase III studies with semuloparin for VTE prophylaxis

Study	n	Comparator (Dose and Interval)	Prophylaxis (Days)	Total VTE or All-Cause Death Semuloparin vs. Comparator OR (95% CI)	Clinically Relevant Bleeding[a] Semuloparin vs. Comparator OR (95% CI)
SAVE-KNEE	1,150	Enoxaparin (30 mg bid)	7–10	24.5% vs. 28.1%	2.6% vs. 1.8%
				0.83 (0.60–1.14)	1.50 (0.67–3.49)
SAVE-HIP1	2,326	Enoxaparin (40 mg od)	7–10	6.3% vs. 11.1%	1% vs. 2.2%
				0.54 (0.38–0.76)	0.48 (0.23–0.94)
SAVE-HIP2	1,003	Enoxaparin (40 mg od)	7–10	17.7% vs. 22%	2% vs. 0.8%
				0.77 (0.53–1.12)	2.59 (0.83–9.57)
Meta-analysis	4,479	Enoxaparin	7–10	13.4% vs. 17.6%	1.7% vs. 1.8%
				0.70 (0.58–0.85)	0.95 (0.60–1.50)
SAVE-HIP3	509	Placebo	19–23	3.9% vs. 18.6%	0.6% vs. 0%
				0.18 (0.07–0.45)	NA
SAVE-ABDO	4,414	Enoxaparin (40 mg od)	7–10	6.3% vs. 5.5%	1.2% vs. 1.2%
				1.16 (0.84–1.59)	NA
SAVE-ONCO	3,212	Placebo	Variable, at least 90	1.2% vs. 3.4%	2.8% vs. 2.0%
				0.36 (0.21–0.60)	NA

SAVE-KNEE, elective knee replacement surgery; SAVE-HIP1, total hip replacement surgery; SAVE-HIP2, hip fracture surgery; meta-analysis, meta-analysis of three studies (SAVE-KNEE, SAVE-HIP1, and SAVE-HIP-2); SAVE-HIP3, hip fracture surgery (extended prophylaxis); SAVE-ABDO, major abdominal surgery; SAVE-ONCO, medical patients undergoing chemotherapy; bid, twice daily; od, once daily; VTE, venous thromboembolism; OR, odds ratio; CI, confidence interval; NA, not available.
[a]Clinically relevant bleeding is major bleeding plus clinically relevant nonmajor bleeding.

and/or clopidogrel received an intravenous bolus of pegnivacogin at doses of 15, 30, 50, or 75 mg. Subsequent administration of anivamersen (30, 60, 100, or 150 mg) rapidly restored the aPTT to baseline levels.[105] In the second study, 24 patients undergoing nonurgent PCI were randomized in a 5:1 ratio to receive pegnivacogin or heparin with immediate or delayed reversal of pegnivacogin after the procedure.[106] All procedures were successful, and both reversal strategies enabled successful sheath removal.

In the phase II RADAR study, 640 ACS patients were randomly allocated to receive open-label pegnivacogin or heparin in a 4:1 ratio prior to cardiac catheterization. Patients given pegnivacogin then received various doses of anivamersen at the end of the procedure. Doses of anivamersen that produced at least 50% reversal were associated with rates of major plus minor bleeding similar or lower than that with heparin. The plans for phase III investigation of pegnivacogin have not yet been disclosed.

TTP889. An orally active partial inhibitor of factor IXa, TTP889 exhibited antithrombotic activity in rat and porcine arteriovenous shunt models. A small phase I study showed a predictable pharmacokinetic profile after single- or multiple-dose administration and a half-life of about 20 hours. In a phase II study, 261 patients undergoing surgery for hip fracture were given conventional anticoagulant prophylaxis for 1 week and were then randomized to receive once-daily oral TTP889 (300 mg) or placebo for 3 weeks at which point bilateral venography was performed.[107] The primary efficacy outcome was VTE, which occurred in 32.1% of patients randomized to TTP889 and in 28.2% of those given placebo ($P = 0.58$). There were no major bleeding events and only two clinically relevant nonmajor bleeds with TTP889. Because of the negative results, development of TTP889 was halted.

Factor Xa Inhibitors

New factor Xa inhibitors include agents that block factor Xa indirectly or directly (FIGURE 110.4). Indirect inhibitors are given parenterally and act by catalyzing factor Xa inhibition by antithrombin. In contrast, direct factor Xa inhibitors bind directly to the active site of factor Xa, thereby blocking its interaction with its substrates. New direct factor Xa inhibitors include otamixaban, which is given intravenously, and a number of orally active drugs. Unlike the heparin/antithrombin complex, which has limited capacity to inhibit factor Xa incorporated into the prothrombinase complex,[108,109] direct factor Xa inhibitors inhibit both free and platelet-bound factor Xa.[110,111] This property may endow these agents with an advantage over indirect factor Xa inhibitors.

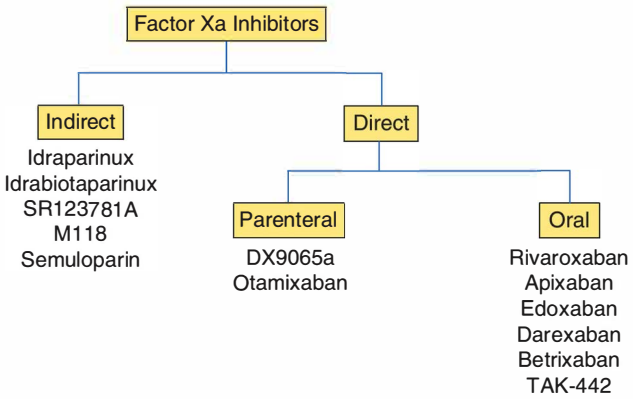

FIGURE 110.4 Classification of the new factor Xa inhibitors. The new factor Xa inhibitors exert their anticoagulant activity indirectly or directly. Indirect inhibitors bind to antithrombin and accelerate the rate at which it inhibits factor Xa. In contrast, direct factor Xa inhibitors, which include parenteral and oral agents, bind directly to factor Xa and prevent it from activating prothrombin.

Indirect Factor Xa Inhibitors

The prototype for most of the new indirect factor Xa inhibitors is fondaparinux, a first-generation synthetic analog of the antithrombin-binding pentasaccharide found in heparin or LMWH. Three of the newer indirect factor Xa inhibitors, idraparinux, idrabiotaparinux, and SR123781A, are second- and third-generation variants of fondaparinux, whereas M118 and semuloparin are LMWH derivatives.

Idraparinux. A hypermethylated derivative of fondaparinux, idraparinux binds antithrombin with such high affinity that its plasma half-life of 80 hours is similar to that of antithrombin.[112] Because of its long half-life, idraparinux can be given subcutaneously on a once-weekly basis. In a phase II dose-finding trial, idraparinux was compared with warfarin in 659 patients with proximal DVT.[113] Patients given the lowest dose of idraparinux, 2.5 mg, had less bleeding than those randomized to warfarin ($P = 0.029$), and this dose was chosen for further trials.

The phase III van Gogh DVT and PE trials[114] randomized 2,904 patients with acute symptomatic DVT and 2,215 patients with PE to either a 3- or 6-month course of once-weekly subcutaneous idraparinux or to conventional therapy with LMWH or heparin followed by a VKA with the dose adjusted to achieve an international normalized ratio (INR) of 2 to 3. In the DVT patients, the rate of recurrent VTE at 3 months was similar in the idraparinux and conventionally treated groups (2.9% and 3.0%, respectively). Clinically relevant bleeds were less common with idraparinux than with conventional treatment (4.5% and 7.0%, respectively; $P = 0.004$). In the PE patients, idraparinux was less effective than conventional therapy at 3 months, with recurrent VTE occurring in 3.4% and 1.6%, respectively. Clinically relevant bleeding events occurred in 5.8% and 8.2%, respectively. Based on the results of these trials, idraparinux appeared to have an acceptable safety profile compared with warfarin. The discordant efficacy results in the DVT and PE trials highlight the importance of adequate levels of anticoagulation for initial PE treatment because the majority of the recurrences occurred early.

The efficacy of long-term idraparinux was evaluated in the van Gogh extension study.[115] In this trial, 1,215 patients who had completed 6 months of initial treatment of DVT or PE with either idraparinux or a VKA were randomized to an additional 6 months of treatment with either once-weekly subcutaneous idraparinux or with placebo. Compared with placebo, idraparinux produced a 72.9% relative reduction in the risk of recurrent VTE ($P = 0.002$). Major bleeding occurred in 3.7% of those given idraparinux, including three fatal intracranial bleeds versus no major bleeds in the placebo group.

In the phase III AMADEUS trial, idraparinux (2.5 mg once weekly) was compared with warfarin (dose-adjusted to achieve an INR of 2 to 3) for stroke prevention in patients with atrial fibrillation.[116] Although the plan was to randomize 5,940 such patients to treatment for 18 months, the trial was stopped after randomization of only 4,576 patients and a mean follow-up of 10.7 months because of an excess of clinically relevant bleeding with idraparinux (19.7 and 11.3/100 patient years, respectively; $P < 0.0001$), including an excess of intracranial bleeding with idraparinux compared with warfarin (1.1 and 0.4/100 patient years, respectively; $P = 0.014$). There were 18 cases of thromboembolism with idraparinux and 27 cases with warfarin (0.9 and 1.3/100 patient years, respectively; HR 0.71, 95% CI 0.39 to 1.30), a result that met the prespecified noninferiority criterion (noninferiority $P = 0.007$).

The results of these studies suggested that although idraparinux was effective, it caused excessive bleeding. Consequently, the development of idraparinux was halted and attention shifted to idrabiotaparinux.

Idrabiotaparinux. A biotinylated form of idraparinux previously known as SSR126517E, idrabiotaparinux exhibits the same pharmacokinetic and pharmacodynamic profile as idraparinux. Like idraparinux, idrabiotaparinux is given subcutaneously on a once-weekly basis. The only difference between the drugs is that the anticoagulant activity of idrabiotaparinux can be neutralized by intravenous administration of avidin. A protein derived from egg white, avidin binds the biotin moiety of idrabiotaparinux with high affinity, thereby forming a 1:1 stoichiometric complex that is rapidly cleared by the kidneys.

In the phase III EQUINOX trial, 757 patients with DVT were randomized to receive equimolar doses of once-weekly subcutaneous idrabiotaparinux or idraparinux (3 and 2.5 mg, respectively) for 6 months.[117] Recurrent VTE occurred in 2.3% and 3.2% of patients given idrabiotaparinux and idraparinux, respectively, while clinically relevant bleeding occurred in 5.2% and 7.3%, respectively ($P = 0.29$). Both agents inhibited factor Xa to a similar extent. Reversibility of idrabiotaparinux was demonstrated in a study in 41 healthy males[118] and in 55 patients with DVT at the end of their planned treatment in the EQUINOX trial. Avidin was well tolerated and reduced the anti-Xa activity by 66% to 97%.

The phase III CASSIOPEA trial compared 3 or 6 months of subcutaneous idrabiotaparinux (3 mg once weekly) with conventional anticoagulant therapy for prevention of recurrent VTE in 3,200 patients with acute PE. At 3 months, the rates of recurrent VTE with idrabiotaparinux and warfarin were 2.1% and 2.7%, respectively, and there was less clinically relevant bleeding with idrabiotaparinux than with warfarin (4.5% and 6.6%, respectively; $P < 0.001$).

The phase III BOREALIS-AF trial, which compared idrabiotaparinux with warfarin for stroke prevention in patients with atrial fibrillation, was stopped early, but the results are not available. With only the EQUINOX and CASSIOPEA trial results to support its use, it is likely that the role of idrabiotaparinux will be limited.

SR123781A. A synthetic hexadecasaccharide, SR123781A is composed of the antithrombin-binding synthetic pentasaccharide plus a thrombin-binding sulfated tetrasaccharide joined together by a central nonsulfated heptasaccharide. SR123781A binds antithrombin with high affinity.[119] In addition to catalyzing factor Xa inhibition by antithrombin, SR123781A is long enough to bridge antithrombin to thrombin, thereby enhancing thrombin inhibition. Like heparin, therefore, SR123781A catalyzes the inhibition of both factor Xa and thrombin.[119] Unlike heparin, however, SR123781A does not bind platelet factor 4 (PF4) or fibrin.[120,121]

SR123781A exhibits almost complete bioavailability after subcutaneous administration and produces a dose proportional increase in anti–factor Xa activity and the aPTT. The drug is cleared by the kidneys.

Subcutaneous SR123781A, at doses of 0.25 to 4 mg once daily, was compared with enoxaparin (40 mg once daily) in 1,023 patients undergoing elective hip arthroplasty.[122] SR123781A reduced the rate of VTE dose dependently, and the rates of VTE with the 2.0 and 4.0 mg doses (7.0% and 4.4%, respectively) were similar to that with enoxaparin (8.7%). Major bleeding occurred in 0.6% and 5.8% of patients given the 2.0 and 4.0 mg doses of SR123781A, respectively, and in 0.6% of those given enoxaparin. A phase II study comparing two different doses of SR123781A with heparin plus a GPIIb/IIIa antagonist in 180 patients with ACS undergoing PCI has been completed, but the results have not been reported.

M118. Obtained by depolymerization of heparin, M118 is a novel LMWH that has a mean molecular weight of 6,500 and an anti–factor Xa to antifactor IIa ratio of 4:1.[123] The drug has a subcutaneous bioavailability of about 70% and a half-life of about 1 hour after intravenous administration and 2 to 3 hours after subcutaneous delivery. The anticoagulant activity is reversed with protamine sulfate and can be monitored using the aPTT.

In the phase II EMINENCE study, 503 patients undergoing PCI were randomized to receive either intravenous M118 or 70 U/kg of heparin.[124] The primary endpoint (a composite of death, MI, stroke, thrombocytopenia, catheter thrombus, bailout use of a GPIIb/IIIa antagonist, or any bleeding up to 20 days) occurred in 31.1% of patients given heparin and in 22.7%, 28.3%, and 30.1% of those randomized to 50, 75, or 110 U/kg of M118, respectively. The rates of adverse events were similar across all study groups.[124] These early results require further confirmation.

Semuloparin. An ultra-LMWH, semuloparin is synthesized from heparin by selective and controlled depolymerization. With a mean molecular weight of 2,000 to 3,000, most of the semuloparin molecules are too short to bridge antithrombin to thrombin. Consequently, semuloparin has high anti–factor Xa activity and minimal activity against thrombin. The drug is administered subcutaneously and exhibits 98% bioavailability. Peak plasma levels are achieved 3 hours after subcutaneous injection and the half-life is 16 to 20 hours, which enables once-daily administration. Excretion of the drug is primarily via the kidneys.

In phase III trials, semuloparin has been evaluated for postoperative thromboprophylaxis after hip or knee arthroplasty or after surgery for hip fracture and for VTE prevention after major abdominal surgery (Table 110.5). Enoxaparin was the comparator in all of these studies. The primary efficacy endpoint in these studies was the composite of VTE and all-cause mortality. Semuloparin also has been compared with placebo for VTE prevention in patients receiving chemotherapy for treatment of cancer. A trial comparing semuloparin with enoxaparin for thromboprophylaxis in hospitalized, medically ill patients was stopped early because of a change in priorities (www.clinicaltrials.gov: NCT00714597).

A meta-analysis of the results of the SAVE-HIP1, SAVE-HIP2 and SAVE-KNEE trials demonstrated an almost one-third reduction in VTE and all-cause mortality with semuloparin compared with enoxaparin (OR 0.70, 95% CI 0.58 to 0.85, $P = 0.0003$); rates of major or clinically relevant nonmajor bleeding were 1.7% and 1.8%, respectively (OR 0.95, 95% CI 0.60 to 1.50).[125] In the SAVE-HIP3 trial, 509 patients undergoing surgery for hip fracture received semuloparin for 7 to 10 days and 469 patients were then randomized in a 2:1 fashion to either continued semuloparin or to placebo for an additional 19 to 23 days.[126] In 332 patients with evaluable venograms, the primary efficacy endpoint occurred in 3.9% of those randomized to extended semuloparin and in 18.6% of those given placebo (OR 0.18, 95% CI 0.07 to 0.45, $P < 0.001$). The rates of major bleeding and clinically relevant nonmajor bleeding with semuloparin in this trial were both 0.3%; there were no bleeding events with placebo.

In the SAVE-ABDO trial, semuloparin (20 mg once daily started 8 hours after surgery) was compared with enoxaparin (40 mg once daily started 12 to 24 hours after surgery) in 4,413 patients undergoing major abdominal surgery.[127] Both treatments were administered for 7 to 10 days. Though it was originally conceived as a superiority trial, there was revision to a noninferiority design after an interim analysis. In the 3,030 patients with evaluable venograms, the primary efficacy endpoint occurred in 6.3% of those randomized to semuloparin and in 5.5% of those given enoxaparin (OR 1.16, 95% CI 0.84 to 1.59), a difference that failed to meet the prespecified noninferiority margin of 1.25. The rates of major VTE (proximal DVT or PE) or all-cause mortality were 2.2% and 2.3%, respectively. Major bleeding occurred in 2.9% and 4.5%, respectively, while the rates of clinically relevant nonmajor bleeding were 1.2% in both groups.

The SAVE-ONCO trial compared semuloparin (20 mg once daily) with placebo in over 3,000 patients with cancer who were receiving chemotherapy (www.clinicaltrials.gov: NCT00694382). Semuloparin reduced the risk of symptomatic VTE or VTE-related death by 64% (from 3.4% to 1.2%, respectively) without producing a significant increase in bleeding (1.1% and 1.2%, respectively).

Overall, the results with semuloparin have been positive, but the need for a new injectable anticoagulant in the orthopedic setting is limited given the availability of the new oral anticoagulants. Although the SAVE-ONCO trial findings are interesting, the disappointing results of the SAVE-ABDO trial suggest that the 20 mg once-daily dose of semuloparin is not appropriate for all thromboprophylactic indications.

Direct Factor Xa Inhibitors

Direct factor Xa inhibitors include parenteral agents, such as DX-9065a and otamixaban, as well as several orally active drugs (FIGURE 110.4). All of the direct factor Xa inhibitors are small molecules that reversibly block the active site of factor

Xa. The large number of oral factor Xa inhibitors highlights the continued focus on development of oral anticoagulants that can replace VKA, such as warfarin.

DX-9065a. A synthetic nonpeptidic direct factor Xa inhibitor, DX-9065a is administered parentally, has a dose-dependent half-life of 40 minutes to 5 hours, and is cleared by the kidneys.[128–130] DX-9065a was evaluated in patients with non–ST-elevation ACS and in patients undergoing PCI. In the ACS trial, 402 patients were randomized to weight-adjusted heparin or to low- or high-dose DX-9065a.[131] The primary efficacy endpoint, a composite of death, MI, urgent revascularization, or ischemia, occurred in 33.6%, 34.3%, and 31.3% of patients, respectively. Major bleeding occurred in 3.3% of those randomized to heparin and in <1% of those who received DX-9065a. In the PCI trial, 175 patients were randomized to open-label DX-9065a or to heparin in one of four sequential phases.[132] Although thrombotic events were rare in all phases of the study, enrolment in the phase evaluating the lowest dose of DX-9065 was stopped because of catheter thrombosis. Major bleeding events were uncommon, and there was no apparent dose response. Although promising, DX-9065a has not undergone further clinical evaluation.

Otamixaban. A parenteral direct factor Xa inhibitor, otamixaban has a rapid onset of action, produces a predictable anticoagulant effect, has a short half-life, and <25% of the drug is cleared by the kidneys.[133,134] These features render otamixaban as a potential candidate to replace heparin in patients with ACS and those undergoing PCI. This possibility was evaluated in two phase II dose-finding trials.

The SEPIA-PCI trial compared five different doses of otamixaban with heparin in 947 patients undergoing nonurgent PCI.[135] The primary outcomes were change in blood levels of F1.2 and anti–factor Xa activity. The highest dose of otamixaban produced greater suppression of F1.2 than heparin. Otamixaban increased bleeding in a dose-dependent fashion, and there was more bleeding with the two highest otamixaban doses than with heparin. Rates of ischemic events were similar across treatment groups.

The SEPIA-ACS-1-TIMI 42 trial compared five different doses of otamixaban with the combination of heparin plus eptifibatide for the prevention of major cardiovascular events in 3,241 patients with non–ST-elevation ACS.[136] All patients received aspirin and clopidogrel and 62.7% underwent PCI. Although the rates of the primary efficacy outcome, a composite of death, MI, urgent revascularization, or bailout GPIIb/IIIa antagonist use at 7 days, were not significantly different across the five doses of otamixaban or compared with heparin plus eptifibatide, rates were numerically lowest with the two intermediate doses of otamixaban. The rates of thrombotic complications were numerically higher with all doses of otamixaban than with heparin plus eptifibatide. Otamixaban was associated with a significant dose-dependent increase in the primary safety outcome, TIMI major or minor bleeding, but bleeding rates with intermediate doses of otamixaban were similar to those with heparin plus eptifibatide. On the basis of these results, a phase III trial of the same design has been initiated (www.clinicaltrials.gov: NCT01076764).

Apixaban. An oral direct factor Xa inhibitor, apixaban is an active drug with an oral bioavailability of about 50%. Apixaban inhibits both free and clot-associated factor Xa activity.[137] In healthy males, levels of apixaban in plasma peak about 3 hours after oral administration, and the drug is cleared with a terminal plasma half-life of 8 to 14 hours.[138] Apixaban is eliminated via multiple pathways, including oxidative metabolism, renal, and intestinal routes. Potent inhibitors of CYP3A4, such as ketoconazole or ritonavir, are contraindicated because they increase plasma drug concentrations.

Prevention of VTE. Apixaban has been evaluated in three phase III randomized controlled trials—two in patients undergoing knee arthroplasty (n = 6,252) and one in patients undergoing hip arthroplasty (n = 5,407). All three trials used the same apixaban dose regimen of 2.5 mg twice daily starting 12 to 24 hours after surgery, which was administered for 10 to 14 days in patients undergoing knee arthroplasty and for 35 days in those undergoing hip arthroplasty. The primary efficacy endpoint in all of the trials was total VTE and all-cause mortality. In the ADVANCE-1 trial, apixaban was similarly effective to enoxaparin 30 mg twice daily (9.0% and 8.8%, respectively; RR 1.02, 95% CI 0.78 to 1.32) in patients undergoing knee arthroplasty, although the prespecified statistical criteria for noninferiority were not met.[139] In the ADVANCE-2 trial, apixaban was superior to enoxaparin 40 mg once daily (15.1% and 24.4%, respectively; RR 0.62, 95% CI 0.51 to 0.78, $P < 0.0001$).[140] Patients undergoing hip arthroplasty were randomized to apixaban or enoxaparin (40 mg once daily) in the ADVANCE-3 trial,[141] where apixaban was superior to enoxaparin (1.4% and 3.9%, respectively; RR 0.36, 95% CI 0.22 to 0.54, $P < 0.001$). Rates of major or clinically relevant nonmajor bleeding were numerically lower with apixaban than with enoxaparin in all three trials, but the differences only reached statistical significance in the ADVANCE-1 trial.

Apixaban was evaluated for thromboprophylaxis in 6,528 medically ill patients in the ADOPT trial,[142] which compared a 30-day regimen of apixaban (2.5 mg twice daily) with enoxaparin (40 mg once daily, given for 6 to 14 days). At 30 days, the primary efficacy outcome (a composite of symptomatic VTE, asymptomatic proximal DVT detected by routine ultrasonography, or VTE-related mortality) occurred in 2.71% of patients given apixaban and in 3.06% of those treated with enoxaparin (RR 0.87, 95% CI 0.62 to 1.23). By day 30, rates of major bleeding in the apixaban and enoxaparin groups were 0.47% and 0.19%, respectively (RR 2.58, 95% CI 1.02 to 7.24, P = 0.04). Therefore, extended thromboprophylaxis with apixaban was not superior to a shorter course with enoxaparin, and there was more bleeding with apixaban.

Treatment of VTE. The phase III multicenter, double-blind, randomized AMPLIFY trial is comparing apixaban (10 mg twice daily for 1 week followed by 5 mg twice daily thereafter) with conventional anticoagulant therapy (heparin or LMWH followed by dose-adjusted warfarin) for treatment of patients with acute VTE. The phase III multicenter, double-blind, randomized AMPLIFY-EXT trial is comparing apixaban (at doses of either 2.5 or 5 mg twice daily) with placebo for prevention of recurrent VTE in patients who have completed a minimum of a 6-month course of anticoagulation therapy for a first episode of VTE (www.clinicaltrials.gov: NCT00633893 and NCT00643201, respectively).

Stroke prevention in atrial fibrillation. The AVERROES trial, which compared apixaban with aspirin, is discussed in Chapter 92. The multicenter, double-blind, randomized phase III ARISTOTLE trial[143] compared apixaban (5 mg twice daily) with warfarin (dose adjusted to achieve an INR of 2.0 to 3.0) in 18,201 atrial fibrillation patients with at least one additional risk factor for stroke. Compared with warfarin, treatment with apixaban reduced stroke or systemic embolism from 1.60% to

1.27% (RR 0.79, 95% CI 0.66 to 0.95). Rates of major bleeding were lower with apixaban than with warfarin (2.13% and 3.09%, respectively; RR 0.69, 95% CI 0.60 to 0.80).[143]

Acute coronary syndromes. In the phase II APPRAISE study, varying doses of apixaban (2.5 mg twice daily, 10 mg once daily, 10 mg twice daily, or 20 mg once daily) were compared with placebo in 1,715 patients with recent ST-elevation or non–ST-elevation ACS.[144] Nearly all patients received aspirin and 76% also received clopidogrel. The primary outcome was bleeding. Recruitment into the two higher dose arms of apixaban was stopped early because of excess bleeding. Compared with placebo, apixaban (2.5 mg twice daily or 10 mg once daily) increased bleeding in a dose-dependent fashion. There was a trend for fewer ischemic events in patients given apixaban, a phenomenon that was attenuated in those taking aspirin plus clopidogrel compared with those receiving aspirin alone.

The multicenter, double-blind, randomized, phase III APPRAISE-2 trial compared apixaban (5 mg twice daily) with placebo as adjuncts to antiplatelet therapy with aspirin with or without a thienopyridine. The trial was stopped early after recruitment of 7,392 patients because of excess bleeding (including intracranial bleeding) with apixaban and no compensatory reduction in ischemic events. Thus, the primary efficacy outcome, a composite of cardiovascular death, MI, or ischemic stroke, occurred in 7.5% of patients given apixaban and in 7.9% of those assigned to placebo, whereas major bleeding occurred in 1.3% and 0.5%, respectively (HR 2.59, 95% CI 1.50 to 4.46, $P = 0.001$).[145]

Rivaroxaban. Rivaroxaban is an active compound with 80% oral bioavailability. Plasma levels of rivaroxaban peak 2 to 3 hours after administration and the terminal half-life is 7 to 11 hours.[146,147] One-third of the administered drug is cleared as unchanged active drug by the kidneys, one-third is metabolized by the liver via CYP3A4-dependent and independent pathways and then excreted in feces, and one-third is metabolized to inactive metabolites, which are then excreted by the kidneys. The pharmacokinetic and pharmacodynamic profile of rivaroxaban is predictable and dose dependent and is not influenced by age, gender, or body weight. Potent inhibitors of both CYP3A4 and P-glycoprotein (P-gp), such as ketoconazole or ritonavir, are contraindicated because they increase plasma drug concentrations.

Prevention of VTE. Rivaroxaban has been approved in the European Union, USA, Canada, and several other countries for the prevention of VTE in adult patients undergoing hip or knee arthroplasty, and the results of the studies are reviewed in Chapter 86.

The MAGELLAN trial compared extended prophylaxis with oral rivaroxaban (10 mg once daily for 35 days) with short-term prophylaxis with subcutaneous enoxaparin (40 mg once daily for 10 days) in 8,101 hospitalized, medically ill patients.[148] The primary efficacy outcome, VTE and VTE-related mortality at 35 days, occurred in 4.4% of patients given rivaroxaban and in 5.7% of those treated with enoxaparin (HR 0.77, 95% CI 0.62 to 0.96). However, there was more major plus clinically relevant bleeding with rivaroxaban than with enoxaparin (5.3% and 2.1%, respectively).

Treatment of VTE. In the phase III, open-label EINSTEIN-DVT trial, 3,449 patients with acute, symptomatic DVT were randomized to treatment with rivaroxaban (15 mg twice daily for 21 days, followed by 20 mg once daily) or conventional therapy (enoxaparin followed by a VKA) for 3, 6, or 12 months based on the treating physician's assessment at baseline.[149] Rivaroxaban was noninferior to conventional treatment for the prevention of recurrent VTE, which occurred in 2.1% and 3.0% of patients, respectively (HR 0.68, 95% CI 0.44 to 1.04). Major or clinically relevant nonmajor bleeding, the primary safety endpoint, occurred in 8.1% of patients in both groups, while major bleeding occurred in 0.8% and 1.0% of patients given rivaroxaban and conventional treatment, respectively. The rates of all-cause mortality were similar in both treatment groups (2.2% and 2.9%, respectively). The EINSTEIN PE trial, which compared the same rivaroxaban regimen with conventional anticoagulant treatment in 4832 patients with PE, showed similar rates of recurrent venous thromboembolism (2.1% and 1.8%, respectively; HR 1.12, 95% CI 0.75 to 1.07). Rates of major or clinically relevant nonmajor bleeding were 10.3% and 11.4% in the rivaroxaban and standard therapy groups, but there was significantly less major bleeding with rivaroxaban (1.1% and 2.2%, respectively).

The EINSTEIN EXT trial randomized 1,196 patients who had completed a 6- or 12-month course of anticoagulant therapy with rivaroxaban or a VKA for a first episode of VTE to either rivaroxaban (20 mg once daily) or placebo for an additional 6 or 12 months.[149] Compared with placebo, rivaroxaban reduced the risk of recurrent VTE from 7.1% to 1.3% (HR 0.18, 95% CI 0.09 to 0.39, $P < 0.001$). Major or clinically relevant nonmajor bleeding occurred in 5.4% and 1.4%, respectively ($P < 0.001$).

Stroke prevention in atrial fibrillation. As reviewed in Chapter 92, the ROCKET-AF trial compared rivaroxaban with dose-adjusted warfarin. Rates of stroke or systemic embolism with rivaroxaban and warfarin were 2.1% and 2.4%, respectively, and rates of major bleeding were 3.6% and 3.5%, respectively.[150] Rivaroxaban has now been licensed in the United States, Europe and Canada for this indication.

Acute coronary syndromes. In a phase II study, varying doses and regimens of rivaroxaban (5 to 20 mg once daily, or the same total daily doses given twice daily) were compared with placebo in 3,491 patients who were stabilized after ACS.[151] The primary outcome, clinically significant bleeding, increased in a dose-dependent fashion with rivaroxaban compared with placebo. Rates of the primary efficacy endpoint, a composite of death, MI, stroke, or severe recurrent ischemia requiring revascularization with rivaroxaban and placebo were 5.6% and 7.0%, respectively. The rate of the secondary efficacy endpoint, a composite of death, MI, or stroke, was reduced with rivaroxaban compared with placebo (3.9% and 5.5%, respectively; HR 0.69, 95% CI 0.50 to 0.96, $P = 0.027$).

The phase III, ATLAS ACS TIMI 51 trial compared rivaroxaban (at doses of 2.5 or 5 mg twice daily) with placebo as adjuncts to aspirin with or without a thienopyridine for prevention of recurrent ischemic events in 15,526 patients with a recent ACS.[152] Rivaroxaban significantly reduced the primary efficacy outcome (a composite of cardiovascular death, MI, or stroke) compared with placebo, with respective rates of 8.9% and 10.7% (HR 0.84, 95% CI 0.74 to 0.96, $P = 0.008$). Both the 2.5 and the 5 mg doses of rivaroxaban significantly reduced the primary efficacy endpoint compared with placebo (9.1%, 8.8%, and 10.7%, respectively) and the 2.5 mg dose reduced the rates of death from cardiovascular causes (2.7% and 4.1%, $P = 0.002$) and from any cause (2.9% and 4.5%, $P = 0.002$). Compared with placebo, rivaroxaban increased the rates of major bleeding (2.1% and 0.6%, $P < 0.001$) and intracranial hemorrhage (0.3% and 0.2%, $P = 0.009$). There were fewer fatal bleeds with the 2.5 mg dose of rivaroxaban than with the 5 mg dose (0.1% and 0.4%, $P = 0.04$). Therefore, the 2.5 mg twice-daily dose regimen of rivaroxaban reduces mortality in patients with ACS, but increases the risk of major and intracranial bleeding.

Edoxaban. Formerly known as DU-176b, edoxaban is an active drug with an oral bioavailability of at least 50%. Edoxaban is rapidly absorbed from the gastrointestinal tract such that plasma concentrations peak 1 to 2 hours after dosing. Elimination follows a biphasic pattern and the terminal elimination half-life is approximately 8 to 10 hours.[153] There is a dual mechanism of elimination; approximately 35% of the total administered oral dose is excreted via the kidneys, while the remainder is eliminated in the feces.[153]

Prevention of VTE. Oral edoxaban (30 mg once daily) was compared with subcutaneous enoxaparin (20 mg twice daily) in two double-blind phase III trials conducted in Japan. Overall, edoxaban was more effective for VTE prevention and was not associated with more bleeding. On the basis of these studies, edoxaban has been licensed in Japan for this indication.

Treatment of VTE. The phase III double-blind HOKUSAI trial is comparing edoxaban 60 mg once daily (reduced to 30 mg once daily in selected patients) with warfarin (dose adjusted to achieve an INR of 2 to 3). Patients are randomized to edoxaban or warfarin after they have completed an initial course of therapy with heparin or LMWH for at least 5 days. Study drug is administered for 3, 6, or 12 months depending on whether the VTE is provoked or unprovoked (www.clinicaltrials.gov: NCT00986154).

Stroke prevention in atrial fibrillation. In a phase II trial, edoxaban (at doses of 30 or 60 once daily or 30 or 60 mg twice daily) was compared with warfarin in 1,146 patients with atrial fibrillation. The rates of the primary endpoint, the composite of major plus clinically relevant nonmajor bleeding, were significantly higher in the groups randomized to 30 or 60 mg of edoxaban twice daily than the rate in patients given warfarin (7.8%, 10.6%, and 3.2%, respectively). In contrast, the rates with 30 or 60 mg of edoxaban once daily were similar to that with warfarin (3.0% and 3.8%, respectively).[154] Based on these data, the phase III double-blind ENGAGE-AF-TIMI 48 trial is comparing two doses of edoxaban (30 or 60 mg once daily with dose adjustments for renal impairment, low body weight or use of potent P-gp inhibitors) with warfarin (INR of 2 to 3) for stroke prevention in 21,500 patients with atrial fibrillation.[155] Enrollment into the trial has been completed, and patients are now being followed in this event-driven trial.

Darexaban. Formerly known as YM150, darexaban has high oral bioavailability and drug levels peak about 2 hours after oral drug administration. The half-life of darexaban is 14 to 18 hours; consequently, the drug is given once daily. Darexaban has a dual mechanism of elimination; one-half is eliminated via the kidneys and the remainder in the feces.

Prevention of VTE. Darexaban was first evaluated in 174 patients undergoing elective hip arthroplasty.[156] At once-daily doses of 3, 10, 30, or 60 mg, darexaban produced a statistically significant dose response for efficacy. No major bleeds were reported, and there was no dose–response trend for clinically relevant nonmajor bleeding. In a second phase II dose-finding study, 1,017 patients undergoing elective hip arthroplasty were randomized to once-daily oral darexaban (at doses of 5, 10, 30, 60, or 120 mg) or to subcutaneous enoxaparin (40 mg once daily starting 12 hours prior to surgery) for 5 weeks. The primary efficacy endpoint, a composite of VTE or all-cause mortality, occurred in 18.9% of patients given enoxaparin. Darexaban reduced the rate of VTE in a dose-dependent fashion and the rates in the 30, 60, and 120 mg dose groups were 19.3%, 13.3%, and 14.5%, respectively. There was one major bleed with the 60 mg dose of darexaban and one with enoxaparin.

Stroke prevention in atrial fibrillation. In a phase II dose-finding study, once-daily darexaban was compared with warfarin in 448 patients with atrial fibrillation. The warfarin dose was adjusted to achieve an INR of 2.0 to 3.0 for those up to an age 69 years and an INR of 1.6 to 2.6 for those aged 70 years or older. Recruitment into the 240 mg darexaban dose arm was stopped early because of excess bleeding. Rates of major or clinically relevant nonmajor bleeding with the 30, 60, and 120 mg doses of darexaban were 2.2%, 2.2%, and 3.2%, respectively, while the rate was 2.1% with warfarin.

Acute coronary syndrome. In the phase II RUBY-1 study, 1,279 patients with recent ACS were randomized to receive one of six dosage regimens of darexaban or placebo in addition to antiplatelet therapy.[157] The primary outcome of the study, major or clinically relevant nonmajor bleeding, was higher with all doses of darexaban than with placebo (pooled HR 2.27, 95% CI 1.13 to 4.60). There was no apparent decrease in the rate of recurrent ischemic events with any of the regimens. Because of these results and the lack of a clear advantage of darexaban over other oral factor Xa inhibitors, development of darexaban has been halted.

Betrixaban. With oral bioavailability of 47% and a half-life of 19 hours, betrixaban is reported to exhibit minimal renal excretion. Betrixaban had antithrombotic activity in animal models and was well tolerated in humans in a phase I trial that included 64 subjects.[158]

Prevention of VTE. In the phase II EXPERT trial, oral betrixaban, at doses of 15 or 40 mg twice daily, was compared with subcutaneous enoxaparin (30 mg twice daily) for thromboprophylaxis in 215 patients undergoing elective knee arthroplasty.[159] Randomization was done in a 2:2:1 fashion and treatment was given for 10 to 14 days. VTE occurred in 20% and 15% of patients given 15 or 40 mg of betrixaban, respectively, and in 10% of those given enoxaparin. There were no major bleeds in the 171 patients given betrixaban versus, one in the 43 patients given enoxaparin.

Stroke prevention in atrial fibrillation. In the phase II, EXPLORE-Xa study, oral betrixaban was compared with warfarin (INR of 2 to 3) in 506 patients with atrial fibrillation. Major or clinically relevant nonmajor bleeding occurred in 1, 5, and 4 patients in the groups of patients given 40, 60, or 80 mg of betrixaban, respectively, and in 4 of those given warfarin. Betrixaban is now undergoing phase III evaluation for extended thromboprophylaxis in medically ill patients.

TAK-442. With good oral bioavailability, TAK-442 produced a predictable and dose-proportional level of anticoagulation in healthy volunteers. Plasma concentrations peaked 1 to 2 hours after oral administration and the elimination half-life was 9 to 13 hours.[158] In a phase II dose-finding study, oral TAK-442 (in doses of 40 or 80 mg once daily or 10, 20, 40, or 80 mg twice daily) was compared with subcutaneous enoxaparin (30 mg twice daily) in 1,038 patients undergoing knee arthroplasty.[160] Both treatments were given for 10 to 14 days. The primary efficacy endpoint was composite of total VTE and all-cause mortality, while the primary safety endpoint was major bleeding. Recruitment into the TAK-442 10 and 20 mg twice-daily arms of the study was stopped early because the rates of the primary efficacy endpoint were higher than that with enoxaparin (39.0%, 38.4%, and 22.0%, respectively). The primary efficacy endpoint occurred in 23.5%, 21.4%, 26.8%, and 14.3% of those receiving TAK-442 40 mg once daily, 40 mg twice daily, 80 mg once daily, or 80 mg twice daily, respectively. Rates of major

bleeding were low and the rates of major plus clinically relevant nonmajor bleeding were similar across all treatment groups.[159]

A phase II dose-finding study compared varying doses and regimens of TAK-442 with placebo in ACS patients, most of whom were also taking aspirin and clopidogrel (www.clinicaltrials.gov: NCT00677053). Although the study has been completed, the results have not been reported.

LY-517717. With oral bioavailability of 25% to 82%, LY-517717 has a half-life of about 25 hours and is given once daily.[158] LY-517717 was evaluated in a phase II noninferiority study that randomized 511 patients undergoing hip or knee arthroplasty to one of six doses of LY-517717 (25, 50, 75, 100, 125, or 150 mg started 6 to 8 hours after wound closure) or to once-daily subcutaneous enoxaparin (40 mg) started the evening before surgery.[161] Both treatments were administered for a total of 6 to 10 doses. Randomization to the three lower doses of LY-517717 was stopped early due to lack of efficacy. The three higher doses of LY-517717 had efficacy similar to that of enoxaparin (17.1% to 24.0% and 22.2%, respectively). Adjudicated major bleeding events were uncommon in all study arms. Although promising, further development of LY-517717 has been halted.

Inhibitors of Factors Va and/or VIIIa

Factor Va and, to a lesser extent, factor VIIIa are the targets of activated protein C (APC). APC acts as an anticoagulant by proteolytically degrading and inactivating factors Va and VIIIa, key cofactors in thrombin generation (FIGURE 110.5). Factors Va and VIIIa are directly inhibited by drotrecogin alfa (activated), a recombinant form of APC. Recomodulin, previously known as ART-123, is a recombinant analogue of the extracellular domain of thrombomodulin that binds thrombin and enhances its capacity to activate protein C. TB-402 is a factor VIIIa–directed monoclonal antibody.

Drotrecogin alfa (activated). A recombinant form of APC, drotrecogin is licensed for treatment of patients with severe sepsis. Approval for this indication was based on a trial comparing drotrecogin with placebo in 1,690 patients with severe sepsis.[162] The phase III trial and postapproval trials are discussed in Chapter 123.

Recomodulin. A recombinant analogue of the extracellular domain of thrombomodulin,[163] recomodulin binds thrombin and converts it from a procoagulant enzyme into a potent activator of protein C. Recomodulin has nearly 100% bioavailability after subcutaneous administration and a half-life of 2 to 3 days. In a phase IIa dose-ranging study in patients undergoing elective hip arthroplasty, the primary endpoint (a composite of venographically detected DVT and symptomatic PE) occurred in 4.3% of the 94 patients given lower dose recomodulin and in none of the 99 patients receiving the higher dose.[164] Major bleeding occurred in 1.6% and 5.7% of patients receiving low- or high-dose recomodulin, respectively. In a double-blind study, recomodulin was compared with placebo in 750 patients with sepsis associated with disseminated intravascular coagulation. Although the study has been completed, the results are not available.

TB-402. A human IgG_4 monoclonal antibody that partially inhibits factor VIIIa, TB-402 was generated by introducing a point mutation into the gene encoding MAB-LE2E9, a factor VIII–directed monoclonal antibody.[165,166] TB-402 does not affect the interaction of factor VIII with vWF, but it inhibits

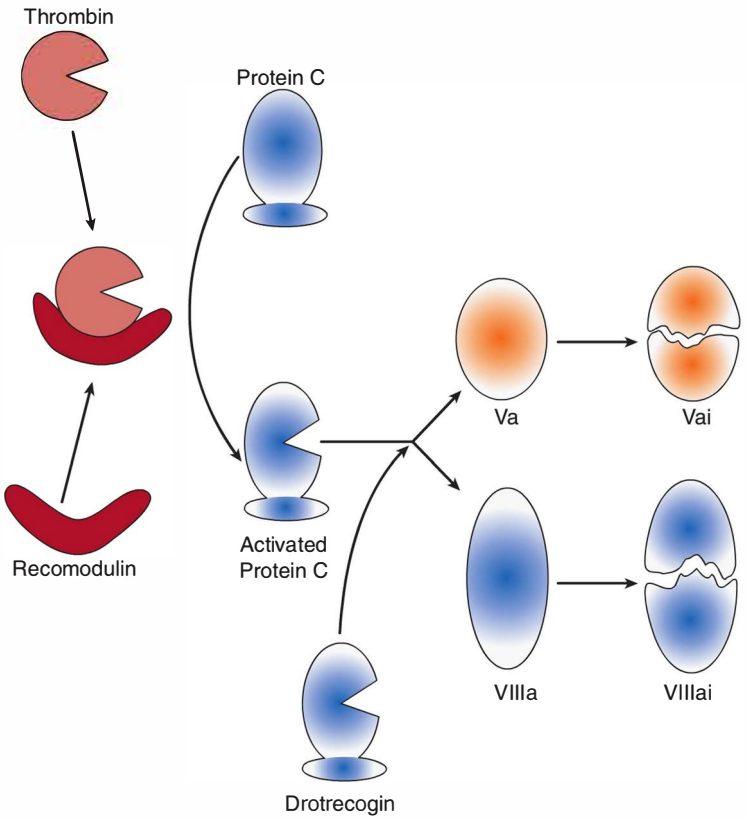

FIGURE 110.5 Inhibitors of factors Va and VIIIa. Recomodulin and solulin, which are analogues of the extracellular domain of thrombomodulin, bind thrombin and alter its substrate specificity such that it becomes a potent activator of protein C. Activated protein C (APC) acts as an anticoagulant by degrading factors Va and VIIIa, key cofactors in coagulation, into inactive fragments (Vai and VIIIai, respectively). Drotrecogin, a recombinant analogue of APC, bypasses the need for activation of endogenous protein C and directly inactivates factors Va and VIIIa.

factor VIII activity by about 40%.[167] The antibody has a half-life of about 3 weeks, which may enable prolonged VTE prophylaxis with a single dose.

In a phase I study in healthy volunteers, increasing doses of TB-402 decreased factor VIII activity to plateau levels that were one-third to two-thirds lower than baseline.[167] The aPTT was correspondingly prolonged and remained so for at least 4 weeks, consistent with the long half-life. In a phase II study, a single dose of intravenous TB-402 was compared with enoxaparin in 316 patients undergoing knee arthroplasty.[168] All patients received a 40 mg subcutaneous dose of enoxaparin 12 hours prior to surgery; they were then randomized to receive either TB-402 or enoxaparin 18 to 24 hours after the procedure. The primary outcome, major or clinically relevant nonmajor bleeding, occurred in 3.8% of patients given enoxaparin and in 4.0%, 5.4%, and 8.0% of those given TB-402, 0.3, 0.6, and 1.2 mg/kg, respectively. The primary efficacy outcome, total VTE, occurred in 39.0% of the patients given enoxaparin and in 16.7%, 23.9%, and 24.1% of those receiving TB-402 at doses of 0.3, 0.6, and 1.2 mg/kg, respectively. Thus, there was no dose–response trend for efficacy with TB-402, but there was a dose-dependent increase in bleeding. At a dose of 0.3 mg/kg, TB-402 appeared to have efficacy and safety similar to that of enoxaparin. Additional studies are needed to confirm these findings.

Inhibitors of Thrombin

Thrombin is a logical target for new anticoagulants because in addition to converting fibrinogen to fibrin, thrombin also amplifies its own generation by activating factors V, VIII, and XI and serves as a potent platelet agonist. Thrombin can be inhibited indirectly or directly. Indirect inhibitors that are specific for thrombin act by catalyzing heparin cofactor II. In contrast, direct thrombin inhibitors bind to the enzyme and block its interaction with substrates.

Direct thrombin inhibitors have properties that give them potential mechanistic advantages over indirect inhibitors.[169,170] First, because direct thrombin inhibitors do not bind to plasma proteins, they produce a more predictable anticoagulant response. Second, unlike heparin, direct thrombin inhibitors do not bind to PF4. Consequently, the anticoagulant activity of direct thrombin inhibitors is unaffected by the large quantities of PF4 released in the vicinity of platelet-rich thrombi. Finally, direct thrombin inhibitors inactivate fibrin-bound thrombin, as well as fluid-phase thrombin.

Flovagatran and pegmusirudin are parenteral direct thrombin inhibitors that have undergone phase II evaluation; since neither agent has advanced to phase III, they will not be discussed further. There are three new oral thrombin inhibitors; odiparcil, an indirect inhibitor whose development has been halted, and dabigatran etexilate and AZD0837, which are oral direct thrombin inhibitors.

Dabigatran etexilate. A prodrug of dabigatran, dabigatran etexilate has an oral bioavailability of about 6%.[170] After oral administration, dabigatran etexilate is rapidly and completely converted by esterases to dabigatran. Plasma levels of dabigatran peak 2 hours after drug administration. Dabigatran has a half-life of 14 to 17 hours, which permits once-daily or twice-daily administration. The elimination of dabigatran occurs predominantly via the kidneys, with approximately 80% of the drug excreted unchanged in the urine. Dabigatran exhibits predictable pharmacokinetics and pharmacodynamics with little effect of food. Drug–drug interactions are limited; potent P-gp inhibitors, such as ketoconazole, are contraindicated.[170]

Prevention of VTE. As discussed in Chapter 86, dabigatran has been evaluated for VTE prevention in four phase III randomized controlled trials in patients undergoing hip arthroplasty (n = 5,549) or knee arthroplasty (n = 4,691). Based on the results of these trials, dabigatran has been licensed in many countries, but not the United States, for short-term thromboprophylaxis after elective hip or knee arthroplasty.

Treatment of VTE. In the phase III, double-blind RE-COVER trial, 2,539 patients with acute VTE were randomized to treatment with dabigatran etexilate (150 mg twice daily) or warfarin (INR of 2 to 3) for 6 months after an initial course of therapy with a parenteral anticoagulant for at least 5 days.[171] Dabigatran was noninferior to warfarin for the prevention of recurrent VTE or VTE-related mortality (HR 1.10, 95% CI 0.65 to 1.84). The rate of major bleeding was 1.6% with dabigatran and 1.9% with warfarin (HR 0.82, 95% CI 0.45 to 1.48). A second phase III trial with the same design is underway (RE-COVER-2; www.clinicaltrials.gov: NCT00680186). Other phase III trials compared dabigatran etexilate with placebo for the long-term secondary prevention of recurrent VTE in patients who have completed a course of anticoagulant treatment with a VKA or dabigatran for 6 to 18 months (RE-SONATE; www.clinicaltrials.gov: NCT00558259), or dabigatran with warfarin in patients who have completed a 3- to 12-month course of anticoagulant therapy for acute VTE (RE-MEDY; www.clinicaltrials.gov: NCT00329238).

Stroke prevention in atrial fibrillation. Based on the results of the RELY trial, which is reviewed in Chapter 92, dabigatran etexilate is licensed as an alternative to warfarin for stroke prevention in patients with atrial fibrillation in the United States, Europe, and Canada.

Acute coronary syndromes. Although a phase II dose-finding study in ACS patients has been completed,[172] a phase III trial has yet to be initiated for this indication.

AZD0837. A follow-up to ximelagatran, AZD0837 is a prodrug, which undergoes rapid metabolism by CYP isoenzymes, including CYP2C9, CYP2C19, and CYP3A4, to AR-H69927, an intermediate that is then converted to AR-H067637, a selective and reversible direct inhibitor of thrombin.[167] The half-life of AR-H067637 is 9 to 14 hours in healthy subjects and AZD0837 and its metabolites are excreted in the urine and the feces. The oral bioavailability of AZD0837 is in the range of 22% to 55%, and drug levels in plasma peak about 1.5 hours after oral drug administration. Both immediate release and extended release formulations of AZD0837 have been developed.[173] The extended release form has the potential for once-daily administration. In a phase II study, the immediate release formulation of AZD0837 (in doses of 150 or 350 mg twice daily) was compared with warfarin (dose-adjusted to achieve an INR of 2 to 3) in 250 patients with atrial fibrillation.[173] Total bleeding events were reported in 6 patients receiving the 150 mg dose of AZD0837, in 15 patients given the 350 mg dose, and in 8 of those treated with warfarin. Elevations in alanine aminotransferase levels were infrequent and the rates were similar with AZD0837 and warfarin.

The extended release formulation has been evaluated in two phase II studies. The first compared the extended release formulation of AZD0837 (150, 300, and 450 mg once daily and 200 mg twice daily) with warfarin in 955 patients with atrial fibrillation.[174] Total bleeding events were similar or lower with

all doses of AZD0837 than with warfarin (5.3% to 14.7% and 14.5%, respectively).

The second phase II study compared the extended release formulation of AZD0837 (150 or 300 mg once daily) with standard therapy (consisting of either nothing, aspirin, or clopidogrel), in 131 patients with atrial fibrillation who were unwilling or unable to take a VKA.[175] Treatment was given for a median duration of 6 weeks. There were no major bleeds.

Side effects of AZD0837 include dyspepsia, diarrhea, and nausea and a reversible elevation in the serum creatinine level that appears to be the result of decreased inhibition of the tubular secretion of creatinine.[176] Despite the promising clinical results with AZD0837, phase III trials have not been initiated.

CONCLUSIONS AND FUTURE DIRECTIONS

The last decade has seen an explosion of new antithrombotic drugs enter the clinical arena. Several of these agents have already been licensed. On the antiplatelet front, ticagrelor was recently approved in Europe and Canada. Following on the heels of prasugrel, the benefit-to-risk profile of ticagrelor appears to be more favorable. But will prasugrel or ticagrelor replace clopidogrel? Compared with clopidogrel, both prasugrel and ticagrelor reduce the risk of stent thrombosis in patients undergoing PCI, but the risk of this complication is low and may be reduced even further with double-dose clopidogrel. In addition to lowering the risk of stent thrombosis in PCI patients, however, ticagrelor also is associated with reduced mortality compared with clopidogrel. This is an important finding; whether it reflects greater ADP receptor inhibition or a $P2Y_{12}$-independent phenomenon remains to be established.

PAR-1 is a promising target because its inhibition does interfere with platelet adhesion or activation in response to collagen. The efficacy and safety of PAR-1 inhibitors when added on top of aspirin and clopidogrel remain to be established. The recent discontinuation of vorapaxar in stroke patients entered in the TRA2P-TIMI 50 trial highlights the potential for bleeding with triple antiplatelet therapy. Safety will be even more of a concern for add-on therapy if there is a shift from clopidogrel to more potent ADP receptor antagonists.

Perhaps the greatest advance has been in the area of new oral anticoagulants. Rivaroxaban, dabigatran, and apixaban have been licensed in many countries as alternatives to LMWH or fondaparinux for thromboprophylaxis after knee or hip arthroplasty. Although these agents streamline out-of-hospital prophylaxis by eliminating the need for daily injections, these drugs were designed for longer term use as alternatives to VKA. The elusive goal of developing oral anticoagulants that can be administered in fixed doses without the need for routine coagulation monitoring has finally been realized with the recent licensing of dabigatran and rivaroxaban as alternatives to warfarin for stroke prevention in patients with atrial fibrillation. Apixaban and other oral factor Xa inhibitors are likely to follow for this and other indications. Therefore, we are on the verge of a new era of oral anticoagulation therapy as we move from VKA to fixed dose unmonitored treatments.

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CHAPTER

111 Antiplatelet Therapy

CARLO PATRONO

As reviewed in other chapters, platelet activation and aggregation play a key role in atherothrombosis.[1] Antiplatelet drugs target enzymes or receptors involved in the platelet synthesis of important agonists (e.g., thromboxane [TX] A_2) or in mediating their activating/amplifying effects, respectively (FIGURE 111.1).[2,3] Because of the multifactorial nature of atherothrombosis, antiplatelet drugs can only avoid a fraction (typically, one-quarter to one-third) of major vascular complications. This protective effect against the occurrence of a first (primary prevention) or recurrent (secondary prevention) myocardial infarction or ischemic stroke cannot be dissociated from enhanced risk of bleeding complications, because of the important role played by the same drug targets in primary hemostasis. Moreover, antiplatelet therapy should be viewed within the context of a multifactorial approach to cardiovascular prevention that includes lifestyle modifications as well as other pharmacologic interventions.

This chapter reviews the pharmacologic basis and clinical evidence supporting the efficacy and safety of antiplatelet drugs for the treatment and prevention of atherothrombosis.

ASPIRIN

Aspirin (acetylsalicylic acid) was originally developed by the Bayer Company as an analgesic, antipyretic, and antiinflammatory agent at the end of the 19th century, and represented the prototypic nonsteroidal anti-inflammatory drug (NSAID). Following the discovery of its mechanism of action and characterization of its unique pharmacodynamic properties in inhibiting platelet function (reviewed in Ref.4), aspirin was developed as an antiplatelet agent during the past 30 years, a process largely driven by the medical–scientific community (reviewed in Ref.5).

Pharmacokinetics and Pharmacodynamics

Aspirin is rapidly absorbed in the stomach and upper intestine. Peak plasma levels occur 30 to 40 minutes after oral intake, and maximal inhibition of platelet function is achieved by 1 hour.[2] In contrast, it can take up to 3 to 4 hours to reach peak plasma levels after the oral administration of enteric-coated aspirin. The oral bioavailability of regular aspirin tablets is approximately 50% over a wide range of doses,[6] because of deacetylation of the drug during passage through the liver (first-pass effect). However, substantial variability has been reported in the oral bioavailability of enteric-coated aspirin formulations.[7] Poor absorption from the higher pH environment of the small intestine and lower bioavailability of some enteric-coated aspirin preparations may result in inadequate platelet inhibition, particularly in heavier subjects.[7]

The plasma concentration of aspirin decays with a half-life of 15 to 20 minutes.[2] Despite the rapid clearance of the intact drug moiety from the circulation, aspirin induces a long-lasting functional defect in human platelets. This appears to be primarily, if not exclusively, due to permanent inactivation by aspirin of a key enzyme in platelet arachidonate metabolism (FIGURE 111.2). This enzyme, prostaglandin (PG) H-synthase 1, is responsible for the formation of PGH_2, the precursor of TXA_2. In human platelets, TXA_2 provides a mechanism for amplifying the activation signal by being synthesized and released in response to various platelet agonists (e.g., collagen, adenosine diphosphate [ADP], platelet-activating factor, thrombin) and, in turn, inducing irreversible aggregation.[1]

Aspirin selectively acetylates the hydroxyl group of a single serine residue at position 529 (Ser^{529}) within the polypeptide chain of platelet PGH-synthase 1. This enzyme exhibits two distinct catalytic activities: a bis-oxygenase (cyclooxygenase [COX]) involved in formation of PGG_2 and a hydroperoxidase (HOX) allowing a net two-electron reduction in the 15-hydroperoxyl group of PGG_2, thus yielding PGH_2 (FIGURE 111.2). An inducible form of PGH-synthase has been identified and termed PGH-synthase 2 or COX-2.[8] While newly formed platelets can express both COX-1 and COX-2,[9] mature platelets primarily display COX-1 activity. As a consequence of *O*-acetylation of Ser^{529} by aspirin, the COX activity of the enzyme is lost permanently, whereas the HOX activity is not affected. The highly hydrophobic environment of the COX-1 channel stabilizes the aspirin-modified serine side chain against hydrolysis. As a result of this permanent modification of the enzyme, COX activity can only resume through new protein synthesis (occurring within a few hours in nucleated cells) or cell turnover (occurring at a daily rate of 10% to 12% in the case of human platelets).[2] Aspirin is equally potent in acetylating COX-1 and COX-2, when tested *in vitro*.[10] However, its unique mechanism of action and unusual pharmacokinetic features[6] (short half-life and presystemic encounter with the platelet target in the portal blood prior to first-pass liver metabolism) allow selective, cumulative inhibition of platelet COX-1 at low doses while substantially sparing endothelial COX-2.[2] Whereas the effect of aspirin on COX-1–dependent TXA_2 production is saturable at daily doses as low as 30 mg,[11] its inhibitory effect on COX-2–derived PGI_2 production is dose dependent up to daily doses of 650 to 1,300 mg.[12]

The strikingly nonlinear relationship between inactivation of platelet COX-1 and inhibition of TXA_2-dependent platelet function by low-dose aspirin (i.e., relatively limited inhibition of platelet function between 0% and 95% inhibition of COX-1; exponential increase in platelet inhibition between 97% and 100% COX-1 inactivation)[13,14] has important clinical implications: (a) a substantial loss of platelet inhibition is associated

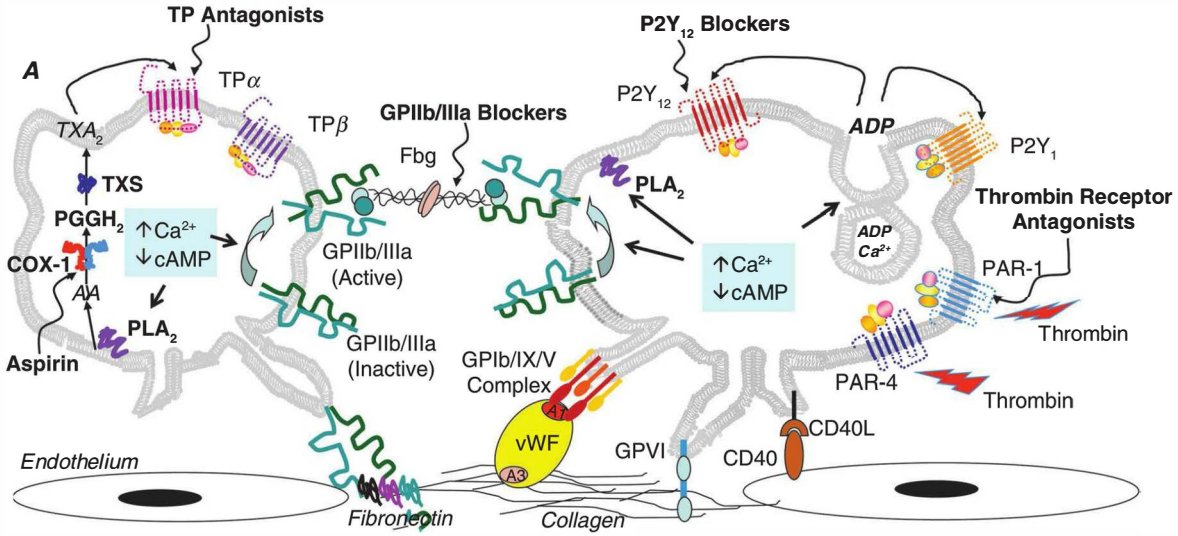

FIGURE 111.1 Platelet activation and aggregation: agonists, receptors, and site of action of antiplatelet drugs. The activation, adhesion, and aggregation of platelets are triggered by several agonists interacting with specific receptors on the platelet membrane. The GPIb/IX/V complex plays an essential role in the initial phase of platelet–vessel wall interaction that finally converges to GPIIb/IIIa activation. vWF binds to GPIb and collagen, while the subendothelial collagen binds to GPIa/IIa and GPVI as well. Platelet activation by agonists results in the decrease in cAMP and/or increase in intracellular Ca^{2+}, leading to the inside-out change in the ligand-binding properties of GPIIb/IIIa, which conformationally switches to a high-affinity state for adhesive proteins such as fibrinogen, vWF, fibronectin, and vitronectin. Fibrinogen-mediated bridging of adjacent platelets via activated GPIIb/IIIa is the final mediator of aggregation. This response is amplified by further release of ADP and TXA_2 and degranulation, which induce additional platelet activation/recruitment, changes in morphology and aggregation stability via outside-in signaling events mediated by activated GPIIb/IIIa. Antiplatelet drugs target enzymes (e.g., COX-1) or receptors (e.g., $P2Y_{12}$) involved in the platelet synthesis of endogenous agonists (e.g., TXA_2) or in mediating their activating/amplifying effects, respectively. ADP, adenosine diphosphate; cAMP, cyclic adenosine monophosphate; TX, thromboxane; TP, thromboxane receptor; PKC, protein kinase C; PAR, protease-activated receptor; TXS, thromboxane synthase; PGH_2, prostaglandin H_2; COX, cyclooxygenase; AA, arachidonic acid; PLA_2, phospholipase A_2; GP, glycoprotein; vWF, von Willebrand factor; CD40L, CD40 ligand; Fbg, fibrinogen.

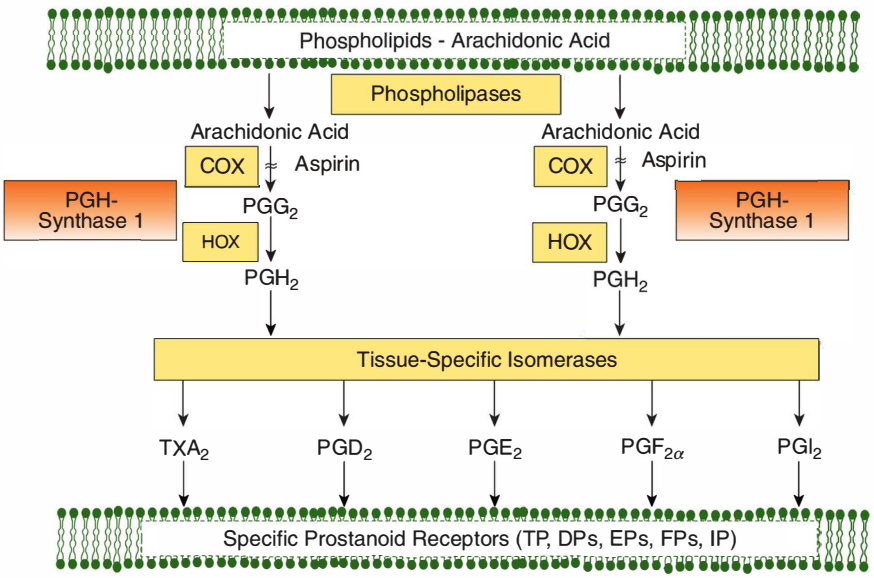

FIGURE 111.2 Arachidonic acid metabolism through the PG H-synthase pathways. Arachidonic acid, a 20-carbon fatty acid containing four double bonds, is liberated from the *sn*2 position in membrane phospholipids by several forms of phospholipase A_2, which are activated by diverse stimuli. Arachidonic acid is converted by cytosolic PGH-synthases, which have both COX and HOX activity, to the unstable intermediate PGH_2. The synthases are colloquially termed COXs and exist in two forms, COX-1 and COX-2. Aspirin inhibits both COX-1 and COX-2. PGH_2 is converted by tissue-specific isomerases to multiple prostanoids. These bioactive lipids activate specific cell-membrane receptors of the superfamily of G-protein–coupled receptors. TP denotes the TXA_2 receptor, DP the PGD_2 receptor, EP the PGE_2 receptor, FP the $PGF_{2\alpha}$ receptor, and IP the PGI_2 receptor.

with less than maximal inactivation of COX-1; (b) recovery of platelet function is disproportionately rapid upon drug withdrawal; and (c) the requirement for virtually complete and persistent inhibition of platelet COX-1 cannot be met by most traditional NSAIDs, allowing their COX-2–dependent cardiotoxicity to be unmasked.[15]

Efficacy and Safety

A very large database of randomized clinical trials (RCTs) now offers the most compelling evidence that prevention of myocardial infarction and ischemic stroke by aspirin is largely due to permanent inactivation of platelet COX-1.[2,5] These studies, which tested the efficacy and safety of the drug when given at daily doses ranging from as low as 30 mg to as high as 1,500 mg, have established two important facts. First, the antithrombotic effect of aspirin is saturable at doses in the range of 50 to 100 mg, as would be expected from human studies of platelet COX-1 inactivation.[11] Second, despite a half-life of approximately 20 minutes in the human circulation, the antithrombotic effect of aspirin is observed with a dosing interval of 24 to 48 hours, reflecting the permanent nature of platelet COX-1 inactivation and the duration of TXA_2 suppression following oral dosing in human.[11] Other mechanisms of action that have been suggested to contribute to the antithrombotic effect of aspirin, such as a direct anti-inflammatory effect of the drug, are incompatible with these unique properties, because they require higher doses and shorter dosing interval.[2]

Aspirin's unique feature in inhibiting platelet COX-1—its ability to inactivate the enzyme permanently through a short-lived active moiety—is ideally suited to its role as an antiplatelet drug, because it limits the extent and duration of extraplatelet effects of the drug, including the inhibition of PGI_2.[12] Moreover, the cumulative nature of platelet COX-1 acetylation by repeated low doses of aspirin[11] explains the clinical efficacy of doses as low as 30 to 50 mg daily,[2] the predictable high-grade inhibition of platelet TXA_2 biosynthesis,[14] and the persistence of the drug's effect.[2] These features, in turn, may limit the consequences of less-than-ideal compliance in a real world setting.

Permanent inactivation of platelet COX-1 by aspirin may lead to the prevention of atherothrombosis as well as to excess bleeding. At least two distinct COX-1–dependent mechanisms contribute to the increased risk of upper gastrointestinal (GI) bleeding associated with aspirin exposure: inhibition of TXA_2-mediated platelet function and impairment of PGE_2-mediated cytoprotection in the GI mucosa.[2] Whereas the former effect is dose independent for daily doses >30 mg, the latter effect is clearly dose dependent.[2] Inhibition of platelet function is largely responsible for the twofold increase in the risk of upper GI bleeding associated with daily doses of aspirin in the range of 75 to 100 mg, inasmuch as a similar relative risk (RR) is associated with other antiplatelet agents (e.g., clopidogrel) that do not act on COX and therefore do not affect PGE_2-mediated cytoprotection.[16] Inhibition of COX-1–dependent cytoprotection of the GI mucosa amplifies the risk of bleeding/perforation by causing new mucosal lesions or aggravating existing ones and is associated with a RR of four to six at the higher, analgesic, or anti-inflammatory doses of aspirin.[2]

The hypothesis that both the reduction in atherothrombotic complications and the increase in bleeding events are primarily due to inhibition of platelet TXA_2 biosynthesis is supported by the recent finding that a highly selective TXA_2 receptor (TP) antagonist, terutroban, and low-dose (100 mg daily) aspirin were associated with virtually identical rates of recurrent vascular events and major bleeds in an RCT of over 19,000 patients with a recent stroke.[17] The possibility that aspirin may exert "pleiotropic" effects at low doses is not incompatible with a single mechanism of action, that is, inactivation of platelet COX-1, explaining antithrombotic, anti-inflammatory, and antiproliferative effects of the drug. In fact, there may be "pleiotropic" consequences of platelet inhibition by low-dose aspirin, resulting from reduced release of platelet-active prostanoids, inflammatory cytokines, and growth factors.[1] The recent reports[18,19] of long-term reduction in the risk of colorectal (and other) cancer and death associated with low-dose aspirin in RCTs using as low as 75 mg daily are consistent with this hypothesis.[20]

Assessing the benefit/risk profile of aspirin requires an estimation of the absolute risk of the individual patient for atherothrombotic and hemorrhagic complications. In individuals at low risk for vascular occlusion (e.g., <1% per year), a very small absolute benefit may be offset by exposure of very large numbers of healthy subjects to undue serious bleeding complications (see Balance of Benefits and Risks). As the risk of experiencing a major vascular event increases, so does the absolute benefit of antiplatelet prophylaxis with aspirin (FIGURE 111.3), and, above a certain threshold, benefit clearly outweighs risk of bleeding (Table 111.1).[2,5]

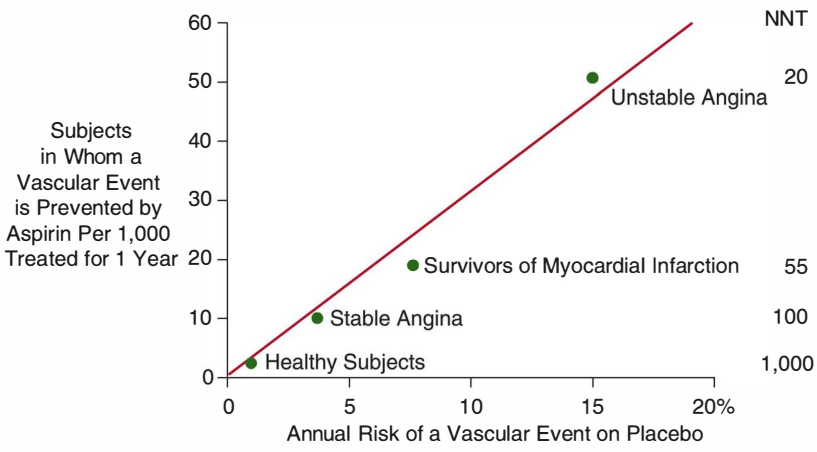

FIGURE 111.3 The risk of vascular complications is the major determinant of the absolute benefit of antiplatelet therapy. Data are plotted from placebo-controlled aspirin trials in different clinical settings. For each category of patients, the abscissa denotes the absolute risk of experiencing a major vascular event as recorded in the placebo arm of the trial(s). The absolute benefit of antiplatelet treatment is reported on the ordinate as the number of subjects in whom an important vascular event (nonfatal MI, nonfatal stroke, or vascular death) is actually prevented by treating 1,000 subjects with aspirin for 1 year. Numbers needed to treat (NNT) are also reported for each clinical setting.

Table 111.1

Benefit/risk ratio of antiplatelet prophylaxis with aspirin in different settings

Clinical Setting	Benefit[a]	Risk[b]	Benefit/Risk Ratio
	Number of Patients in Whom a Major Vascular Event is Avoided per 1,000/y	Number of Patients in Whom a Major GI Bleeding Event is Caused per 1,000/y	
Men and women at low cardiovascular risk	1–2	1–2	1
Essential hypertension	1–2	1–2	1
Chronic stable angina	10	1–2	5–10
Prior stroke or TIA	10	1–2	5–10
Prior myocardial infarction	15	1–2	7.5–15
Unstable angina	50	1–2	25–50

[a]Benefits are calculated from randomized trial data reviewed in Refs. 2, and 5 and depicted in FIGURE 111.3.
[b]Risks of upper GI bleeding are estimated from a background rate of 1 event per 1,000 per year in the general population of nonusers and a RR of 2.0 to 3.0 associated with aspirin prophylaxis. Such an estimate assumes comparability of other risk factors for upper GI bleeding, such as age and concomitant use of NSAIDs, and may actually underestimate the absolute risk in an elderly population exposed to "primary" prevention. The absolute excess of major extracranial bleeding complications in the "primary" prevention trials reviewed in Ref. 39 ranged between 0.2 and 2.0 per 1,000 patient-years.
Modified from Patrono C, Baigent C, Hirsh J, et al. Antiplatelet drugs: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines (8th Edition). *Chest* 2008;133:199S–233S.

The efficacy and safety of aspirin has been evaluated in numerous placebo-controlled RCTs of patients at variable risk of atherothrombotic complications, from asymptomatic low-risk individuals to patients presenting with an acute myocardial infarction or an acute ischemic stroke[2,5]; trials have extended from as short as a few weeks' duration to as long as 10 years. In the Second International Study of Infarct Survival,[21] a single tablet of aspirin (162.5 mg) started within 24 hours of the onset of symptoms of a suspected myocardial infarction and continued daily for 5 weeks produced highly significant reductions in the risk of vascular mortality (by 23%), nonfatal reinfarction (by 49%), and nonfatal stroke (by 46%). There was no increase in hemorrhagic stroke or GI bleeding in the aspirin-treated patients and only a small increase in minor bleeding.[21] Treatment of 1,000 patients with suspected acute myocardial infarction with aspirin for 5 weeks will result in approximately 40 patients in whom a vascular event is prevented,[22] with a proportional odds reduction of 30%. Two separate trials with a similar protocol, the International Stroke Trial[23] and the Chinese Acute Stroke Trial,[24] tested the efficacy and safety of early aspirin use in acute ischemic stroke. Approximately 40,000 patients were randomized within 48 hours of the onset of symptoms to 2 to 4 weeks of daily aspirin therapy (300 and 160 mg, respectively) or placebo. An overview of the results of both trials suggests an absolute benefit of nine fewer deaths or nonfatal strokes per 1,000 patients in the 1st month of aspirin therapy.[22] The proportional odds reduction in fatal or nonfatal vascular events was only 10% in this setting. Although the background risk of hemorrhagic stroke was threefold higher in the Chinese Acute Stroke Trial than in the International Stroke Trial, the absolute increase in this risk associated with early use of aspirin was similar in the two studies (excess 2 per 1,000 patients).[23,24]

Balance of Benefits and Risks

Long-term aspirin therapy confers conclusive net benefit on the risk of subsequent myocardial infarction, stroke, or vascular death among subjects at high risk of vascular complications. These include patients with chronic stable angina,[25] patients with prior myocardial infarction,[22] patients with unstable angina,[26–29] and patients with transient ischemic attack (TIA) or minor stroke[30–35] as well as other high-risk categories.[22] The proportional effects of long-term aspirin therapy on vascular events in these different clinical settings showed substantial heterogeneity, ranging from no statistically significant benefits in patients with peripheral vascular disease[36] to approximately 50% risk reduction in patients with unstable angina.[22] In terms of absolute benefit, these protective effects of aspirin translate into avoidance of a major vascular event in 50 per 1,000 patients with unstable angina treated for 6 months and in 36 per 1,000 patients with prior myocardial infarction, stroke, or TIA treated for approximately 30 months.[22]

For patients with different manifestations of ischemic heart or brain disease, a consensus exists in defining a rather narrow range of recommended daily doses (i.e., 75 to 160 mg) for the prevention of atherothrombotic complications.[37,38] This is supported by separate trial data in patients randomized to treatment with low-dose aspirin or placebo as well as by an overview of all antiplatelet trials showing no obvious dose dependence, from indirect comparisons, for the protective effects of aspirin.[22] There is no convincing evidence that the dose requirement for the antithrombotic effect of aspirin varies in different clinical settings.[2]

Among most high-risk patient groups, the expected number avoiding a serious vascular event by using aspirin substantially exceeds the number experiencing a major bleed (Table 111.1). However, it is unclear whether aspirin might benefit people who, although asymptomatic, are at low to intermediate risk of serious vascular events. The question of whether aspirin is effective and safe for the primary prevention of vascular events has been addressed in a meta-analysis of six randomized trials.[39] It is interesting to compare the effects of low-dose aspirin in primary prevention with the well-known benefits in secondary prevention.[39] In the six primary prevention trials among 95,000 low-risk individuals, with a mean follow-up of 6.9 years, aspirin allocation yielded a 12% RR reduction in serious vascular events,

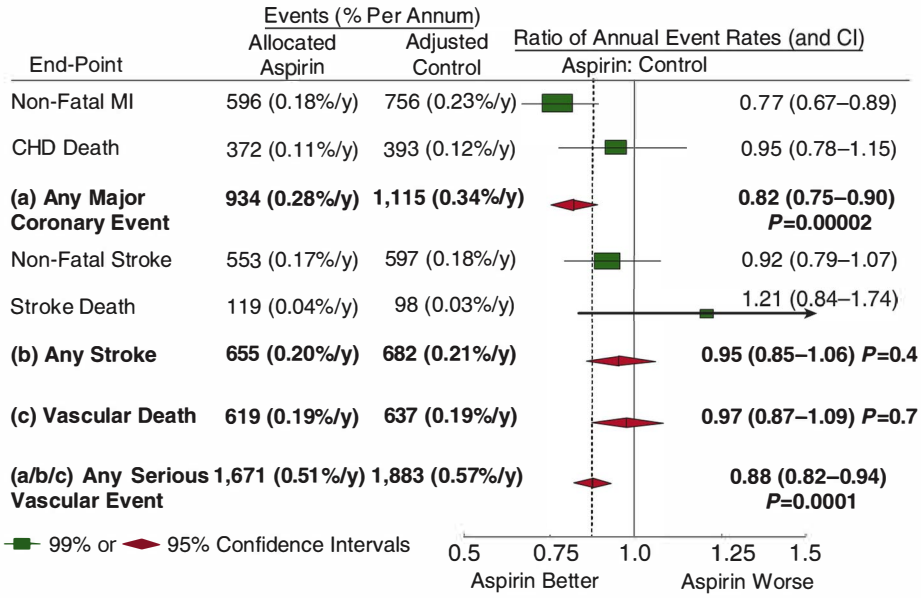

FIGURE 111.4 Serious vascular events in primary prevention trials—proportional effects of aspirin allocation. Actual numbers for aspirin-allocated trial participants, and adjusted numbers for control-allocated trial participants, are presented, together with the corresponding mean yearly event rate (*in parentheses*). Rate ratios for all trials are indicated by *squares* and the 99% CIs by *horizontal lines*. Subtotals and the 95% CIs are represented by *diamonds*. *Squares* or *diamonds* to the left of the *solid line* indicate benefit. MI, myocardial infarction; CHD, coronary heart disease. (Modified from ATT Collaboration. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. *Lancet* 2009;373:1849–1860.)

from an annual rate of 0.57% to 0.51% (FIGURE 111.4). This effect was mainly due to a reduction in nonfatal myocardial infarction, from 0.23% to 0.18% per year. The net effect on stroke was not significant (from 0.21% to 0.20% per year), reflecting a small reduction in presumed ischemic stroke and counterbalancing effects on hemorrhagic stroke and other stroke.[39] There was no significant reduction in vascular mortality (from 0.19% to 0.19% per year). Aspirin allocation increased GI (or other extracranial) bleeds by about half, from 0.07% to 0.1% per year.[39]

In 16 long-term, secondary prevention trials in 17,000 high-risk patients with prior myocardial infarction, or prior stroke or TIA, with a mean follow-up of 2.5 years, aspirin allocation yielded 19% fewer serious vascular events (from 8.2% to 6.7% per year), with similar proportional reductions in coronary events (20% RR reduction) and ischemic stroke (22% RR reduction), but a nonsignificant increase in hemorrhagic stroke.[39] Although the 19% proportional reduction in serious vascular events was only slightly higher than that observed in primary prevention, the absolute benefit of aspirin was 25 times larger in secondary prevention (15 vs. 0.6 fewer vascular events per 1,000 per year).[39] In both primary and secondary prevention trials, the proportional reductions in serious vascular events appeared similar for men and women and for older and younger people.[39] In the primary prevention studies, the risks of serious vascular events and of major extracranial bleeds were predicted by the same independent risk factors (age, male gender, diabetes mellitus, current smoking, blood pressure, and body mass index), so those at higher risk of vascular complications also had a high risk of bleeding.[39]

While for secondary prevention the net benefits of adding aspirin to other preventive measures (e.g., statin) would substantially exceed the bleeding hazards, irrespective of age and gender, in people without preexisting vascular disease the benefits of adding long-term aspirin would be of similar magnitude as the hazards.[39] However, the recent reports[18,19] of additional long-term effects of low-dose aspirin on the risk of death due to cancer, recorded in patients participating in both primary and secondary cardiovascular prevention trials, may lead to reconsidering the benefit/risk profile of the drug in people without preexisting vascular disease.

A low ankle brachial index (ABI) is a relatively simple measure of peripheral atherosclerosis and predicts cardiovascular events and mortality.[40] The Aspirin for Asymptomatic Atherosclerosis trial[41] aimed to determine whether screening the general population for a low ABI could identify a higher-risk group who might benefit from antiplatelet therapy. Its largely negative findings in 3,350 men and women with a low ABI (≤0.95), after a mean follow-up of 8.2 years, are most likely due to lack of statistical power possibly amplified by relatively poor compliance.[42]

CLOPIDOGREL AND OTHER P2Y$_{12}$ BLOCKERS

Ticlopidine, clopidogrel, and prasugrel are structurally related thienopyridines with platelet inhibitory properties. These drugs selectively inhibit ADP-induced platelet aggregation, with no direct effects on the metabolism of arachidonic acid.[43] Thienopyridines also can inhibit platelet aggregation induced by collagen and thrombin, but these inhibitory effects are abolished by increasing the agonist concentration and, therefore, likely reflect blockade of ADP-mediated amplification of the response to other agonists.[43]

Pharmacokinetics and Pharmacodynamics

Thienopyridines inhibit ADP-dependent platelet function by irreversible modification of platelet P2Y$_{12}$[44] through short-lived

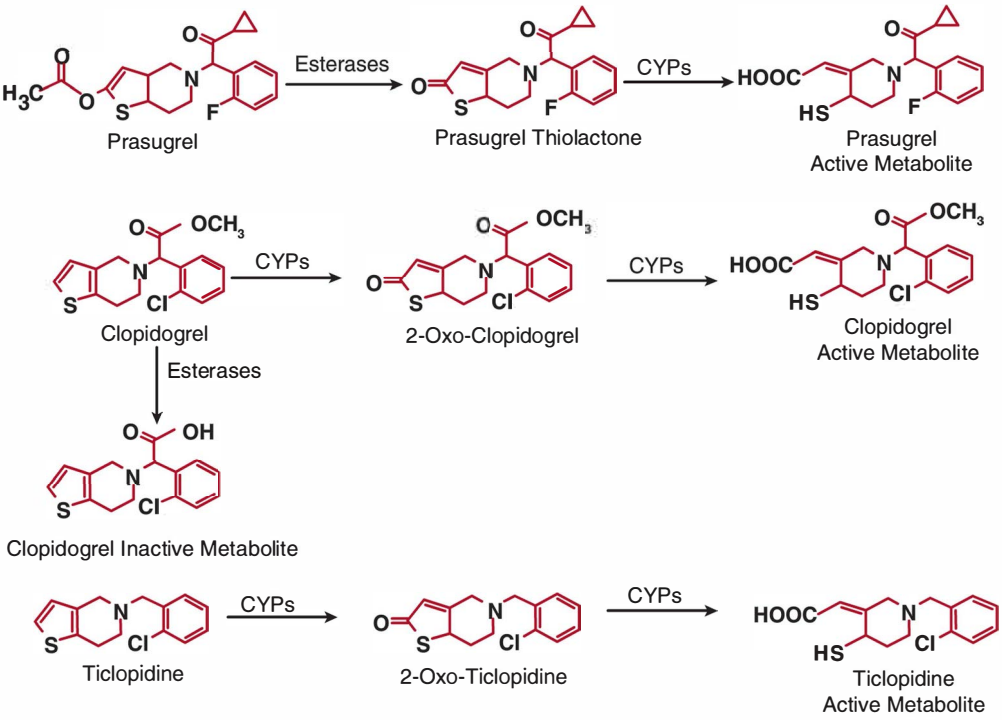

FIGURE 111.5 Pathways of thienopyridine metabolism. Metabolic activation of prasugrel, clopidogrel, and ticlopidine by the liver cytochrome P450 (CYP) system to form thiol-containing AMs is illustrated. The latter are responsible for the antiplatelet effects of thienopyridines.

active metabolites (AMs) generated by liver cytochrome P450 (CYP) isozymes[45] (FIGURE 111.5). Although conceptually analogous to aspirin in terms of pharmacokinetic/pharmacodynamic dissociation (short half-life, long duration of action), the inhibition of ADP-dependent platelet function by clopidogrel is less predictable than the inhibition of TXA_2-dependent platelet function by aspirin. Insufficient availability of the AM of clopidogrel at conventional therapeutic doses results in incomplete inactivation of platelet $P2Y_{12}$.[46] However, because of the linear relationship between $P2Y_{12}$ inactivation and inhibition of ADP-dependent platelet function, even incomplete inactivation of the drug target may result in a measurable clinical effect. Genetic variations of the liver enzymes responsible for the metabolism of clopidogrel as well as drug–drug interactions (e.g., with proton pump inhibitors [PPIs]) are important determinants of the highly variable circulating levels of its AM. These, in turn, are associated with variable inhibition of ADP-induced platelet aggregation and variable clinical response to clopidogrel treatment[47,48] (FIGURE 111.6).

Efficacy and Safety of Clopidogrel as Monotherapy

Clopidogrel has undergone an unusual clinical development, with limited phase 2 studies and a single large phase 3 trial (CAPRIE, in over 19,000 patients) to test its efficacy and safety at 75 mg daily compared with aspirin at 325 mg daily in three groups of patients enrolled because of a recent myocardial infarction, recent ischemic stroke, or symptomatic peripheral arterial disease (PAD).[49] Clopidogrel was slightly more effective than aspirin (annual rate of major vascular events: 5.3% vs. 5.8%, RR reduction 8.7%, 95% CI 0.3 to 16.5), and there was some suggestion from a marginally significant ($P = 0.042$) heterogeneity test that clopidogrel might be particularly effective at preventing vascular events in patients with symptomatic PAD (RR reduction = 24% [$P = 0.0028$] in PAD patients vs. RR reduction = 7%, [$P = 0.26$] in stroke patients and an RR increase of 4% [$P = 0.66$] in coronary patients).[49] In CAPRIE, the frequency of severe rash was significantly higher with clopidogrel than with aspirin (absolute excess ~1 to 2 per 1,000), as was the frequency of diarrhea, thus reproducing the characteristic side effects of ticlopidine. No excess neutropenia was associated with clopidogrel, but the frequency of this serious complication was extremely low (0.05%) in this trial.[49] Rates of any bleeding complication and of intracranial hemorrhage were similar for aspirin and clopidogrel, with an absolute excess of severe GI bleeds of approximately 2 per 1,000 for aspirin versus clopidogrel.[49]

DUAL ANTIPLATELET THERAPY

The complementary mechanism of action of clopidogrel and aspirin has led to testing the efficacy and safety of their combination in several high-risk settings.[50–53] The RR reduction of major vascular events associated with the combination of clopidogrel and aspirin, as compared with aspirin alone, was relatively modest in the absence of percutaneous coronary intervention (PCI) and stenting. The additional benefit of dual antiplatelet therapy versus aspirin alone[50–53] was only a fraction of the benefit achieved by aspirin versus placebo in a similar clinical setting (FIGURE 111.7), raising the possibility that the role of ADP in atherothrombosis may have been underestimated on the basis of clopidogrel trials, because of incomplete and variable blockade of ADP-induced platelet aggregation by the drug.[1]

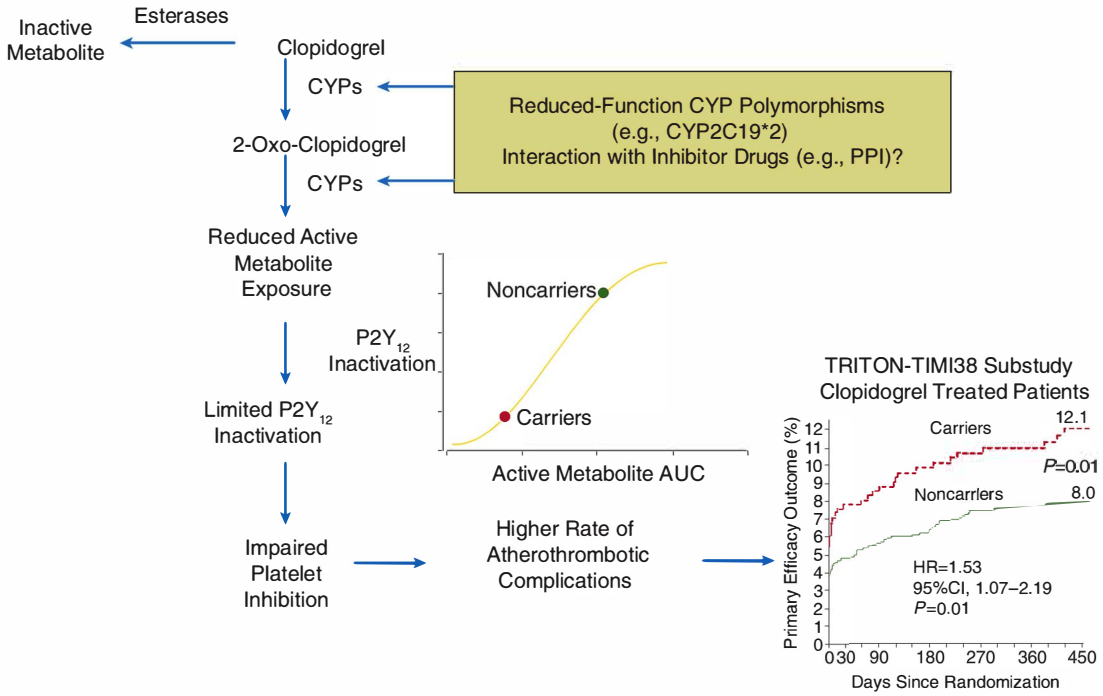

FIGURE 111.6 Mechanisms underlying the interindividual variability in response to clopidogrel. Both pharmacogenetic (e.g., reduced-function polymorphisms of cytochrome P450 [CYP] isozymes) and pharmacokinetic (e.g., drug interactions with inhibitors of CYP isozymes, such as some PPIs) mechanisms may be responsible for reduced conversion of clopidogrel to its AM. The resulting incomplete inactivation of platelet $P2Y_{12}$ is associated with impaired inhibition of ADP-dependent platelet aggregation. In a pharmacogenetic substudy of the TRITON-TIMI 38 trial,[48] clopidogrel-treated carriers of the reduced-function CYP2C19*2 allele had lower levels of the AM of clopidogrel, diminished platelet inhibition, and a higher rate of major cardiovascular events than did clopidogrel-treated noncarriers.

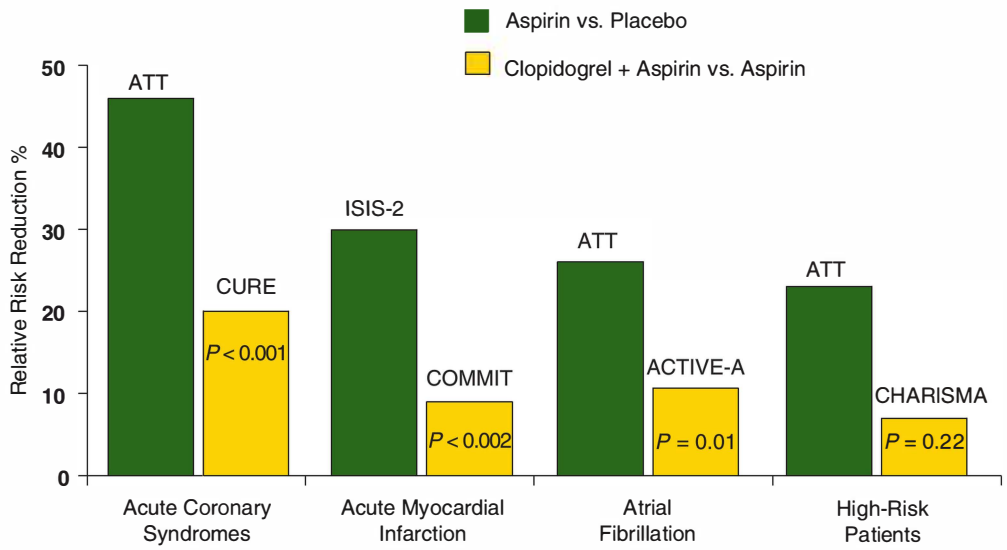

FIGURE 111.7 Additional benefit of dual antiplatelet therapy with aspirin and clopidogrel versus aspirin alone in high-risk settings. RR reductions for the combined endpoint of major vascular events are represented. Data are from individual RCTs and from a meta-analysis of trials of aspirin in high-risk patients. ATT denotes Antithrombotic Trialists' Collaboration,[22] CURE Clopidogrel in Unstable Angina to Prevent Recurrent Events,[50] ISIS-2 Second International Study of Infarct Survival,[21] COMMIT Clopidogrel and Metoprolol in Myocardial Infarction Trial,[51] ACTIVE Atrial Fibrillation Clopidogrel Trial with Irbesartan for Prevention of Vascular Events,[53] and CHARISMA Clopidogrel for High Atherothrombotic Risk and Ischemic Stabilization, Management, and Avoidance.[52]

In the CURE study,[50] blockade of both platelet COX-1 with aspirin and of platelet $P2Y_{12}$ with clopidogrel produced additive effects in patients with acute coronary syndromes without ST-segment elevation (NSTE-ACS), by reducing the rate of the first primary outcome (a composite of cardiovascular death, nonfatal myocardial infarction, or stroke) from 11.4% to 9.3% (RR 0.80, 95% CI 0.72 to 0.90) as compared to aspirin alone, with no evidence of attenuation of the additional benefit over up to 12 months of treatment.[50] As would be expected from more aggressive antiplatelet therapy, there were significantly more patients with major bleeding complications in the aspirin plus clopidogrel group than in the aspirin alone group (3.7% vs. 2.7%, RR 1.38, 95% CI 1.13 to 1.67).[50]

The clinical benefit of dual antiplatelet therapy versus aspirin alone has been confirmed in patients undergoing PCI,[54] in those presenting with acute myocardial infarction with ST-segment elevation (STEMI) within 12[55] to 24 hours[51] after the onset of symptoms, and in patients with nonvalvular atrial fibrillation.[53] In the Clopidogrel and Metoprolol Myocardial Infarction Trial (COMMIT),[51] 45,852 Chinese patients with suspected acute myocardial infarction were studied. Primary PCI was an exclusion criterion, and 55% of the patients received fibrinolytic therapy before or after randomization. Addition of 75 mg/day of clopidogrel to 162 mg/day of aspirin reduced mortality and major vascular events in the hospital by 9% (95% CI 3% to 14%), corresponding to nine fewer events per 1,000 patients treated for about 2 weeks. Overall in this trial, when all transfused, fatal, or cerebral bleeds were considered together, there was no significant excess risk associated with the use of clopidogrel during the scheduled treatment period (0.58% clopidogrel plus aspirin vs. 0.55% aspirin alone; $P = 0.59$), nor was there any excess of major bleeds in patients >70 years of age or in those given fibrinolytic therapy before randomization.[51] Clopidogrel was, however, associated with a small, but significant, excess of 4.7 reported minor bleeds per 1,000 patients treated. Taking major and minor bleeds together, there was no apparent trend with respect to age in the excess risk.[51] Factors that may have contributed to the remarkable safety of dual antiplatelet therapy in the COMMIT trial include the lack of a loading dose of clopidogrel, the uniform use of 162 mg of aspirin, the short duration of treatment with clopidogrel (on average 2 weeks), and the small proportion of elderly patients that received fibrinolytic therapy. In the CLARITY trial, 3,491 STEMI patients <76 years of age received standard fibrinolytic therapy and aspirin and were randomized to a loading dose of clopidogrel 300 mg followed by 75 mg daily or placebo up to and including the day of angiography or day of discharge if no angiography was performed.[55] The mean duration of study treatment was around 3 days. Clopidogrel was associated with higher coronary patency rates at angiography and fewer ischemic complications without a significant increase in major bleeding complications or intracranial hemorrhage. Although long-term treatment with aspirin and clopidogrel for 1 year after STEMI is recommended by various guidelines, irrespective of the acute reperfusion treatment, long-term studies have not been performed in this regard.[43]

At present, there is no sound evidence for defining the optimal duration of dual antiplatelet therapy after drug-eluting stent (DES) placement with current recommendations ranging between 3 and 12 months, depending on the DES type.[56] Several randomized studies, such as OPTIDUAL (Optimal Duration of Dual Antiplatelet Therapy After Drug-Eluting Stent Implantation) and ISAR-SAFE (Safety and Efficacy of Six Months Dual Antiplatelet Therapy After Drug-Eluting Stenting), are currently investigating this issue. Until the results of these studies are available, the potential benefit of extended dual antiplatelet therapy needs to be weighed against the excess risk of major bleeding.

In the ACTIVE-A trial of patients with nonvalvular atrial fibrillation who had an increased risk of stroke and for whom therapy with a vitamin K antagonist was considered unsuitable, dual antiplatelet therapy reduced the risk of major vascular events from 7.6% to 6.8% per year (RR 0.89, 95% CI 0.81 to 0.98) versus aspirin alone (recommended dose, 75 to 100 mg/day).[53] This additional benefit was primarily due to a reduction in the risk of stroke. There was, however, a significant increase in the risk of major bleeding (from 1.3% to 2.0% per year), including a doubling of intracranial bleeding (from 0.2% to 0.4% per year).[53]

In contrast to the consistent finding of a favorable benefit/risk profile of dual antiplatelet therapy in ACS patients[50,51,55] and those undergoing PCI,[54] the same strategy has not proven more effective when compared to clopidogrel alone in patients after a recent ischemic stroke or TIA,[57] or when compared to aspirin alone in patients at high risk for atherothrombotic events.[52] Major bleeding was increased by dual antiplatelet therapy in both MATCH[57] and CHARISMA.[52] In patients with previous stroke or TIA, intensified platelet inhibition seems to be associated with disproportionately increased bleeding risk.[57]

PRASUGREL

Prasugrel is a novel thienopyridine antiplatelet agent. Like ticlopidine and clopidogrel, prasugrel is a prodrug that is inactive *in vitro*.[58] The three compounds are converted to thiol-containing AMs through a corresponding thiolactone[59] (FIGURE 111.5). However, whereas ticlopidine and clopidogrel are metabolized to their thiolactones in the liver by CYP isozymes, prasugrel is converted to its thiolactone via hydrolysis by carboxylesterase 2 during absorption[59] (FIGURE 111.5). Prasugrel thiolactone is efficiently converted to its AM by the liver with a less rigorous dependence on specific CYP isozymes than ticlopidine or clopidogrel. The AM of prasugrel binds specifically and irreversibly to the platelet $P2Y_{12}$ receptor and inhibits ADP-induced platelet aggregation with the same potency as the AM of clopidogrel[58] (FIGURE 111.8). While equimolar concentrations of the AMs of clopidogrel and prasugrel resulted in similar levels of platelet inhibition *in vitro*, the markedly different amounts of each metabolite generated *in vivo* following a loading dose of clopidogrel (300 mg) or prasugrel (60 mg) resulted in approximately 10-fold greater exposure to the latter as compared to the former[58] (FIGURE 111.8). This observation provides a pharmacokinetic basis for the faster, more profound, and less variable inhibition of platelet function observed with prasugrel as compared to clopidogrel in healthy subjects as well as in patients with ischemic heart disease.[46,58] In contrast to clopidogrel, the lack of drug interaction potential and the absence of CYP2C19 genetic effect resulted in a predictable antiplatelet response to prasugrel.[58] This, in turn, helps explain the superior clinical efficacy and enhanced bleeding liability of prasugrel versus clopidogrel in ACS patients undergoing PCI, as established by the TRITON-TIMI 38 trial.[60]

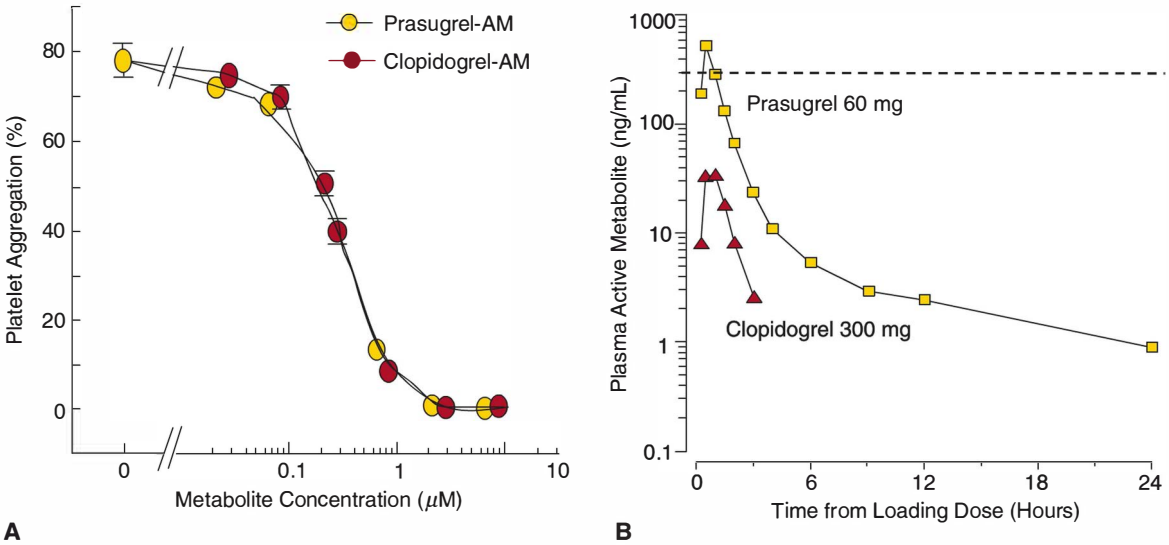

FIGURE 111.8 *In vitro* pharmacodynamics and *in vivo* pharmacokinetics of clopidogrel and prasugrel AMs. **Panel A** illustrates the effects of prasugrel and clopidogrel AM on human platelet aggregation induced by ADP (10 μM). **Panel B** depicts plasma active metabolite concentrations following prasugrel and clopidogrel loading doses. The *broken horizontal line* corresponds to a 1 μM concentration of the active metabolite that is required for profound suppression of ADP-induced platelet aggregation. (Modified from Sugidachi et al. *J Thromb Haemost* 2007;5:1545–1551; Jakubowski et al. *Cardiovasc Drug Rev* 2007;25:357–374.)

REVERSIBLE $P2Y_{12}$ ANTAGONISTS

Three direct and reversible $P2Y_{12}$ antagonists, cangrelor (an intravenous agent), ticagrelor (an oral agent), and elinogrel (available both as an intravenous and as oral agent) are associated with rapid onset and offset of platelet inhibition.[58] Unlike the thienopyridines, they do not require metabolic activation by the liver. Detailed molecular studies of ticagrelor have demonstrated that this selective $P2Y_{12}$ inhibitor displays apparent noncompetitive antagonism toward ADP-induced receptor activation, suggesting the existence of more than one ligand-binding site on $P2Y_{12}$.[61] Ticagrelor is rapidly absorbed and undergoes enzymatic transformation to at least one AM.[58] Peak plasma concentrations of ticagrelor and maximum platelet inhibition are achieved within 1 to 3 hours after dosing. The plasma half-life of ticagrelor is 6 to 13 hours, which dictates a twice-daily regimen of administration.[58]

The full potential of dual antiplatelet therapy with low-dose aspirin and a $P2Y_{12}$ inhibitor in ACS has been unmasked by two recently completed superiority trials of prasugrel and ticagrelor versus clopidogrel, the TRITON-TIMI 38[60] and PLATO[62] studies, respectively. Both trials met their primary endpoint by demonstrating that the 12-month rate of major vascular events can be further reduced by 16% to 19% over clopidogrel with a $P2Y_{12}$ blocker that achieves faster, more profound, and less variable inhibition of ADP-dependent platelet function than clopidogrel[60,62] (FIGURE 111.9). However, important differences between the two trials are worth noting. While the benefit of prasugrel versus clopidogrel was largely confined to a reduction in nonfatal myocardial infarction,[60] the incremental benefit of ticagrelor translated into a statistically significant reduction in vascular as well as total mortality.[62] Moreover, $P2Y_{12}$ blockade with prasugrel was associated with statistically significant increases in both fatal and nonfatal major bleeds,[60] while the effects of ticagrelor were largely confined to nonfatal spontaneous (i.e., non coronary artery bypass graft [CABG]-related) bleeds.[62] In TRITON-TIMI 38, three subgroups of prasugrel-treated patients appeared to be particularly prone to major bleeding complications: the elderly, the underweight, and patients with prior cerebrovascular ischemia.[60] Regardless of the mechanism(s), the increase in death due to hemorrhagic complications of prasugrel treatment may have masked any benefit of the drug on deaths due to vascular causes. The overall safety profiles of the two novel antiplatelet agents displayed additional individual features, with prasugrel being associated with an increase in the diagnosis of colonic neoplasms (13 vs. 4, $P = 0.03$),[60] while ticagrelor caused more frequent episodes of dyspnea, ventricular pauses, and creatinine increases than clopidogrel.[62]

Based on the TRITON-TIMI 38 results, prasugrel has been approved by both the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the reduction of thrombotic cardiovascular events in ACS patients who undergo PCI. A black-boxed warning underscores the increased bleeding risk for patients 75 years of age or older and for patients undergoing urgent CABG. Because of the high probability of a false-positive finding, the imbalance in newly diagnosed cancers is described in the adverse reactions section of the label for prasugrel but is not emphasized as a warning.[63] However, the sponsors have a postmarketing requirement by the FDA to collect baseline and subsequent data on cancer in a large, ongoing clinical trial.[63] The New Drug Application for ticagrelor has been recently approved by EMA and FDA.

INTERINDIVIDUAL VARIABILITY IN RESPONSE TO ANTIPLATELET DRUGS

The term "aspirin resistance" has been introduced some 20 years ago to designate less-than-expected inhibition of platelet aggregation in response to standard doses of the drug.[64,65] Similarly,

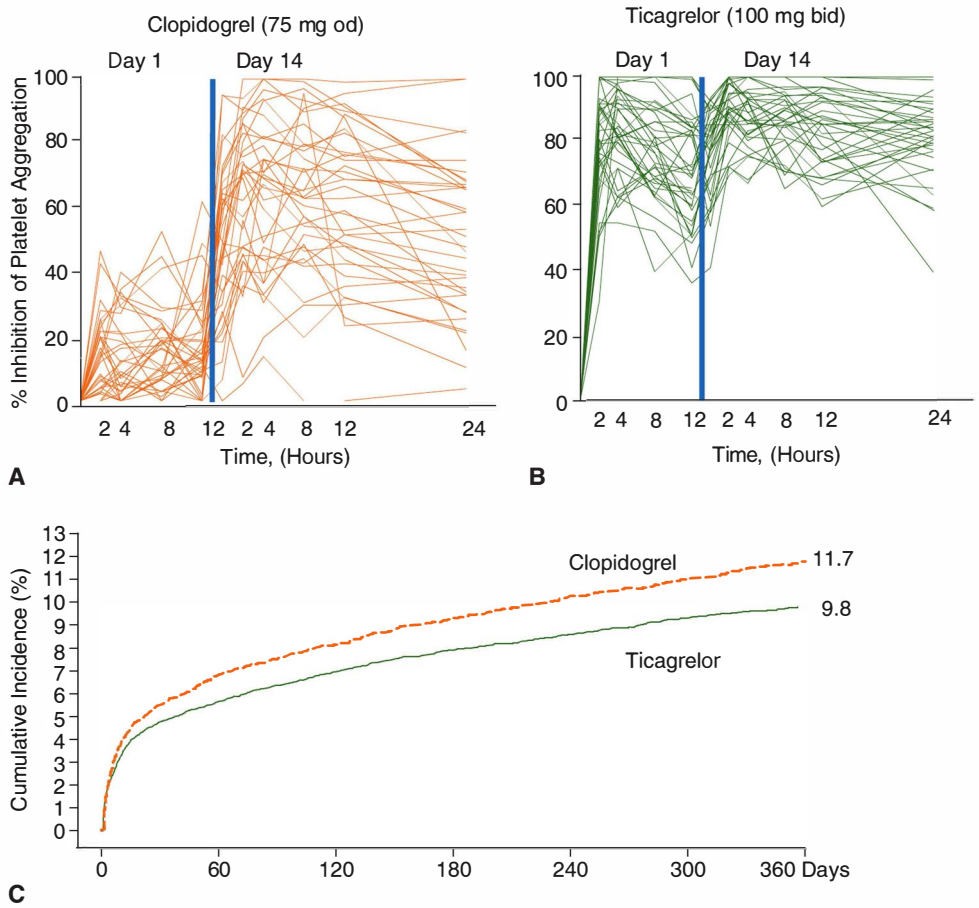

FIGURE 111.9 Pharmacodynamics of clopidogrel and ticagrelor and clinical efficacy in acute coronary syndromes. **Panels A** and **B** illustrate the effects of clopidogrel and ticagrelor, respectively, on ADP-induced platelet aggregation measured *ex vivo* on the 1st day and after 2 weeks of oral dosing. **Panel C** depicts the Kaplan-Meier curves of major vascular events in patients with acute coronary syndromes randomized to clopidogrel or ticagrelor in the PLATO trial. (Modified from Husted et al. *Eur Heart J* 2006;27:1038–1047; Wallentin et al. *N Engl J Med* 2009;361:1045–1057.)

the concept of "clopidogrel resistance" has emerged during the past 10 years based on similar functional measurements.[65,66] Moreover, because these abnormal measurements of platelet aggregation in aspirin- and/or clopidogrel-treated patients have been associated with enhanced risk of atherothrombotic events in a large series of small observational studies and their meta-analyses,[67,68] the same practical corollaries of drug resistance to chemotherapy have evolved as a natural consequence of this clinical paradigm. However, in contrast to antibiotic resistance, there is no agreement on the most appropriate test(s) to diagnose "resistance" to aspirin or clopidogrel. Moreover, there is no randomized clinical evidence that changing antiplatelet therapy in light of such diagnosis is a more effective strategy than maintaining standard therapy.

Aspirin, ticlopidine, clopidogrel, and prasugrel are characterized by individual pharmacokinetic features that may explain the delivery of variable amounts of the active moiety of the drug to its site(s) of action in different individuals. This provides a pharmacokinetic basis for interindividual variability in pharmacologic response. Inadequate bioavailability of intact acetylsalicylic acid or thiol-containing AMs of thienopyridines, because of galenic or genetic reasons, may be sufficient to explain a reduced antiplatelet effect in some individuals without the need for invoking "resistance" of the drug target. This is particularly true for clopidogrel because of its pharmacokinetics that is both unecologic (most of the administered prodrug is wasted) and risky (its metabolic activation is critically dependent on a single CYP).

There seems to be relatively limited room for pharmacodynamic variability in response to aspirin or thienopyridines, with the notable exception of a pharmacodynamic interaction between some reversible COX-1 inhibitors (e.g., ibuprofen and naproxen) and low-dose aspirin.[69,70] These NSAIDs have a higher affinity for and can displace aspirin from Arg-120, a common docking site inside the COX-1 channel, which aspirin has to anchor to in order to acetylate Ser-529.[2] Concomitant treatment with some readily available over-the-counter NSAIDs may limit the cardioprotective effects of low-dose aspirin (as recently outlined by the FDA in a statement for patients and health care professionals), and contribute, together with nonadherence,[71] to many reports of so-called aspirin "resistance."

Based on a pharmacodynamic analysis of the variability in response to aspirin and $P2Y_{12}$ blockers, there seems to be no solid grounds for the practice of phenotyping patients as being

"resistant" or "nonresponders" to these drugs based on a single measurement of platelet function, performed at a variable time point after dosing, and using a largely arbitrary threshold value of response. Moreover, unless the primary mechanism underlying a repeated finding of less-than-expected inhibition of platelet function at a standardized time point (e.g., 24 hours after a witnessed administration) is characterized in the individual patient, changing his/her antiplatelet therapy will represent a purely empirical exercise. Thus, while changing the daily dose of the drug is unlikely to overcome incomplete $P2Y_{12}$ blockade in clopidogrel-treated patients with inadequate metabolic activation of the prodrug, switching to a different thienopyridine (e.g., prasugrel) or reversible $P2Y_{12}$ blocker (e.g., ticagrelor) may effectively address the problem. On the other hand, increasing the daily dose of aspirin or switching from clopidogrel to prasugrel is unlikely to overcome faster renewal of the drug target because of altered platelet production, a problem likely requiring a change in the dosing interval of these irreversible antiplatelet agents or the administration of a reversible inhibitor.

In conclusion, studies of "aspirin resistance" have led to revisiting the clinical pharmacology of platelet COX-1 inhibition in health[14] and disease.[72,73] Serum TXB_2 remains the gold standard test of response to aspirin, and its measurements have clearly indicated noncompliance as well as a pharmacodynamic interaction with other NSAIDs as the main causes of incomplete COX-1 inhibition.[43] The perspective of tailoring aspirin therapy to the individual patient seems unnecessary, except perhaps for changing the pharmaceutical formulation (plain vs. enteric-coated) and/or the dosing interval of aspirin administration where inadequate systemic bioavailability of the drug and/or accelerated renewal of the drug target can be identified as being responsible for less-than-complete inhibition of platelet COX-1. Studies of "clopidogrel resistance," as well as the clinical development of prasugrel and ticagrelor, have been instrumental in characterizing the main determinants of the interindividual variability in response to $P2Y_{12}$ blockers. Pharmacogenetic assessment of clopidogrel response has led to regulatory warning action that requires careful consideration.[74,75] Ideally, tailoring $P2Y_{12}$ blockade to the individual patient would require integrating pharmacokinetic/pharmacodynamic assessment with pharmacogenetics, inasmuch as the therapeutic options might change depending whether inadequate receptor blockade results from a pharmacokinetic interaction with concomitant therapy (e.g., PPI or statin) or reflects genetically determined, poor metabolic activation of the prodrug.

ANTIPLATELET THERAPY: UNCERTAINTIES AND AREAS WHERE WE NEED NEW TRIALS

Although the benefits of low-dose aspirin, clopidogrel, or both are clear among patients with symptomatic vascular disease, the benefit/risk profile of the same preventive strategy is substantially uncertain in low-risk individuals with no clinically apparent vascular disease. The decision to prescribe low-dose aspirin in a person with no history of vascular disease must rely on an individual judgment that the likely benefits of aspirin will exceed any risks. On the basis of the available evidence from a meta-analysis of six primary prevention trials,[39] low-dose aspirin therapy for 4 to 10 years prevents nonfatal myocardial infarction by one-quarter, but it has no clear protective effects against ischemic stroke or vascular death. Therefore, assessing the benefits and risks of low-dose aspirin requires balancing any absolute reduction in nonfatal myocardial infarction (1 to 3 per 1,000 treated for 1 year) against an increased risk of major GI bleeding (1 to 2 per 1,000) and hemorrhagic stroke (0.1 to 0.2 per 1,000).

To maximize the absolute size of benefit versus hazard in primary prevention, most current guidelines[76,77] recommend that aspirin be given to those with coronary risk exceeding a particular threshold. These guidelines implicitly assume, however, either that the absolute risk of bleeding remains approximately constant irrespective of coronary risk or that it depends solely on age. These widely held assumptions have been challenged by the recent Antithrombotic Trialists' meta-analysis of individual participant data from primary prevention trials of antiplatelet prophylaxis,[39] demonstrating that the major risk factors for coronary events, with the notable exception of hypercholesterolemia, are also risk factors for bleeding. As a result, even for people at increased risk of coronary events (i.e., 5-year risk > 10%), the absolute benefit and hazard of adding aspirin to a statin-based primary prevention regimen are likely to be evenly balanced.

A nonfatal stroke or heart attack is more likely to result in long-term disability than is a nonfatal GI (or other extracranial) bleed, but in primary prevention the net absolute reduction in disabling or fatal occlusive events is likely to be small, and at least partially offset by a small increase in serious intracranial and extracranial bleeds.[39] Thus, although it might cost little to add aspirin to any other drugs that are being used for the primary prevention of vascular disease, the additional effectiveness against fatal or disabling outcomes has not been reliably demonstrated for men or women of any age who do not yet have any relevant disease (and, if effectiveness is uncertain then detailed estimates of cost-effectiveness are of limited relevance). Hence, although the currently available trial results could well help inform personally appropriate judgments by individuals about their own use of long-term aspirin, they do not seem to justify general guidelines advocating the routine use of aspirin in all apparently healthy individuals above a moderate level of coronary risk.[39] As noted above, this perspective on primary antiplatelet prophylaxis with low-dose aspirin for cardioprotection may need reevaluation if the recent reports on cancer prevention[18,19] provide the basis for regulatory approval of a new indication.

Further primary prevention trials of aspirin are currently ongoing, with the aim of recruiting people at relatively high cardiovascular risk (expected annual rate of major vascular events ≥ 2%), because of diabetes mellitus (ASCEND and ACCEPT-D), advanced age (ASPREE), or a cluster of risk factors that do not include diabetes (ARRIVE). Although a subgroup analysis of the six primary prevention trials is consistent with some net benefit of aspirin in adults with diabetes but no known vascular disease,[39] the evidence from placebo-controlled trials that recruited only patients with diabetes has been inconclusive.[78] While the unpromising results of these trials may well reflect their relatively limited sample size, it is also conceivable that low-dose aspirin is less effective in people with diabetes because of abnormalities in platelet regeneration potentially limiting the duration of the antiplatelet effect of a standard once-daily regimen of the drug.[79]

Since vascular disease rates rise exponentially above 60 years, and since demographic changes will result in large increases in the elderly population in the coming years, the potential for prevention in this group is substantial. However, the balance of

risk and benefit is uncertain in older individuals (in whom the bleeding risks also increase) because the amount of randomized evidence in those aged 70 or over is limited (around 10% of the primary prevention trial population).[39] Further randomized trials among elderly individuals (e.g., ASPREE) would therefore help to strengthen the evidence in this important group.

Despite virtually complete blockade of platelet COX-1 with low-dose aspirin and $P2Y_{12}$ with prasugrel or ticagrelor, the rate of major vascular events occurring during the 1st year after ACS continues to be unacceptably high, that is, around 10%[60,62] (FIGURE 111.9). The mechanisms underlying these events may include the participation of other pathways of platelet activation (e.g., thrombin-PAR-1) uninhibited by aspirin and $P2Y_{12}$ blockers. Triple antiplatelet therapy with low-dose aspirin, clopidogrel, and vorapaxar, a PAR-1 antagonist, has been tested in ACS patients with limited success,[80] and additional studies are clearly needed to characterize novel drug targets.

Finally, it should be emphasized that tailoring antiplatelet therapy in the individual patient based on his/her genotype and functional response to different $P2Y_{12}$ blockers represents a realistic scenario and may soon become standard of care if ongoing trials establish the efficacy and safety of such a strategy.

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CHAPTER

112 Foundations of Thrombolytic Therapy

VICTOR J. MARDER AND DAPHNE B. STEWART

Thrombolytic therapy represents a conjoint approach to vascular reperfusion, based primarily on the use of fibrinolytic agents delivered systemically or directly into the offending thrombus and complemented by anticoagulation, antiplatelet, and mechanical strategies. Theoretically, any major thrombosed vessel that is the cause of clinically significant disease is amenable to this therapeutic approach, and the decision to utilize thrombolytic therapy requires an informed choice between the potential for clinical benefit and the risk for serious complication. In actuality, thrombolytic therapy represents the acute phase of a prolonged antithrombotic management plan, and the decision about the benefit-to-risk ratio relates to this usually brief but aggressive approach to vascular occlusion.[1,2,3,4–6,7,8,9,10,11–17] This review describes the properties of thrombolytic agents, the physiopathology of thrombolysis and bleeding complications, and the effects of treatment on blood coagulation and fibrinolytic parameters.

THROMBOLYTIC AGENTS

Mode of Action

All of the currently used thrombolytic agents are plasminogen activators (PAs) that induce plasmin action on fibrin contained within a thrombus and an associated state of plasma fibrinogenolysis (lytic state) (FIGURE 112.1). Degradation of fibrin produces the beneficial effect of reducing thrombus size (thrombolysis), but at the same time, PAs may lyse hemostatic plugs at sites of vascular injury[4] and cause bleeding. Susceptible plasma substrates such as fibrinogen are degraded to varying degrees, contributing to a hypocoagulable lytic state. Rethrombosis of the vessel may follow initial reperfusion, generally as a result of a persistent local vascular lesion. The relation of the biologic actions of thrombolysis, altered vascular integrity, rethrombosis, and the plasma lytic state determine the effectiveness and safety of thrombolytic treatment (Table 112.1).

Thrombolytic Agents

Plasminogen Activators

Six PAs have been approved by the US Food and Drug Administration (FDA) for use in major thrombotic diseases, namely, streptokinase (SK),[18] urokinase (UK),[19,20] alteplase (t-PA),[21,22] anistreplase (anisoylated plasminogen streptokinase [APSAC]),[23] reteplase,[24] and tenecteplase (TNK-t-PA),[25] although UK is no longer available in the United States, and anistreplase is rarely utilized (Table 112.2). Certain PAs are considered relatively fibrin selective (e.g., rt-PA and derivatives, staphylokinase, UK) and digest clot with less systemic plasminogen activation, while nonfibrin selective agents (e.g., SK, APSAC) activate systemic and fibrin-bound plasminogen relatively indiscriminately.[26,27]

The t-PA molecule contains four domains, an NH_2-terminal region which is homologous with the finger domains of fibronectin, an epidermal growth factor (EGF) domain, two kringle regions that are homologous with those of plasminogen, and a serine protease domain. Fibrin binding is mediated by the finger and kringle 2 domains, *in vivo* clearance is regulated by the finger and growth factor domains and by the carbohydrate side chains, and plasminogen activator inhibitor-1(PAI-1) inhibition occurs via interaction with the positively charged amino acid sequence at position 296 to 304.[21,22,27]

Tissue-type PA variants have been synthesized by recombinant DNA technology in attempts to attain improved properties such as greater activity and fibrin specificity, prolonged half-life, or an attenuated lytic state (FIGURE 112.2). Reteplase is a single-chain nonglycosylated deletion variant consisting only of the kringle 2 and the proteinase domain of human rt-PA and has a prolonged half-life (15 minutes) that allows for administration in a double bolus regimen.[28] Tenecteplase[25] is altered at three t-PA amino acid sites, conferring resistance to inhibition by PAI-1[29] and a prolonged half-life, allowing administration as a single bolus injection.[30] Lanoteplase (nPA) has a deletion of the finger and EGF domains and the carbohydrate at amino acid 117, resulting in a very prolonged half-life.[31] Vampire bat salivary PA is 70% homologous to t-PA, differing in that it has one kringle domain rather than two,[32,33] conferring greater fibrin specificity. A recombinant version of the protein, desmoteplase, has been tested in a phase III trial of acute ischemic stroke.[34]

UK is secreted as a 54-kDa single-chain molecule (scuPA; pro-UK)[35,36] and converted to a two-chain form (tcuPA) by proteolytic cleavage.[37] It contains an NH_2-terminal growth factor domain, a protease domain, and one kringle structure homologous to those of plasminogen and t-PA. In the presence of a fibrin clot, scuPA, but not tcuPA, induces fibrin-specific clot lysis.[38–41]

Staphylokinase (Sak) is a PA produced by *Staphylococcus aureus* strains that forms a 1:1 stoichiometric complex with plasmin that activates thrombus-bound plasminogen.[42,43] As with SK, Sak is immunogenic, and patients may develop neutralizing antibodies.[44]

The major distinctions between PAs relate to their origin (antigenicity), half-life, potential for inducing a lytic state, and hemorrhagic potential (Table 112.3). PAs that are derived from a human protein (UK, alteplase, reteplase, saruplase, tenecteplase, lanoteplase) are minimally or nonantigenic, whereas those from a bacterial species, whether purified from the *Streptococcus* as SK, complexed with human plasminogen as anistreplase, or prepared

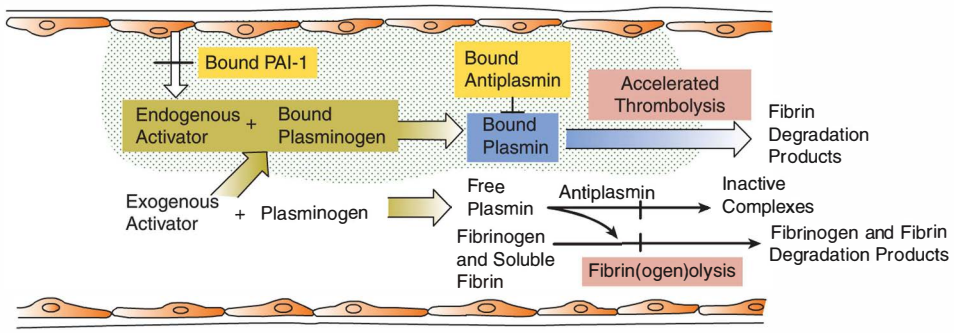


FIGURE 112.1 Molecular interactions of physiologic and therapeutic thrombolysis. Therapeutic administration of plasminogen activator accelerates thrombolysis. Free plasmin in the blood exceeds the capacity of antiplasmin to neutralize the protease activity, resulting in fibrinogen degradation and the so-called plasma proteolytic state. PAI-1, plasminogen activator inhibitor-1.

by recombinant technology from *Staphylococci* as Sak,[45] can induce antibody formation and an allergic response. Modified recombinant forms of Sak and SK with lessened antigenicity and retained thrombolytic potency have been developed.[46,47]

The half-life of each PA determines whether it can be administered as a bolus injection, short infusion, or continuous infusion. The most suitable agents for bolus injections are anistreplase (half-life, ~40 minutes),[48] reteplase,[49] tenecteplase,[50] and lanoteplase,[51] and the least suitable is alteplase (half-life, 5 minutes),[52] which best utilizes a continuous infusion for therapy. Clinical results suggest that some of the newer agents will have less of a plasma lytic state, for example, tenecteplase,[53] but to date, all PAs are associated with a significant bleeding risk. The rate of bleeding is roughly equivalent for all agents, except for the higher rate of intracranial hemorrhage (ICH) with t-PA[54] or t-PA mutant derivatives,[49,55] relative to results using SK. The anticipation of greater safety with a new PA, based on biochemical studies, must be considered cautiously, because no PA has yet been shown to be free of hemorrhagic risk.

Recombinant forms of UK,[35] saruplase (pro-UK, scu-PA),[36] Sak,[37] and BAT-PA[33] (from the salivary gland of *Desmodus rotundus*), chimerics of t-PA and pro-UK,[56] and bifunctional agents composed of antifibrin or antiplatelet antibodies complexed to PA[57] are at various stages of clinical testing. Desmoteplase has marked homology with rt-PA and a much longer half-life of 21 minutes,[58] but it showed no clinical benefit and increased ICH (3.5% to 4.5%) in acute stroke patients at 3 to 9 hours after symptom onset.[34] A recombinant plasminogen that is activated by thrombin, rather than by a PA, has been developed as an agent that would work only at sites of fresh thrombus formation.[59] It has an injection half-life of >4 hours, and early studies have not shown increased ICH.[60,61]

Direct-Acting Thrombolytics

An old agent has recently been revisited as a therapeutic thrombolytic: the direct-acting thrombolytic enzyme plasmin.[62,63,64] The potential for safety is based upon regional administration of the agent by catheter. PAs are administered in sufficient concentration that the inhibitory capacity of PAI-1 is overwhelmed, so they lyse hemostatic plugs and cause hemorrhagic complications, even after local (catheter) delivery (FIGURE 112.3, middle-left and bottom-left panels). In contradistinction, plasmin is rapidly and irreversibly neutralized by α_2-antiplasmin,[65] so it lacks thrombolytic activity upon systemic infusion (FIGURE 112.3, middle right). However, plasmin binds to and dissolves thrombus when administered by catheter, and, after thrombolysis, plasmin would be quickly neutralized by α_2-antiplasmin, thereby preventing its circulation to sites of vascular injury, and avoiding bleeding

Table 112.1

Principal biologic effects of thrombolysis therapy

Clinical Result	Process	Cellular and Biochemical Events
Therapeutic	Thrombolysis	Degradation of fibrin and disruption of platelets in the thrombus, restoration of blood flow
Side effect	Systemic lytic state	Plasma fibrinogenolysis and derangement of platelet function
Complications	Bleeding	Degradation of fibrin in hemostatic plugs and of matrix in abnormal vessels, plus blood hypocoagulability
	Rethrombosis	Persistent local vascular lesions plus permissive status of blood coagulation

Table 112.2

Food and drug administration approvals of PA

Agent	Clinical Indication	FDA-Approved Regimen	Common Usage
SK (Streptase)	Acute MI	IV or IC	Used primarily by IV route
	PE	IV over 12 h	Effective given for shorter courses, even 2 h
	DVT	IV for up to 36 h	Shorter infusions safer, for example, over 18 h
	PAO	IV	IV route not as effective as regionally
	Thrombosed arteriovenous cannulae	Local installation	
Reteplase (Retavase)	Acute MI	Two bolus injections 30 min apart	Approved, 1999
	PAO	Not approved	Usage "off-label" regionally
Alteplase (Activase)	Acute MI	3 h or 90 min	Accelerated dosage used most often
	Acute ischemic stroke	90 mg over 1 h	Limited usage, as must be applied within 3 h of onset
	PE	100 mg/2 h	UK over 2 h, equal efficacy
Anistreplase (Eminase)	Acute MI	30 U over 2–5 min	Infrequently used
Tenecteplase (TNKase)	Acute MI	Single bolus, weight adjusted	Approved, 2000
UK (Abbokinase)	PE	12-h IV infusion	Agent not currently available
	Acute MI	Intracoronary only	Agent not currently available
	Thrombosed central line or shunt	5,000 U in 1 mL	Agent not currently available

PE, pulmonary embolism; IV, intravenous; MI, myocardial infarction; PAO, peripheral arterial occlusion; DVT, deep-vein thrombosis; IC, intracoronary.

that could result from hemostatic plug degradation.[66,67] Animal studies support this hypothesis, showing that more than six-fold the therapeutic dose of plasmin does not induce bleeding, whereas t-PA causes bleeding at the therapeutic dose, and even at dosages as low as 25% of the optimal therapeutic amount.[68] Thrombolysis under conditions of limited plasminogen content (restricted blood flow) is superior following plasmin compared with t-PA.[66] Plasmin is safe and efficacious in preclinical models of cerebral artery occlusion,[69] has been assessed in patients with thrombosed hemodialysis shunts,[70] and is currently in clinical trial in patients with peripheral arterial or graft occlusion (NCT 00418483)[71] and acute ischemic stroke (NCT 01014975).[72]

Other direct-acting thrombolytic agents are shown schematically in FIGURE 112.4.[63] A truncated plasmin, "miniplasmin," consists of the plasmin serine protease domain and kringle 5[73,74] and is inhibited by α_2-antiplasmin 100 times slower than is plasmin; thus its principal inhibitor is α_2-macroglobulin.[75,76] Miniplasmin has shown efficacy in a canine femoral artery thrombolysis model[77] and in the middle cerebral artery ligation stroke model in mouse and hamster,[78] but there are no reports of this agent in human clinical trial.[63] Microplasmin is produced chemically by cleavage of the kringle domains from the serine protease domain[79] or by recombinant technology.[80] Microplasmin does not bind to fibrin and is inhibited by α_2-antiplasmin 100 times slower than is plasmin.[81] Microplasmin showed rapid thrombolysis but no clinical benefit in patients with peripheral arterial or graft occlusion,[82] and trials in ischemic stroke have been curtailed after a phase II study[83]; studies in vitreoretinal disease have shown positive results.[84] Delta-plasmin is a recombinant molecule composed of plasmin kringle 1 spliced to the serine protease domain; it retains the same fibrin-binding capacity and fibrinolytic effect as plasmin and is likewise rapidly inhibited by α_2-antiplasmin.[85] Delta-plasmin has a similar safety profile as full-length plasmin in a preclinical model of bleeding[86] but has not yet been assessed in clinical trials.

A recombinant derivative of fibrolase (alfimeprase), isolated from the venom of the Southern copperhead snake, degrades fibrinogen and fibrin and is inhibited relatively slowly by α_2-macroglublin.[87] Alfimeprase showed promise in an early trial[88] in patients with peripheral arterial or graft occlusion, but results from a subsequent study were not better than placebo, and release of bradykinin from low molecular weight kininogen caused hypotension in some recipients.[89]

ADJUNCTIVE AGENTS AND APPROACHES

Inadequate response to PA therapy occurs if a thrombosed vessel does not manifest full reperfusion or if reocclusion quickly follows initial success. Generally, t-PA and recombinant derivatives have potent thrombolytic activity and achieve early vascular patency with relatively mild effects on blood coagulation, but are utilized along with anticoagulants to counteract any ongoing procoagulant state. PAs such as SK elicit a greater effect on plasma coagulation factors and reperfuse vessels more gradually but tend to maintain an anticoagulant state. Acknowledging that early and persistent patency is a principal objective of treatment, considerable effort has been directed to adjunctive management with antiplatelet agents and antithrombins.

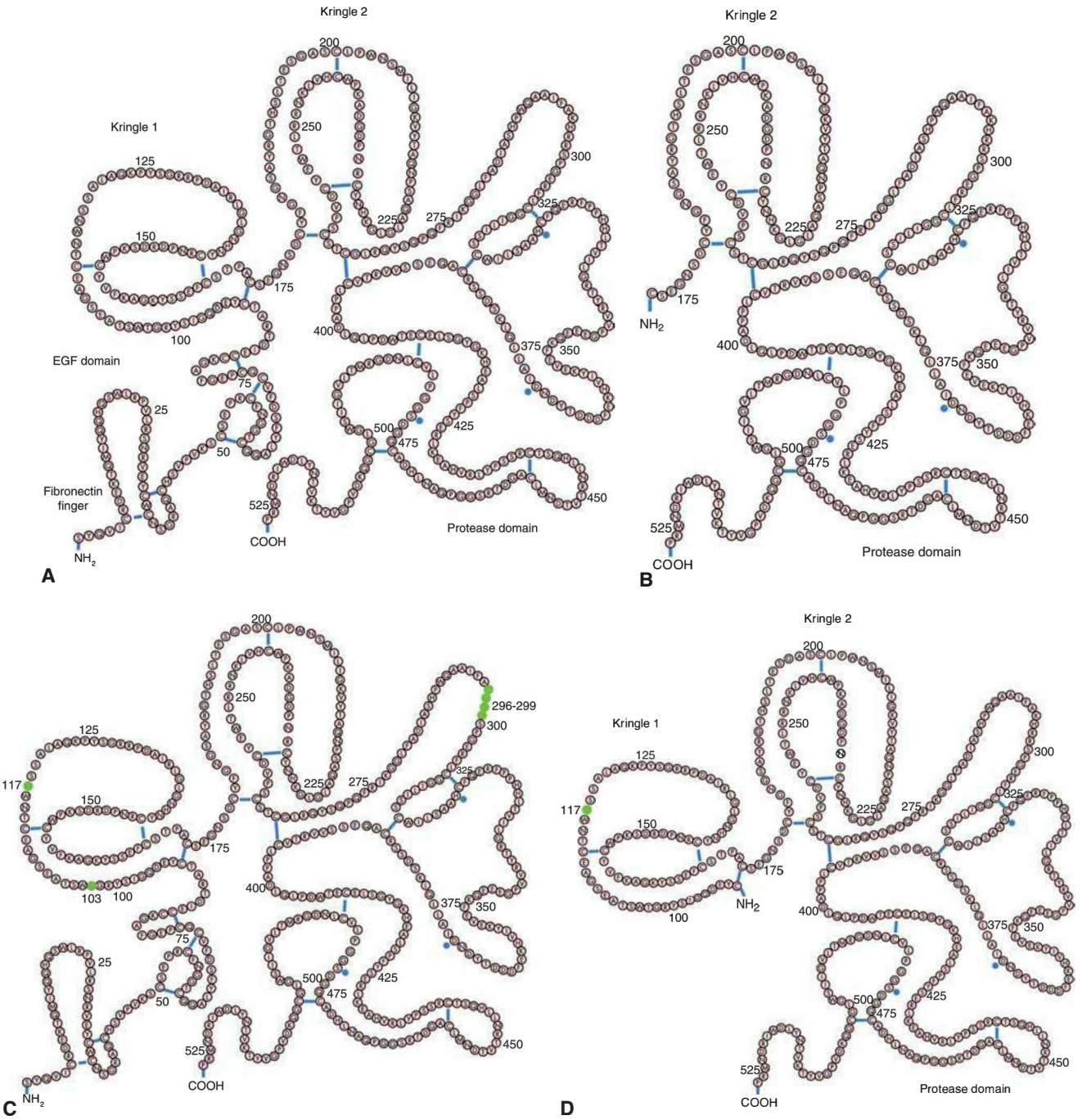


FIGURE 112.2 Schematic representation of t-PA and three mutant derivatives synthesized by recombinant technology. **Panel A** shows t-PA (alteplase), **panel B** shows reteplase, **panel C** shows TNK (tenecteplase), **panel D** shows lanoteplase. (Reproduced from Nordt TK, Bode C. Thrombolysis: new thrombolytic agents and their role in clinical medicine. *Heart* 2003;89:1358–1362, with permission.)

Antiplatelet Function Approaches

Aspirin

The first definitive demonstration of antiplatelet efficacy in the treatment of an acute thrombotic event was with the use of a simple aspirin regimen of 160 mg/day for 30 days to decrease mortality after acute myocardial infarction (AMI) (Second International Study of Infarct Survival, ISIS-2).[90] In patients treated within 6 hours, aspirin reduced the 35-day mortality by 23%, equal to the reduction achieved by SK alone, and both agents together had an additive effect amounting to a 39% relative risk reduction compared to patients who received neither SK nor aspirin. Clopidogrel, an oral antiplatelet agent that blocks platelet activation and aggregation by inhibiting the

Table 112.3
Comparison of PAs

Agent	Source	Antigenic	Half-Life (min)	Regimen	Fibrin Selectivity	Bleeding
SK	*Streptococcus*	Yes	20	Infusion	1+	4+
Staphylokinase	Recombinant	Yes	6	Infusion	1+	4+
Anistreplase	*Streptococcus* + plasminogen	Yes	70	Bolus	1+	4+
Alteplase	Recombinant	No	5	Infusion	2+	4+
Reteplase	Recombinant	No	15	Double bolus	1+	4+
Tenecteplase	Recombinant	No	15	Bolus	3+	4+
UK	Cell culture	No	15	Infusion	1+	4+
Saruplase	Recombinant	No	5	Infusion	1+	4+
Lanoteplase	Recombinant	No	30	Bolus	1+	4+
Desmoteplase	Recombinant	Minimal	21	Bolus	4+	4+

$P2Y_{12}$ adenosine diphosphate receptor, was randomly added to a fibrinolytic and aspirin regimen versus placebo and showed a 36% reduction in the rate of death or recurrent ischemia and represents the current standard of care.[91]

Glycoprotein IIb/IIa Inhibitors

Effective GP IIb/IIIa inhibition can be achieved by the monoclonal antibody 7E3 (Abciximab [Centocor Inc., Malvern, PA]) and by small molecule inhibitors, which effectively block fibrinogen binding to platelets and prevent the cascade of biochemical events leading to aggregation.[92–101] These agents are effective in preventing adverse outcomes after unstable angina and angioplasty,[102,103] and appropriate dosing of concomitant anticoagulant therapy prevents the excessive bleeding associated with these procedures.[104] The rationale for combining GP IIb/IIIa inhibition with fibrinolytic therapy would be that passivation of arterial wall by a long-acting or bound GP IIb/IIIa inhibitor could prevent vascular reocclusion, especially that which follows t-PA therapy.[105] Early angiographic patency studies showed that abciximab combined with low-dose PA led to higher rates of full recanalization (thrombolysis in myocardial infarction [TIMI] 3 flow), with one trial showing a higher rate of bleeding using the combination.[106,107] Concern for greater bleeding risk led to large trials comparing reduced doses of PA combined with a GP IIb/IIIa inhibitor. In the Assessment of the Safety and Efficacy of a New Thrombolytic Regimen (ASSENT)-3 mortality trial, reduced-dose tenecteplase plus abciximab and low-dose unfractionated heparin (UFH) showed similar mortality rates compared with tenecteplase plus low molecular weight heparin (LMWH) or UFH,[108] although the combination was associated with significantly higher rates of major bleeding and requirement for transfusion in patients older than 65 years. The GUSTO-V trial showed no difference in mortality between low-dose reteplase plus abciximab versus standard reteplase, but there was a higher rate of ICH in patients older than 75 years of age in the combination therapy group.[109] Meta-analysis shows no benefit of adding abciximab to fibrinolytic regimens,[110] and the 8th Conference of the American College of Chest Physicians (ACCP) guidelines recommend against the combination.[111]

Several phase II trials in patients with ischemic stroke have looked to the combination of attenuated-dose PA and GPIIb/IIIa antagonists in order to increase vascular patency and lower the risk for ICH. The CLEAR trial[112] randomized 94 patients to low-dose alteplase (0.3 to 0.45 mg/kg) plus eptifibatide (75 μg/kg bolus, then 0.75 μg/kg/min infusion for 2 hours), versus standard intravenous alteplase (0.9 mg/kg). The primary safety endpoint was rate of ICH, and there was no suggestion of more bleeding in the combination arm, although efficacy analysis suggested slight superiority of standard-dose alteplase.

Anticoagulants

Heparin

UFH was included in fibrinolytic regimens, and the Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO) study[113] documented that early intravenous heparin is no more effective than delayed subcutaneous heparin in patient survival at 30 days after AMI following fibrinolysis with SK or alteplase. Analysis of 21 small randomized trials demonstrated improved survival (11.4% vs. 14.9% in-hospital mortality following treatment with a fibrinolytic agent plus heparin versus placebo).[114] However, the large trials that combined fibrinolytic therapy with aspirin and heparin or placebo showed the benefit of adding heparin was minimal (in-hospital mortality 6.8% vs. 7.3%).[115] Coronary artery patency studies show that intravenous heparin, in addition to SK or APSAC (plus adequate aspirin), does not increase vascular patency[116,117] over fibrinolytics alone, but does increase bleeding complications.[117] The rate of ICH is 0.92% to 2.8% with accelerated t-PA regimens combined with intravenous heparin at 5,000 units bolus followed by 1,300 U/h, but are reported to be lower (0.7% to 1.16%) using a reduced heparin infusion rate of 1,000 U/h.[118]

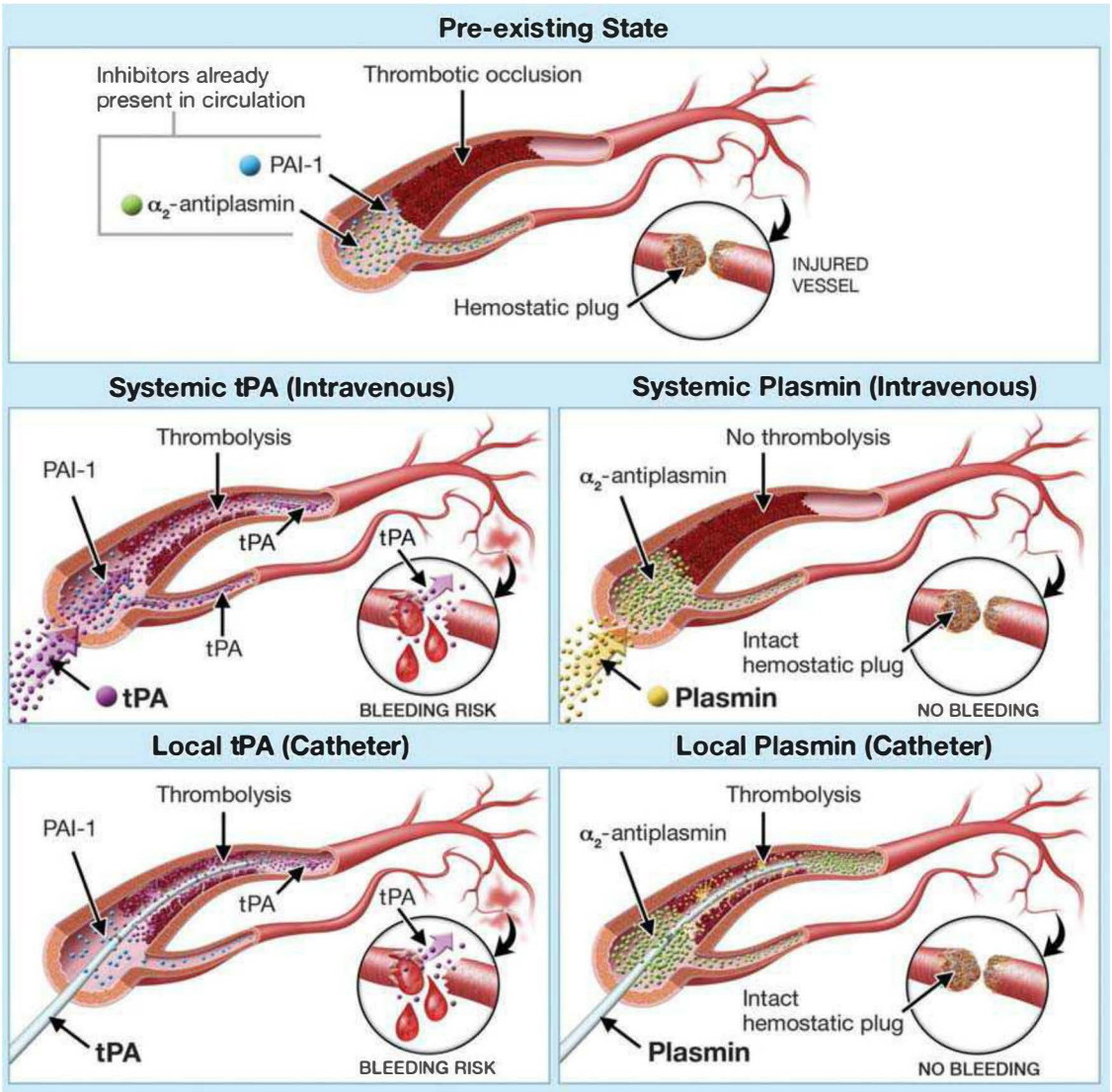


FIGURE 112.3 Modes of action of plasmin and t-PA on vascular thrombi and hemostatic plugs at vascular injury sites, after intravenous or catheter administration. (Reproduced from Marder VJ. Historical perspective and future direction of thrombolysis research: the re-discovery of plasmin. *J Thromb Haemost* 2011;9(Suppl 1):364–373, as modified from Marder VJ, Novokhatny V. Direct fibrinolytic agents: biochemical attributes, preclinical foundation and clinical potential. *J Thromb Haemost* 2010;8:433–444.) **Top panel**: Thrombus occludes an artery or vein and a hemostatic plug is present at a site of vascular injury. Inhibitors of plasmin (α_2-antiplasmin) and of t-PA (PAI-1) in the circulation are shown as *green* and *blue spheres*, respectively. **Middle panels**: Systemic delivery of t-PA exceeds the inhibitory capacity of PAI-1, allowing it to reach and dissolve thrombus; t-PA also reaches and dissolves hemostatic plugs, which can result in bleeding. Plasmin delivered systemically is safe but ineffective, as it is neutralized by α_2-antiplasmin. **Bottom panels**: Catheter delivery of t-PA is effective for dissolving thrombi, but t-PA enters the circulation despite its "local" administration and can cause bleeding. Catheter delivery of plasmin induces thrombolysis. Plasmin that enters the circulation after thrombolysis is rapidly neutralized by α_2-antiplasmin, thus preventing lysis of hemostatic plugs and avoiding hemorrhage.

The benefit of UFH depends on ASA use and dosage: when the dosage of aspirin is 160 mg/day or more, the addition of heparin produces little or no improvement of patency,[119–121] but with 80 mg/day or less of aspirin, heparin exerts a markedly improved vascular patency.[122,123] In spite of these trials, the recommendation of the ACCP is to use weight-adjusted heparin with t-PA and also suggests weight-adjusted intravenous or subcutaneous UFH with SK.[111]

Low Molecular Weight Heparin

The ASSENT-3 PLUS trial,[93] which assessed LMWH versus UFH in combination with tenecteplase, favored LMWH in the composite efficacy endpoint (mortality, reinfarction, ischemia) offset by a higher rate of ICH for LMWH. The ExTRACT-TIMI 25 trial randomized more than 20,000 patients with acute MI treated with thrombolytic therapy to enoxaparin versus UFH,

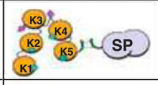
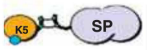
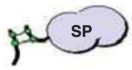
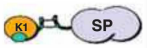
NAME	SCHEMATIC	MASS (kDa)	FIBRIN BINDING	INHIBITOR
Plasmin		82	Yes	Very fast (α2-antiplasmin)
Mini-plasmin		37	Yes	Fast (α2-macro-globulin)
Micro-plasmin		26-29	No	Fast (α2-macro-globulin)
Delta-plasmin		37	Yes	Very fast (α2-antiplasmin)
Alfimeprase		23	No	Fast (α2-macro-globulin)

FIGURE 112.4 Direct-acting thrombolytics. (Modified from Marder VJ, Novokhatny V. Direct fibrinolytic agents: biochemical attributes, preclinical foundation and clinical potential. *J Thromb Haemost* 2010;8:433–444.) SP, serine protease; K, kringle; MP, metalloprotease. *Small circles* on kringles represent lysine-binding sites. Diamonds represent carbohydrate, which are present only on plasmin. The likely main inhibitor of microplasmin is considered to be α_2-macroglobulin, given the absence of kringles.

and the results showed improvement in the net composite endpoint (30-day death, nonfatal MI, major bleeding) after enoxaparin treatment (11% vs. 12.8%).[124] Patients older than 75 years, or with impaired renal function, had increased bleeding complications. The current ACCP recommendation favors the use of enoxaparin over UFH in patients younger than 75 with preserved renal function.[111] The use of fondaparinux, an indirect thrombin inhibitor that selectively binds fXa, is not superior to UFH when combined with fibrin-specific PA therapy for the treatment of ST-elevation-MI.[125]

Antithrombin-Independent Anticoagulants (Hirudin and Related Agents)

Among the newer potential adjunctive agents, two groups have received the most attention: the hirudin family of small molecule inhibitors of thrombin and argatroban.[94–97] Pilot studies in patients with AMI showed encouraging increases in 90-minute patency rates with hirudin rather than with heparin,[126–128] but the relatively high dosages of anticoagulant resulted in unacceptable rates of ICH.[129–131] The advantages in the use of a direct thrombin inhibitor over indirect antithrombin include the inhibition of thrombin-mediated platelet activation and the capability of inactivating thrombin bound to fibrin derivatives. With a scaled-back dosage regimen of anticoagulant in patients receiving t-PA or SK along with aspirin, a large patient trial of 3,002 patients (TIMI-9B) showed no significant difference in clinical outcome using heparin or hirudin.[132] A patency study of 412 patients showed significantly greater TIMI-3 patency at 90 minutes (48% vs. 35%) and less bleeding using Hirulog (bivalirudin) rather than heparin after SK therapy.[133] More emphatically, retrospective analysis of the GUSTO-IIb trial[134] showed that hirudin had a dramatic mortality benefit over heparin in AMI in the patients treated with SK but not in the patients treated with t-PA. It is possible that the relatively high 90-minute patency rate attained with t-PA plus heparin and aspirin approaches a maximal response, whereas the slower reperfusion that occurs with SK plus heparin and aspirin can be ameliorated by the more effective anticoagulant action of hirudin. A definitive study of 17,000 patients with AMI (Hirulog Early Reperfusion/Occlusion 2) assessed whether such increased coronary artery patency is translated to a lower mortality using Hirulog rather than heparin (plus aspirin) before SK therapy.[135] Despite a higher 90-minute patency rate compared with heparin, there was no difference in mortality, and a slight (insignificant) increase in ICH occurred with bivalirudin use. Hirudin or bivalirudin is indicated in combination with fibrinolytic therapy in patients with heparin-induced thrombocytopenia (see Chapter 108).

OTHER MODALITIES TO MAXIMIZE THERAPEUTIC EFFECT OF THROMBOLYTICS

Prehospital Administration of Agent

Since the Fibrinolytic Therapy Trialists collaborative study established the time to reperfusion as a crucial determinant of mortality benefit to any intervention in AMI,[7] efforts to deliver therapy as near to the onset of symptoms have been undertaken. Several trials of prehospital treatment with fibrinolytic therapy have demonstrated a shortened time to definitive therapy and trends toward decreased mortality. The Early Retavase-Thrombolysis in Myocardial Infarction 19 study showed delivery of bolus reteplase produced a decrease in time to initial treatment of 32 minutes.[136] The ASSENT-3 PLUS trial analyzed a prehospital strategy of delivering fibrinolytic therapy, showing 53% of patients were able to receive treatment within 2 hours of symptoms if delivered in the field.[137] When compared with percutaneous coronary intervention (PCI), prehospital fibrinolysis in patients with symptom onset of <2 to 3 hours had equivalent 30-day mortality.[138,139]

Plasminogen Supplementation

Because fibrinolysis depends on conversion of plasminogen to plasmin, the rate is determined at least in part by the plasminogen concentration incorporated into a clot.[140] The decrease in plasma plasminogen varies according to the type of PA that is administered, decreasing by 80% with SK,[141] by 50% to 75% with rt-PA,[141–143] and by 10% to 20% with Sak or TNK.[142,143] Enrichment of plasminogen enhances the thrombolytic rate

in vitro[144] and in animal models,[145] but despite the theoretical advantage of supplemental plasminogen administration, clear-cut clinical benefit has not been obtained.[146–148]

Ultrasound

Ultrasound has been applied to thrombolysis, alone by remote or direct application, and with or without targeted delivery of microbubbles.[149] Direct application by thin wire[150] is effective but less amenable for many thrombi, and appropriate conditions of frequency and energy must be utilized to disrupt and disintegrate clots.[151] Percutaneous, transvascular ultrasonic disruption of a femoral artery thrombus has been reported in animals[152,153] and patients.[154] Experiments suggest that transcutaneous ultrasound may induce thrombolysis when combined with systemic infusion of microbubbles, even in the absence of PA administration[152]; another potential mechanism of action may involve accelerated transport of plasminogen into the thrombus.[155] Ultrasound accelerates recanalization induced by systemic rt-PA in stroke patients.[156] The phase II CLOTBUST trial randomized patients with ischemic stroke to t-PA with or without continuous 2 MHz transcranial Doppler ultrasound, observing complete early recanalization or significant clinical recovery at 2 hours in 49% of treated patients versus 30% of controls ($P = 0.03$), but with no clinical difference at 24 hours and no difference in ICH (4.7% in both arms).[157] Optimism for improved results with better methodology is anticipated upon further work in this field,[158] which has even been applied with an intravascular device for effective lysis of dural sinus thrombosis, even in the face of preexisting subarachnoid and intraparenchymal hemorrhages.[159]

Catheter-Directed (Regional) Infusion

A recurrent issue concerns the administration of PA directly through a catheter onto or into a thrombus. The rationale for such regional therapy is compelling for arterial occlusions, because the thrombus is usually localized to a single vessel downstream from the catheter tip, and the catheter also provides a means for angiographic documentation of progress. Regional treatment of arterial thrombi does achieve better thrombolysis than systemic therapy, but activity of the PA is not restricted to the region in question. During the period of infusion, the lytic state may be comparable to that following systemic administration, and bleeding or ICH may complicate therapy.[160] Local therapy for venous thromboembolic disease achieves an impressive degree of vascular reperfusion,[161–163] although bleeding complications, including ICH, are not avoided.[164] Local intraarterial (IA) administration of fibrinolytics for the treatment of acute ischemic stroke has been studied in randomized trials.[165] Meta-analysis of five randomized trials of 395 patients treated with IA t-PA, UK, or pro-UK found an increased rate of complete recanalization (19% vs. 1.4%) versus controls as well as a doubling of beneficial neurologic outcomes.[166] There was an increased risk of symptomatic ICH with IA fibrinolysis, but no difference in overall mortality.[166] An approach of IV followed by IA delivery of rt-PA (IMS trials) is under assessment,[167] including a trial that combines IB/IA rt-PA with catheter-applied ultrasound.[168]

The main decision about regional treatment is the clinical exigency of the patient. For example, regional infusion into the coronary artery may dissolve clots more effectively, but systemic administration is more efficient for first-line treatment of patients with MI. Still, with maximization of effort such as has been applied to early angioplasty for AMI,[169] mechanical revascularization therapy rendered the use of local coronary artery delivery of thrombolytic agent obsolete.[170] Regional treatment of slower progressing illnesses such as peripheral arterial occlusion (PAO) and especially deep-vein thrombosis (DVT) and occluded central venous access devices is attractive. Catheter-directed therapy (CDT) for DVT applies multiple approaches to recanalize the thrombosed vessel, in addition to local PA infusion, including thrombectomy, thrombus disruption, angioplasty, and stent placement, an approach that has been termed "minimally invasive endovascular strategies."[171] Patency rates are high (up to 70%),[172,173] although a direct comparison with IV PA administration, which induces a 45% to 50% recanalization rate,[174] has never been performed. The CDT approach likely provides a better outcome in preventing the postphlebitic syndrome[175] and in managing difficult cases of DVT such as in the May-Thurner syndrome.[176] In the CaVenT study,[175] the 6-month iliofemoral patency rate was 66% using CDT versus 47% with conventional anticoagulant therapy ($P = 0.012$), and the 24-month incidence of the postphlebitic syndrome was 41% for CDT versus 56% for anticoagulant therapy ($P = 0.047$). Clinically relevant bleeding events occurred in 9% of patients, noted as an "acceptable" complication rate, but one that still merits careful attention to contraindications to such therapy.

Endovascular intervention in acute ischemic stroke, aiming to mechanically extract an occlusive thrombus, may be a useful adjunct ("rescue") following incomplete intravenous or IA thrombolysis.[177] In the RECANALISE trial, a prospective registry trial that compared outcomes using systemic t-PA versus systemic t-PA plus mechanical thrombectomy demonstrated improved functional outcome with the combined approach, with equivalent rates of ICH.[178] A multitude of novel retrieval or combined stent/retrieval devices has been manufactured and approved for clinical use in acute thromboembolic stroke. While high recanalization rates of 50% to 80% can be achieved, functional benefit is not universal, at least partly due to their use after delay of up to 8 hours after stroke onset.[166] Furthermore, despite high rates of recanalization, there is a 26% to 36% incidence of symptomatic ICH, and about one-third of cases end in death.[17] As for the treatment of PAO and DVT, CDT for stroke applies a multitude of recanalization approaches ("multimodal reperfusion therapy"),[179] including IA agent delivery,[165] clot retrieval,[177] ultrasound,[157] stent placement,[180] and mechanical disruption.

EFFECTS OF THROMBOLYTIC TREATMENT ON THE BLOOD

The lytic state is a general description of the effects of PA therapy in the circulation[181] (Table 112.4), beginning with a shortening of the euglobulin lysis time, a test that measures free activator. Laboratory parameters reflect the biochemical events that culminate in the degradation of plasma fibrinogen and a hypocoagulable state. The simplest definition of the lytic state is a decrease in plasma fibrinogen that is not due to laboratory artifact,[3] such as contamination of the sample with heparin. Platelet glycoprotein degradation may cause

Table 112.4

Development of the plasma proteolytic (lytic) state

Biochemical State	Laboratory Finding
Circulating plasminogen activator	Short euglobulin lysis time, platelet hyperreactivity
Plasminogen activation	Decreased plasminogen
Antiplasmin inhibits plasmin	Plasmin–antiplasmin complexes
Free plasmin	Increased chromogenic activity
Degradation of fibrinogen	Decreased clottable protein, increased D-dimer
Platelet dysfunction	Decreased platelet aggregation
Hypocoagulable state	Prolonged coagulation tests

decreased platelet aggregation with ADP or ristocetin,[182–184] sometimes following an initial hyperaggregability.[184] Patients may experience sequential hypercoagulability and hypocoagulability or even the simultaneous occurrence of these opposite influences, depending upon the phase of lytic state that exists.

Predictive Value of Laboratory Changes

Laboratory markers of hematologic events that occur after PA administration have potential relevance for predicting vascular events (Table 112.5). According to the concept that the actions of PAs in the blood and on the thrombus are separate events,[4] successful dissolution of the pathologic thrombus through fibrin degradation would not necessarily correlate with the extent of fibrinogenolysis (lytic state). Likewise, bleeding that would result from hemostatic plug disruption or vascular matrix degradation could occur independently of changes in blood hemostatic measurements.[3,4] An ongoing question is whether the degree of hypocoagulable state contributes to hemorrhagic events. Proponents of this thesis would state that a PA with fibrinogen-sparing properties causes less bleeding, because the induced hypocoagulability is minimized. Similar questions have been asked about the effect of blood changes on vascular reperfusion and reocclusion. The potential markers of bleeding could include any change in the lytic state, including a decrease in plasminogen, hypofibrinogenemia, increased D-dimer, and deranged platelet aggregation. Markers of vascular reperfusion as a surrogate of clinical recovery could potentially utilize lytic state markers, including changes in platelet function, or an increased D-dimer level (FIGURE 112.1).[185]

Bleeding Associated with Thrombolytic Therapy

Although patients who receive thrombolytic agents are at risk of bleeding, such complications are at best only weakly correlated with laboratory parameters of the lytic state. For example, UK or SK treatment of pulmonary embolism (PE) or DVT regularly induces a marked lytic state, but the degree of such changes in fibrinogen concentration, plasminogen, or euglobulin lysis time is not significantly different in patients with or without a hemorrhagic complication.[174,186] The direct comparison of t-PA and SK in the TIMI trial–phase I showed significantly lower fibrinogen values with SK than with t-PA, but the incidence of major and minor bleeding complications was the same with both treatments.[187] Furthermore, patients with ischemic (thrombotic) or hemorrhagic cerebrovascular accident (CVA) who were treated with t-PA had the same nadir concentration of fibrinogen.[188]

Table 112.5

Hematologic and vascular parameters of thrombolysis and potential for laboratory monitoring

Hematologic	Vascular	Potential Marker
Thrombolysis	Reperfusion	Hypofibrinogenemia, elevated D-dimer
Hemostatic plug or matrix disruption	Bleeding	Long bleeding time, hypofibrinogenemia
Hypercoagulable state	No reperfusion or occlusion	Increased prothrombin fragment 1+2, thrombin–antithrombin complexes, soluble fibrin
Hypocoagulable state	Late bleeding (with new injury)	Long bleeding time, persistent lytic state

Table 112.6

Rate of ICH in four studies of accelerated regimen alteplase (t-PA)

	GUSTO-I (1990–1993)		GUSTO-III (1995–1997)		COBALT		ASSENT-2	
	SK (17,804)	t-PA (9,039)	Reteplase (10,138)	t-PA (4,921)	Bolus t-PA (3,585)	t-PA (3,585)	Bolus tenecteplase (8,461)	t-PA (8,488)
Age <75 y	0.4	0.5	0.7	0.8	0.7	0.6	0.8	—
Age >75 y	1.2	2.5	2.5	1.7	3.6	2.6	1.7	2.6
Overall	0.5	0.7	0.9	0.9	1.1	0.8	0.93	0.94

GUSTO, Global Use of Strategies to Open Occluded Coronary Arteries; COBALT, Continuous Infusion Versus Double-Bolus Administration of Alteplase; ASSENT, Assessment of the Safety and Efficacy of a New Thrombolytic Regimen; SK, streptokinase; t-PA, tissue-type plasminogen activator.

The only predictive blood value for hemorrhagic complication during t-PA treatment is the plasma t-PA concentration (3.4 μg/mL in bleeders vs. 2.2 μg/mL; $P = 0.002$).[189] This suggests that t-PA has a direct effect on susceptible hemostatic plugs or vascular sites, more with higher concentrations, independent of changes in fibrinogen concentration. This effect helps to explain the higher rate of ICH with high therapeutic dosages of t-PA[190] and supports the concept that bleeding occurs independent of PA effect on blood coagulation, probably the result of action at sites of vascular injury or malformation.[4] A retrospective analysis of more than 40,000 patients in the GUSTO-I study found independent predictors of hemorrhage to be of older age, lighter body weight, female sex, and of African origin, rather than the degree of laboratory derangement.[191]

The template bleeding time is prolonged during thrombolytic therapy, either as a direct result of the PA or as a result of concomitant aspirin therapy, but there is no distinction in patients with or without hemorrhagic complication.[192] Thrombocytopenia of $<100^9$/L has been noted in 2.4% to 3.9% of patients undergoing coronary artery angioplasty and receiving abciximab, with approximately one-fourth of these patients having severe decreases of $<50^9$/L.[193] Thrombocytopenia was associated with more unfavorable outcomes, including bleeding episodes, and with lower baseline counts, increasing age, and decreased weight.

The occurrence of ICH with PA treatment is relatively infrequent but of great clinical importance in that more than 50% of patients have lethal outcomes or significant neurologic deficits. Although major hemorrhagic complications other than ICH are induced with approximately equal frequency by all of the PAs, the more potent agents such as t-PA have a consistently higher risk of inducing ICH. The reason is not clear; it is not likely to reflect the presence of a fresh hemostatic plug that is dissolved, but may be related to a vascular abnormality associated with amyloid deposition.[194] As with other bleeding complications, the degree of lytic state does not explain the event, as the fibrinogen level is the same in patients with ICH or with a thrombotic (ischemic) CVA.[188] Subcutaneous heparin added to aspirin during PA infusion increases the risk slightly (0.6% vs. 0.4%),[7] but the use of t-PA is the most important predictor,[54] along with age >65, weight <70 kg, hypertension on admission, and a prior history of transient ischemic attack or CVA.[190] A retrospective comparison of SK (41,000 patients), anistreplase (21,000 patients), and t-PA (41,000 patients) showed the incidence of ICH to be 0.2%, 0.6%, and 0.6%, respectively.[12] Recent observations of accelerated regimen t-PA versus SK, reteplase, or double bolus t-PA show an overall risk of approximately 0.8% for accelerated t-PA, with the risk in elderly patients (older than 75 years of age) being significantly higher (2.1%) (Table 112.6).[113,195,196]

Vascular Patency and Reocclusion

Laboratory results also do not predict vascular reperfusion or degree of patency. A reasonable hypothesis for noninvasive measurement of successful thrombolysis would be to measure plasma levels of crosslinked fibrin-degradation products (FDPs), but the data so far are not conclusive, with some showing high predictability for successful vascular reperfusion[197] whereas others fail to document such a correlation.[198] In the Urokinase Pulmonary Embolism Trial (UPET) study in which a large systemic dose of UK was administered, there was no correlation of laboratory results with decrease of embolus size,[186] and DVT trials also showed a lack of correlation of venographic change after PA therapy with degrees of alteration in fibrinogen, thrombin time, plasminogen, and FDP.[174] Even intracoronary administration of SK for treatment of AMI showed no difference in nadir plasma fibrinogen in patients who did or did not reperfuse,[199] and similar measurements in patients with AMI receiving t-PA also failed to show a correlation with posttreatment coronary artery patency.[189] Although there is no universal agreement, the data do suggest that changes in blood coagulation or fibrinolytic assays following PA administration do not directly reflect the degree of thrombus dissolution.

Prediction of coronary artery reocclusion in an individual patient is problematic, but trials using t-PA provide instructive information.[189] Among 55 patients with reocclusion, the depth of fibrinogen nadir was less (120 vs. 180 mg/dL) than in patients whose vessels stayed patent. The data suggest that a more profound lytic state protects against reocclusion and may partially explain the lower tendency for reocclusion with SK, UK, and anistreplase than with t-PA.[113,200,201]

Laboratory Monitoring

The major role for monitoring thrombolytic therapy with a PA would be to document that a given dose of agent has achieved a lytic state. There is no need to regulate the dose of PA based

Table 112.7
Absolute contraindications to fibrinolytic therapy

Risk	Condition
Intracranial bleeding	Hemorrhagic CVA, intracranial neoplasm, recent cranial surgery or trauma (within 10 d), uncontrolled severe hypertension
Massive hemorrhage	Major surgery of thorax or abdomen (within 10 d), prolonged cardiopulmonary resuscitation, current severe bleeding site

on laboratory markers, as these values neither improve the chance of thrombolysis nor decrease the risk of hemorrhagic complication. The exception to this rule is for therapy using direct thrombolytic agents such as plasmin, for which there is a direct relation of dose with plasma fibrinogen, and an apparent threshold of plasma fibrinogen above which hemostatic safety is anticipated.[48]

MANAGEMENT OF BLEEDING COMPLICATIONS

Prevention of Bleeding

The absolute contraindications to PA therapy are those that may result in ICH or massive, life-threatening hemorrhage (Table 112.7). Decisions about PA use must weigh the expected severity of a complication with the potential benefit of accelerated thrombolysis. Therefore, some patients with obvious life-threatening thrombotic disease, for example, massive PE with profound shock or a large anterior wall AMI, may warrant potentially life-saving thrombolytic therapy even in the face of "absolute" contraindications. On the other hand, patients with a relatively less threatening thrombotic disease such as DVT would reasonably not receive PA therapy if there is a relevant history that significantly increases the risk of ICH, for example, transient ischemic attack, prior thrombotic stroke, or current hypertension. The risk of major bleeding after recent surgery of the head, thorax, or abdomen is obvious, but some clinical situations are associated with an increased risk of symptomatic but not serious bleeding, for example, patients with superficial wounds, arteriotomy or venotomy, active menses, and minor surgical or biopsy procedures. Elderly patients have a higher risk of bleeding, especially ICH, but they also have a higher risk of death from AMI than do younger patients. Therefore, the survival benefit in patients older than 70 years is relatively more than in patients younger than 60 years (80 vs. 25 lives saved per 1,000 treated).[90,202]

Management of Acute Bleeding and Posttreatment Surgery

In patients who have life-threatening bleeding or sudden emergencies that require surgical intervention, normal hemostasis can quickly be reestablished by discontinuing PA infusion, replenishing plasma fibrinogen with either whole plasma or cryoprecipitate, and providing fresh platelets to correct a prolonged bleeding time (Table 112.8). If the PA is not yet cleared from the circulation, fibrinolytic activity can be neutralized by infusion of fibrinolytic inhibitor such as ε-aminocaproic acid.

There are cases of coronary artery bypass graft surgery after SK usage, indicating that the operation can be performed immediately,[203,204] but that such therapy may result in bleeding complications and the need for blood transfusion.[205] Such results are relevant for invasive procedures after t-PA treatment as well, even if performed after a delay of 12 and sometimes 24 hours.[206] In the TIMI-IIA study,[189] angioplasty performed immediately after stopping t-PA resulted in a considerably greater need for blood transfusion than if performed 18 to 48 hours later. The reason for bleeding, coincident with interventional procedures after t-PA, which is cleared quickly from the circulation, is that the nadir fibrinogen remains low for 24 hours.[189] Plasma fibrinogen concentrations are ultimately achieved by hepatic synthesis of fresh fibrinogen, a process that requires 24 to 36 hours to accomplish, irrespective of the PA that was administered.

In anticipation of invasive surgery immediately after PA treatment, it is reasonable to replenish fibrinogen with cryoprecipitate because essentially no normal fibrinogen is present during SK[207] or t-PA infusion[208] and to restore platelet function with fresh platelet transfusion for up to 24 hours after PA administration. In addition, antifibrinolytic therapy should be administered prophylactically if activator is still circulating, an expected situation for variable times after the infusion is stopped.[209] Once a patient is to be treated with a PA, unnecessary trauma to the

Table 112.8
Hypocoagulable state induced by PA

	During Treatment	Immediately After Treatment	6–36 h After Treatment
Plasma fibrinogen	Low	Nadir	Recovery
Need for cryoprecipitate	Yes	Yes	Variable
Bleeding time	Long	Variable	Normal[a]
Need for platelets	Yes	Yes	No
Circulating PA	Present	Variable	Absent
Need for antifibrinolytics	Yes	Yes	Variable

[a]Unless aspirin has been administered, in which case the potential for bleeding persists.

patient should be avoided. One should administer medication by mouth or intravenously, but not intramuscularly. Phlebotomy sites should be compressed for 5 to 10 minutes, unnecessary arteriotomy should be postponed or avoided, and rough manual manipulation of the patient should be minimized. The use of intravenous or subcutaneous heparin in addition to aspirin and a PA increases the risk of bleeding.[90,118]

Bleeding that occurs during PA infusion should be managed appropriately with blood replacement and, if necessary and possible, by discontinuing the PA treatment. If bleeding is to be controlled immediately, the PA must be neutralized by a fibrinolytic inhibitor, and cryoprecipitate and platelets should be transfused. Recent and unhealed vascular trauma sites are susceptible to bleeding for approximately 10 days. Depending upon their location, a bleeding complication can be unimportant (ecchymoses and hematuria), bothersome and requiring transfusion (duodenal ulcer and accessible surgical site), or life-threatening (thoracic or cranial surgery, recent or even remote stroke).

Other Side Effects and Complications

Termination of treatment may be necessitated by allergic or pyrogenic side effects, which could also be managed with corticosteroids, antihistamines or adrenergic agents for anaphylaxis, and antipyretics for fever. Allergic reactions are three to five times more frequent using bacterial derived products than rt-PA-type derivatives, but patients who receive t-PA may rarely manifest allergic reactions such as bronchospasm or angioneurotic edema.[210,211] A second side effect is hypotension, especially in patients with AMI, occurring in approximately 12% of patients exposed to SK or anistreplase and 7% of those exposed to t-PA, in comparison with only 1.5% of patients not exposed to a PA.[212] Hypotension occurred in a significant proportion of patients treated during clinical trial of alfimeprase, which is known to cause bradykinin release.[88]

Embolic phenomena of clinical importance are rare in patients with venous thromboembolic disease who receive PA treatment. In the UPET trial,[186] recurrent PE occurred in 15% of patients who received either UK or heparin. A venous thrombus breaking off to become a fatal pulmonary embolus is distinctly unusual and may have alternative explanations other than lysis of a vulnerable, free-floating thrombus. On the other hand, distal systemic embolization from a cardiac source during PA treatment, although unusual, does occur, and up to 20% of patients with PAO are affected by sudden new ischemia of the distal extremity.[213] In this circumstance, the best treatment approach is continued local instillation of PA, which usually succeeds in dissolving the new embolus. Rarely, systemic treatment with PA results in cholesterol crystal embolization, usually in the setting of extensive preexisting atherosclerotic disease.[214]

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CHAPTER

113

Anticoagulant and Procoagulant Proteins in the Animal Kingdom

ANGELINA WEI LING TAN, SHIYANG KWONG, AND R. MANJUNATHA KINI

Blood circulation has a crucial role in the survival of vertebrate animals, including man. It transports oxygen and nutrients to every tissue and organ and carries waste products and carbon dioxide to be removed via the liver, kidneys, and lungs. It also has a vital role in defense against a wide range of pathogens by recruiting leukocytes and other immune cells to the appropriate site in case of a pathogen attack. Thus, any problem with the hemostatic system can lead to death or severe debility, and therefore, it is normally tightly regulated by a complex system of proteins and cells that prevent blood loss from injuries or major blockages by pathologic thrombi and emboli.

Various animals have developed mechanisms to target the hemostatic system and exploit this vulnerability for their own ends. In some species (e.g., snakes), such mechanisms are used to immobilize and kill the prey/victim, and these are also used as defensive strategies against attacks by larger animals. In others (e.g., leeches, or insects such as mosquitoes and ticks), they are used to provide a continuous supply of blood as food. In all these animals, the mechanisms have evolved to perfection over millions of years, in parallel with the development of their hosts, to allow their survival. Since many of the components present in venom and saliva target critical hemostatic pathways, they have been of great interest in basic research into the mechanisms of these pathways, as well as in potential therapeutic applications to modify disease. Over the last three to four decades, due to the efforts of scientists from various backgrounds including biology, protein chemistry, molecular biology, pharmacology, hematology, and structural biology, significant progress has been made in understanding the structure–function relationships, as well as the mechanism of action of a number of exogenous factors from various animals, that affect blood coagulation. These exogenous factors have contributed significantly to the development of research tools as well as providing new therapeutic agents. In this chapter, we describe anticoagulant and procoagulant proteins that have been reported from the animal kingdom. Their mode of inhibition or activation of the blood coagulation cascade together with their structure–function relationships is also discussed.

ANTICOAGULANTS

Anticoagulants may inhibit the blood coagulation cascade through either direct or indirect mechanisms. In nature, animals make use of anticoagulants to their advantage, to kill their prey or to feed off their hosts. As these anticoagulants evolved independently and for different purposes, they differ greatly in function and structure.

Anticoagulants from Snake Venoms

Snakes feed on a variety of animals, including snails, fishes, frogs, toads, lizards, chickens, mice, rats, and even other snakes. As they lack the ability to tear apart the flesh of their prey, they resort to swallowing them whole. Hence, the venoms of snakes have evolved as a complex mixture of pharmacologically active proteins and peptides that have the ability to incapacitate, immobilize, and even digest their prey. These molecules include neurotoxins, cardiotoxins, hemotoxins, and cytotoxins, which target crucial physiologic systems of the prey as well as a number of hydrolytic enzymes including phospholipases, proteinases, and nucleotidases. Hence, exposure to these toxins during a snake bite often leads to symptoms ranging from tissue damage and paralysis to hypotension and hemolysis. Over the years, numerous toxins that contain anticoagulant properties have been isolated and characterized. These proteins and glycoproteins have molecular masses ranging from 6 to 90 kDa and inhibit blood coagulation through a variety of mechanisms. They can be classified under enzymatic or nonenzymatic anticoagulant proteins. As not all of the mechanisms of these anticoagulant proteins are entirely well understood, further research of these proteins is essential to delineate their mechanism and structure–function relationships.

Anticoagulant Enzymes

Phospholipases A_2

Phospholipases A_2 (PLA_2) enzymes are esterolytic enzymes that specifically hydrolyze the *sn*-2 acyl bond of phospholipids, releasing lysophospholipids and fatty acids. Snake venoms are a rich source of these PLA_2 enzymes, and hundreds of them have been previously purified and characterized.[1,2] Approximately 13 kDa in size, they usually consist of 116 to 124 amino acid residues and have 6 to 7 disulphide bonds. Typically, the structure of PLA_2 enzymes comprises a core of three α-helices, a calcium-binding loop, and a β-wing composed of antiparallel β-sheets (FIGURE 113.1). Although they share this common scaffold with nontoxic mammalian enzymes, most snake venom PLA_2 enzymes are toxic and induce diverse pharmacologic effects, including cardiotoxic, myotoxic, neurotoxic, hemolytic, convulsive, anticoagulant, antiplatelet, and edema-inducing and tissue-damaging effects.[3,4] Based on their anticoagulant properties, PLA_2 enzymes have been classified into strongly, weakly, or nonanticoagulant enzymes.[5,6] Strongly anticoagulant PLA_2 enzymes inhibit blood coagulation below 2 μg/mL, whereas weakly anticoagulant enzymes inhibit between 3 and 10 μg/mL. Nonanticoagulant enzymes do not display anticoagulant activities even at 15 μg/mL. As many coagulation complexes

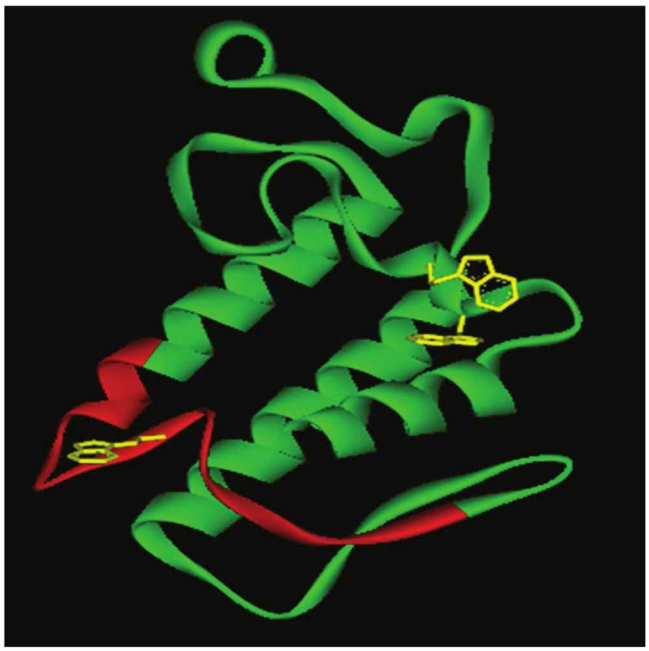

FIGURE 113.1 Three-dimensional structure of snake venom PLA_2. The ribbon model of *Naja naja* PLA_2 was generated from the PDB file #1POA. The predicted anticoagulant region is highlighted in *red*. Trp residues are shown in *yellow*. (Reprinted from Kini RM. Structure-function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. *Toxicon* 2005;45(8):1147–1161, with permission.)

depend on phospholipids for their formation and function, it was thought that PLA_2 enzymes exhibit their anticoagulant function primarily through their ability to hydrolyze phospholipids. However, the results from the experiments to study the role of phospholipid hydrolysis in the anticoagulant effects of PLA_2 enzymes were contradictory. For example, several studies indicated that the enzymatic activity of PLA_2 enzymes is essential for their anticoagulant activities. (a) The modification of active site His48 by *p*-bromophenacyl bromide led to the loss of both enzymatic and anticoagulant activities. (b) The replacement of hydrolyzable phospholipids by nonhydrolyzable phospholipids led to loss of anticoagulant activities. (c) Longer times of incubation of PLA_2 enzymes with plasma led to increased phospholipid hydrolysis and clotting times. On the other hand, several other studies suggested that the enzymatic activity of PLA_2 enzymes is *not* essential for their anticoagulant activities. (a) The catalytic efficiency of anticoagulant PLA_2 enzymes did not correlate with their anticoagulant potency. (b) There is a lack of correlation between plasma lipid hydrolysis and anticoagulant effects of the PLA_2 enzymes. (c) The addition of antibodies to PLA_2-hydrolyzed phospholipids reversed the anticoagulant effects. (d) Chemical modifications have shown that the loss of enzymatic activity led to little effect on anticoagulant effects, and *vice versa* (for details, see review[7]). The controversies in the field were essentially due to two critical but wrong assumptions, namely, the anticoagulant mechanism of different PLA_2 enzymes is the same and the phospholipid requirements of different coagulation complexes are alike.

To resolve the controversies and decipher the mechanism of anticoagulant activity, three anticoagulant PLA_2 enzymes—CM-I, CM-II, and CM-IV from the venom of the black-necked spitting cobra (*Naja nigricollis*), were studied.[8] Based on their anticoagulant properties, CM-I and CM-II are classified as weakly anticoagulant enzymes, whereas CM-IV, which is at least 100-fold more potent than CM-I and CM-II, is classified as strongly anticoagulant enzyme.[9] As a first step, the coagulation complexes that are inhibited by these enzymes were identified using the "dissection approach" (see FIGURE 113.2), followed by their effects on reconstituted coagulation complexes. CM-IV was reported to inhibit the coagulation cascade at two different points—the extrinsic tenase complex as well as the prothrombinase complex.[10] Further studies have identified that while it inhibits the extrinsic tenase complex through a combination of enzymatic and nonenzymatic mechanisms, the inhibition of the prothrombinase complex, however, is independent of phospholipid hydrolysis.[11] This inhibition is achieved through competing with factor Va (FVa) in binding to factor Xa (FXa), hence blocking the formation of the prothrombinase complex. It forms a 1:1 complex with FXa, with a dissociation constant (K_d) of 500 nM.[12] On the other hand, CM-I and CM-II inhibits only at a single point in the coagulation cascade, at the extrinsic tenase complex, solely through enzymatic hydrolysis of phospholipids. Unlike CM-IV, CM-I and CM-II display no effect toward the prothrombinase complex.[13]

As mentioned previously, although PLA_2 enzymes possess similar folds and three-dimensional (3-D) structures, they display a vast array of biologic functions. This indicates that their structure–function relationships are subtle and complex. By a systematic analysis, the anticoagulation site was identified to be situated between residues 54 and 77 in strongly anticoagulant PLA_2 enzymes.[13] This predicted site was well supported by subsequent experiments.[9,14–16] This region is positively charged and is located on the surface of the molecule, thus accessible for interactions (FIGURE 113.1). This region contains four positively charged lysine residues that may play a role in intermolecular interactions.[9,16] In the case of protein CM-IV, a comparison of amino acid sequences with bovine FX, FV, prothrombin, and tissue factor (TF) revealed that half of CM-IV's anticoagulant region was homologous with the light chain of FVa and the other half with a section of TF.[11] These two sites are hypothesized to be responsible for targeting the prothrombinase and the extrinsic tenase complexes respectively.[17]

Metalloproteinases

Metalloproteinases are zinc-dependent endoproteolytic enzymes that cleave peptide bonds in the middle of the proteins. They form three different classes, P-I, P-II, and P-III, based on their domain structure and size.[18,19,20] The snake venom metalloproteinases exert digestive (of prey), hemorrhagic, procoagulant, anticoagulant, and antiplatelet effects.[18] Most snake venom metalloproteinases are α-fibrinogenases that selectively cleave the Aα chain of fibrinogen. They inhibit blood coagulation as they produce truncated fibrinogen, which is unable to form a fibrin clot as stable as the native fibrinogen. The structure–function relationships of these metalloproteinases with respect to their anticoagulant effects have not yet been studied.

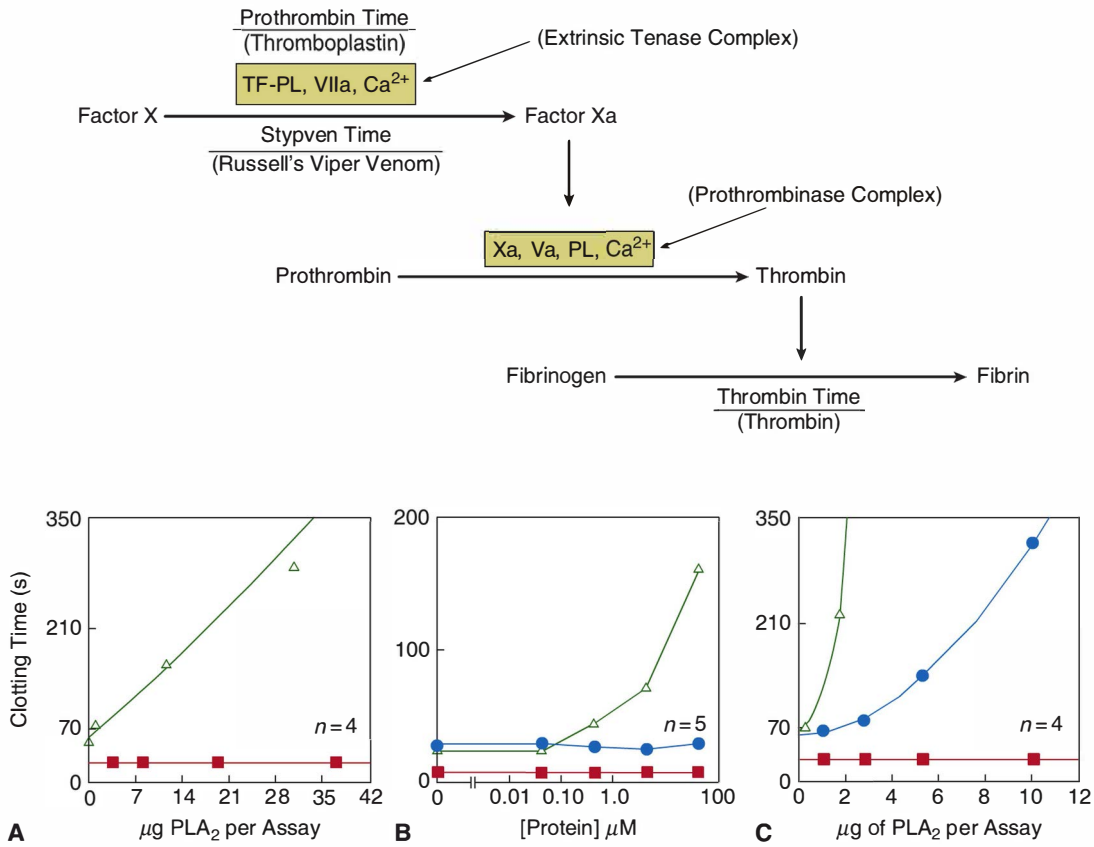

FIGURE 113.2 The dissection approach. This is a simple strategy designed to determine which particular step an anticoagulant is interfering with in the extrinsic coagulation pathway **(upper panel)**. It comprises three common clotting assays—prothrombin time, Stypven time, and thrombin time. The concept is if the cascade is initiated "upstream" from the point of inhibition, clotting times will be prolonged, whereas if initiated "downstream," clotting times will not be affected. In the example **(lower panels)**, the strongly anticoagulant CM-IV (*C*) prolonged the prothrombin time (Δ) and Stypven time (•), but not the thrombin time (❑). Hence, it can be concluded that CM-IV does not inhibit the conversion of fibrinogen to fibrin clot, but may affect either the prothrombinase complex alone or both the extrinsic tenase and prothrombinase complexes. In contrast, weakly anticoagulant CM-I (*A*) and a three-finger toxin, protein A (*B*) prolonged only the prothrombin time, but not the Stypven time and the thrombin time. These results suggest that these proteins affect only the extrinsic tenase complex but not the prothrombinase complex and the conversion of fibrinogen to fibrin clot. This strategy is applicable to any anticoagulant affecting the extrinsic coagulation cascade. (Reprinted from Kini RM, Banerjee Y. Dissection approach: a simple strategy for the identification of the step of action of anticoagulant agents in the blood coagulation cascade. *J Thromb Hemost* 2005;3:170–171, with permission.)

Serine Proteases

Protein C activators are the only serine proteases that indirectly inhibit coagulation. Under normal physiologic conditions, protein C is activated by thrombin and thrombomodulin to activated protein C (APC). APC exerts its anticoagulant activity by degrading FV/FVa and FVIII/FVIIIa. It also stimulates fibrinolysis by interacting with plasminogen activator inhibitor.[21] Protein C activators are approximately 36 to 40 kDa in molecular weight, and they activate protein C in the absence of Ca^{2+} with low salt concentrations.[22–24] A protein C activator isolated from the venom of *Agkistrodon contortrix contortrix* activates protein C through thrombomodulin-independent mechanisms.[22] The stretch of positively charged residues on its interfacial surface, together with three carbohydrate moieties surrounding the catalytic site, are pivotal in protein C recognition, binding, and activation.[25]

Nonenzymatic Anticoagulants

C-type Lectin-related Proteins

C-type lectins are calcium-dependent carbohydrate-binding proteins that agglutinate red blood cells. They usually exist as homodimers. On the other hand, C-type lectin-related proteins are usually heterodimers or oligomeric complexes of heterodimers, and they do not have lectin-like activity.[26,27,28] Anticoagulant C-type lectin-related proteins were one of the first few nonenzymatic proteins isolated and characterized from snake venoms.[17] From the venoms of the hundred-pace pit viper (*Deinagkistrodon acutus*) and Stejneger's bamboo viper (*Trimeresurus stejnegeri*), anticoagulant proteins that inhibit prothrombinase activation were isolated and purified.[29–32] Although they were shown to perform inhibition through nonenzymatic mechanism, details on their mechanism and structure–function relationships are still unclear. A specific FIX-binding protein

and FX-binding protein from the venoms of *Protobothrops flavoviridis* (formerly, *Trimeresurus flavoviridis*) and *D. acutus*, respectively, were isolated and characterized. Both are heterodimeric, with α- and β-chains, and both chains are structurally similar to C-type lectins. They were shown to bind and inhibit the coagulation factors FIX and FX respectively, with nanomolar and subnanomolar affinities. The dimeric interface created by domain swapping recognizes the Gla domain of FX and FIX.[33]

Another group of anticoagulant C-type lectin-related proteins interact specifically with thrombin/prothrombin. This includes bothrojaracin[34,35] from *Bothrops jararaca* and bothroalternin[36] from *Bothrops alternatus* venoms. Bothrojaracin inhibits thrombin directly through both exosite 1 and exosite 2, which accounts for its high affinity toward thrombin with a K_d of 0.6 nM. However, it does not bind to the active site of thrombin. Due to its binding to thrombin's exosite 1, it also inhibits FV activation by thrombin.[37] In prothrombin, bothrojaracin binds to partially exposed anion-binding exosite (pro-exosite 1) with a K_d of 175 nM, thus inhibiting the activation of prothrombin to thrombin.[38]

Three-finger Toxins

Three-finger toxins are a family of nonenzymatic proteins that are commonly found in the venom of snakes.[39] They range from 60 to 74 amino acid residues in length. Their structure is unique, with three β-stranded loops extending from a central core, which is held by four to five conserved disulphide bridges. This gives it an appearance of three extended fingers, hence its name. Despite the overall similarity in structure, three-finger toxins display a great diversity of biologic functions. Members of this family include α-neurotoxins,[40,41] κ-bungarotoxins,[42] muscarinic toxins,[43] fasciculins,[44] calciseptine,[45,46] cardiotoxins (cytotoxins),[47,48] dendroaspins,[49] and anticoagulant proteins.[50,51] An example of an anticoagulant three-finger toxin is the Hemextin AB complex, isolated from the ring-necked spitting cobra, *Hemachatus haemachatus*.[51] This complex consists of two subunits, Hemextin A and Hemextin B, and inhibits the extrinsic tenase complex with a K_i of 50 nM. Interestingly, subunit Hemextin B by itself does not display any anticoagulant effects, but instead synergistically enhances the anticoagulant effects of Hemextin A by forming a complex with it.[52]

Anticoagulants from Hematophagous Animals

In hematophagous organisms, blood meals from their hosts are crucial for survival. This process is not trivial as the hosts defend themselves by a variety of responses at the feeding site, which involves platelet activation and aggregation, local vasoconstriction, blood coagulation, and raising an immune response. To overcome such host responses, blood-feeding organisms produce a pharmacopeia of molecules in their saliva, from anticoagulants and platelet aggregation inhibitors to vasodilators, anti-inflammatory, and immunomodulatory molecules. The blood-feeding habit is found in several thousands of species in nature, and in arthropods alone blood-feeding habit has evolved at least 20 times independently, resulting in a vast molecular diversity of antihemostatic molecules. Thus, it is not uncommon to observe functional diversity among closely related proteins and functional convergence among structurally unrelated proteins. The saliva of these animals provides an excellent source of novel anticoagulant proteins and peptides, which differ in structure and mechanism of action. Based on this mechanism of action of the anticoagulants, they can be broadly classified into thrombin inhibitors, FXa inhibitors (FXaIs), extrinsic tenase complex inhibitors, and intrinsic tenase complex inhibitors.

Thrombin Inhibitors

There are several classes of thrombin inhibitors isolated from hematophagous animals (FIGURE 113.3). Each class of thrombin inhibitors shows distinct protein folds, and hence, they have evolved through convergent evolution. They also inhibit thrombin by different mechanisms.

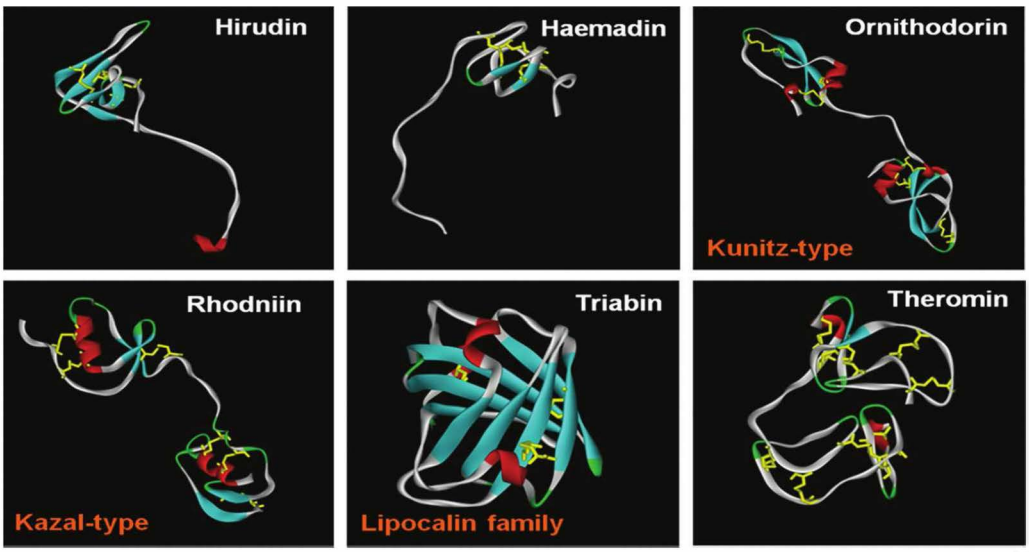

FIGURE 113.3 Structural diversity of thrombin inhibitors. Thrombin inhibitors have been isolated from a multitude of hematophagous animals. As the feeding habit of these animals evolved independently at least 20 times in over 15,000 species of arthropods in more than 400 genera, it is of no surprise that nature has produced such a wide array of thrombin inhibitors. As seen in the figure, although all six molecules inhibit thrombin, they come in different shapes and sizes, each binding to thrombin in their own unique ways. There are at least nine other structural classes of thrombin inhibitors whose 3-D structures are not yet known.

Hirudin

Hirudin, isolated from the medicinal leech, *Hirudo medicinalis*, is probably the most prominent and well-characterized thrombin inhibitor. Reported more than 100 years ago, hirudin is a 65-residue protein that is specific for thrombin, with a K_i of 22 fM.[53] A sulphated tyrosine (Tyr64) on hirudin has been identified to play a significant role in interactions with thrombin, which, when removed, results in a binding to thrombin 10 times weaker.[54] Hirudin's first three *N*-terminal residues bind to the hydrophobic pocket of thrombin's active site in a noncanonical form, through hydrogen bonds. The *C*-terminus on the other hand is rich in acidic residues and through specific electrostatic interactions binds to exosite-I of thrombin.[55–59] Thus, via these two interactions, hirudin inhibits specifically thrombin as a tight-binding inhibitor. Apart from hirudin, there have also been many inhibitors from other species of leeches that belong to this family of proteins that were subsequently isolated.[60,61]

Haemadin

Another molecule, haemadin, was also isolated from leeches. Found in *Haemadipsa sylvestris*, the Indian leech, haemadin is 57-amino acid residue in length, slightly smaller than hirudin.[62] It is a slow and tight-binding inhibitor of thrombin, with a K_i of 210 fM. Although haemadin's sequence similarity with hirudin is low, they share a common 3-D fold, where its globular *N*-terminal core is stabilized by three disulphide bridges with an extended *C*-terminal tail.[63] Like hirudin, the first three *N*-terminal residues bind to active site of thrombin in a noncanonical form. However, haemadin's acidic *C*-terminus binds to thrombin's exosite-II, instead of exosite-I as in the case of hirudin[63,64] (see FIGURE 113.4).

Kunitz-type Thrombin Inhibitors

The Kunitz-type inhibitors are a family of proteins belonging to the serine protease inhibitors.[65] They have a characteristic reactive-site loop, which binds and runs antiparallel to the enzyme active site residues. This type of inhibitors is commonly found in ticks. However, based on their sequences, there appears to be two different subclasses of these inhibitors, which originate from the two different families of tick—Ixodidae (hard ticks) and Argasidae (soft ticks). From the hard ticks, inhibitors that have been isolated include amblin from *Amblyomma hebraeum*,[66] boophilin from *Boophilus microplus*,[67] and haemalin from *Haemaphysalis longicornis*.[68] With two tandem Kunitz domains, these proteins generally have a lower affinity for thrombin as compared to those from the soft ticks (see below), with a K_i of 20 nM for amblin[66] and 1.8 nM for boophilin.[67] On the other hand, Kunitz-type inhibitors from soft ticks include ornithodorin from *Ornithodoros moubata*,[69] savignin from *Ornithodoros savignyi*,[70,71] and monobin from *Argas monolakensis*.[72] They are slow, tight-binding, and competitive inhibitors of thrombin, with a K_i of 4.89 pM for savignin[70] and 7 pM for monobin.[72]

Kazal-type Thrombin Inhibitors

Another common family of serine protease inhibitors are the Kazal-type inhibitors.[65] While the classical Kazal domains have the first two cysteine residues separated by seven to eight residues, the nonclassical Kazal domains has only one to two spacer residues. Generally, thrombin inhibitors belonging to this family of proteins contain tandem nonclassical Kazal domains and bind to thrombin in a slow, tight-binding, and competitive mode. In these inhibitors, the first domain binds to the active site of thrombin canonically while the second domain, together with interdomain linkers, binds to exosite-I. Examples of exogenous Kazal-type thrombin inhibitors include rhodniin from the triatomid bug, *Rhodnius prolixus*,[73,74] dipetalogastin from the blood-sucking bug, *Dipetalogaster maximus*,[75] and infestin from the assassin bug, *Triatoma infestans*.[76] Kinetically, the K_i values for rhodniin, dipetalogastin (domain 3 to 4), and infestin (domain 1 to 2) are 0.2,[73] 0.05,[75] and 25 pM,[76] respectively.

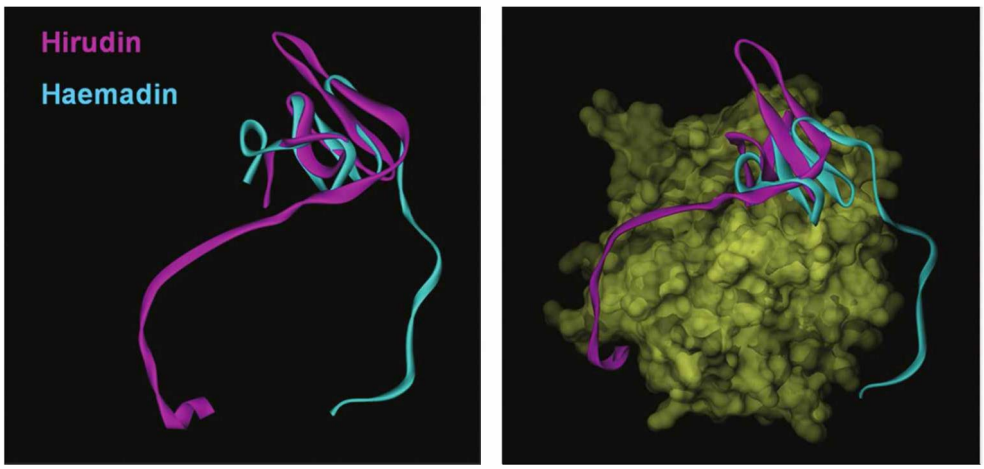

FIGURE 113.4 Identical fold but distinct mechanisms: hirudin versus haemadin. These two molecules are thrombin inhibitors isolated from the medicinal leech and the Indian leech, respectively. Although the two molecules display low sequence similarity, they share a common 3-D structure. Despite this, both molecules inhibit thrombin through distinct mechanisms. Hirudin targets thrombin's active site and exosite-I, whereas haemadin binds to the thrombin's active site and exosite-II. This is due to marked differences in haemadin's *C*-terminus where it lacks a sulphated tyrosine and complex formation with thrombin is independent of ionic strength, as in the case of hirudin. This classic example highlights how proteins of different amino acid sequences are able to have similar 3-D folds, while proteins of similar structures are able to bind and inhibit their target molecule through different mechanisms.

Variegin

Recently, a novel class of thrombin inhibitors have been identified from the tropical bont tick, *Amblyomma variegatum*. Named variegin, this fast and tight-binding thrombin inhibitor is one of the smallest thrombin inhibitors found in nature, with a length of only 32 residues.[77] Structurally, variegin is flexible as it lacks secondary structures. Despite this and its small size, variegin is highly specific to thrombin and binds with a K_i of 10.4 pM.[77] It does this by binding to thrombin's exosite-I with its *C*-terminus and the active site with its middle region. Its *N*-terminus is found to be responsible for its fast-binding kinetic properties. Interestingly, comparing structure and function, variegin resembles bivalirudin,[78] which is the product of a human-designed, bivalent thrombin inhibitor. It is fascinating to envisage the independent development of bivalirudin through rational drug design[78] and compare it with nature's own design of variegin through evolution and natural selection.

FXa Inhibitors

Kunitz-type FXa Inhibitors

Two anticoagulants, tick anticoagulant peptide (TAP)[79] and FXaI,[80] were isolated from the soft ticks *O. moubata* and *O. savignyi* respectively. These two inhibitors belong to the Kunitz-type FXaIs. Unlike the Kunitz-type thrombin inhibitors, TAP and FXaI contains only a single Kunitz domain with 60 and 56 amino acid residues, respectively. Both anticoagulants are slow, tight-binding, and competitive inhibitors of FXa.[79,81] TAP inhibits FXa through a noncanonical active site inhibition, where its first three *N*-terminal residues are in multiple contacts with the FXa active site and catalytic triad.[82] TAP also interacts with some of the residues on the Na^+-binding site and on the autolysis loop of FXa.[82]

Ascaris-type FXa Inhibitors

The Ascaris family of serine protease inhibitors is distinguished by 10 cysteine residues that form a unique disulphide pattern in a single domain.[83] Several Ascaris-type FXa-binding proteins have been reported from the hookworms *Ancylostoma caninum* (NAP5/6 and NAPc2/3/4)[84,85,86] and *Ancylostoma ceylonicum* (AceAP1).[87] While NAP5/6 and AceAP1 bind to the active site of FXa,[86,87] NAPc2/3/4 binds to an exosite.[84,86] As NAPc2/3/4 and AceAP1 exploit the binding of FXa to inhibit the FVIIa–TF complex, they are considered as extrinsic tenase complex inhibitors (see below for details). NAP5 inhibits FXa canonically at the active site, with a K_i of 43 pM.[86] Similar to TAP, it also interacts with the Na^+-binding site and autolysis loop of FXa.[88]

Antistasin-like FXa Inhibitors

Many leeches have anticoagulants that contain antistasin-like domain. This class of FXaIs includes antistasin,[89,90] ghilanten,[91] and therostasin.[92] Antistasin is a 119-amino acid protein that was isolated from the Mexican leech, *Haementeria officinalis*. It contains two tandem antistasin-like domains, which have 10 cysteines that form five intradomain disulphide bridges. Antistasin is a slow, tight-binding, and competitive inhibitor, with a K_i of 0.3 to 0.6 nM. Inhibition of the active site of FXa is achieved through the canonical reactive-site loop residing in the *N*-terminal domain.[93] In therostasin, although this canonical reactive-site loop and the domain signature are conserved, its overall sequence similarity with antistasin is low.[92] It is noted that therostasin is more potent than antistasin, with a K_i of 34 pM.

Serpin Family of FXa Inhibitors

Serpins are a superfamily of proteins (45 to 55 kDa) that undergo huge conformational changes and form a covalent complex with the target protease, thereby inhibiting it irreversibly.[94] An example of a FXaI that belongs to this class of proteins is found in the saliva of the yellow fever mosquito, *Aedes aegypti*. The crude salivary gland extract of female *A. aegypti* was found to inhibit the FXa active site reversibly and noncompetitively.[95] Fifty-four kilodalton in size, this protein, named as anticoagulant factor Xa (AFXa), contains posttranslational modifications (four *N*-linked glycosylation sites), which are likely to be important for its activity. However, as compared to typical serpins, AFXa has a shorter reactive-site loop and different hinge residues.

Extrinsic Tenase Complex Inhibitors

There are two main classes of extrinsic tenase complex inhibitors that have been isolated from hematophagous animals. Both classes act through a similar, but not identical, mechanism as the physiologic TF pathway inhibitor. The first class of inhibitors is the Kunitz-type inhibitors. Two such inhibitors have been isolated from the hard tick, *Ixodes scapularis*—ixolaris[96] and penthalaris.[97] Ixolaris, 15.7 kDa in size, has two tandem Kunitz domains. It was postulated that the second Kunitz domain binds first to FX/Xa, then followed by the FVIIa–TF complex via the first Kunitz domain.[96] Ixolaris binds to FX and FXa with affinities between 0.5 and 10 nM, but not at the FXa active site.[98] The surface amino acid residues of F X/Xa that are involved in the binding of ixolaris overlap largely with the heparin-binding proexosite/exosite.[98,99] This binding of ixolaris to FX and FXa impairs their interactions with FVIIIa and prothrombin, respectively.[98,99] Penthalaris, on the other hand, has five Kunitz domains, as compared to only two in ixolaris. Penthalaris inhibits the FVIIa–TF complex in the same way as ixolaris, making use of FX or FXa as a scaffold. The contribution of the three additional Kunitz domains of penthalaris to the interaction/function has yet to be elucidated.[97]

The second class of extrinsic tenase complex inhibitors is the Ascaris-type inhibitors, isolated from the hookworms *A. caninum*[84] and *A. ceylanicum*.[84,100] They are single-domain Ascaris-type inhibitors, such as NAPc2 and AceAP1. NAPc2 binds to the FXa heparin-binding exosite with its extended *C*-terminus, before binding to the FVIIa–TF complex.[101] In contrast, AceAP1 uses FXa as a scaffold by binding to both its active site and exosite and forms a quaternary complex with the FVIIa–TF complex.[84,100]

Intrinsic Tenase Complex Inhibitors

To date, nitrophorin-2 (or prolixin-s), isolated from *R. prolixus* (kissing bug), is the only intrinsic tenase complex inhibitor that has been identified from animals.[102] Approximately 20 kDa in molecular weight, it belongs to the lipocalin family. By targeting the Gla-domain of FIX/FIXa, nitrophorin-2 binds specifically to FIX/FIXa with a K_d of about 13 nM. This binding interferes with FIX activation (by both the FVIIa–TF complex and FXIa) and with FIXa activity in the intrinsic tenase complex.[103] The surfaces on nitrophorin-2 involved in FIX/FIXa binding have been suggested to be within its B-C and E-F strands as well as its *C*-terminus.[104]

PROCOAGULANTS

Procoagulants are agents that promote coagulation of blood. Often, these molecules either mimic the action of coagulation factors or activate them. Most procoagulant toxins characterized are proteinases, and majority of them are found in snake venoms. Until 1992, it was thought that only snake venoms had procoagulant activities.[105] This was due to difficulty in collecting venoms of other species, as their venoms were low in yield. However, with the progresses in technology, procoagulant toxins are also being isolated and characterized from spiders and scorpions. This section describes procoagulant toxins found in snakes and other venomous species.

Procoagulants from Snakes

In addition to the plethora of anticoagulant molecules, snake venoms are also a rich source of procoagulant toxins, many of which have been purified and characterized. These procoagulant toxins are serine proteases or metalloproteinases, and they promote blood clotting by activating zymogens, such as FX or prothrombin, to initiate blood clotting.

FX Activators

Venom toxins, which activate FX, are either metalloproteinases or serine proteases. These toxins can be found in venoms of snakes in the Viperidae, Crotalidae, and Elapidae families. The FX activator (RVV-X) from Russell's viper (*Daboia russelli*) venom[106] is a metalloproteinase with a mass of 93 kDa. Structurally, it consists of a heavy chain and two distinct light chains.[107] The heavy chain, which is the catalytic subunit, consists of a metalloproteinase, a disintegrin, and a cysteine-rich domain.[108] The light chains are homologous to C-type lectin-related proteins.[109] Functionally, RVV-X specifically cleaves the Arg52–Ile53 bond in the heavy chain of FX, and it requires Ca^{2+} for optimal activity. Several serine proteases that activate FX have also been isolated from the venoms of *Ophiophagus hannah*, *Bungarus fasciatus*, *Cerastes cerastes*, and *Cerastes vipera*.[110] However, the structural details of these serine proteases are not yet known.

Prothrombin Activators

A number of snake venoms possess prothrombin activators (PAs) in their venom. This group of toxins activates prothrombin to thrombin, which then causes fibrin formation. Based on their structure, cofactor requirements, and end products formed, snake venom PAs have been classified into four groups (Table 113.1, FIGURE 113.5).[111] Both group A and group B PAs are metalloproteinases, and they convert prothrombin to meizothrombin, which in turn is converted to thrombin through autoactivation. Their main difference lies in their cofactor requirements and structure. Group A PAs, such as ecarin from *Echis carinatus* venom,[112] function efficiently without any cofactor requirements and group B PAs, such as multiactivase from *Echis multisquamatus* venom, require Ca^{2+} for optimal activity.[113] The C-type lectin-related protein subunit confers the Ca^{2+} requirement to group B PAs. Group C and group D PAs are serine proteases, which are found exclusively in Australian elapid snakes, and they activate prothrombin to mature thrombin. Their main difference also lies in their cofactor requirements and structure. Group C PAs, such as pseutarin C from *Pseudonaja textilis* venom, require Ca^{2+} and negatively charged phospholipids but not FVa for maximal activity.[114] Group D PAs, such as trocarin D from *Tropidechis carinatus* venom, require Ca^{2+}, activated FV, and negatively charged phospholipids for their optimal activities.[115] Pseutarin C consists of two subunits, pseutarin C catalytic subunit and pseutarin C nonenzymatic subunit, which are structurally and functionally similar to FXa and FVa, respectively.[116–118] In contrast, group D PAs are structurally and functionally similar to FXa. Both these PAs activate prothrombin by targeting identical cleavage sites.[119,120] Thus, group C and group D PAs are similar to blood coagulation factors (FIGURE 113.5) and are probably evolved from coagulation factors by gene duplication.[121,122–124]

Procoagulants from Lonomia Caterpillar Venoms

Lonomia caterpillars are found in South America, and their venom found in the bristles affects mainly the coagulation system.[125] Victims get envenomed upon contact with the bristles. *Lonomia oblique* and *Lonomia achelous* venoms have procoagulant activities. Kelen et al.[126] were the first to examine the hemotoxic effect of *L. oblique* and found that its crude venom induces dose-dependent procoagulant activities, which increase in the presence of calcium. *Lonomia oblique* venom activates FX and prothrombin.[127] Subsequently, two serine proteases were isolated and characterized from the *L. oblique* venom: an FX activator termed as Losac (*L. obliqua* Stuart-factor activator) and Lopap (*L. obliqua* PA protease).[128,129] *Lonomia achelous* venom possesses procoagulant activities. Two PAs from this venom

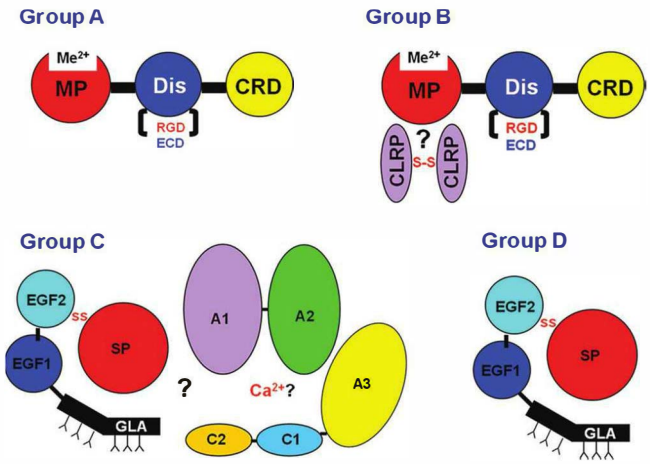

FIGURE 113.5 Schematic representation of snake venom PAs. Groups A and B PAs are metalloproteinases and are unrelated to blood coagulation factors. Group B PAs have a light chain made up of C-type lectin-related proteins. Individual domains are MP, metalloproteinase domain; Dis, disintegrin-like domain; CRD, Cys-rich domain; CLRP, C-type lectin-related protein domain. Groups C and D PAs are serine proteases. Group D PAs are structurally similar to FXa. They have a light chain containing Gla, EGF1, and EGF2 domains and a heavy chain containing serine protease domain. Group C PAs are structurally similar to FXa–FVa complex. The catalytic subunit is similar to FXa and group D PAs, whereas the nonenzymatic subunit is similar to FVa. Individual domains are Gla, γ-carboxyglutamic acid domain; EGF1 and EGF2, epidermal growth factor domains; SP, serine protease domain; A1, A2, A3, C1, and C2, respective domains of FVa.

Table 113.1

Classification of snake venom prothrombin activators

Group	Cofactor Requirements	Type of Proteinase	Product Formed	Estimated Size	Similar to Blood Coagulation Factors	Examples
A	None	Metalloproteinase	Meizothrombin	~47 kDa	None	Ecarin
B	Ca^{2+}	Metalloproteinase	Meizothrombin	Two subunits of ~25 and 60 kDa	None	Carinactivase, multactivase
C	Ca^{2+} *plus* phospholipids	Serine protease	Thrombin	Two subunits of ~60 and 220 kDa	"FXa–FVa" complex	Oscutarin, pseutarin C
D	Ca^{2+} *plus* phospholipids *plus* FVa	Serine protease	Thrombin	~60 kDa	FXa	Trocarin D, hopsarin D, notanarin D

were isolated and characterized[130]: Lonomin III, which directly activates prothrombin independently of the prothrombinase complex, and Lonomin IV, which has FXa-like activity.[130]

Procoagulants from Scorpion Venoms

Scorpion stings are a major threat to public health in many underdeveloped tropical countries, such as South India, the Middle East, and Latin America. The estimated annual number of scorpion stings is 1.2 million leading to 3,250 deaths.[131] Besides the painful local lesion of a scorpion sting, severe systemic complications have been also observed. Some of the hemotoxic symptoms include blood-stained tears and urine, intraperitoneal hemorrhage, and priapism.[132] The crude venom of *Palamneus gravimanus* has been shown to have procoagulant activities at low concentrations by significant shortening of the clotting time of recalcified plasma.[133] The crude venom was partially fractionated, and three fractions were shown to reduce recalcification time in plasma deficient of blood coagulation factors XII, IX, VIII, and VII, but not in those deficient of factors X, V, and prothrombin. It is concluded that the *P. gravimanus* venom promotes blood coagulation by activating blood coagulant FX.[133] Further characterization of these procoagulant factors is still needed.

Procoagulants from Spider Venoms

Venomous spiders use their toxins to immobilize their prey. Their venoms induce a variety of clinical symptoms related to hemorrhage, edema, hemolysis, inflammation, neurotoxicity, cytotoxicity, and platelet aggregation, and they interfere in plasma coagulation.[134] Although the majority of spider bites cause minor discomforts in humans, a few groups of spiders such as the Australian funnel web *Hadronyche* species may cause lethal bites.[135] Recently, a serine protease from the venom of *Hippasa agelenoides* was isolated.[136] This protease is 16,350 Da in mass and is termed Hag-protease. This enzyme is nonlethal but shows procoagulant properties when characterized with plasma recalcification times.[136] It reduces activated partial thromboplastin time and prothrombin time and hence targets the common pathway of the blood coagulation cascade.[137] It does not clot purified fibrinogen but is able to coagulate FX-deficient human plasma.[137] Thus it was concluded that this enzyme has FXa-like activity. In addition, Hag-protease induces aggregation of human platelets in platelet-rich plasmas at low concentrations but induces spontaneous clumping at high concentrations. This Hag-protease is similar to the group C and/or D snake venom PAs as it is a serine protease and has maximal activity in the presence of $CaCl_2$ and phospholipids. However, the lack of structural details and low molecular weight does not allow its grouping with them.

FUTURE PROSPECTS

Since ancient times, plants and animals have been exploited for medicinal purposes. A classic example is that of development of antimalarial drugs based on the alkaloid quinine from the barks of *Cinchona* trees. Only in the last couple of decades, scientists have developed drugs based on animal proteins. For example, angiotensin-converting enzyme inhibitors (Captopril, Enalapril family) were designed based on bradykinin-potentiating peptides isolated from Brazilian snake venom. Similarly, based on hirudin from medicinal leech, Hirulog or bivalirudin was

Snake Venom Proteins as Diagnostic Agents

Toxins from snake venoms that target hemostatic systems have greatly facilitated numerous routine assays for coagulation factors and the study of hemostasis in general. For instance, the venom of Russell's viper, *D. russelli*, is used in standard practice to assay the blood clotting factors V, VII, and X, as well as lupus anticoagulants. The venom contains a component that directly activates FX, turning FX into FXa, in the presence of calcium ions, FVa, and phospholipids. This complex then activates prothrombin, which will initiate the fibrin clot formation. As the clotting time, known as the Stypven time, is proportional to the concentration of FX, this assay is commonly used as a diagnostic test for FX deficiency in patients.

designed to inhibit thrombin. So far, a myriad of anticoagulants from other animals have been identified and characterized. With further development, some of these anticoagulants may potentially play a role in the treatment and prevention of thromboembolic diseases. On the other hand, procoagulants from animals have been used as diagnostic agents in the laboratory settings (Box 113.1). There is still a large number of anticoagulant and procoagulant compounds to be characterized. Thus, proteins from animal sources have a tremendous potential in developing new therapeutic agents, research tools, and diagnostic agents.[138]

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CHAPTER

114 Mechanical Devices and Embolectomy

HERVÉ DECOUSUS, PATRICK MISMETTI, AND NICOLAS MENEVEAU

VENA CAVA FILTERS AND VENOUS THROMBOEMBOLISM

Interruption of the vena cava by filter insertion was initially suggested by Armand Trousseau in 1868, and these devices have been used since the 1970s to reduce the risk of pulmonary embolism (PE).[1,2] These devices have been used for the treatment of venous thromboembolism (VTE) either alone, in patients with a contraindication to anticoagulation, or in combination with conventional anticoagulation, especially in case of failure of the antithrombotic therapy. Inferior vena cava (IVC) filters could also be proposed as VTE prophylaxis in certain patient populations at high thromboembolic risk. Despite these rather limited indications and despite current guidelines, IVC filters are frequently used especially in North America with 49,000 insertions in the United States in 1999.[3,4] Forty-five percent of the filters are inserted for patients with deep vein thrombosis (DVT), 36% for those with PE, and 19% are used as primary prophylaxis.[3] An increasing proportion of all filter insertions are outside the recommendations of current guidelines.[5] The use of these devices has been extensively studied with more than 2,000 publications during the past 10 years. However, these publications are mainly case reports or retrospective cohorts. Several prospective cohort studies with a limited number of patients have been performed, and only two randomized clinical trials have been published[6,7]: the first comparing IVC filter plus anticoagulant therapy with anticoagulant therapy alone in patients with proximal DVT and no contraindication to anticoagulation[6] and the second comparing two types of IVC filters in patients requiring filters for different reasons.[7]

Different Types of Filters

Several types of IVC filters have been developed, and at least 10 variants of vena cava filters are currently in use in North America and Europe. The original filters were permanent, with no possibility of removal, such as the Greenfield (Boston Scientific, Boston, MA, USA), Bird's Nest (Cook, Bloomington, IN, USA), Venatech (B Braun, Boulogne, France), and Simon nitinol (Bard, Tempe, AZ, USA) filters (FIGURE 114.1).

One of the main complications of these filters is a twofold increase in DVT recurrences.[6,8] Due to this risk, the filter placement requires prolonged anticoagulation, and despite this, the rate of filter thrombosis, of new lower extremity DVT, and of new PE was 12%, 20%, and 5%, respectively.[9] This complication may occur late (≥3 months after filter insertion)[6] and could be avoided if the filter is retrieved, since the indications for IVC filters are mainly temporary. Accordingly, temporary filters were then developed, but they need a permanent venous access to be retrieved (Tempofilter II, B. Braun, Boulogne, France). This permanent venous access could be associated with septic complications, and this reduced the duration of IVC filter implantations. More recently, retrievable filters, such as the Gunther Tulip (Cook, Bloomington, IN, USA), Recovery (Bard, Tempe, AZ, USA), OptEase (Johnson and Johnson, Cordis Endovascular, Miami, FL, USA), and ALN (ALN Implants Chirurgicaux, France) filters, have been developed (FIGURE 114.2). These filters can be left in place indefinitely or retrieval can be performed several weeks and up to 1 year after insertion.[10]

Complications of IVC Filters

Before discussing their efficiency and potential indications, it seems important to note that apart from the thromboembolic complications mentioned above, the iatrogenic effects of these filters can be serious, and the incidence of these complications is not clearly established. Indeed the occurrence of adverse events after filter implantation is not negligible, as shown in several cohorts.[11] Moreover failures of filter removal can reach 12% in observational studies.[11]

These complications are listed in Table 114.1.

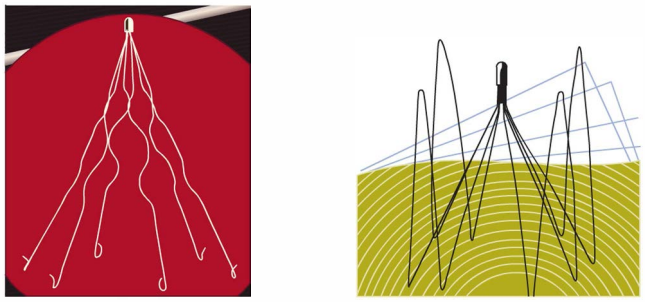
Recommendations for Use

As with all invasive procedures, insertion of a vena cava filter may be associated with a risk of serious adverse events,[3] justifying careful consideration of the benefit-to-risk ratio for all indications. Due to the limited data currently available regarding the benefit-to-risk ratio of these devices, their use even for validated indications should be restrictive.

The eighth edition of the American College of Chest Physicians (ACCP) guidelines published in 2008 recommends[12]

- For the use of IVC filters in patients with acute proximal lower extremity DVT, in whom anticoagulant therapy is not possible because of the risk of bleeding (grade 1C)
- Against the routine use of filter in addition to anticoagulants for most patients with PE and for patients with proximal DVT (grade 1A)
- Against the routine use of filter as primary prophylaxis in trauma patients (grade 1C) and in spinal cord injury (grade 1C)

Despite these recommendations, filters are increasingly prescribed especially in the United States either to prevent or to treat VTE.[13] This is probably due to potential indications that are insufficiently assessed. These potential indications correspond in particular to patients supposed to be at risk of VTE despite anticoagulant therapy and/or to be at high risk of bleeding while receiving anticoagulants.[4,13]



A B

FIGURE 114.1 Permanent filters. **A:** SGF Greenfield. **B:** Vena Tech LP-B Braun.

Recommended and Potential Indications of IVC Filters

Treatment of VTE

For this indication, there are considerable discrepancies between the United States and Europe in terms of frequency of use. Two North American cohorts showed that a filter[4,13,14] was inserted in about 15% of patients hospitalized for an episode of VTE compared with only 2% of patients in a similar cohort in Europe.[15]

In one of the North American cohorts,[4] three experts in the subject reviewed each of the indications for the filters, and only 50% of the indications were considered to comply with ACCP guidelines. The indications outside of the guidelines were essentially related to the perception of a high risk of bleeding or to a recurrence of VTE despite anticoagulant therapy.

Treatment of VTE in Patients with a High Risk of Bleeding

In patients presenting with a recent VTE episode and a contraindication for anticoagulant treatment, the risk of PE recurrence or PE-related death may reach 20%.[16,17] With this risk in mind, it is not ethically acceptable to perform a randomized trial to validate use of the filter.

There is, however, a European cohort study that supports this indication for the IVC filter. In a population of 17,368 VTE patients, the incidence of major hemorrhagic incidents was 2.3%, and the mortality rate for the patients who experienced bleeding was 33%. Among these patients with a hemorrhagic incident, a filter was inserted in 9% and multivariate analysis identified the filter as the only independent protective factor with a reduced risk of death after a hemorrhagic incident, with an odds ratio of 0.2 (95% confidence interval [CI], 0.1 to 0.6).[18]

These results would at first sight justify use of the vena cava filter in all VTE patients with a hemorrhagic incident contraindicating anticoagulant therapy. Indeed, when these hemorrhagic incidents are transient or curable, use of a retrievable filter is fully justified, provided that removal of the filter is effectively scheduled.

Treatment of VTE in Patients at a High Risk of Fatal PE

IVC filters could be used when the risk of death due to PE is high (>5% at 3 months) despite anticoagulation,[4] that is,

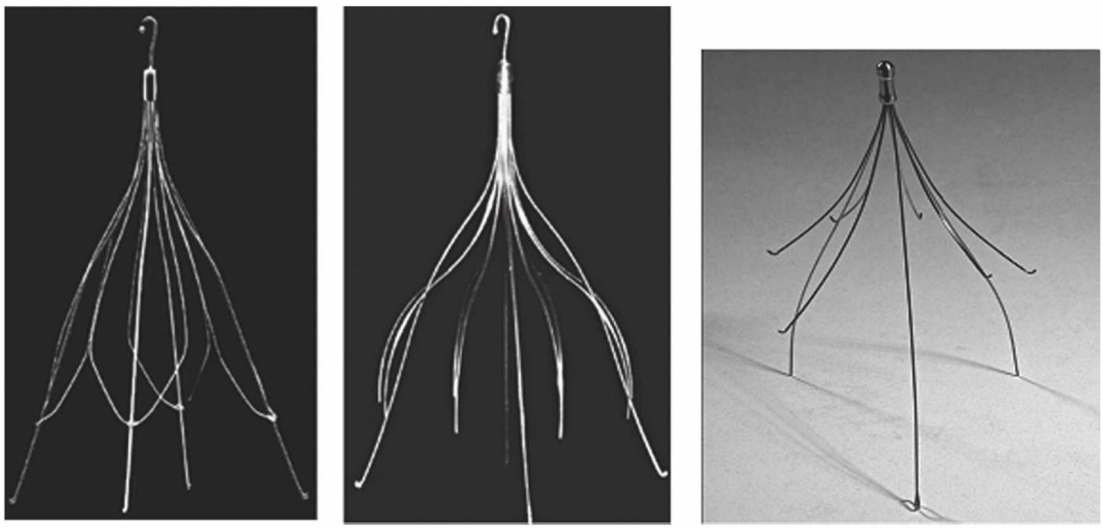
- In hemodynamically unstable patients with PE
- In patients at risk of recurrence of life-threatening PE, such as those with chronic pulmonary heart disease, especially if a surgical treatment if proposed

In these specific situations, indications for IVC filter placement are intuitive, and a randomized clinical trial is unrealistic and not feasible. In the absence of more convincing data, the indications are not discussed in the most recent guidelines.[12]

Treatment of VTE Recurrences Despite Anticoagulant Treatment

In VTE patients presenting with a recurrent PE in spite of optimal anticoagulant treatment, use of vena cava filters appears to be appropriate. However, there are no scientific data to support this indication. Moreover, it is necessary in this case to discuss the notion of optimal anticoagulant treatment.

For example, in studies in cancer patients with an episode of VTE, continued administration of low molecular weight



A B C

FIGURE 114.2 Retrievable filters. **A:** Gunther Tulip. **B:** Celect Filter-Cook. **C:** ALN Filter.

Table 114.1

Different types and rates of adverse events associated with filter implantation

Hematoma and infection related to filter insertion	4%–10%
Thrombosis at filter insertion site	2%–28%
Arteriovenous fistula at the insertion site	<1%
Filter tilt	6%
Filter migration	3%–69%
Acute perforation of vena cava	3%–25%
Filter thrombosis	5%–30%
Failure of filter removal	0%–12%
Mortality rate related to the filter insertion procedure	0.1%

Imberti D, Prisco D. Retrievable vena cava filters: key considerations. *Thromb Res* 2008;122(4):442–449

heparin for 3 to 6 months seemed to be more effective than vitamin K antagonists (VKAs), even when the international normalized ratio (INR) was optimally controlled.[19,20] Thus, for recurrent PE during VKA therapy with an INR between 2 and 3, it may be more appropriate to resume heparin treatment rather than to use a vena cava filter. It is likely that the arrival of the new anticoagulants will provide another alternative to VKAs and filters in this situation.

Treatment of VTE in Patients at High Risk of Recurrences Despite Anticoagulant Treatment

The only well-conducted randomized trial, the PREPIC study was designed for patients with this indication.[6] In this study, patients with a proximal DVT were randomly allocated to receive either anticoagulation with insertion of a filter or anticoagulant treatment alone. The filters assessed in this study were permanent versions. The results after 8 years of follow-up showed a significant reduction in the risk of PE (hazard ratio [HR] 0.37; 95% CI, 0.17 to 0.79; $P = 0.008$), but this beneficial effect was accompanied by a significant rise in the risk of DVT in the lower limbs (HR 1.57<; 95% CI, 1.02 to 2.27; $P = 0.042$) and notably with a 13% risk of filter thrombosis.[21] These contradictory results justify the fact that the guidelines do not recommend routine use of filters.[12]

The beneficial effects with respect to PE are evident essentially during the 1st 3 months of treatment, while the negative effects are observed mostly after the sixth month.[6] Therefore, a retrievable filter left in place for only 3 months could achieve the beneficial effects regarding PE while avoiding the extra risk of DVT recurrence. In addition, in order to optimize the beneficial effects of the filter, it could be inserted in patients with a higher risk of PE than in the PREPIC population. In fact, when recruited to the study, 38% of the PREPIC patients presented with DVT alone without any initial PE,[6] whereas the presence of an initial PE multiplies by five the risk of a fatal PE at 3 months compared to isolated DVT.[8,22]

With this in mind, a randomized study, PREPIC 2, is under way to assess the effect of a retrievable vena cava filter, systematically removed after 3 months in patients with an initial PE and thrombosis of the lower limbs, all of whom are treated with an anticoagulant agent (ClinicalTrials.gov identifier: NCT 00457158).

Optimal Duration of Anticoagulant Treatment in VTE Patients with an Inserted Filter

The insertion of a filter is associated with a doubling of recurrent of DVT.[6,21] Furthermore, full efficacy of vena cava filters is not guaranteed, with an estimated residual incidence of PE ranging from 0.5% to 6% despite filter insertion.[11] According to these data, the optimal duration of anticoagulant treatment should be discussed independently from the filter insertion and based on the current guidelines.[12]

Prevention of VTE in High-risk Situations

For this prophylactic indication, the ACCP guidelines[23] recommend against the routine use of IVC filters (grade 1C). There is no randomized comparative trial evaluating the systematic use of filters as primary prophylaxis after trauma or spinal cord injury. However, certain recommendations suggest that these filters may be used for the following indications in patients with a high thromboembolic risk.[24]

Prophylaxis of VTE in Trauma Patients

Filters present potential advantages in situations where there is a high risk of VTE and a simultaneous risk of hemorrhage, limiting the use of anticoagulant treatment even at prophylactic levels. With this hypothesis in mind, filters are in widespread use for patients at particular high risk for PE immediately after a traumatic injury including spinal cord injury, pelvic fracture, lower extremity fracture, venous repair, injury severity score >9 or >15, and history of VTE, coma (Glasgow coma scale <8). These indications are even supported by EAST guidelines.[24]

Prophylaxis of VTE in Bariatric Surgery

In spite of the recommendations against filters by the ACCP[23] and American Society of Hematology,[25] clinicians suggest the use of retrievable filters in bariatric surgery insofar as the efficacy of prophylactic anticoagulant treatments appears to be limited in obese patients, notably those with a BMI > 50 kg/m^2.[26] Accordingly, these filters are used today in about 50% of bariatric surgery cases in the United States.[25] Nevertheless, the efficacy of these filters along with the iatrogenic effects of the insertion and retrieval procedures is not well established, with up to 30% failures to retrieve and 15% cases of filter thrombosis.[27] Furthermore, the problem with insufficient prophylactic effect from low molecular weight heparin can most likely be obviated by a modest increase of the dose for this group of patients.[28]

Prophylaxis of VTE in Other Indications

Vena cava filters are also used in other situations with a high risk of thromboembolism, such as major orthopedic surgery (total hip or knee replacement).[29] Other indications such as cancer surgery have been studied.[30] However, in these indications, the noninvasive prophylactic measures are efficient and the benefit–risk ratio is well established, which is not at all true for vena cava filters.[23]

Filter Retrieval Procedures in Prophylactic Situations

The primary prophylactic indications for filters are situations with a temporary risk, and filters may appear appropriate in

that they are removable. This requires they are indeed removed once the VTE risk period is over. The iatrogenic effects of the retrieval procedures are not well established, and the rate of successful retrieval is <100%, and all the more so given that retrieval is more difficult and takes longer than insertion.[31] Retrieval more than 3 months after insertion of the filter seems to be associated with a 50% failure rate. The procedure also requires that the person who performs the procedure has a good level of experience.[32] Unfortunately, about 50% of these filters that were inserted precisely because they are retrievable are never removed.[33] This nonretrieval may be due to filter thrombosis, failure to remove, patients lost to follow-up, or apprehension concerning the retrieval procedure.[32] It would thus appear essential to establish guidelines and routines at each center that uses filters in order to optimize the rate of retrieval and limit the long-term risk of complications of these filters, notably IVC thrombosis and postthrombotic syndromes.[32]

Considerations Regarding the Different Types of Retrievable IVC Filters

There is only one randomized study comparing two types of filters: Greenfield (Boston Scientific, Natick, MA, USA) and TrapEase (Cordis, Bridgewater, NJ, USA).[7] This study showed that the TrapEase filter appears to be associated with a higher risk of thrombosis than the Greenfield filter.

Observational series also suggest that certain types of filters may be associated with a higher risk of fracture (FIGURE 114.3) and embolization, for example, the first- and second-generation Bard Recovery filters (Bard Peripheral Vascular, Tempe, AZ, USA).[34]

Apart from these observations, there are not sufficient data to recommend one type of filter rather than another.

FIGURE 114.3. Complications of vena cava filters. Rupture of one leg of a filter.

Conclusion

Permanent vena cava filters, as well as retrievable filters, may be considered valuable aids to the management of patients at very high risk of VTE recurrence and/or patients with a contraindication to anticoagulant treatment. Fortunately, the majority of patients seem to benefit from less invasive methods of VTE prophylaxis or treatment. The use of vena cava filters is increasing, probably due to the availability of more easy-to-insert implants, but more trials are needed to confirm their benefit and accurately assess their safety and the feasibility of removal in clinical practice. While awaiting these data, it seems justified to recommend against the routine use of these devices when conventional prophylactic and therapeutic anticoagulation is not contraindicated.

STENTS AND ACUTE VENOUS THROMBOEMBOLISM

Until recently, the gold standard for the treatment of superior vena cava syndrome (SVCS) was surgical repair or surgical bypass. However, surgery for SVCS is fraught with high morbidity and mortality. Previously, malignant causes of SVCS were dominating, accounting for up to 90% of cases. Nowadays, an increasing proportion of SVCS (up to 40%) are of nonmalignant origin, due to more widespread use of central venous catheters and pacemaker/defibrillator leads in clinical practice. In this context, a percutaneous approach with endovascular stent implantation has recently been advocated as a viable alternative to surgery to treat SVCS in both malignant and nonmalignant causes. A number of small, nonrandomized studies have reported excellent feasibility, with technical success rates of up to 95%, and immediate- and long-term (1 year) patency rates exceeding 80%.[35,36–38] Symptom relief is immediate, with accompanying functional improvement. The complication rates reported with percutaneous stenting for this indication are extremely low, at a maximum of 4% to 5%, and no deaths.[36] The potential benefit of additional thrombectomy prior to stenting is currently being explored.[39]

SURGICAL AND PERCUTANEOUS EMBOLECTOMY

Current guidelines suggest that the prognosis of PE should be analyzed as an individual estimate of the risk of early PE-related death rather than the anatomical distribution of intrapulmonary emboli.[40] Patients are now classified as high risk versus intermediate or low risk, replacing the former terminology of acute massive, submassive, or nonmassive PE. High-risk PE accounts for 5% of all cases of acute PE, and constitutes a life-threatening emergency, since most deaths occur during the first few hours after admission. We will review here the current state of the art on surgical and catheter embolectomy in the management of acute PE.

Embolectomy

High-risk PE is associated with acute right-ventricular (RV) failure, which results in shock and severe arterial hypotension. In this setting, potentially life-saving therapies include rapid initiation of thrombolysis, surgical embolectomy, or catheter

thrombectomy, in addition to conventional anticoagulant therapy. Surgical embolectomy and catheter thrombectomy are alternative treatments to thrombolysis, particularly in the presence of an increased risk of bleeding. Indeed, approximately one-third of all high-risk PE patients are not eligible for thrombolysis because of major contraindications, including trauma, prior surgery, stroke, or concomitant active bleeding.[41]

Surgical Pulmonary Embolectomy

Up until the past few years, pulmonary embolectomy was restricted to clinically desperate circumstances in moribund patients who required cardiopulmonary resuscitation. However, the use of expanded indications for pulmonary embolectomy has been associated with a reduced operative risk. A case series with 47 patients with high- and intermediate-risk PE subjected to surgical embolectomy showed interesting results with 92% early survival.[42] Almost half the study population had a contraindication to thrombolytic therapy, and 10% of the patients had not responded to previous thrombolysis. Thus, widening the indications for surgical embolectomy in patients with intermediate-risk PE is associated with a prognosis similar to that observed after conventional treatment.

Unless intracardiac thrombi or a patent foramen ovale are present, surgical embolectomy should be performed avoiding aortic cross-clamping and cardioplegic cardiac arrest.[42,43] After median sternotomy and pericardiotomy, patients are heparinized and cannulated for cardiopulmonary bypass. The arterial cannula is placed in the ascending aorta with either a bicaval or a single-venous cannula placed through the right atrium. The procedure is performed under normothermia without cardiac arrest and with vacuum-assisted venous drainage. The clot is extracted through a longitudinal arteriotomy in the main pulmonary artery, under direct inspection using forceps.

The indications for surgical embolectomy are given in Table 114.2. Surgical embolectomy with cardiopulmonary bypass is recommended for the management of patients with high- or intermediate-risk PE in whom contraindications preclude thrombolysis. Other indications include patients who require surgical extraction of intracardiac thrombi or closure of a patent foramen ovale, as well as rescue in patients who do not respond adequately to thrombolysis. Indeed, rescue surgical embolectomy leads to a better in-hospital course when compared with repeat thrombolysis in the setting of unsuccessful previous thrombolysis. Registry data on 488 patients with PE and failed thrombolysis at a single center demonstrated that uneventful in-hospital evolution was significantly more common in the surgical group, due to a lower in-hospital mortality rate, less frequent episodes of major bleeding, and fewer recurrent PE.[44] Consequently, the transfer of patients who have not responded to thrombolysis to tertiary cardiac surgery centers could be considered as an option in this context. Prolonged periods of postoperative cardiopulmonary bypass and weaning may be necessary until recovery of RV function.[40] This surgical approach is of course associated with potentially severe complications. Particularly, extraction of old thrombi that have been in place for several days and become adherent to the pulmonary artery can lead to possible pulmonary hemorrhage.

Catheter Embolectomy

Percutaneous catheter embolectomy is the only alternative to thrombolysis or surgical embolectomy to reverse PE-related right heart failure and cardiogenic shock. Catheter embolectomy can be particularly useful when contraindications to thrombolytic therapy exist, or when surgical embolectomy is either unsuitable or unavailable. Unfortunately, no randomized controlled trial has ever compared catheter embolectomy with surgical embolectomy or thrombolytic therapy in this setting. Available data are limited to small cohort studies or case series.[45] In many cases, these various devices are used in association with local thrombolytic therapy. This combined approach makes it difficult to analyze the efficacy of the percutaneous catheter devices in isolation. The rate of clinical success observed with this approach, and defined as immediate hemodynamic improvement, appears to be over 80% and is associated with mortality rates from 0% to 25%.[46]

Various catheter-directed interventions are currently available, including clot fragmentation, suction thrombectomy, and rheolytic thrombectomy (Table 114.3).[47] The Greenfield Embolectomy (Boston Scientific/Meditech; Watertown, MA, USA) device is a 10F steerable catheter that was specifically designed for the treatment of massive PE.[48] The Amplatz thrombectomy device (Bard-Microvena, White Bear Lake, MN, USA) is a 7F catheter device with a distal impeller mounted on a drive shaft that rotates at high speed (150,000 revolutions per minute). The rotation creates a vortex of circulating blood that attracts the clots toward the rotating impeller, pulverizing and recirculating fresh thrombus.[49] Hydrodynamic or rheolytic catheter

Table 114.2

Current indications for surgical and catheter embolectomy in high-risk PE

Surgical Embolectomy
Valuable therapeutic option in patients with high-risk PE in whom thrombolysis is absolutely contraindicated or has failed, as well as in compromised patients with insufficient time for systemic thrombolysis to be effective
Catheter Embolectomy
Consider as an alternative to surgical embolectomy (either unsuitable or unavailable) in high-risk PE patients when thrombolysis is absolutely contraindicated or has failed, as well as in compromised patients with insufficient time for systemic thrombolysis to be effective.

Torbicki A, Perrier A, Konstantinides S, et al. Guidelines on the diagnosis and management of acute pulmonary embolism: the Task Force for the Diagnosis and Management of Acute Pulmonary Embolism of the European Society of Cardiology (ESC). *Eur Heart J* 2008;29(18):2276–2315

Table 114.3

Catheter embolectomy devices for high-risk PE

Device	Manufacturer	Size, F	Thrombus Fragmentation	Clinical Success (%)
Greenfield suction catheter	Boston Scientific, US	10	Thrombus aspiration	81
Pigtail rotational catheter	Cook Europe, the Netherlands	6	Thrombus fragmentation	67
Amplatz thrombectomy device	BARD-Microvena, US	7	Thrombus pulverization and recirculation	88
Angiojet Xpeedior	Possis Medical, US	6	Thrombus fragmentation and aspiration	75
Hydrolyser	Cordis Europe, the Netherlands	6	Thrombus fragmentation and aspiration	92

Clinical success was defined as immediate hemodynamic improvement.
Adapted from Kucher N. Catheter embolectomy for acute pulmonary embolism. *Chest* 2007;132(2):657–663.

devices also create a vortex that causes fragmentation and aspiration of the clots (Hydrolyzer device, Cordis, Warren, NJ, USA; Oasis catheter, Boston Scientific/Meditech; AngioJet Xpeedior, Possis, Minneapolis, MN, USA).[50–52] The 11F Aspirex catheter thrombectomy device (Straub Medical, Wangs, Switzerland) is a high-speed rotational coil (40,000 revolutions per minute) that creates negative pressure at the catheter tip, macerates aspirated thrombus, and removes thrombus fragments.[53]

Percutaneous catheter device interventions are limited by a number of potential drawbacks that can cause serious complications. Indeed, it must be underlined that these procedures necessitate a vascular approach with selective catheterization of the pulmonary arteries and injection of contrast medium. The most serious complication that can arise is perforation of the myocardium or of a branch of the pulmonary artery. This can rapidly lead to tamponade or massive intrapulmonary hemorrhage, precipitating death in a large majority of cases. The risk of perforation is increased when the diameter of the pulmonary artery is below 6 mm. To minimize the risk of perforation and dissection, embolectomy should only be carried out in the main and lobar pulmonary arteries, and not in the segmental pulmonary arteries. Similarly, the procedure should be stopped as soon as there is an improvement in hemodynamic conditions, regardless of the angiographic result.[53] Some procedures can be complicated by distal embolization of proximal thrombi, which can exacerbate the patient's hemodynamic instability. Certain devices can also lead to substantial blood loss in case of prolonged aspiration, or even mechanical hemolysis. Finally, other complications can arise related to the procedure itself, including anaphylactic reaction to iodine contrast medium, contrast-induced nephropathy, ventricular arrhythmia, or vascular access complications.[46]

Catheter-directed interventions are therefore only appropriate when thrombolysis is contraindicated or has failed to improve the patient's clinical condition. A catheter-based approach might be particularly suitable in patients who are too frail for surgery, or in any patient with severe underlying pulmonary disease that makes successful cardiopulmonary bypass unlikely. Evidently, operators need to be very experienced, in view of the severity of the potential complications of these approaches. The current indications for catheter embolectomy are provided in Table 114.2. Overall, the use of percutaneous catheter devices should only be envisaged in high-risk PE, in the presence of at least one absolute contraindication to thrombolysis, in addition to the presence of subtotal or total filling defect in the left and/or right main pulmonary artery determined by CT scan or by conventional pulmonary angiography.[54]

Conclusion

Management of acute high-risk PE encompasses several therapeutic options, which must be selected on a case-by-case basis according to the patient's clinical profile. Systematic evaluation of the expected risk–benefit ratio is mandatory. Thrombolytic treatment should be first-line therapy in patients with high-risk PE presenting with cardiogenic shock and/or persistent arterial hypotension, with very few absolute contraindications. Both surgical and catheter pulmonary embolectomy are valuable therapeutic options in patients in whom thrombolysis is absolutely contraindicated or has failed.

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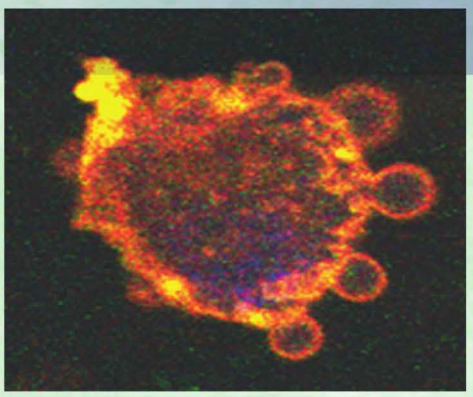
PART V



Hemostatic and Thrombotic Disorders Associated with Systemic Conditions

CHAPTER

115 Overview of Special Clinical Groups

VICTOR J. MARDER AND JOEL S. BENNETT

The majority of this textbook is devoted to basic aspects of hemostasis and thrombosis and specific hemostatic and thrombotic diseases. But more common in clinical practice are disorders in which hemostasis and thrombosis are complicating features of another pathologic process. In latter disorders, the homeostatic balance that maintains the fluidity of the blood, while at the same time leaving it poised to prevent hemorrhage after trauma, is perturbed by systemic processes to cause bleeding, thrombosis, or both. This section of the textbook considers the potential for hemorrhagic or thrombotic manifestations in select patient groups, from conception to old age. The goal of these chapters is to provide a conceptual framework for understanding pathogenesis. Details of treatment are provided in chapters devoted to individual therapeutic agents or to specific thrombotic and hemorrhagic events.

PREGNANCY AND THE POSTPARTUM STATE

Issues related to pregnancy, the postpartum period, and the use of oral contraceptive agents are discussed in Chapters 116, 117, and 118. The hormonal changes that accompany pregnancy and the use of oral contraceptive agents represent a particular challenge to the hemostatic balance since they favor thrombosis. While this may be advantageous for the integrity of the fetal–placental circulation and for the prevention of postpartum hemorrhage, it otherwise puts women at risk for venous thromboembolism, especially during the postpartum period; for placental thrombosis associated with fetal growth retardation, spontaneous abortion, and eclampsia; for the exacerbation of concurrent hemostatic disorders such as immune thrombocytopenia; and for diseases that commonly complicate pregnancy such as thrombotic thrombocytopenic purpura and disseminated intravascular coagulation.

HEMOSTASIS DURING INFANCY AND CHILDHOOD

As discussed in Chapter 119, the components of the hemostatic system undergo significant changes during the neonatal period and infancy, reaching levels found in adults at variable intervals, generally but not exclusively by 1 year of age. Nonetheless, the presence of normal concentrations of clotting factors and platelets belies the unique clinical presentations of bleeding and thrombosis in children, features of which are often unknown or unappreciated by "adult" hematologists. Similarly, therapy of children often requires appreciation of issues other than simply dosing modification due to the small size of children, issues that are discussed in detail in Chapters 120 and 121.

HEMOSTATIC AND THROMBOTIC DISORDERS ASSOCIATED WITH SYSTEMIC ILLNESS

The contribution of perturbed hemostasis to the morbidity of systemic illness becomes immediately apparent to the consulting hematologist who is "barraged" with questions regarding the basis for, and management of, the almost "ambient" thrombocytopenia in hospitalized patients; the preoperative management of the hemostatic disorders of patients with severe renal and liver disease; the treatment of sepsis; the management of stroke and acute chest syndrome in patients with sickle cell disease; the treatment of the ever-present venous thrombosis in patients with malignancy; and the hemostatic complications associated with iatrogenic vascular trauma (surgery) and the use of cardiopulmonary bypass devices. Moreover, with the appearance of facilities devoted to the treatment of traumatic injury, hematologists are now required to understand the hemostatic derangements that accompany trauma and issues regarding their management. Chapters 122 to 129 provide detailed discussion of each of these topics.

HEMOSTASIS AND THROMBOSIS IN THE ELDERLY

With the aging of the population, it has become apparent that both hemostasis and thrombosis are deranged in elderly patients. For example, not only are the elderly more prone to thrombosis, but they present challenges for the safe and effective administration of antithrombotic therapy when thrombosis does occur. Issues regarding the diagnosis and management of hemostatic and thrombotic disorders in elderly patients are considered in Chapter 130.

CHAPTER

116

Venous Thromboembolism during Pregnancy and with Hormonal Contraceptives

SASKIA MIDDELDORP, MATHILDE NIJKEUTER, AND MICHIEL COPPENS

PART I: PREGNANCY-RELATED VENOUS THROMBOEMBOLISM

Epidemiology

Venous thromboembolism (VTE) complicates 1 to 2 per 1,000 pregnancies.[1,2–4] Furthermore, VTE is the leading cause of maternal death in Western countries, accounting for 1 to 2 deaths per 100,000 pregnancies and 20% to 30% of all maternal deaths.[5,6,7] Compared to age-matched nonpregnant women, this implies a 4- to 16-fold risk increase during pregnancy.[1] In the postpartum period, the relative risk of VTE has been found to be as high as 60.[8]

Deep vein thrombosis (DVT) accounts for 85% of pregnancy-related symptomatic VTE, and the majority, that is 65%, occur during pregnancy.[9] Contrary to this, the majority (85%) of episodes of pulmonary embolism (PE) occur in the postpartum period.[1] Approximately 22% of episodes of VTE during pregnancy occur in the first, 34% in the second, and 48% in the third trimester.[10] Given the much longer duration of the antepartum period than the postpartum period, the daily absolute risk of VTE is highest postpartum.

Inherited thrombophilias also increase the risk, and absolute risk estimates are summarized in Table 116.1. The presence of a positive family history for VTE modifies the risk of a first pregnancy-related VTE and in this setting ranges between 1.7% and 14.0%, depending on the type of hereditary thrombophilia.[11,12] No data are available for the risk of a first pregnancy-related VTE in women with antiphospholipid syndrome. Outside of pregnancy, the persistent presence of lupus anticoagulants including anticardiolipin or β2-glycoprotein antibodies is associated with a 2- to 10-fold risk increase for a first VTE.[13,14] If all pregnancy-related VTE is considered, the presence of antiphospholipid syndrome is associated with a 15.8 (95% CI 10.9 to 22.8) times higher risk.[6] It should be noted that in this study, antiphospholipid syndrome was classified by hospital admission code and that this risk increase, at least in part, is likely due to the coexistent history of VTE, which in itself was the strongest risk factor for pregnancy-related VTE (odds ratio [OR] 24.8, 95% CI 17.1 to 36.0).[6] It is unclear what the risk of VTE is in women with antiphospholipid syndrome diagnosed on reproductive clinical criteria such as recurrent miscarriage. In a small study in women with recurrent miscarriage and antiphospholipid syndrome, the risk of subsequent thrombotic events was not increased compared to women with unexplained recurrent miscarriage.[15] The risk of pregnancy-related VTE also increases with age, mode of delivery, and presence of comorbid conditions.[1,6,16]

Despite these strong risk increases, a strong evidence base for the management of pregnancy-related VTE is missing. It is striking that, contrary to nonpregnant patients, diagnostic strategies, therapeutic options, and preventive measures for VTE in pregnant and postpartum women have not been addressed adequately in well-sized observational or intervention studies. Therefore, management is not standardized between physicians, centers, and countries.

Pathophysiology

The elements of Virchow's triad, that is, venous stasis, hypercoagulability, and vascular damage, are all more or less present during pregnancy and the postpartum period. This predisposition to develop VTE has probably evolved to protect women from hemorrhage during childbirth. Venous stasis already begins in the first trimester and is assumed to be caused by progesterone-induced venodilation.[17] Compression of the pelvic veins by the gravid uterus becomes more important in the second and third trimester of pregnancy. A third component leading to increased venous stasis is the crossing of the right iliac artery over the left iliac vein that may lead to pulsatile compression of the iliac vein (FIGURE 116.1). Pregnancy is a hypercoagulable state caused by decreased anticoagulant activity, increased procoagulant activity, and decreased fibrinolysis. The decrease in anticoagulant activity is due to a decrease in protein S concentration and an increase in activated protein C (APC) resistance. The increase in procoagulant activity is a result of an increase in factor V, VII, VIII, IX, X, and fibrinogen concentrations.[18] Fibrinolytic activity is decreased because of an increase in plasminogen activator inhibitor 1 and 2 activity and a decrease in tissue plasminogen activator (tPA). The third element of Virchow's triad, that is, vascular damage, is induced by delivery. Forceps, vacuum extraction, and surgical delivery can aggravate this vascular injury.

Clinical Presentation of VTE in Pregnancy

DVT in pregnant patients has a different distribution along the venous system as compared to nonpregnant patients. The predisposition for DVT to occur in the left leg, that is, almost 90% in pregnant patients, is much stronger than in nonpregnant patients (~60%).[10,19–21] Isolated iliac vein thrombosis occurs in 17% of all DVT in pregnant women, whereas this is very uncommon in nonpregnant patients.[20] Furthermore, in contrast to nonpregnant patients, more than 60% of DVT is restricted to the iliac and/or femoral vein, without calf vein involvement.[20]

Table 116.1

Risk of pregnancy-related VTE in thrombophilic women stratified by family history for VTE

Thrombophilic Defect	Prevalence in Population, %[145–149]	Estimated RR OR (95% CI)	Absolute Risk of VTE[a], % of Pregnancies (95% CI)	
			Family Studies	Nonfamily Studies
Factor V Leiden, heterozygous	2.0–7.0	8.3 (5.4–12.7)[150]	3.1 (2.1–4.6)[114,115]	1.2 (0.8–1.8)
Factor V Leiden, homozygous	0.2–0.5	34.4 (9.9–120)[150]	14.0 (6.3–25.8)[11,151]	4.8 (1.4–16.8)
Prothrombin, heterozygous	2.0	6.8 (2.5–18.8)[150]	2.6 (0.9–5.6)[112,152]	1.0 (0.3–2.6)
Prothrombin, homozygous	Very rare	26.4 (1.2–559)[150]	—	3.7 (0.2–78.3)
Antithrombin deficiency	<0.1–0.6	4.7 (1.3–17.0)[150]	3.0 (0.08–15.8)[153]	0.7 (0.2–2.4)
Protein C deficiency	0.2–0.3	4.8 (2.2–10.6)[150]	1.7 (0.4–8.9)[153]	0.7 (0.3–1.5)
Protein S deficiency	<0.1–0.1	3.2 (1.5–6.9)[150]	6.6 (2.2–14.7)[153]	0.5 (0.2–1.0)
Lupus anticoagulants (persistent)[b]	No consistent data	2 to 10 (wide confidence intervals)[13,14]	—	0.3 to 1.4 (95% CI uncertain)

[a]Observed in family studies, estimated from multiplying the baseline risk of 1.40 per 1,000 by the RR in nonfamily studies.[12]
[b]Risk increase is stronger for lupus anticoagulant than for anticardiolipin or β2-glycoprotein antibodies. Data are very limited; hence, the estimated absolute risk should be interpreted with caution.

Diagnosis of VTE in Pregnant Women

Clinical Decision Rules and D-Dimer Testing

Studies on the diagnostic management strategies of DVT and PE have excluded pregnant women. Currently, no single study has adequately demonstrated the safety of ruling out VTE by clinical probability assessment combined with the use of D-dimers. In pregnancy, as in nonpregnant patients, D-dimer tests should not be used as a stand-alone test because of the small but important risk of a false-negative result. Objective imaging remains therefore the cornerstone of diagnosis and is crucial to avoid treating women who do not have VTE.[22]

Safety of Diagnostic Imaging in the Pregnant Patient

Imaging techniques to diagnose or exclude VTE in pregnancy should take into account the radiation exposure of the fetus (Table 116.2). These are much lower than the threshold dose for induction of malignancies (100 mSv) in the fetus and clearly justify the use of diagnostic testing involving radiation in pregnancy for the exclusion of potentially fatal VTE.[23]

Concerns have been raised that breast tissue of pregnant women, which receives particularly high doses of radiation, is supposedly especially susceptible to its carcinogenic effects, but evidence supporting this concern is not available.[24]

The iodinated contrast agents used in CT scanning and venography cross the placenta. Fetal exposure to iodinated contrast media is thought to be relatively short-lived. Although no effect on fetal thyroid function has ever been reported with clinical doses of iodinated contrast media, it is standard care that neonates of all mothers exposed to iodinated contrast media during pregnancy are screened for hypothyroidism.[25,26] Gadolinium contrast agents are used during MRI and do cross the placenta, but no neonatal adverse effects have been described and tests postpartum are not necessary.[25] Only tiny amounts of iodinated or gadolinium contrast media are excreted in breast milk and only a minute proportion entering the baby's gut is absorbed. This is considered insufficient to warrant stopping breast-feeding for 24 hours following either iodinated or gadolinium contrast agents.[25]

Diagnosing Deep Vein Thrombosis in Pregnancy

Compression ultrasonography (CUS) should be performed as a first test in a pregnant patient with a clinical suspicion of DVT (FIGURE 116.2). CUS has a sensitivity of 97% to 100% and a specificity of 98% to 99% outside of pregnancy, is noninvasive, and carries no radiation exposure.[27,28] Serial CUS testing after an initial normal test is of less value in pregnant patients, since DVT in pregnancy usually occurs in proximal veins without involvement of the calf veins. If doubt about the presence of DVT persists, limited venography with abdominal shielding should be considered. If iliac or pelvic vein thrombosis is suspected because of abdominal, groin, or back pain combined with swelling of the entire leg, CUS needs to be followed by duplex Doppler ultrasound or, alternatively, full venography can be performed. An absent waveform on modulation of respiration strongly suggests iliac vein thrombosis, but due to slow flow and external compression by the gravid uterus, false-positive ultrasound results may occur as well as false-negatives due to nonocclusive thrombi or collaterals. In centers with the availability of, and experience with, MRI, MR venography is a reasonable alternative test to venography in demonstrating iliac or pelvic vein thrombosis. However, the safety of excluding DVT when MR venography is normal has not been prospectively studied.

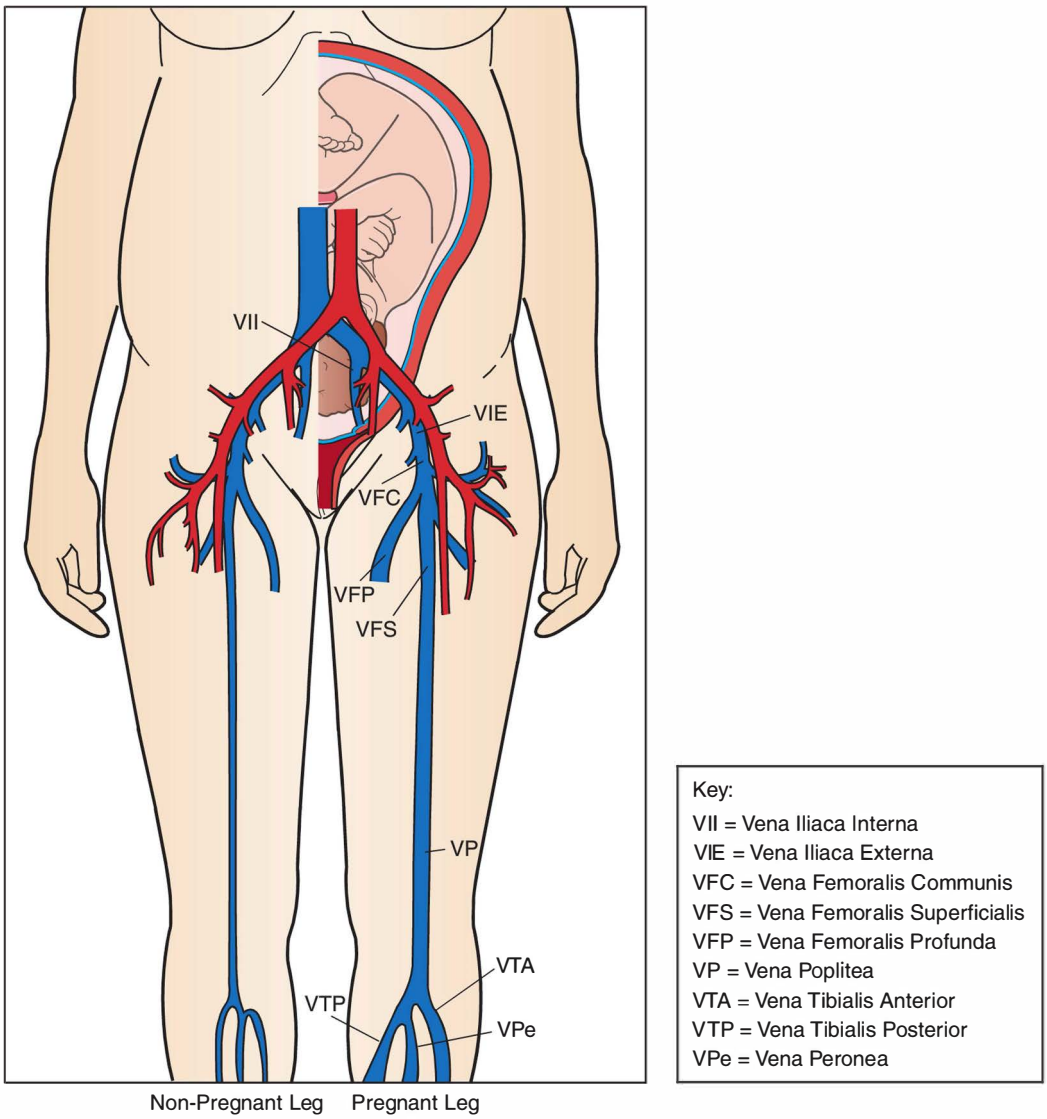


FIGURE 116.1 Venous stasis during pregnancy. The left leg represents the pregnant leg, while the right leg represents the nonpregnant leg. Venous stasis in pregnancy is caused by progesterone-induced venodilation and compression of the pelvic veins by the gravid uterus (here depicted posterior of the vessels for clarity, but in reality anterior of the blood vessels). These components of increased venous stasis during pregnancy add to the anatomic crossing of the right iliac artery over the left iliac vein that may lead to pulsatile compression of the iliac vein. VII, vena iliaca interna; VIE, vena iliaca externa; VFC, vena femoralis communis; VFS, vena femoralis superficialis; VFP, vena femoralis profunda; VP, vena poplitea; VTA, vena tibialis anterior; VTP, vena tibialis posterior; VPe, vena peronea.

Diagnosing Pulmonary Embolism

FIGURE 116.3 depicts several algorithms that can be used in pregnant patients with a suspicion of PE, depending on local availability and expertise.[22,29] Although the diagnostic yield of CUS (algorithm a) of the legs is low in asymptomatic women, it is a reasonable approach to avoid radiation in women suspected of PE. When CUS demonstrates DVT, further testing is not necessary and the patient can be considered to have a VTE. In a recent study, CUS showed proximal DVT in 39 of 139 (31%) nonpregnant patients suspected of PE but without symptoms of DVT.[30] In pregnant women, this percentage is probably much lower since the overall prevalence of VTE in suspected patients is lower, but there are no data available. Obviously, ultrasound testing should not lead to diagnostic delay and if negative must prompt objective diagnostic testing of the lungs. Furthermore, the possibility of a false-positive result should be taken into account, especially if the patient has had an ipsilateral DVT in the past.

Advantages of the algorithm starting with a CT scan (FIGURE 116.3, algorithm b) are the feasibility to reach a diagnostic conclusion and the possibility to detect an alternative diagnosis, combined with a low fetal radiation exposure. An ongoing prospective diagnostic management study in which CT as the single test was used to exclude PE during pregnancy in 85 women showed no symptomatic VTE during follow-up of 3 months (95% CI 0% to 3.5%; Nijkeuter et al., 2012). CT scan

Table 116.2

Radiation dose to the fetus by radiologic examination

	Radiation (mSv)
Unilateral venography without shielding	3.14
Unilateral venography with shielding	<0.5
Pulmonary angiography via femoral route	2.21–3.74
Pulmonary angiography via brachial route	<0.5
Perfusion scintigraphy (^{99m}Tc MAA, 200 MBq)	0.2–0.6
Perfusion scintigraphy (^{99m}Tc MAA, 40 MBq)	0.11–0.20
Ventilation scintigraphy (^{99m}Tc aerosol)	0.1–0.3
Ventilation scintigraphy (81 mKr, 600 MBq)	0.0001
Single-detector row helical CT	0.026
Multidetector row helical CT	0.013
Chest x-ray	0.001

Estimates of radiation exposure are derived from McMaster University and University Medical Centre Leiden.[154,155]
To convert mSv to rads: 1 mSv ~ 0.1 rad.

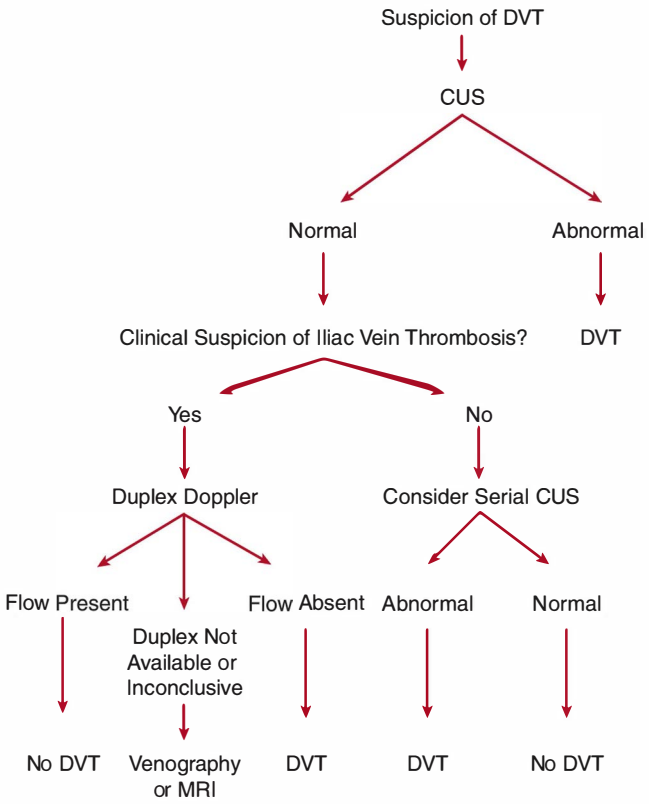


FIGURE 116.2 Algorithm of a clinical suspicion of DVT in pregnancy. CUS, compression ultrasonography; DVT, deep vein thrombosis; LMWH, low molecular weight heparin; MRI, magnetic resonance imaging.

results were inconclusive in 4 of 94 patients, that is, 4.2%. This percentage is much lower than the 19% and 35% reported in two retrospective studies but higher than in the nonpregnant population (0.9%).[31–33]

The algorithm containing perfusion scintigraphy (FIGURE 116.3, algorithm c) should bear in mind that a chest x-ray is advised to rule out other diagnoses and to increase the chance of a conclusive perfusion scan. Four studies evaluated the performance of lung scintigraphy in pregnancy; diagnostic scan results ranged from 75% to 94%.[32,34–36] Selection of patients by normal chest x-ray and no history of asthma or chronic obstructive pulmonary disease resulted in the higher percentage of diagnostic tests.[32,36] However, these were all retrospective studies that were subject to bias since no uniform diagnostic protocol was used. Hence, in up to a quarter of pregnant patients with suspected PE, perfusion scintigraphy needs to be followed by additional testing. This strategy obviously is associated with a higher amount of fetal radiation exposure.

Risks of Anticoagulant Drugs in Pregnant and Postpartum Women

In the next paragraphs, we discuss specific fetal and maternal safety issues for anticoagulant agents. Furthermore, as in the nonpregnant population, the most obvious maternal safety issue of any anticoagulant is the risk of bleeding, both antepartum as well as around delivery and in the postpartum period. These risks are described in the paragraphs of the specific agents if data are available in pregnant women.

Vitamin K Antagonists

Vitamin K antagonists (VKAs) cross the placenta and the induced vitamin K deficiency in the fetus may cause fetal wastage, bleeding, and coumadin embryopathy, a disorder that is characterized by nasal hypoplasia and stippled epiphyses.[37] This teratogenic effect is only present when VKAs are taken in the 6th to 12th weeks of gestation (defined as weeks after the 1st day of the last menstrual cycle). When VKAs are used throughout pregnancy, as is sometimes done in women with mechanical heart valves, the risk of congenital abnormalities is estimated between 3.7% and 6.4%.[37–39] Although exposure during the second trimester of pregnancy does not lead to overt handicaps, careful examination of school-aged children who had been exposed to VKA in utero showed that 18 out of 274 exposed children (7%) had an increased risk for a "multiple adverse outcome," that is, growth impairment, minor neurologic dysfunction, IQ < 80, and behavioral problems, in comparison to 2 children out of 231 nonexposed controls (risk difference 5.7%, RR 7.6, 95% CI 1.8 to 32.4).[40,41] The adverse outcomes were mainly found in boys, and of the affected children, 94% had been exposed during the second and third trimester only. From approximately 36 weeks gestation, the bleeding risk for the fetus during delivery is a reason to avoid VKA during this time.

Women can breast-feed during the use of VKAs; particularly nonlipophilic types such as acenocoumarol and warfarin.[42,43] Of the lipophilic phenprocoumon, only minute amounts are

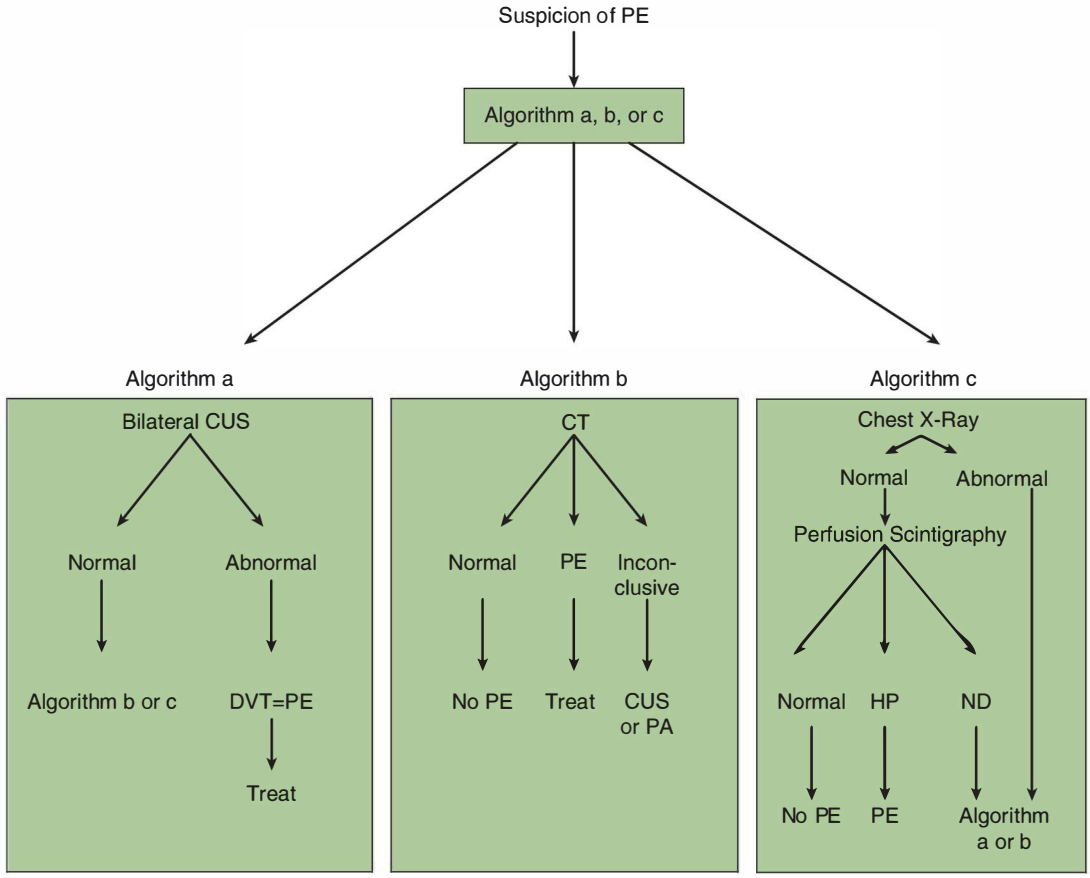


FIGURE 116.3 Algorithms of a clinical suspicion of PE in pregnancy. CT, computed tomography; CUS, compression ultrasonography; HP, high probability; ND, nondiagnostic; PE, pulmonary embolism; PA, pulmonary angiography.

excreted in the breast milk that are insufficient to prolong the prothrombin time in infants.[44]

In a systematic review of VKA use in pregnant women with mechanical heart valves, major bleeding events occurred in 2.5% (95% CI 1.7% to 3.5%) of all pregnancies, most at the time of delivery.[38]

Low Molecular Weight Heparin

There is ample experience with low molecular weight heparin (LMWH) in pregnant women. These agents do not cross the placenta and are considered safe to use in pregnancy, based on numerous observational studies.[12,45] The need for, usually once daily, injections is a nuisance of LMWH, and the long-term use in pregnant women frequently leads to skin reactions, which are mainly type IV delayed hypersensitivity reactions at the injection site.[46–48] Switching to another LMWH preparation is reported to be indicated in 25% of patients, and of these, about one-third also developed complaints with the use of another LMWH.[46] The incidence of heparin-induced thrombocytopenia (HIT) in pregnant patients who are only treated with LMWH is considered very low (<0.1%),[45] and therefore, the weak, grade 2C recommendation in the most recent American College of Chest Physicians (ACCP) guideline is to not routinely monitor platelet counts. The risk of HIT is assumed to be somewhat higher, between 0.1% and 1%, in women who have been exposed to unfractionated heparin prior to LMWH treatment and monitoring of platelet counts is advised.[49] Small studies of bone density in pregnant women receiving prophylactic doses of LMWH suggest that the use of this medication does not induce more bone loss as compared to pregnant women who did not receive LMWH.[50,51]

Although small amounts of LMWH are excreted into breast milk, LMWH is not absorbed after oral intake and this is unlikely to have any relevant effect in breast-fed infants.[12,52]

The risk of significant bleeding with the use of LMWH in pregnant women is generally reported to be around 2% and as being mostly related to obstetric causes.[45] These data are based on observational studies including a wide range of doses of LMWH with the majority of prophylactic and intermediate doses. Furthermore, these reports may be biased by selective publication of successful patient histories and underreporting of complications.[53–55] When therapeutic doses are employed, the incidence of major bleeding was estimated to be 1.7% (95% CI 0.4% to 5.0%) in a systematic review of 15 studies (including 6 case reports) reporting 174 women who had been treated for acute VTE.[45] In a prospective evaluation of 126 pregnant women with acute VTE, the risks for bleeding during pregnancy was 6%, with none of the episodes considered by the authors as major bleeding, although these included episodes of rectal bleeding from endometriosis and haemoptysis.[56] The risk of postpartum hemorrhage (>500 mL of blood loss) was 5% in the first 24 hours after delivery, and secondary major postpartum

hemorrhage occurred in 2% of the women in the days thereafter. In our own study of 83 women who had been treated with therapeutic doses of LMWH, the incidence of postpartum hemorrhage was around 10%, which was not statistically different from a cohort of women who delivered in the same hospital and who were not treated with anticoagulants.[57]

Unfractionated Heparin

Unfractionated heparin can be administered subcutaneously and dose adjustments can be made based on activated partial thromboplastin times (APTTs), but the short half-life leads to the need for a twice daily dosing regimen. Furthermore, unfractionated heparin is more frequently associated with osteoporosis, HIT, and type I allergic reactions than LMWH.[12] Long-term unfractionated heparin use is known to cause symptomatic osteoporosis in up to 2% of patients.[58] Thus, unfractionated heparin should be reserved for women with a contraindication to LMWH (mostly chronic renal failure with a creatinine clearance below 30 mL/min) or when LMWH is not available.[12]

Unfractionated heparin has a high molecular weight and a strong negative charge and does not pass into breast milk.

In a retrospective cohort study of 100 pregnancies in 77 women, the rate of major antepartum bleeding in pregnant women treated with unfractionated heparin was 1% (95% CI 0.2% to 5.4%), which is consistent with reported rates of bleeding associated with heparin therapy in nonpregnant patients.[59]

Other Heparin-Like Anticoagulants

Danaparoid and fondaparinux have been and are increasingly being used in pregnancy, although the experience in human pregnancies is limited.[12,60] Furthermore, minor anticoagulant activity could be detected in fetal cord blood in five neonates of mothers who were treated with fondaparinux, indicating some placental transfer of the pentasaccharide.[61] Thus, in the absence of extensive safety data, danaparoid and fondaparinux should be reserved for women who do not tolerate heparin, most often when all registered heparins lead to type I allergy, skin problems, or HIT.[62]

Very little is known about the passage of fondaparinux or danaparoid into human breast milk. Since both agents are not absorbed after oral intake, it is unlikely to have any anticoagulant effect in breast-fed infants.[63]

Thrombin and Factor Xa Inhibitors

A few case reports have described the use of parenteral thrombin inhibitors in pregnancy, but these data are insufficient to conclude on their safety in pregnant women.[12] There is no place for new oral anticoagulants, since the human reproductive risks of these medications, that is, dabigatran, rivaroxaban, and apixaban, are unknown. The summaries of product characteristics for dabigatran and rivaroxaban describe animal reproductive toxicity.

It is recommended by the manufacturers that breast-feeding women do not use these medications.[12]

Thrombolytics

Although thrombolysis is contraindicated in pregnancy, a recent literature review identified 13 case reports that described its use in pregnant patients with massive PE.[64,65] Recombinant tPA and streptokinase are large molecules that do not cross the placenta, whereas urokinase does.[64] In these severely ill women, fetal death occurred in 15% and preterm delivery in approximately 30% of cases.

The risk of maternal major bleeding complications was 30.8%; 95% CI (9.1 to 61.4).[64]

Treatment of Acute VTE in Pregnant Women

Pregnant women have been excluded from all clinical trials investigating various treatment regimens in acute VTE. In pregnancy, the volume of distribution of LMWH changes and glomerular filtration rate increases in the second trimester. Dosing therapeutic LMWH in pregnant women is associated with uncertainties about whether a prepregnancy weight or actual body weight needs to be used to determine the appropriate dose, whether dose adjustments are required as the pregnancy progresses, and whether a twice daily regimen should be preferred over a once daily regimen. Data from pharmacokinetic studies of various LMWHs in pregnant women have shown conflicting results with regard to the need for dose escalation to maintain levels within the therapeutic range.[66–71] The ACCP guidelines do not provide specific advice about anti-Xa level monitoring, in the absence of large studies using clinical endpoints demonstrating that there is an optimal therapeutic anti-Xa LMWH range or that dose adjustments increase the safety or efficacy of therapy.[12] Nevertheless, in our practice, we prescribe therapeutic doses of LMWH throughout pregnancy and monitor anti-Xa levels to achieve a 4-hour postinjection peak level of 0.8 to 1.6 U/mL with a once daily regimen of LMWH (0.6 to 1.0 if a twice daily regimen is used).[72] Prospective observational studies have not demonstrated an increase in the risk of recurrence with the once daily regimen over the twice daily regimen,[56,73] and a once daily regimen likely enhances adherence. Women should be treated at least until 6 weeks postpartum, since the daily risk in any woman to develop VTE is highest during this period.[8] Extrapolating the optimal duration of anticoagulation of nonpregnant patients with a VTE provoked by a major temporary risk factor, the minimum duration should be 3 months, so this postpartum treatment may be longer if VTE occurred in late pregnancy.[74]

For women in whom the need for rapid counteraction of the anticoagulant effect is anticipated, intravenous unfractionated heparin to achieve a therapeutic APTT is a reasonable short-term option. Unfractionated heparin has a short half-life (3 hours) and can be completely counteracted by protamine sulfate, although there are no solid safety data of the latter agent in pregnancy. In our experience, these presumed benefits are often counterbalanced by difficulty in achieving APTTs in the desired range, with the obvious risk of extending thrombosis as a result of undertreatment in a woman with acute VTE and overanticoagulation in a woman in whom this option is chosen because of an increased risk of bleeding.

In case of life-threatening PE, that is, pregnant women with an established diagnosis who are hemodynamically unstable, systemic thrombolysis with either recombinant tPA or streptokinase (whatever protocol is in place) is justified.[64]

Prevention of VTE

We identify three categories of women in whom pharmacologic prophylaxis of pregnancy-related VTE should be considered: (a) women with a history of VTE; (b) asymptomatic women with

an increased baseline risk of VTE, that is, known thrombophilia and/or a positive family history of VTE; and (c) women who have undergone a cesarean section.

LMWH is the agent of choice to prevent pregnancy-related VTE. The optimal dose of LMWH, however, is controversial, since no evidence from adequate randomized controlled trials are available.[12] Most centers use a prophylactic dose of LMWH in women who have an indication for thrombosis prophylaxis in pregnancy and the postpartum period. However, numerous treatment failures have been reported,[53,75,76] with an estimated risk of recurrent VTE despite the use of low-dose LMWH as high as 5% to 6%.[76–78] Potential benefits of the intermediate dose of LMWH consist of superior efficacy as compared to the low dose of LMWH. Harms consist of an increased risk of bleeding, mostly associated with delivery and neuraxial anesthesia. However, we observed no VTE recurrences in a retrospective study of 95 women who used therapeutic dose LMWH, whereas the risk of serious bleeding was not increased compared to 524 women who had delivered in the same hospital without LMWH use.[57] In the absence of evidence, the ACCP guidelines suggest either a prophylactic or an intermediate dose of LMWH to prevent recurrent VTE in pregnancy and the postpartum period, but it is clear that a randomized controlled trial between different doses of LMWH is needed.[12]

Prevention of Recurrent VTE

Women with a history of VTE have a three- to fourfold higher risk of VTE during subsequent pregnancies than outside of pregnancy.[79] The absolute risk of recurrent VTE during pregnancy, in the absence of pharmacologic prophylaxis, is estimated to be between 2.4% and 10%.[76,80,81] Data regarding prognostic factors for recurrent VTE during pregnancy, including the presence of provoking risk factors during the first event and hereditary thrombophilia, are inconsistent. In two retrospective studies, women who had their first episode of VTE provoked by use of oral contraceptives or related to pregnancy or the postpartum period had a nonsignificant higher risk of recurrent VTE in a subsequent pregnancy than women whose first VTE was unprovoked or associated with a nonhormonal transient risk factor.[76,81] Likewise, in a large population-based cohort study, women who had their first VTE associated with pregnancy or the postpartum period had a higher risk of recurrence during a subsequent pregnancy than women with an unprovoked first VTE, that is, 4.5% versus 2.7% (RR 1.71, 95% CI 1.0 to 2.8).[82]

According to the ACCP guidelines, patients can be categorized into groups at low risk (major transient risk factor for VTE), intermediate (hormone- or pregnancy-related or unprovoked VTE), or high risk (multiple prior unprovoked VTE or persistent risk factors such as paralysis) during pregnancy. All women with a history of VTE should receive prophylaxis with LMWH for 6 weeks postpartum. For antepartum prophylaxis, the ACCP guidelines suggest to withhold this in women with a low risk of pregnancy-related recurrence (major nonhormonal transient risk factor for VTE).[12] In women at moderate-to-high risk of recurrence who are not on long-term anticoagulant therapy, prophylaxis with LMWH is recommended during the entire pregnancy. The recommendations are summarized in Table 116.3.

Finally, in women who are on long-term anticoagulant therapy switching to full adjusted dose LMWH as soon as a urine pregnancy test is positive (i.e., before 6 weeks gestational age) and supplying vitamin K 5 mg orally for 3 days is a feasible regimen (for more details about the full-dose LMWH regimen, see the above section on treatment of acute VTE). Postpartum, women can switch back to their usual anticoagulant therapy with bridging of LMWH until the target international normalized ratio (INR) is reached.

Prevention of VTE in Asymptomatic Women with Thrombophilia

In women with a positive family history and/or hereditary thrombophilia who do not have a personal history of VTE, the decision to use pharmacologic prophylaxis is based on the absolute risk estimate of having VTE during pregnancy or in the postpartum period. The increase in risk begins early in pregnancy and when antepartum prophylaxis is utilized, it should be commenced as early as possible in the first trimester. The threshold to use antepartum prophylaxis is higher given the burden of self-injecting with LMWH over several months as opposed to 6 weeks in the postpartum period.

Table 116.3

Summary of recommendations to prevent a first or recurrent pregnancy-related VTE[a]

Antepartum and Postpartum Prophylaxis	Postpartum Prophylaxis During 6 Weeks[b]	No Pharmacologic Prophylaxis[b]
Women with a single unprovoked episode of VTE, or provoked by the use of oral contraceptives, pregnancy, or postpartum	Women with a history of a single episode of VTE related to a major nonhormonal transient risk factor	General population
Women with a history of recurrent VTE	Women with hereditary thrombophilia and a positive family history[c] of VTE	Women with a positive family history[c] of VTE
Women who are homozygous for factor V Leiden or prothrombin mutation who have a positive family history[c] of VTE	Women who are homozygous for factor V Leiden or prothrombin mutation who do *not* have a positive family history[c] of VTE	Women who are heterozygous for factor V Leiden or prothrombin mutation who do *not* have a positive family history[c] of VTE

[a]Recommendations are weak, based on a low level of evidence leaving room to individualize prophylactic strategies based on the patient's preferences.[12]
[b]Unless women can be categorized into one of the more aggressive prophylactic strategies in this table.
[c]A positive family history is defined as having a first-degree relative with VTE.

Based on the risk estimates in Table 116.1, the ACCP guidelines suggest both antepartum and postpartum prophylaxis only in homozygous carriers of the factor V Leiden or prothrombin gene mutations who have a positive family history of VTE.[12] In women with the other inherited thrombophilias who have a family history of VTE, or in homozygous carriers of the factor V Leiden or prothrombin gene mutations without a positive family history of VTE, clinical vigilance antepartum and postpartum prophylaxis with LMWH is suggested.[12] For postpartum prophylaxis, VKA is an alternative option in women without protein C or S deficiency (who are at risk of developing warfarin-induced skin necrosis) but is impractical because of the time needed to reach the target INR. The recommendations are summarized in Table 116.3.

Prevention of VTE After Cesarean Section

The absolute risk estimate of VTE after cesarean section ranges from 1 to 18 per 1,000.[12] The ACCP guidelines extrapolate from general surgery patients that the balance of desirable and undesirable consequences of thrombosis prophylaxis would suggest prophylaxis for women with an absolute VTE risk of 30 per 1,000 or higher.[12] With a baseline risk of 5/1,000 VTE postcesarean delivery, the presence or absence of risk factors will determine the absolute benefit of thrombosis prophylaxis. Table 116.4 provides an overview of major and minor risk factors to identify women with a high risk of VTE after cesarean section (30/1,000).[12] In women with no other risk factors for VTE except cesarean section, no prophylaxis is recommended apart from early ambulation. For women at increased risk of VTE after cesarean section because of the presence of one major or at least two minor risk factors, the ACCP guidelines suggest a prophylactic dose of LMWH (or mechanical prophylaxis in those with contraindications to anticoagulants) for as long as their hospital stay.[12] The optimal duration of prophylaxis after cesarean section is not established. A commonly used strategy is to continue prophylaxis until discharge from hospital and extend prophylaxis until 6 weeks postpartum in women with significant ongoing risk factors.

Management of Delivery and Postpartum Period

A well-described plan, made by a multidisciplinary team, should be available and depends on indication for and dose of LMWH, as well as local preferences and experience, rather than evidence. Several options are possible, including spontaneous labor and delivery, induction of labor, and elective cesarean section. If there is no obstetric indication for an induced delivery, women can be instructed to not inject LMWH as soon as labor starts with either contractions or rupture of the membranes. With this approach, most women will deliver within 24 hours, and with the peak anti-Xa level at 4 hours after the last injection, most women remain somewhat, but not fully, anticoagulated while in labor. Active management of the third stage of labor, that is, the delivery of the placenta, is necessary to minimize the risks of obstetric hemorrhage. In women who are expected to deliver fast or have a history of peripartum bleeding, switching to a twice daily regimen can be considered in the last weeks prior to expected delivery (e.g., from the gestational age of 37 weeks), although there is no pharmacokinetic rationale in pregnant women to support this approach.

A planned delivery should be considered in women at very high risk for extension or recurrent VTE (arbitrarily those in whom acute VTE was diagnosed within a month prior to expected delivery), so that the duration of time without anticoagulation can be minimized. These women can be switched to therapeutic intravenous unfractionated heparin, which is then discontinued 4 hours prior to the expected time of delivery or the use of neuraxial anesthesia (see also the above section on Treatment of VTE during pregnancy). In exceptional cases, for instance, in women who also have a contraindication to anticoagulation transiently (need for a cesarean section), the use of a temporary inferior vena cava filter may be considered. Still, experience with these devices during pregnancy is limited and filter migration and inferior caval vein perforation have been described in pregnant patients.[83,84]

Besides the potential increased risk for bleeding from obstetric causes, it is important to note that the use of LMWH precludes the use of neuraxial anesthesia, because of the (very low) risk of neuraxial hematoma. In prophylactic doses, LMWH should be discontinued at least 12 hours prior to neuraxial anesthesia and should not be restarted until 8 hours after catheter removal.[85] In therapeutic doses, LMWH should be discontinued at least 24 hours prior to neuraxial anesthesia and should not be restarted until 24 hours after catheter removal.[85] Discontinuation of anticoagulation for 48 hours or longer is not attractive in the setting of the treatment of acute VTE (arbitrarily defined as within 1 month).

Postpartum Management

Anticoagulation should be restarted after delivery, as soon as possible, but depending on the amount of estimated vaginal blood loss and the type of delivery. Generally, restarting anticoagulation 6 to 12 hours after delivery is feasible, but this period should be longer if hemostasis is not adequate. For women in whom resumption of VKA is planned, we restart LMWH at first, while the first dose of VKA is initiated at least 1 day later.

PART II: HORMONAL CONTRACEPTION AND VENOUS THROMBOEMBOLISM

Introduction

Combined oral contraceptives contain both an estrogen and a progestagen. They were first approved in the United States in 1960 and it is estimated that over 100 million women worldwide currently use an oral contraceptive pill.[86] Almost immediately after their introduction, it became clear that the use of these female hormones increased the risk of VTE and it is currently considered an important acquired risk factor for this disease.[87] Combined oral contraceptive pills are the most widely used contraceptives, but progestagen-only pills and nonoral hormone contraceptives, such as hormone releasing intrauterine devices (IUDs), injectables, subcutaneous implants, or skin patches, are also available. Apart from contraceptive purposes, female hormones can also be used for treatment of menstrual disorders and in lower doses as hormone replacement therapy

Table 116.4

Risk factors for VTE after cesarean section

Major Risk Factors (ORs >6): Presence of at Least One Risk Factor Suggests a Risk of Postpartum VTE of >3%
• Immobility (strict bed rest for a week or more in the antepartum period)
• Postpartum hemorrhage ≥1,000 mL with surgery
• Previous VTE
• Preeclampsia with fetal growth restriction
• Thrombophilia
- Antithrombin deficiency[a]
- Factor V Leiden (homozygous or heterozygous)
- Prothrombin G20210A (homozygous or heterozygous)
• Medical conditions
- Systemic Lupus Erythematodes (SLE)
- Heart disease
- Sickle cell disease
• Blood transfusion
• Postpartum infection
Minor Risk Factors (ORs >6 when combined): Presence of at Least Two Risk Factors or One Risk Factor in the Setting of Emergency Cesarean Section Suggests a Risk of Postpartum VTE of >3%
• BMI > 30 kg/m^2
• Multiple pregnancy
• Postpartum hemorrhage >1 L
• Smoking >10 cigarettes per day
• Fetal growth restriction (gestational age + sex adjusted birth weight <2.5th percentile)
• Thrombophilia
- Protein C deficiency
- Protein S deficiency
• Preeclampsia

[a]Although the OR in a systematic review was 4.69, confidence intervals were wide and numbers small.[150] Further, other retrospective studies have calculated ORs of 282 (95% CI 31–2,532) for type I antithrombin deficiency and 28 (95% CI 5.5–142) for type II deficiency.[159] Thus, antithrombin deficiency has been left as a major risk factor. Table from Bates SM, Greer IA, Middeldorp S, et al. Venous thromboembolism, thrombophilia, antithrombotic therapy and pregnancy: ACCP evidence-based clinical practice guidelines (ninth edition). *Chest* 2012;141:e691S–e736S. Used with permission. Original data from other studies.[6,16,73,150,156–158]

(HRT) to improve quality of life in women with perimenopausal complaints. The next section discusses the effects of female hormones on blood coagulation and the risk of VTE in women using them.

Hormonal Contraceptives

Risk of First VTE

The majority of risks estimates of VTE associated with hormonal contraceptives are derived from case–control or cohort studies that provide relative risk increases for users as compared to nonusers. In the interpretation of these relative risks, one first needs to know the absolute baseline risk of VTE. If no valid observations on the absolute risk associated with hormone use are available, one can estimate the absolute risk of VTE by multiplying this baseline risk with the relative risks of VTE associated with hormone use. This indirect estimation of the absolute risk is valuable for clinical practice but likely leads to a modest overestimation of the risk, because the age- and sex-specific baseline risks are derived from cohorts in which a proportion of the women used oral contraceptives.

The absolute risk of a first VTE increases sharply with age, in particular after the age of 45.[88–90] The absolute incidence of first VTE in women aged 20 to 44 was 0.39 per 1,000 person-years, increasing to 1.00 per 1,000 person-years in women aged 45 to 54 in a large Norwegian cohort.[89] Hence, the excess risk caused by hormonal contraceptive use is very modest for most women on the individual level and needs to be balanced against the beneficial effects of these hormones, such as avoidance of an unintended pregnancy. However, even a small increase of VTE risk is relevant due to the large numbers of women using oral contraceptives worldwide, leading to a significant population-attributable risk. Importantly, the relative risk of VTE is highest

in the first 3 months of use and gradually decreases during the 1st year. This was elegantly illustrated in a large case–control study of patients with a first VTE that showed an OR of 13 (95% CI 7 to 22) during the first 3 months of oral contraceptive use, decreasing to an OR of 5 (95% CI 4 to 6) after the 1st year.[91] Of note, despite this "starter effect," the increased risk of VTE associated with oral contraceptive use remains, even after long-term exposure (i.e., more than 5 years).[91] Furthermore, with increasing length of oral contraceptive use, the baseline risk of VTE increases with age, so that in absolute terms, the starter effect may have less absolute impact than the persisting relative risk.

Oral Contraceptives

Most currently available oral contraceptives are combined preparations containing both an estrogen (i.e., ethinylestradiol) and a progestagen. Numerous types of oral contraceptives are available containing different doses of estrogen and different types of progestagens. The first available preparations contained a high dose of estrogen of more than 50 μg. However, after the reported increased thrombotic risk associated with combined oral contraceptive use was attributed to the amount of estrogen in the contraceptive pill, the dose of estrogen was stepwise reduced. The initial lowering of the estrogen dose to 30 μg indeed decreased the risk of venous thrombosis by 30% to 50%.[91,92,93] Further reduction of the estrogen dose to 20 μg was shown to reduce the risk of VTE by another 20%.[91,94]

The progestagens in combined oral contraceptives modulate the prothrombotic effect of the estrogens. This leads to different thrombotic risks between contraceptives with different progestagens.[95] Numerous different types of progestagens with different chemical compositions are available. The oldest types of progestagens are lynestrenol and norethisterone. These so-called first-generation progestagens are not used very often nowadays. Combined oral contraceptives that contain the progestagens levonorgestrel or norgestrel are currently most widely used worldwide. The use of these second-generation oral contraceptives is associated with a three- to fourfold increased risk of VTE.[91,96] Newer types of oral contraceptives contain the progestagens gestodene, desogestrel, or norgestimate and these are often referred to as third-generation oral contraceptives. The use of these newer oral contraceptives is associated with a higher risk of VTE (OR 5 to 8 compared to nonusers) than levonorgestrel-containing oral contraceptives (OR 3 to 4 compared to nonusers).[91,96,97] Preparations containing cyproterone acetate are used for treatment of acne vulgaris, seborrhea, or mild hirsutism and have antiovulatory action similar to that of a progestagen. Preparations containing drospirenone, which is an antimineralocorticoid, also inhibit ovulation. These were heavily marketed arguing that these pills reduce side effects of older contraceptives including bloating and mood swings. However, the risk of VTE with these oral contraceptives is increased six- to sevenfold, that is, similar to the risk of the third-generation contraceptives.[91,96]

Progestagen-only preparations used for therapeutic reasons (mainly menstrual disorders), containing different progestagens or higher doses of progestagens than used in combined oral contraceptives, are associated with an approximately fivefold increased risk of VTE.[98,99] Contrary to this, progestagen-only pills used for contraception, so-called minipills, do not seem to increase the risk of VTE.[94,98,100] Table 116.5 shows the estimated relative and absolute risks of different types of oral contraceptives.

Nonoral Contraceptives

The levonorgestrel-releasing IUD is a T-shaped plastic contraceptive that, after insertion into the uterine cavity, releases 10 to 20 μg levonorgestrel per day, which leads to plasma levels of 150 to 200 pg/mL in the peripheral blood, as compared to a maximum level of 800 pg/mL during the use of a 30-μg levonorgestrel-only pill.[101] The use of this IUD was not associated with an increased risk of VTE in two studies.[96,102]

Depot medroxyprogesterone (DMPA) is an injectable contraceptive that is used once per 12 weeks. Although its effect on VTE has not been widely studied, its use seems to be associated with a mild-to-moderate increased risk of VTE.[100,102]

The etonogestrel-containing implant is a progestagen-only contraceptive that is implanted under the skin. Etonogestrel is an active metabolite of the third-generation progestagen desogestrel. The delivery dose varies over time from 60 to 70 μg/day in the 1st weeks of use to 25 to 30 μg/day after 3 years. Very little is known about the thrombotic risks associated with the use of this implant. Studies on coagulation markers in patients with implants do not show prothrombotic changes, providing some indirect evidence that there may not be an increased risk of VTE.[103,104]

Patches deliver hormones through the skin and are thereby uninfluenced by the first-pass metabolism in the gut wall and liver. The patch designed for contraception delivers 15 to 20 μg/day of ethinylestradiol combined with norelgestromin. The risk of VTE in women with the patch has not been compared to nonusers. However, two case–control studies compared the risk of this patch to the risk of a levonorgestrel-containing combined oral contraceptives showing a similar to twofold increased risk.[105–107]

Finally, the hormone-releasing vaginal ring delivers 15 μg/day of ethinylestradiol combined with etonogestrel. No studies on the risks of VTE in women who use this vaginal ring are available. Scarce studies on coagulation markers in women with a vaginal ring show conflicting results.[101,108,109] Table 116.5 shows the estimated relative and absolute risks of these nonoral contraceptives.

Risk of VTE in Women with an Increased Baseline Risk

The presence of hereditary thrombophilia increases the risk of a first VTE in carriers. The relative risk of VTE associated with the use of hormonal contraceptives is similar in women with or without thrombophilia, but due to the higher baseline risk in the former, the absolute risk increase is larger in women with thrombophilia. The baseline risk of VTE in women with thrombophilia depends on the setting in which they were tested. Women with thrombophilia, selected from families with a tendency for VTE, have a higher absolute risk than women with the same defect identified by population testing.[110] Furthermore, individuals with a first-degree relative with VTE have a twofold increased risk of VTE and women with more than one first-degree relative with VTE have a fourfold increased risk of VTE, regardless of the presence of thrombophilia.[111] For women with inherited thrombophilia and a positive family history of VTE, family cohort studies provide useful estimates of the absolute risk of VTE during oral contraceptive use.[112–116] However, due to the relatively small number of women using oral contraceptives in these family studies, no distinction between the risks of different types of oral contraceptives can be made. Table 116.6 shows the observed absolute incidences per year of

Table 116.5

Estimates of relative and absolute risks in women without previous VTE for different forms of hormonal contraceptives and HRT

	Odds Ratio (95% CI)	Absolute Risk (/ 1,000 Person-Years)[a]
Hormonal Contraceptives		
Strong Risk Increase (OR 5–8)		
• Ethinylestradiol[b]/desogestrel	7.3 (5.3–10.0)[91]	2.8 (2.1–3.9)
• Ethinylestradiol[b]/cyproterone	6.8 (4.7–10.0)[91]	2.7 (1.8–3.9)
• Ethinylestradiol[b]/drosperidone	6.3 (2.9–13.7)[91]	2.5 (1.1–5.3)
• Ethinylestradiol[b]/norgestimate	5.9 (1.7–21.0)[91]	2.3 (0.7–8.2)
• Ethinylestradiol[b]/gestodene	5.6 (3.7–8.4)[91]	2.2 (1.4–3.3)
• Ethinylestradiol[b]/lynestrenol	5.6 (3.0–10.2)[91]	2.2 (1.2–4.0)
• Oral progestagen only, high dose (5–30 mg)	5.3 (1.5–18.7)[98]	2.1 (0.6–7.3)
Moderate Risk Increase (OR 2–5)		
• Ethinylestradiol[b]/noresthisterone	3.9 (1.4–10.6)[91]	1.5 (0.5–4.1)
• Ethinylestradiol[b]/levonorgestrel	3.6 (2.9–4.6)[91]	1.4 (1.1–1.8)
• Injectable DMPA	3.6 (1.8–7.1)[102]	1.4 (0.7–2.8)
• Transdermal ethinylestradiol/norelgestromin[c]	1.0 (0.7–1.5)[107]	1.5 (0.5–4.1)
No Risk Increase		
• Levonorgestrel-releasing IUD	0.3 (0.1–1.1)[102]	0.1 (0.0–0.4)
• Progestagen only, low dose		
• Norethisteron 350 μg or levonorgestrel 30 μg	0.6 (0.3–1.0)[94]	0.2 (0.1–0.4)
• Desogestrel 75 μg	0.6 (0.2–1.9)[96]	0.2 (0.1–0.7)
Unknown[d]		
• Etonogestrel subcutaneous implant	n/a	n/a
• Vaginal ring (ethinylestradiol/etonogestrel)	n/a	n/a
Hormone Replacement Therapy		
Modest Risk Increase (OR 1.5–3.0)		
Oral combined estrogen/progestagen pills	2.6 (2.0–3.2)[130]	2.6 (2.0–3.2)
Oral estrogen only	2.2 (1.6–3.0)[130]	2.2 (1.6–3.0)
No Risk Increase		
Transdermal (combined estrogen/progestagen and estrogen only)	1.2 (0.9–1.7)[130]	1.2 (0.9–1.7)
Tibolone	0.9 (0.8–1.1)[133]	0.9 (0.8–1.1)

[a]Estimates of the absolute risk were obtained by multiplying the OR with the baseline incidence of VTE of 0.39/1,000 person-years for women aged 20–44 for hormonal contraceptives and of 1.00/1,000 person-years for women aged 45–54 for HRT.[89]
[b]Ethinylestradiol in the most commonly used dose of 30–40 μg daily.
[c]OR for VTE as compared to OCP: EE2/norethisterone. Therefore, the OR of EE2/norethisterone was used to estimate the absolute risk.
[d]No studies with clinical outcome available; studies assessing coagulation markers only were disregarded in this table.
95% CI, 95% confidence interval; OR, odds ratio.

oral contraceptive use derived from those family studies. With these absolute incidences, rough estimates can be calculated of the number of women with thrombophilia that need to refrain from oral contraceptive use in order to prevent one VTE. For deficiencies of antithrombin, protein C, or protein S, this number is around 50 and for the factor V Leiden and prothrombin 20210A mutations, this number is around 200 to 400. Finally, approximately 2,500 women with a family history of VTE need to refrain from oral contraceptive use in order to prevent one VTE, while this number is approximately 5,000 in women from the general population.[117]

As mentioned before, increasing age is another important factor that increases the baseline risk of VTE.[89] This implies that the excess risk increase induced by hormonal contraceptives, in absolute terms, is larger for older women than for younger women.

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Table 116.6

Absolute risk of VTE per year of oral contraceptive use in asymptomatic women with a positive family history for VTE, according to the type of thrombophilia

	Oral Contraceptive Use (% per Year of Use, 95% CI)[a]
Hereditary deficiencies of antithrombin, protein C, or protein S	4.3 (1.4–9.7)[115]
Factor V Leiden	0.5 (0.1–1.4)[114]
Prothrombin 20210A	0.2 (0.0–0.9)[112]
Elevated factor VIII	0.6 (0.2–1.5)[113]
Mild hyperhomocysteinemia	0.1 (0.0–0.7)[116]
Positive family history of VTE (≥1 first-degree relative)	0.1 (0.1–0.1)[b]

[a]Due to relatively small numbers of women using oral contraceptives in these studies, no risk differentiation of different oral contraceptives could be calculated.
[b]Estimated absolute risk by multiplication of the OR of 2.2 (95% CI 1.9–2.6) of a positive family history[111] with the baseline incidence of 0.039% per year.[89]
Observed in retrospective family studies that investigated first-degree family members of patients with VTE and a thrombophilia.
CI, confidence interval.

Risk of Recurrent VTE

Observational data indicate that women who resume oral contraceptive use after VTE have an increased risk of recurrence. In a relatively small follow-up study, women who used an oral contraceptive after VTE had a nonsignificant two- to threefold increased risk of recurrence, regardless of whether their first VTE was unprovoked or provoked by oral contraceptives.[118] Although oral contraceptive use is a clear and removable risk factor for developing VTE, there is some debate whether oral contraceptive use during a (first) VTE, either recently started or after long-term exposure, should be classified as a provoked VTE that would have a lower recurrence rate than an unprovoked VTE. As compared to women with an unprovoked VTE, the relative risk of recurrence in women with a first VTE associated with an oral contraceptive ranges from 0.3 to 1.2.[30,118–121]

Hormone Replacement Therapy

Initially, HRT was prescribed to improve cardiovascular risk factors and to protect women against the postmenopausal rise in the incidence of arterial cardiovascular disease.[122,123] This radically changed after large randomized controlled trials demonstrated that HRT does not prevent arterial cardiovascular disease and even has a detrimental effect in the first year of use.[124–126]

With regard to the risk of VTE, it is important to reemphasize that, due to increasing age, the population baseline risk of VTE is higher for women using HRT than for women using hormonal contraception.[89] Hence, a risk factor with an OR of 2.5 has a much larger impact on the absolute risk in older women.

Similar to hormonal contraceptives, HRT is available in various forms and generally provides a low dose of estrogen (either conjugated equine estrogens or esterified, synthetic estrogens), combined with a progestagen or progestin. HRT can be taken orally, or delivered via patches, creams, gels, or injection. Both observational studies and randomized controlled trials have consistently shown an approximately two- to threefold increased risk of VTE in women using oral HRT.[127–129,130] Although only a limited number of observational studies, with inconsistent results, have assessed the risk of VTE associated with transdermal HRT, the pooled risk estimate for first VTE was 1.2 (95% CI 0.9 to 1.7) in a metaanalysis of these studies.[130] Tibolone, which is used as an oral HRT, is a synthetic steroid whose metabolites have estrogenic, progestagenic, and androgenic activities. Its use does not seem to be associated with an increased risk of VTE.[131–133] However, there are concerns of other harmful effects, that is, an increased risk of stroke or recurrent breast cancer.[131,132] Table 116.5 shows the estimated relative and absolute risks of VTE for women using HRT.

HRT also increases the risk of recurrent VTE. A randomized controlled trial of HRT in women with prior VTE was terminated early because of a markedly increased risk of recurrence in women who were given HRT.[134]

Effects of Female Hormones on the Coagulation System

Hormonal contraceptive use is associated with changes in the coagulation system at different levels. Coagulation studies have demonstrated an increase in clotting factors II, VII, VIII, and X in women using oral contraceptives,[135] a decrease of the levels of the natural anticoagulant protein S,[136] and a decrease of fibrinolytic activity, mainly through an increase of thrombin-activatable fibrinolysis inhibitor.[137] The use of oral contraceptives leads to increased resistance to the natural anticoagulant activity of APC resistance, which is partly explained by decreases of free protein S and free tissue factor pathway inhibitor.[138,139]

Although it is clear that the use of oral contraceptives is associated with a prothrombotic state as measured by coagulation and fibrinolysis assays, one may question if these intermediate endpoints indicate a true increased risk of VTE associated with hormone use. For example, combined oral contraceptives that contain progestagens such as desogestrel or gestodene induce a more pronounced APC resistance than combined oral contraceptives containing levonorgestrel.[140,141] In line with this difference in APC resistance, there is convincing evidence that users of combined oral contraceptives with desogestrel or

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gestodene have an increased risk of VTE as compared to users of contraceptives with levonorgestrel.[97] All studies using these intermediate endpoints point in the same direction and are in line with observed risks of VTE, indicating that these coagulation markers may be valid as surrogate endpoints when studies with clinical endpoints are not yet available.

Implications for Use of Hormonal Contraception and Hormone Replacement Therapy

Hormonal Contraception

If an oral contraceptive is considered, a combined oral contraceptive containing levonorgestrel or norethisterone should be preferred over other combined oral contraceptives, because of the lower risk of VTE. Alternatively, a levonorgestrel-releasing IUD, low-dose progestagen-only pills, the contraceptive patch, or the DMPA injectable can be considered. However, costs, adverse effects, and patient preference should also be taken into account in the choice of the contraceptive method.

A common question is whether women with an increased baseline risk of VTE, especially hereditary thrombophilia, are allowed to use hormonal contraception. Although, in absolute terms, the risk increase caused by hormonal contraceptives is larger for women with a higher baseline risk, one should also take into account the risk of VTE caused by an unintended pregnancy. For example, condom use has a much higher failure rate than the use of combined oral contraceptives (12 vs. 0.2 per 100 women-years).[142] A recent study demonstrated that the risk of first VTE in women with hereditary thrombophilia is the same for women who use a condom for contraception as compared to women who use a combined oral contraceptive, due to the additional risk of VTE conferred by unintended pregnancies.[142] Therefore, although a contraceptive method with a low risk of VTE should be preferred, we strongly feel that women should be counseled about the best contraceptive method for their specific situation, taking into account patient preferences. This implies that there should not be an absolute contraindication for hormonal contraceptives.

After an episode of VTE, we strongly advise to refrain from further or future use of hormonal contraceptives. It is noteworthy that there is no immediate need to stop oral contraceptives in women at the time of diagnosis of VTE, as anticoagulant therapy effectively prevents extension and recurrence of VTE[143] and adequate contraception is crucial in women treated with VKA in order to prevent warfarin embryopathy.[12] Thus, oral contraceptives may be continued until shortly before discontinuation of anticoagulant therapy.

Hormone-releasing IUDs are a rational choice for women with previous VTE. Data suggest that this indeed is a safe contraceptive method with regard to VTE risk, although these studies were limited to first thrombotic events and the safety has not been tested in women with a history of VTE.[96,102]

Hormone Replacement Therapy

In the Women's Health Initiative, the harms of HRT (modest increase in breast cancer, arterial cardiovascular disease, and VTE) outweighed the benefits of HRT (improvement of perimenopausal symptoms and a small decrease in osteoporotic hip fractures and colorectal cancer).[124] The most common remaining indication for postmenopausal HRT is improvement of perimenopausal symptoms, especially hot flushes.[144] This benefit needs to be balanced against the increased risks of adverse events, of which VTE is the most important in terms of absolute risk. The shortest possible duration should therefore be preferred. Women with an increased baseline risk of VTE (i.e., hereditary thrombophilia or a positive family history of VTE) are generally advised to refrain from HRT, although risk estimates for these women are less precise.[130] Furthermore, women with a personal history of VTE should not use HRT, as this increases the risk of recurrence.[134] In women with an increased risk of VTE who wish to use HRT despite having been counseled about its risks, transdermal use is preferred over oral use.

Overall Conclusions

Pregnancy and the postpartum period, as well as the use of hormonal contraceptives and HRT, all impact on a woman's lifetime risk of VTE. Identifying women at risk based on a personal or family history of VTE facilitates prevention of pregnancy-related VTE and prudent prescribing of hormones, taking into account patient preferences. Particularly in the area of pregnancy and VTE, there is an urgent need to establish a strong evidence base to guide clinical practice.

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CHAPTER

117 Gestational Vascular Complications

BENJAMIN BRENNER, ANAT AHARON, AND JEAN-CHRISTOPHE GRIS

PLACENTAL HEMOSTASIS IN NORMAL PREGNANCY AND GESTATIONAL VASCULAR COMPLICATIONS

The placenta is a highly vascularized organ receiving blood supply from fetal and maternal circulations. Maternal blood flows in the intervillous spaces, while fetal blood is confined in the intravillous blood vessels. This may result in potential hemostatic problems, mainly the risk of hemorrhage or placental vascular complications[1] (FIGURE 117.1).

The placental villus is lined with trophoblast cells, specialized epithelial cells, that invade through the maternal endometrial stroma, migrate down the lumen of spiral arteries, replace the vascular endothelial lining, and become embedded in the arterial walls.[2,3,4] The trophoblasts are involved in local hemostatic mechanisms as well as in maternal and placental crosstalk (FIGURE 117.1).

TISSUE FACTOR

During pregnancy, placenta and deciduas are putative sources of tissue factor (TF), which is localized on the cell membranes, can bind to factor VII, and initiate the coagulation cascade.[5,6] TF is also essential for embryogenesis, angiogenesis, invasion, and implantation.[7–9] Normally, TF is not presented on cells that are in contact with blood circulation; however, the placenta is a unique organ—its surface is in direct contact with maternal circulation and trophoblast cells constitutively express high levels of TF antigen and activity.[3,10] In addition, cytotrophoblast (CTB) differentiation and fusion to multinucleated cells—syncytiotrophoblasts (STB)—require initiation of apoptosis and exposure of negatively charged phospholipids on their membrane surface.[11] Membrane "flip-flop" and exposure of negatively charged phospholipids are known to promote coagulation. Blockade of TF with a monoclonal antibody prevents activation of the coagulation pathway, restores placental blood flow, precludes placental oxidative stress, inhibits release of soluble vascular endothelial growth factor (VEGF) receptor 1 (sFlt-1), and consequently rescues pregnancy.[12] The procoagulant nature of placental trophoblast and decidual cells may reflect the physiologic need for immediate inhibition of hemorrhage in the placental intervillous spaces, protecting against placental abruption (PA) and meeting the hemostatic demands of labor and delivery.

PHYSIOLOGIC ANTICOAGULANTS

Despite the above-mentioned observations, coagulation does not normally occur across the entire villous surface.[3] The high levels of TF are tightly regulated by a variety of physiologic anticoagulants—tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), thrombomodulin (TM), and annexin V—which act by a variety of mechanisms.

TFPI is highly expressed in placental tissues from the 10 weeks of pregnancy up to term.[13,14] TFPI is produced and pooled (50% to 80%) in the microvascular endothelial cells[15] and in trophoblast cells.[10,13] In normal pregnancy, free TFPI antigen in plasma increased between first trimester and delivery but remained lower in nonpregnant controls.[16]

TFPI-2, (placental protein 5—PP5) is prominently expressed in human placenta[17] and in isolated villous CTB cells,[18] but its physiologic role in the placenta is currently unclear. Inactivation of the murine TF or TFPI genes results in embryonic lethality, indicating that both of these genes are required for embryonic development. Remarkably, the expression of low levels of TF in transgenic mice (1% of human TF) rescues TF-null embryos. However, low-TF mice (mTF(−/−)/hTF+) develop hemostatic defects in the uterus, placenta, heart, and lungs.[19]

TF–TFPI balance in human placenta may be disturbed, resulting in gestational vascular complications (GVCs). It has been found that placental trophoblasts obtained from GVC pregnancies have a disturbed balance between TF and TFPI. While some studies have demonstrated an increase in TF, others have revealed a decrease in TFPI, with underlying mechanisms remaining unclear.[14,20] Inflammatory cytokines, whose levels are occasionally elevated in GVC, contribute to this imbalance.[10] Low molecular weight heparins (LMWHs) stimulate expression, synthesis, and release of TFPI in endothelial cells and may exert their effect in pregnant women at risk for GVC, by modulating local hemostasis at the placental STB surface.[14]

TM is a cell-surface receptor for thrombin that converts thrombin from procoagulant activity into the protein C (PC) anticoagulant pathway. Studies in mice demonstrated that abortion of TM-deficient embryos is caused by TF-initiated activation of the blood coagulation cascade at the fetomaternal interface. Activated coagulation factors induce cell death and growth inhibition of placental trophoblasts.[21] The TM amount observed on vascular endothelium and STB rose significantly with growing gestational age, and the higher levels found at term microvilli were associated with an increase in TM ability to activate PC.[22] TM-mRNA expression increased in villous vessels of intrauterine growth restriction (IUGR) placentas[23] and reduced in placentas of recurrent miscarriages compared to those of healthy pregnancies.[24]

Endothelial PC receptor enables PC activation by the thrombin–TM complex. In mice, EPCR is detected in the giant trophoblast cells at the fetomaternal boundary from E7.5. Disruption of the EPCR gene in mice causes placental thrombosis and early embryonic lethality.[25] $EPCR^{-/-}$ embryos that had been removed

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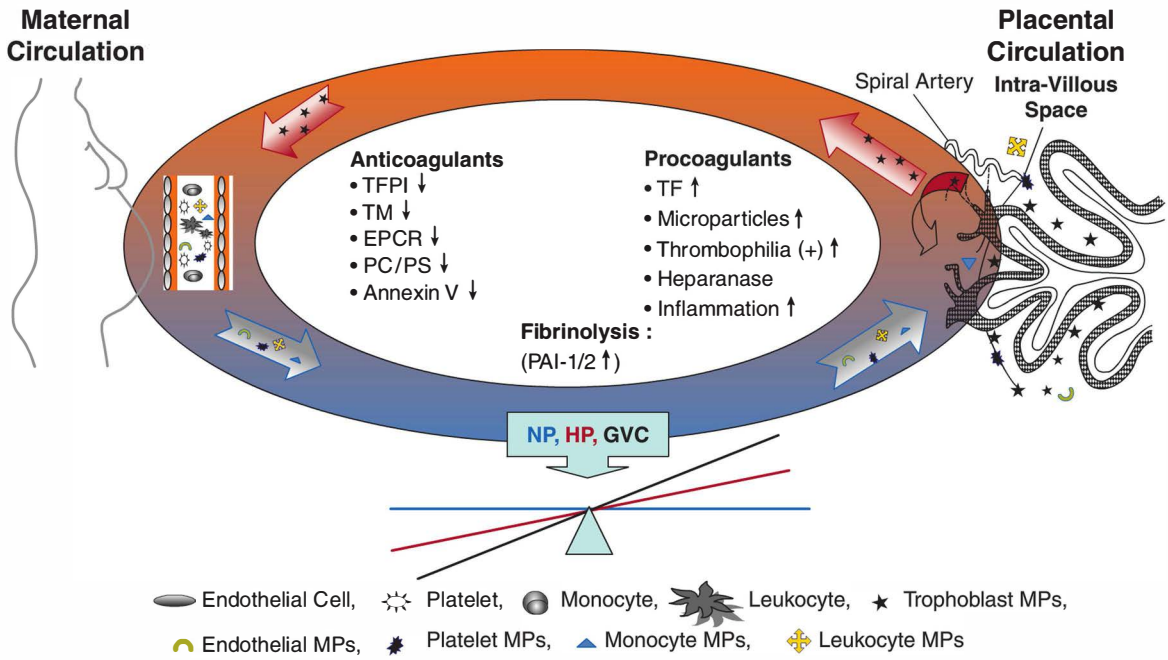

FIGURE 117.1 Placental–maternal crosstalk and hemostatic balance model. In pregnancy, the high levels of tissue factor (TF) on trophoblast cells and microparticles (MPs) are tightly regulated by a variety of physiologic anticoagulants: tissue factor pathway inhibitor (TFPI), endothelial protein C receptor (EPCR), thrombomodulin (TM), and annexin V, which act by a variety of mechanisms. In gestation vascular complications (GVCs), there are further increases in procoagulant properties, including maternal and placental MP levels, and further decreases in anticoagulant proteins, which perturb placental and maternal hemostatic balance. NP - non-pregnant HP - healthy pregnancy

from extraembryonic membranes and tissues at day E7.5 and cultured *in vitro* developed beyond E10.5, suggesting a role of EPCR in the normal function of the placenta and/or at the maternal–embryonic interface.[25]

The common variations in EPCR *gene* (c.323–20T>C, c.717+16G>C, c.323–9_336 duplication, c.655A>G) are rare and are not a major cause of recurrent miscarriage. However, the EPCR A6936G polymorphism was found to be associated with the risk of unexplained fetal loss in Mediterranean European couples and paternal EPCR 219Gly variant was found to be a mild and limited risk factor for deep vein thrombosis during pregnancy.[26–28]

Annexin V, an anticoagulant protein, is highly expressed on the apical surfaces of placental STBs. Binding of annexin V to the trophoblast procoagulant surfaces is crucial for the maintenance of blood flow through the placenta and consequently for fetal viability.[3,29] Reduced annexin V expression has been demonstrated in some placentas from women with preeclampsia (PE)[30] and fetal growth restriction.[31] Anti–annexin V antibodies are associated with higher incidence of intrauterine fetal loss, PE, as well as arterial and venous thrombosis. *In vivo* study demonstrated that anti–annexin V antibody induced trophoblast apoptosis and significantly reduced trophoblast gonadotropin secretion.[32]

Heparanase. Heparan sulphate proteoglycans are abundant in the extracellular matrix (ECM) of placenta and deciduas. Heparanase cleaves heparan sulfate at specific sites, leading to a release of growth factors that may be involved in placentation and trophoblast invasion decidualization and remodeling of the maternal vasculature in humans and mice. Heparanase is expressed in normal and abnormal placenta, in small fetal vessels, and in a variety of trophoblast subpopulations with different invasive potentials.[33,34] A high level of heparanase was observed in isolated first-trimester trophoblasts; however, failure of blocking antibody to inhibit trophoblast invasion suggests another role for heparanase during pregnancy.[35] Heparanase upregulates TF expression and interacts with TFPI on endothelial cells, resulting in increased cell-surface coagulation activity.[36] The regulatory effect of heparanase on TFPI and TFPI-2 in trophoblasts suggests a potential involvement of heparanase in early miscarriages.[37]

Fibrinolysis. Fibrin accumulation, characterizing term placenta, can cause occlusion and infarcts in placental blood vessels and intravillous spaces and may result in placental insufficiency and pregnancy loss (PL). Fibrinolysis is a process of clot degradation by plasmin that is converted from plasminogen by a tissue-type activator (tPA) or a urokinase-type plasminogen activator (uPA). Fibrinolysis is regulated by plasminogen activator inhibitor-1 and -2 (PAI-1, PAI-2) and by thrombin-activatable fibrinolysis inhibitor.[38] Both decidual and extravillous trophoblast cells of human placentas are capable of producing tPA, uPA, PAI-1, and PAI-2 to a different extent. Variations in the distribution of tPA and PAI-1 may be related to fibrinolytic activity at early stages of placentation and to separation of placenta at term, while differences in the distribution of uPA and PAI-2 may be promoted by degradation of trophoblast cell-associated ECM at early stages of placentation.[39] mRNA and protein levels of PAI-1 and PAI-2 were found to be higher in placental

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tissue of preeclamptic women compared to healthy pregnancies. Exposure of trophoblast cell culture to tumor necrosis factor-α results in an increased expression and secretion of PAI-1, with no significant alteration in tPA.[40,41] The change in PAI-1and PAI-2 could explain the imbalance of local fibrinolysis at the placental trophoblast level in gestational complications.

MICROPARTICLES IN NORMAL PREGNANCY AND GESTATIONAL VASCULAR COMPLICATIONS

Microparticles (MPs), membrane vesicles, are found in blood circulation under normal physiologic conditions, and their levels increase in a variety of disease states. MPs are shed from cell membranes upon activation or apoptosis (FIGURE 117.2) and vary in size (0.1 to 1 μm) as well as in phospholipid and protein composition, reflecting those of their cell origin. MPs can induce cell signaling that may lead to invasion, angiogenesis, or apoptosis[42–45] and are involved in thrombosis, inflammation, and vascular dysfunction.[46,47] Therefore, MPs may have a potential significant role in the maternal placental crosstalk.

An overview of the studies measuring circulating levels of MP and their cell origin in normal and complicated pregnancy demonstrates high variation.[48] Some of the trials showed an increase in MP subpopulations in women with PE compared to healthy pregnant females, including increase in the total number of MPs[49] or in total negatively charged phospholipid-bearing MPs,[50] elevation in platelet MPs[50] and activated platelet MPs,[51,52] as well as increase in endothelial MPs[53,54] and white blood cell MPs.[50,55,56] Other studies revealed no differences in the total number of MPs or negatively charged phospholipid-bearing MPs and platelet MPs between normal pregnant and preeclamptic women[53,55,57] or decrease in the total negatively charged phospholipid-bearing MPs and platelet MPs[51,58] and activated platelet MPs[59] in preeclamptic women compared to those with healthy pregnancies. The broad variation in the origin of cell MPs observed in healthy pregnancy and PE may be related to the differences in the characteristics of GVC as well as to the diversity in study methods used for MP characterization.[60]

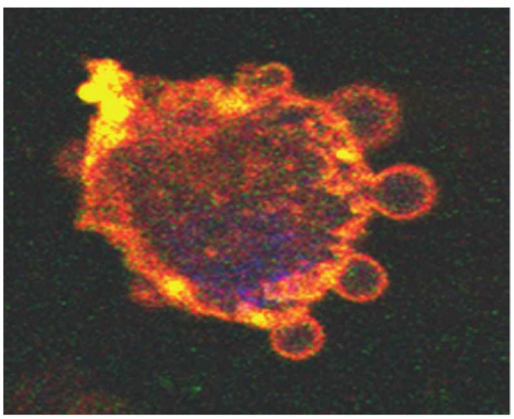

FIGURE 117.2 MP blebs from the membrane surface of umbilical vein endothelial cells (HUVECs) labeled with fluorescent-tagged antibody against CD31 and previously stimulated with monocyte MPs for 2 hours. This photograph was by confocal microscopy (60×).

MICROPARTICLE THROMBOGENICITY

MPs play a dominant role in coagulation initiation and thrombus formation.[47] MPs bearing TF and other coagulation factors may reflect the delicate hemostatic balance between maternal and placental cells (FIGURE 117.1).

MPs obtained from healthy pregnant women demonstrate high procoagulant activity compared to nonpregnant females. The procoagulant activity further increases in MPs of women with GVC without a change in the TF expression, but with reduction in TFPI.[61] On the other hand, other trials demonstrated that MP-associated thrombin generation was similar in normal and preeclamptic pregnancies,[62] the procoagulant activity generated by the total annexin V MPs was unchanged in pathologic pregnancies,[58] and prothrombin fragments 1 + 2 and subsequent thrombin generation were nearly similar in women with recurrent spontaneous abortion and controls.[63] The variance between the studies may be related, not only to the differences in the GVC characteristics and MP study methods, but also to the difference in coagulation assay sensitivity and diversity in the externalization patterns of negatively charged phospholipids and TF on MPs.[64]

TROPHOBLAST-DERIVED MICROPARTICLES

STB MPs are shed into the maternal circulation (FIGURE 117.1) and can be detected in normal pregnancies by the second trimester, while their numbers increase during the third trimester.[65,66] Excessive shedding of STB-derived MPs is a feature of preeclamptic women,[65] mainly in early-onset PE, but not of normotensive IUGR.[67] While normal pregnancy, labor, and placental separation have no effect on the shedding of STB MPs, PE is associated with an increase in STB MP shedding at full dilatation, compared to prelabor levels.[68]

Trophoblast differentiation is assumed to yield massive shedding of TF-bearing MPs that can be detected in the circulation of healthy pregnant women, and the percentage of total TF-bearing MPs significantly decreases in GVC. This represents an increase in the maternal source of TF-bearing MPs that potentially reflects the systemic nature of such pathologies.[61]

PE is characterized by a rise in inflammation markers. Higher amounts of circulating STB MPs in maternal blood might lead to endothelial dysfunction monocyte stimulation and excessive maternal inflammatory reaction.[65,66,69,70] Additionally, preeclamptic MPs have been shown to induce nuclear factor-κB activation and nitric oxide (NO) synthase expression and to enhance oxidative stress.[71]

In summary, the balance between the procoagulant TF and the physiologic anticoagulants is crucial for pregnancy and influences the placenta, embryo, and maternal crosstalk. The placental hemostatic balance has a major role in normal pregnancy, and the balance disruption may result in pregnancy complications. MPs affect physiologic processes (cell migration, invasion, and angiogenesis) and can promote pathologic states including thrombus formation, inflammation, and endothelial dysfunction. MPs participate in the placental and maternal crosstalk in normal pregnancies as well as in GVCs (FIGURE 117.1).

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HEMOSTATIC CHANGES IN NORMAL PREGNANCY

Normal pregnancy is characterized by a marked increase in the procoagulant activity, manifested by an elevation of coagulation factors VII, X, and VIII; fibrinogen; and von Willebrand factor[72] and an elevation in prothrombin fragments 1 + 2, and thrombin–antithrombin (AT) complexes,[73] which is maximal around term. There is a profound decrease in physiologic anticoagulants, manifesting by a significant reduction in protein S (PS) activity[74] and by acquired activated protein C resistance.[75] The overall fibrinolytic activity is impaired during pregnancy but returns rapidly to normal following delivery,[76] largely due to placenta-derived PAI type 2, which is present in substantial quantities during pregnancy.[77] Levels of D-dimer, a specific marker of fibrinolysis resulting from breakdown of cross-linked fibrin polymer by plasmin, increase as pregnancy progresses.[78]

IMPLANTATION AND HEMOSTASIS

Pregnancy requires successful implantation of competent embryo into a receptive endometrium. Monthly fecundity rates in humans compared to other mammalian species are relatively low at approximately 20%.[79] Of note, clinical pregnancy rates per embryo transfer for the year 2006 in Europe were around one-third.[80] Current data suggest that thrombophilia can be associated with repeated implantation failure. One study reported significantly higher prevalence of thrombophilias in women with four or more failed IVF cycles when compared with spontaneous conceptions (OR 3.6, 95% CI 1.25 to 10.6) or women who conceived after their first cycle (OR 2.9, 95% CI 1.02 to 8.4).[81] The mechanisms underlying these associations are less clear, partly due to the limited knowledge regarding the earliest stages of human implantation.

HEMOSTATIC CHANGES IN PREECLAMPSIA

Thrombocytopenia is a consistent finding in severe cases of PE closely correlating with activation of coagulation. A fall in platelet count in women with gestational hypertension may herald the onset of PE.[82] The platelet membrane shows an abnormal lipid composition with a raised cholesterol to phospholipid ratio in PE.[83] Assessment of platelet hyperactivity in whole blood may reveal elevated aggregation. Release of inflammatory mediators from activated platelets may trigger inflammatory reactions in endothelial cells. In theory, platelets are a potential regulator of trophoblast infiltration into maternal spiral arteries, thereby contributing to vascular remodeling.[84] While at present the role of activated platelets in the pathophysiology of PE remains to be established, evidence of platelet activation preceding the onset of PE is emerging.[85]

Alterations in the hemostatic system leading to excessive uteroplacental thrombosis are thought to be an important feature at the first stage in the pathogenesis of PE, involving reduced placental perfusion due to failed vascular remodeling in early pregnancy within the placental bed.[86] Coagulation factor levels including TF, factor VIII, fibrinogen, and von Willebrand factor are significantly higher in the peripheral circulation in PE compared with normal pregnancy. TF and free TFPI concentrations are significantly higher in maternal plasma of PE women compared with normal pregnancy.[87,88] The TFPI to TF ratio is significantly lower in patients with PE than in normal pregnancy, and there is a higher maternal plasma concentration of PAI-1 in PE compared to a normal pregnancy. Of interest, increased PAI-1 levels are detected in patients who show early evidence of placental dysfunction on uterine artery Doppler studies, while the low PAI-2 levels in PE may reflect placental dysfunction.

EPIDEMIOLOGY AND PATHOGENESIS OF PREGNANCY COMPLICATIONS

Unexplained PL and GVC, including PA, PE, and small-for-gestational age (SGA) pregnancies, are heterogeneous gestational pathologies associated with devastating maternal and fetal complications, variably affecting 5% to 15% of all pregnancies.

Up to 9% to 13% of women experience one clinically recognized PL, 5% two or more PLs, and 1% to 2% three or more losses.[89–93] Up to 50% of recurrent PLs remain unexplained, with unclear initial categorization[94] (Table 117.1). Early measurements of the fetus crown-rump length (CRL) and detection of fetal heart beats led to the definition of gestational age based on ultrasound (US)[95] assessment ("fetus" meaning fetal heart activity and/or a CRL > 10 mm), with four PL categories being recognized[95] (Table 117.2). Women with idiopathic PL tend to have recurrences at the same gestational age period,[96] with a likelihood of PL causes to be gestational age specific. Placental bed biopsies from karyotyped, late[97] and early[98] sporadic miscarriages suggest that only late miscarriages are associated with impaired trophoblast invasion and inadequate transformation of spiral arteries, similar to PE. Most recurrent PLs are nonfetal cases resulting from random repetition of aneuploidic pregnancies; normal embryonic karyotypes increase with the number of previous PLs ($n = 2$: <50%; >6: 75%)[99] and predict a subsequent PL.[100] First-trimester idiopathic recurrent PL occurs in 22%, 2%, and 0.6% before 6, 8, and 10 gestational weeks, respectively with only 3% following the detection of fetal heart activity.[101] Recurrent PL means at least three losses, usually of unknown cytogenetics: two euploid PLs identify couples at higher recurrence risk, likely to bear a causal noncytogenetic factor.[102]

PE complicates 4% to 10% of nulliparous pregnancies and is twofold more frequent in primigravidas.[103] The recurrence risk is around 15% after one previous PE, increasing to 30% after two consecutive PEs.[104] A severe recurrent early-onset type, with delivery before 34 weeks, affected by chronic genetic/environmental factors, opposes a milder sporadic form affected by transient factors (two-third of cases).[104] Severe PE subtypes are associated with various recurrence risks. Eclampsia generates a

Table 117.1

Classical definitions of PL types

Type of PL	Typical Gestation, Weeks
Miscarriage	<20[a]
First-trimester PL	5–12[a]
Second-trimester PL	13–24
Third-trimester PL	≥25

[a]Heterogeneous: before 10 wk, embryonic loss; after 10 wk: fetal loss.

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Table 117.2

Contemporary definitions of PL types based on US measurements

Type of PL	Typical Gestation, Weeks (Range)	US Findings	Fetal Heart Activity	βHCG Levels and Kinetics
Biochemical loss	6 (0–6)	Pregnancy is not located	Never detected	Low then fall
Early PL, embryonic PL	Between 6 and 8 (4–10)	Empty sac or large sac with minimal structures	Not detected	Rise then fall
Late PL, fetal PL	From week 12 (10–20)	CRL > 10 mm	Detected then lost	Rise then are static or fall
Stillbirth	>20 (viable)	Crown-heel length > 25 cm Estimated weight > 500 g	Detected, confirmed then lost	—

mean 22% PE recurrence risk and a 2% eclampsia risk. In primigravidas, early-onset PE before 34 weeks generates a mean 25% PE risk and a 5% early-onset PE risk during the second pregnancy. Moreover, a second-trimester PE generates a 66% PE risk on the next pregnancy. Likewise, HELLP syndrome (hemolysis, elevated liver enzymes and low platelet counts) generates a mean 43% PE and a 27% HELLP syndrome recurrence risk. While smoking during pregnancy is associated with spontaneous abortion, stillbirth, preterm labor, fetal growth restriction, and PA, the incidence of PE is reduced in smokers.[105] Abnormal placentation and an imbalance in angiogenic factors are essential for the development of the PE syndrome,[106] with high levels of trophoblast-derived circulating antiangiogenic factors mediating maternal endothelial dysfunction, soluble fms-like tyrosine kinase-1, and soluble endoglin.

In PE, the loss of endothelial NO synthase activity promotes endothelial dysfunction due to production of superoxide instead of NO.[107] PE may arise due to loss of VEGF activity and from the possible elevation of soluble Flt-1 (sFlt-1).[108] In support of this, adenoviral overexpression of sFlt-1 in pregnant rats resembled manifestations of the disease[109] and serum levels of sFlt-1 and placenta growth factor were found to be associated with the clinical presentation of PE.[110–112] Finally, reducing circulating levels of free sFlt-1 below a certain threshold in women with PE could alleviate the clinical signs of the syndrome.[113]

PA, defined as premature separation of a normally inserted placenta prior to delivery of the fetus, complicates 1% of pregnancies. The maternal effect depends on PA severity, whereas the fetal PA effect is determined both by its severity and by its gestational age onset. PA involving more than 50% of the placenta leads to fetal death. Conditions associated with acute inflammation (premature rupture of membranes and intrauterine infection) are more prevalent in preterm PA than in-term gestation abruptions, whereas chronic processes inducing vascular dysfunction or chronic inflammation are present throughout gestation.[114]

PE, SGA pregnancies (<10th percentile adjusted on gestational age; severe if <3rd percentile), and PA are closely related heterogeneous diseases, with reduced trophoblast invasion, uteroplacental underperfusion, chronic hypoxia, and placental ischemia as the principal mechanism involved in their cause. Reasons for preterm births include first of all PE, then SGA, fetal distress, and PA (around 50% of cases).[115] A retrospective cohort study showed women with PE, SGA, and PA in their first pregnancy having an increased risk of recurrence of any of the GVCs,[116] suggesting some similarity of causative mechanisms.

THROMBOPHILIA AND FETAL LOSS

Acquired thrombophilia is associated with fetal loss, mainly first-trimester PL, and its rate is increased in women with essential thrombocythemia (ET).[117–119] The *JAK2*V617F mutational status was inconsistently found to be related to a higher incidence of ET pregnancy-associated complications. The incidence of the *JAK2* V617F mutation was increased in PL cases in a large cohort of women in first pregnancy,[120] with small series showing discrepant results.[121]

Antiphospholipid antibodies (aPL-Abs) and the primary antiphospholipid antibody syndrome (APS) are emblematic models.[122,123] Fetal loss at or beyond week 10 and recurrent consecutive embryonic losses before week 10 are classical features entering the obstetrical APS definition. A meta-analysis assessed the strength of association of lupus anticoagulant (LA), anticardiolipin antibodies (aCL-Abs), and anti-β_2 glycoprotein-1 antibodies (aβ_2GP1-Abs) with non-SLE–related recurrent miscarriages. LA was strongly associated with late PL (<24 weeks) with no evidence of association with early PL (<13 weeks); similar results were obtained for aβ_2GP1-Abs.[124] In contrast, aCL-Abs were associated with both early and late recurrent PLs. Retrospective studies have shown a stronger association with recurrent fetal loss >10 weeks in women positive for all the three tests.[125,126] Positivity for aβ_2GP1-Abs is found to correlate

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with a positive LA in women with PL.[127] Association of LA and aβ_2GP1-Abs with late PL is thus probably more reasonable.[128,129] The role and significance of other autoantibodies is currently under investigation. An observational study in a cohort of women with a first embryonic PL suggested that aPL-Abs–positive women have a higher risk of various GVCs during their second pregnancy.[130]

Anticoagulants, such as hirudin or fondaparinux, did not prevent PL in a mouse model of APS, suggesting that thrombosis is not the cause of fetal death in this model.[131] TF on maternal neutrophils is critical for the pathogenesis of aPL-induced fetal loss, with a suggested functional linkage between complement component C5a, TF, and neutrophil activation. Activated neutrophils release reactive oxygen species and proteolytic enzymes, leading to decidual damage and fetal wastage.[132] TF inhibition decreased sFlt-1 release, restored placental blood flow, prevented placental oxidative damage, and rescued pregnancies, suggesting that TF may be an interesting target for therapy in this model.

Constitutional thrombophilias. Scattered data suggest that pregnancy outcome in maternal homocystinuria is usually normal.[133,134] Mild hyperhomocystinemia, resulting from common polymorphism (homozygosity for the *MTHFR* C677T) and nutritional conditions (folate, vitamin B_6 and B_{12} deficiency), is associated with a noncausal enhanced risk of embryonic and fetal PL,[129,135] with vitamin supplementation attempts being unsatisfactory.[136]

Dysfibrinogenemia cases were associated with a high PL rate and, to a lesser extent, with stillbirths,[137] with prophylactic use of fibrinogen concentrates suggested to be of value. Fibrinogen supports the development of fetal–maternal interface and stabilizes embryo implantation in animal models.

The first systematic analysis of a possible association between PL and AT, PC, PS deficiency, or factor V Leiden (FVL) variant focused on cohorts of women from thrombotic families with a documented thrombophilia.[138,139] The EPCOT study has demonstrated the risk of PL to be increased in thrombophilic women, with a stillbirth risk being higher than other PL risk types. Additionally, combined defects were associated with a higher risk for PL. While AT and PS deficiencies reached significance for stillbirth, PC deficiency appeared to be significant for miscarriage.[139] The follow-up of females participating in the EPCOT trial[140] showed a slightly increased risk of fetal loss in untreated women with thrombophilia without a previous history of PL. The relative risk varied minimally from 1.4 for FVL to 1.6 for AT deficiency.

Case–control studies thereafter focused on women with various PL types[141–152] and generally found positive associations. However, in some of the studies, these associations were limited to the fetal-type PL,[146–152] while other reports failed to show significance.[26,153–155]

A crucial new function of the PC system in controlling the growth and survival of trophoblast cells had emerged from mouse models.[24] In human couples, presence of maternal and paternal *PROCR* polymorphism in the EPCR gene, a cell-surface trophoblastic receptor, could predict a first fetal loss.[156]

Several reviews and meta-analyses attempted to clarify the observed discrepancies regarding the prevalence of thrombophilic risk factors and GVCs[157,158,159] (Table 117.3). Conclusions of the TREATS study[159] pointed toward a significant, albeit modest, increase in the relative risk. The major findings included a higher risk for a late PL in the third trimester, stronger associations with recurrent PL, and a higher risk of second-trimester loss than of recurrent first-trimester loss for FVL and *F2* 20210A heterozygotes; finally, late PL was most strongly associated with PS deficiency. However, potential confounders and biases in design and conduction of available studies may have an impact on the results, thereby limiting the strength of conclusions.[94] A meta-analysis of prospective cohort studies, claimed to provide a superior methodologic design, showed the probability of PL in FVL women to be 52% higher, corresponding to a small absolute increase in risk of nonembryonic PL (3.2% to 4.2%), but lacked power to detect increased risks in women with *F2* 20210A polymorphism.

Evidence linking constitutional thrombophilias to PLs is still insufficiently characterized, lacking the systematic integration of categorization and cofactors. Routine screening of pregnant women cannot be recommended. Screening of previously symptomatic women depends on the assessment of beneficial therapeutics in women harboring thrombophilia. Even if thrombophilias are not strongly causative, they may contribute to miscarriage potential. In this regard, regulating hemostasis may have potential clinical advantage.[94]

Table 117.3

Constitutional thrombophilias and PL

PL Type	AT-def	PC-def	PS-def	MTHFR-H	FVL-h	FVL-H	PTM-h
Early PL, first and second trimester[159]	0.9 (0.2–4.5)	2.3 (0.2–26)	3.6 (0.4–36)	1.4 (0.8–2.6)	1.7 (1.1–2.6)	2.7 (1.3–5.6)	2.5 (1.2–5.0)
Recurrent first-trimester PL[159]				0.9 (0.4–1.7)	1.9 (1.0–3.6)		2.7 (1.4–5.3)
Nonrecurrent PL, second trimester[159]					4.1 (1.9–8.8)		8.6 (2.2–34)
Late PL, third trimester[159]	7.6 (0.3–196)	3.1 (0.2–39)	20 (3.7–109)	1.3 (0.9–1.9)	2.1 (1.1–3.9)	2.0 (0.4–9.7)	2.7 (1.3–5.5)
All PL[161]					1.5 (1.06–2.2)		1.1 (0.64–2.0)

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THROMBOPHILIA AND GESTATIONAL VASCULAR COMPLICATIONS

Acquired thrombophilias. Clinical criteria for the diagnosis of APS include one or more premature births of a morphologically normal neonate before the 34th week of gestation as a result of (a) eclampsia or severe PE, or (b) recognized features of placental insufficiency.[122] Definitive prospective studies examining the contribution of aPL-Abs to preterm births associated with placental insufficiency or severe PE are currently not available. The TREATS study found aCL-Abs, but not LA, to be associated with PE.

Constitutional thrombophilias. Genetic thrombophilias were first reported to be associated with various GVCs in late 1990s.[160] Further studies analyzed specific GVC subtypes. The TREATS study[159] (Table 117.4) showed the global risk of PE to be associated with heterozygous FVL or *F2* 20210A polymorphisms and homozygous *MTHFR* 677T polymorphism. The paucity of data related to AT, PC, and PS deficiencies is mainly due to the low prevalence of these risk factors. Evidence of heterogeneity was demonstrated in the analysis of heterozygous FVL, which was found to be related to severe PE only (OR 2.04 [1.23 to 3.36]), but not to mild PE.[158] Of note, the Ottawa review on prospective cohort studies[161] found a trend but no significant association of GVC and thrombophilia (Table 117.4).

While the global risk of PA was found to be significant for a heterozygous FVL or a heterozygous *F2* 20210A polymorphism,[159] it was not reported in prospective studies[161] (Table 117.4). A recent analysis[162] performed on 22 articles showed the random-effect OR for FVL to be 3.4 (1.4 to 8.3) and the fixed-effect OR for the *F2* 20210A polymorphism to be 6.7 (3.2 to 13).

Finally, the global risk for SGA pregnancies was nonsignificant for a heterozygous FVL or *F2* 20210A and for a homozygous *MTHFR* 677T polymorphism[159,161] (Table 117.4).

A large population-based nested case–control study performed in Finland showed FVL but not *F2* 20210A polymorphism to be a risk factor for late preterm birth at or after 32 weeks—OR 2.9 (1.5 to 5.6), with history of venous thrombosis increasing the risk.[163]

The evidence linking constitutional thrombophilias to GVCs is heterogeneous, lacks systematic integration of severity and cofactors, shows modest OR, and is mainly sustained in PA. Routine screening of pregnant women for thrombophilia cannot be recommended. Screening of previously symptomatic women will depend on conclusive therapeutic developments in women harboring thrombophilia.

ANTITHROMBOTIC THERAPY DURING PREGNANCY

Anticoagulant therapy is indicated during pregnancy for the prevention and treatment of venous thromboembolism (VTE), systemic embolism in patients with mechanical heart valves and, in combination with aspirin, in women with aPL-Abs and recurrent PL.[164] Currently available antithrombotics include unfractionated heparin (UFH), LMWH, and antiplatelet agents. In the past, UFH was the drug of choice for the prevention and treatment of VTE during pregnancy. However, because of its significant adverse effects, such as osteoporosis and heparin-induced thrombocytopenia (HIT), as well as the need for laboratory monitoring, UFH is being replaced by LMWH in gestational settings.[165] Both UFH and LMWH do not cross the placenta and are not secreted in breast milk; however, LMWHs exhibit a number of clinical advantages over UFH, including superior bioavailability, longer plasma half-life allowing once-daily dosing, and an improved safety profile.[166] The anticoagulant effect of heparin is mediated largely through its interaction with AT; this produces a conformational change in AT and so markedly enhances its ability to inactivate the coagulation enzymes thrombin, factor Xa, and factor IXa.[167] In addition, LMWH increases production and secretion of TFPI from endothelial cells.[168,169]

SAFETY OF LMWH DURING PREGNANCY

Although women with prior VTE have an increased risk of disease recurrence during gestation, no large clinical trials assessing the role of prophylaxis in pregnant women with previous VTE

Table 117.4

Constitutional thrombophilias and GVCs

GVC Type	AT-def	PC-def	PS-def	MTHFR-H	FVL-h	FVL-H	PTM-h
Preeclampsia[159]	3.9 (0.2–97)	5.2 (0.3–102)	2.8 (0.8–11)	1.4 (1.1–1.8)	2.2 (1.5–3.3)	1.9 (0.4–7.9)	2.5 (1.5–4.2)
Preeclampsia[161]					1.23 (0.89–1.70)		1.25 (0.79–1.99)
Placental abruption[159]	1.1 (0.1–18)	5.9 (0.2–151)	2.1 (0.5–9.4)	1.5 (0.4–5.4)	4.7 (1.1–20)	8.3 (0.4–171)	7.7 (3.0–20)
Placental abruption[161]					1.85 (0.92–3.70)		2.02 (0.81–5.02)
Small for gestational age[159]				1.2 (0.8–1.8)	2.7 (0.6–12)	4.6 (0.2–116)	2.9 (0.6–14)
Small for gestational age[161]					1.00 (0.89–1.25)		1.25 (0.92–1.70)

Results of the TREATS study, a systematic review of retrospective and prospective studies[159] and of the Ottawa meta-analysis of prospective cohort studies[161]: overall average odds ratios and 95% confidence intervals.
AT, antithrombin; PC, protein C; PS, protein S; def, deficiency; h, heterozygous; H, homozygous; MTHFR, *MTHFR* 677T polymorphism; FVL, *FV* 1691A polymorphism; PTM, *F2* 20210A polymorphism.

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are available.[164] A recent review of close to 2,800-treated pregnancies evaluated safety and efficacy of LMWH in pregnancy.[170] The main indications were prophylaxis of VTE and prevention of PL. The rate of bleeding complications was low and thrombocytopenia was rare, with no cases of HIT. Likewise, clinically significant osteoporosis was extremely rare. Live birth rates were 85% to 96%, depending on the indication for treatment.[171] A good safety profile in the use of enoxaparin during 624 pregnancies was also documented in a retrospective French study.[171]

ANTIPHOSPHOLIPID SYNDROME

In women with recurrent miscarriage associated with the antiphospholipid syndrome, the use of a combination of low-dose UFH and low-dose aspirin, compared to aspirin alone, showed an absolute increase of live birth rate from 41% to 72% in a randomized controlled trial (RCT),[172] with two other trials also reporting a benefit of this combination compared to aspirin alone.[173,174] Two RCTs where LMWH was added to aspirin did not demonstrate benefit of this combination therapy in this setting.[175,176] Notably, there are hardly any placebo-controlled trials to assess the efficacy of aspirin alone in women with antiphospholipid syndrome.[177] Currently, the grade 1B level of recommendation by the American College of Chest Physicians guidelines is not shared by other guidelines.[178]

PREGNANCY LOSS IN THROMBOPHILIC WOMEN

In an earlier study, 50 women who had thrombophilia and were presented with recurrent fetal loss were treated with the LMWH enoxaparin throughout gestation and 4 to 6 weeks in the postpartum period.[179] Of the 61 pregnancies, 46 (75%) resulted in a live birth, compared with a success rate of only 20% in these women in previous gestations without antithrombotic therapy.[180]

A study by Gris et al.[181] demonstrated that in women who had thrombophilia and previous one PL after a 10-weeks gestation, enoxaparin at a dose of 40 mg daily resulted in a significantly better live birth rate compared with low-dose aspirin (86% vs. 29%, respectively). The differences were found in women who had FVL and *F2* 20210A as well as in those who had PS deficiency.

LIVE-ENOX is a multicenter, prospective, randomized study comparing two doses of enoxaparin, 40 mg/day and 40 mg/every 12 hours, in women with thrombophilia and a history of PL.[182,183] Of the 180 women enrolled, live birth rate before the study was only 28%, but during the study, live birth rates were 84% for the 40 mg/day group and 78% for the 80 mg/day group. Late gestational complications decreased after enoxaparin treatment. Both doses of enoxaparin appeared to be safe and well tolerated, with low incidence of postpartum bleeding and enoxaparin-related allergic local skin reactions.

While these studies are encouraging, large prospective RCTs have not been preformed.

UNEXPLAINED PREGNANCY LOSS

An earlier study comparing enoxaparin 40 mg/day to low-dose aspirin reported similar live birth rate in women with unexplained PL in whom thrombophilia was excluded.[184] Three recent prospective multicenter randomized trials, ALIFE, SPIN, and HABENOX, reported that antithrombotic prophylaxis did not improve pregnancy outcome in women with at least two unexplained PLs.[185,186] Thrombophilia cases were a small minority in these trials. Indeed, the majority of cases of unexplained PL could be attributed to other causes, including aneuploidy.

ANTITHROMBOTIC PROPHYLAXIS FOR GESTATIONAL VASCULAR COMPLICATIONS

A pilot randomized trial compared dalteparin 5,000 U/day versus no prophylaxis in 110 women without identifiable thrombophilia who had prior placenta-mediated complications.[187]

Dalteparin was associated with a lower rate of the composite primary outcome (severe PE, birth weight < 5th percentile, major PA), which occurred in 5.5% of those in the dalteparin arm compared to 23.6% in the no prophylaxis arm (OR 0.15, 95% CI 0.03 to 0.70, NNT 5.5, $P = 0.016$).

In another study, 160 women with prior objectively confirmed PA were randomized to enoxaparin 4,000 IU/day until 36 weeks or to no drug intervention.[188] The primary composite outcome consisted of any of the following: PE, SGA PA, or IUFD. The primary outcome was significantly reduced from 31.3% (25/80) in the no intervention group to 12.5% (10/80) in the enoxaparin group. In a subsequent study, 224 women with prior objectively confirmed severe PE were randomized to enoxaparin 4,000 IU/day plus low-dose aspirin or to low-dose aspirin.[189] The primary outcome was the composite one described above. This primary outcome was significantly reduced from 25% (28/112) in the no intervention group to 8.9% (10/112) in the enoxaparin group. While promising, subsequent studies will be required to solidify these findings.

A small placebo controlled RCT demonstrated that enoxaparin 40 mg/day at the time of embryo transfer was associated with a significant improvement in live birth compared with placebo (23.8% vs. 2.8%, respectively; $P < 0.05$).[190] RCTs are needed in the setting of recurrent implantation failure to asses this clinical relevant question.

MONITORING OF LMWH THERAPY DURING PREGNANCY

Since LMWH preparations inhibit preferentially FXa and only to a low extent thrombin and activated partial thromboplastin time, anti-Xa assays have been developed and validated to determine their anticoagulant effect.[191] For the vast majority of patients, LMWH have proved to be effective and safe without the need for anticoagulant monitoring.[192] However, the need to adapt the LMWH dosage to the weight of the pregnant woman or to monitor LMWH treatment during pregnancy remains controversial.[193,194] Plasma levels of anti-Xa activity and TFPI may help to predict the outcome in LMWH-treated pregnancies.[168] Risk assessment of clinical history and thrombophilic risk factors as well as careful monitoring of LMWH therapy can be potentially beneficial for women with high-risk pregnancies.[195]

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CHAPTER

118 Maternal Hemostatic Disorders and Obstetric Hemorrhage

PHILIPPE DE MOERLOOSE, FRANÇOISE BOEHLEN, AND JEAN-CHRISTOPHE GRIS

This chapter reviews conditions associated with disorders of the hemostatic system, which can affect the mother and lead to obstetric hemorrhage, excluding mechanical causes of antepartum hemorrhage (e.g., abnormal placentation, uterine rupture, vasa previa, infections, polyps, or trauma) or postpartum hemorrhage (PPH) (retained placenta, uterine atony, or cervical or vaginal lacerations).[1,2] In the latter situations, coagulation disorders are usually a consequence and not a cause of obstetric hemorrhage.

PLATELET DISORDERS

Thrombocytopenia is the most common hemostatic disorder encountered in pregnancy, and can be considered as isolated and mainly asymptomatic or part of a complex clinical context.

Isolated Platelet Disorders

The differential diagnosis of thrombocytopenia discovered during pregnancy is wide and varies with gestational age and is similar to that of nonpregnant women, but some are unique to pregnancy (FIGURE 118.1),[3,4,5,6] notably the "physiologic" decrease of platelet count.[7–9] Thrombocytopenia, defined as platelet count <150 G/L, is a very common and most often an incidental finding during pregnancy, occurring in 5% to 12% of pregnant women,[7,9–11] but a platelet count <100 G/L is present in only about 1% of pregnant women.[10,12]

Incidental Thrombocytopenia

Gestational thrombocytopenia of pregnancy is responsible for 75% to 80% of all pregnancy-associated thrombocytopenias,[4,10,11,13] occurring mainly in late pregnancy, in distinction to immune thrombocytopenia (ITP), which is the most common diagnosis during the first trimester of pregnancy. It is important to distinguish incidental thrombocytopenia from ITP, the implication for the pregnancy and the newborn being very different, but it often is not possible to distinguish between these two entities. Incidental thrombocytopenia is a diagnosis of exclusion,[14] requiring the following five criteria[15,16]:

- Asymptomatic, mild thrombocytopenia: typically >70 G/L (most often between 130 and 150 G/L)
- Occurrence typically in third trimester
- No prior history of thrombocytopenia (except possibly during a previous pregnancy)
- No fetal thrombocytopenia
- Spontaneous resolution after delivery

According to these criteria, the diagnosis can be confirmed only after birth. Incidental thrombocytopenia being by definition a mild thrombocytopenia, there is no risk for the mother. Indeed a platelet count of more than 70 G/L is usually considered as safe for a locoregional anesthesia and is not associated with an increased hemorrhagic risk during pregnancy and the postpartum period.[17,18] There is no risk for the newborn too since by definition this diagnosis implies that the newborn will have no thrombocytopenia.[10,16,19,20]

The mechanism of this thrombocytopenia is still uncertain. It could be a physiologic condition due to dilution and/or an increased platelet turnover.[21]

Immune Thrombocytopenia

ITP is an acquired immune-mediated disorder characterized by isolated thrombocytopenia,[22] a platelet count of <100 G/L now considered as the threshold for diagnosis.[23] ITP occurs in approximately 1 per 1,000 pregnant women, accounts for 3% to 5% of pregnancy-associated thrombocytopenias,[4,10,11,13,24] and is the most common cause of isolated thrombocytopenia in the first trimester.[4,13] According to the retrospective analysis of Webert et al.,[25] most women with ITP have only a mild to moderate decrease of platelet count; 30% to 50% of women with ITP are first diagnosed during pregnancy.[25,26] The diagnosis of ITP is essentially one of exclusion,[6,15,16,27,28] the most difficult distinction being from incidental thrombocytopenia of pregnancy. Search for antiplatelet antibodies has failed to differentiate these two pathologies.[12] The two most important points are the monitoring of maternal platelet count during pregnancy and after delivery and control of the newborn's platelet count at birth and during the 1st days of life. In general, as detailed in the next paragraphs, (a) the evolution of the platelet count during pregnancy is usually unpredictable;[28] (b) the risk of maternal hemorrhage depends upon the severity of thrombocytopenia;[25] (c) the risk of severe neonatal thrombocytopenia is relatively low;[10] (d) there is no way to predict the severity of fetal/neonatal thrombocytopenia;[10] and (e) maternal treatment does not influence the newborn's platelet count.[29]

Bleeding risk during pregnancy. Women have no bleeding symptoms (65.6%) or only mild or moderate symptoms (respectively 12.9% and 18.1%), and only 3% to 4% of pregnant women have severe symptoms (3.4%).[25] The "safe" platelet number allowing spinal or epidural anesthesia (without risk of epidural hematoma) remains controversial, and there are no data to support a minimum required platelet count.[30] However, as proposed in international consensus, a platelet count ≥70 to 80 G/L is usually considered as safe.[17,18,28]

Management

Treatment is recommended in case of bleeding symptoms or when the platelet count is below 20 to 30 G/L.[15,31] When the delivery date approaches, it is usually recommended to introduce

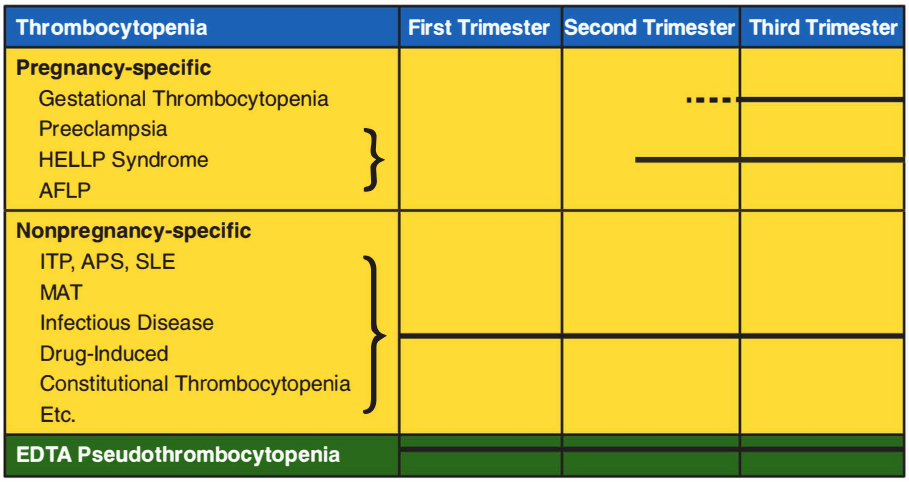

FIGURE 118.1 Differential diagnosis of thrombocytopenia discovered during pregnancy as function of gestational age. AFLP, acute fatty liver of pregnancy; APS, antiphospholipid syndrome; HELLP, hemolysis, elevated liver enzymes, low platelets; ITP, immune thrombocytopenia; MAT, microangiopathic thrombosis; SLE; systemic lupus erythematosus.

treatment to attain a platelet count of >50 G/L to allow good hemostasis during vaginal delivery or cesarean section and to avoid PPH. It is possible that a lower platelet count is acceptable for vaginal delivery without locoregional anesthesia.[6,13,15,31,32] Indeed, in a report of 92 women with ITP (119 pregnancies), the median platelet count at delivery was 85 G/L. In this report, 15% of these women had a platelet count <50 G/L and 6% had a platelet count <20 G/L. Hemorrhagic complications were reported in only four women (nonrequiring red cell transfusions) and were not related to the severity of thrombocytopenia.[25]

Treatment options for the management of ITP in pregnancy are usually similar to those proposed in the general population.[13] First line therapy is usually based on corticosteroids and/or intravenous immunoglobulins.[6,27,28,31] Classically, oral prednisone is initially administered at the dosage of 1 mg/kg/d based on the pregestational weight. This treatment can be associated with side effects for the mother during the gestational period (pregnancy-induced hypertension, gestational diabetes, weight gain, psychosis, etc.) and rarely for the fetus (suppression of the fetal adrenal glands).[31] To limit these side effects, prednisone could be started at a lower dose (e.g., 10 to 20 mg/d), and the dose can be progressively increased to obtain a safe platelet count according to the gestational age.[27] High doses of immunoglobulins (2 g/kg) may be safer than a high dose of prednisone with a similar efficacy but they need to be administered every 3 to 6 weeks.[13,31,33] In case of refractoriness, high dose of methylprednisolone could be used.[28,31,34–36] Azathioprine may be administered safely during pregnancy, but there are no available studies in the case of ITP.[31,37,38,39] Anti-RhD immunoglobulins have been proposed as second line therapy[28,40] but they have to be used with precaution, particularly with a careful monitoring of the neonate although complications are uncommon (neonatal jaundice, anemia, positive direct antiglobulin test after delivery).[28,40] Danazol has been used but only for short-term treatment.[41] Other immunosuppressive agents (such as cyclosporine or cyclophosphamide) cannot be used during pregnancy.[39] Experience with rituximab during pregnancy is limited and its safety for the fetus is uncertain (category C drug for FDA). It is therefore recommended to avoid rituximab during pregnancy unless the mother's health is critically dependent upon use of this medication.[39,42–44] There are no available data on the use of thrombopoietin receptor agonists in pregnancy. Splenectomy can be safely performed during the second trimester.[45,46]

If severe thrombocytopenia is diagnosed near delivery, acute treatment with intravenous immunoglobulin 1 g/kg/d for 2 to 3 days combined with methylprednisolone 1 g/d for 3 days will usually result in an increased platelet count within 24 to 48 hours.[22,32] In case of acute obstetric hemorrhage, for example, in a laboring woman, the treatment will differ. Intravenous immunoglobulin and methylprednisolone have to be considered and in addition platelet transfusion should be given.[47]

Due to the decreased survival of the transfused platelets in the case of ITP, the increase in platelet count will be low. In case of unresponsiveness to this therapy and persistence of hemorrhage, recombinant factor VIIa (rFVIIa) should be considered.[48]

Risk of Neonatal Thrombocytopenia

Maternal antiplatelet antibodies can cross the placenta and cause fetal/neonatal thrombocytopenia. There is no relation between maternal and fetal/neonatal platelet count, a severe neonatal thrombocytopenia can be observed in the newborn of a mother with a mild thrombocytopenia and newborn from a mother with a severe thrombocytopenia can have a normal platelet count at birth.[11,25,49–51] Data about the relation between neonatal thrombocytopenia and the presence or absence of detectable maternal antibodies are controversial.[49,51,52] So the detection of maternal antibodies has not been proven useful for making predictions about the fetal/neonatal platelet count.[12,16,53,54] Corticosteroids given to the mother have no effect on the neonatal platelet count.[29,49,55] Some data have shown that there is an increased risk of neonatal thrombocytopenia in women with a past history of splenectomy, particularly those who are refractory to splenectomy.[55,56,57] The best predictor of neonatal thrombocytopenia is a history of thrombocytopenia in a prior sibling.[25,55,57] Fetal blood sampling by cordocentesis or scalp blood sampling in early labor is not recommended due to the risk of erroneous result and also the risk of fetal distress associated with an invasive procedure.[15,49]

Mode of delivery of women with ITP is controversial. Historically, cesarean section was recommended based on a perinatal mortality of 12% to 21% resulting from birth trauma and intracranial hemorrhage (ICH).[28,58] More recent studies suggested that the neonatal mortality is much lower, around <1%.[25,49] Large prospective studies have then confirmed that the incidence of severe neonatal thrombocytopenia (defined as a platelet count <50 G/L) varied from 5% to 20%, and ICH occurring in 0% to 3% of newborn.[10,25,51–53,56,59,60] There is no evidence that cesarean section is safer for the thrombocytopenic fetus compared with uncomplicated vaginal delivery (which is usually also safer for the mother).[28,53] Cesarean delivery should be determined essentially by obstetric indications.[15,31,56]

Neonatal platelet count should be determined in cord blood immediately after delivery and followed during the 1st week after birth (nadir being often between day 2 and 5 leading to hemorrhagic complication not directly related to the delivery).[6,25,28,31,49] Indeed, it is important to underline that platelet counts of infants born to mothers with ITP may decrease deeply several days after birth.[56,59] It is therefore crucial to alert the pediatricians.[25,31]

Nonisolated Platelet Disorders

Thrombotic Microangiopathies: Thrombotic Thrombocytopenic Purpura—Hemolytic Uremic Syndrome

The term thrombotic microangiopathies (TMAs) encompasses a spectrum of clinical syndromes in which common pathologic feature is the widespread formation of hyaline thrombi in the nonvenous microcirculation, the arterioles, and capillaries. They share thrombocytopenia and Coomb's negative, microangiopathic hemolytic anemia (MAHA) with the presence of schistocytes on the peripheral blood smear. The emblematic thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS) are extremely rare, afflicting 1 in 100,000 pregnancies,[61,62,63] are not placenta related, are often unexpected and initially confused with obstetric diagnosis: pregnancy can be continued without impairing the rate of therapeutic response,[64] and hemorrhagic complications are unusual. Nevertheless, a delay in diagnosis of TTP/HUS may result in life-threatening maternal and fetal consequences.

TTP–HUS is more common in women, being commonly associated with pregnancy and postpartum.[61,63,65] Patients bearing the autosomal recessive TTP, that is, Upshaw-Shulman syndrome, caused by mutations of the *ADAMTS13* gene, can remain asymptomatic until early adulthood (50%) and may present with TMA during pregnancy. Acquired TTP cases are characterized by severely depressed, autoantibody mediated, ADAMTS13 activities (<5%).[66] ADAMTS13 levels may decrease during pregnancy (however, values remaining >40%), but more decreased levels (however, remaining >10%) are associated with other diseases, including severe preeclampsia (PE) and hemolysis–elevated liver enzymes–low platelet (HELLP) syndrome. In contrast to pregnancy-associated TTP, the pathogenesis and presentation of pregnancy-associated diarrhea-negative, atypical HUS still remain ill-defined. A highly prevalent rate of alternative pathway C3 convertase dysregulation has, however, been confirmed.[67] This disease of complement overactivation may display mutations in gene encoding both complement regulators-complement factor H, complement factor I, complement factor H related proteins, membrane cofactor protein (MCP: CD46), the anticoagulant glycoprotein thrombomodulin that plays a role in the inactivation of C3a and C5a−, as well as complement activators—complement factor B, C3—or autoantibodies inactivating factor H.[68,69]

TTP and HUS manifest similar clinical and laboratory findings, with more profound neurologic symptoms in TTP and exaggerated renal abnormalities in HUS. As such, TTP and HUS have been considered by some to be a clinical continuum of diseases summarized by the acronym TTP/HUS, the challenge being their diagnosis in order to initiate plasma exchange. The classic diagnosis of TTP includes the pentad of Coomb's negative MAHA, thrombocytopenia, neurologic changes, renal symptoms, and fever. This pentad is only present in 40% of the time; 75% of patients present with a triad of MAHA, neurologic symptoms and thrombocytopenic purpura. This has changed to merely a dyad[70,71]: onlythrombocytopenia plus MAHA without an alternative apparent etiology is required to consider TTP/HUS, decreased stringency of diagnostic criteria resulting in an increase in the number of patients treated for TTP/HUS.[70] The clinical signs are in most cases subtle and nonspecific, including nausea, vomiting, abdominal pain, weakness, bleeding, easily bruisability, and flu-like symptoms.[62,72,73] TTP/HUS is most often diagnosed in the third trimester or peripartum, when severe PE/HELLP syndrome occur, which are more common diagnosed (0.5% of all pregnancies), both conditions sharing thrombocytopenia and hemolysis, neurologic symptoms with or without hypertension, and latency to treatment impairing maternal–fetal outcomes. Liver enzymes are elevated proportionally to thrombocytopenia in HELLP syndrome but are usually normal or mildly elevated in TTP/HUS in which MAHA associates with elevated lactate dehydrogenase (LDH) levels but minimal elevation of aspartate aminotransferase (AST) (LDH to AST ratio: 29:1)[74]: TTP/HUS coexisting with PE exhibits two to four times higher AST levels (LDH to AST ratio: 13:1)[75] than TTP/HUS alone. TTP/HUS should be suspected if women are normotensive or nonproteinuric during the third trimester or postpartum period, and if hypertension, proteinuria and abnormal laboratory tests are evidenced at <24 weeks gestation when HELLP syndrome is rare. Women diagnosed severe as PE/HELLP syndrome who do not normalize laboratory values within 2 to 3 days postdelivery, with normal coagulation parameters or resolving disseminated intravascular coagulation (DIC), should also be suspected.[71] Severe anemia, severe thrombocytopenia, and elevated LDH levels with minimal elevation of ALT (alanine aminotransferase) levels are sufficient to consider the diagnosis of TTP/HUS[74]: a peripheral blood smear confirms MAHA, the percentage of schistocytes being higher (2% to 5%) than in HELLP (≤1%). ADAMTS13 should be obtained where available and plasmapheresis initiated as soon as possible. The Oklahoma TPP-HUS registry[75] showed only 2 out of 10 pregnant women with ADAMTS13 activity <5% but 8 (7 with acute renal failure) >25%, which may rather indicate HUS than TTP. The risk of recurrent TTP/HUS in subsequent pregnancies is described but unknown. A subsequent pregnancy[75] accounts for 18% of recurrence cases (Table 118.1).

Early consideration of plasma exchanges/plasmapheresis best allows for optimal outcome. A review before the plasma exchange era described a 44% maternal mortality and 80% fetal loss rate.[76] Treatment is indicated for all patients with TTP/HUS whatever the level of ADAMTS13 activity is.[75] A reduced ADAMTS13 activity and anti-ADAMTS13 IgG-antibody in the neonate blood have been described in an autoimmune TTP[77] but without any clinically relevant fetal or neonatal thrombocytopenia.

Table 118.1

TTP—HUS versus severe PE/HELLP syndrome in a woman with thrombocytopenia, hemolysis, neurologic symptoms (shared symptoms)

	TTP/HUS	HELLP Syndrome
Incidence	1:100,000 pregnancies	1:200 pregnancies
Normotensive, nonproteinuric during the third trimester or postpartum period	Likely	Unlikely
Onset before 24 wk of gestation	Likely	Unlikely
Onset before 20 wk of gestation	Likely	No
PE/HELLP without normalization of laboratory values within 2–3 d post delivery	Likely	Unlikely
Normal or mildly elevated AST levels	Likely	Unlikely
LDH to AST ratio >22	Likely	Unlikely
Elevation of liver enzymes proportionally to thrombocytopenia	Unlikely	Likely
Peripheral blood smears: schistocytes 2%–5%	Likely	Unlikely
Peripheral blood smears: schistocytes ≤1%	Unlikely	Likely

Clinical features of pregnant/postpartum women, which must indicate consideration for the diagnosis and intervention with plasma exchange treatment, include mental status abnormalities, focal neurologic abnormalities, seizures, or oliguric acute renal failure.[70] The earlier plasmapheresis is performed in the gestation, the higher the likelihood of a successful maternal–fetal outcome.[71] Larger volumes of plasma exchange are usually required in pregnancy, by virtue of the 45% increase in maternal blood volume. In patients with chronic, relapsing TMAs who may develop recurrent episodes during pregnancy, some anecdotal evidence suggests that prophylaxis with antiplatelet agents or corticosteroids may reduce the frequency or severity of these relapses,[64] other patients being managed with regular transfusion of plasma or plasma-derived fractions[78] and even with monthly prophylactic plasma exchanges.[79] Plasma exchange is the essential therapy, dramatically improves survival, the current available evidence for other treatments being limited.

Some other disorders can mimic TMAs, including HELLP syndromes (see below), microangiopathic antiphospholipid syndromes, and catastrophic antiphospholipid syndromes.[80,81]

Hemolysis–Elevated Liver Enzymes–Low Platelet Syndrome

Although the term "HELLP syndrome" was not coined until 1982 by Weinstein,[82] its pathologic feature had been recognized for at least 100 years. This variant of severe PE is frequent (around 0.8% of pregnancies and one quarter of all severe PE cases) and is associated with increased maternal and perinatal morbidity and mortality rates to imprecise levels, due to the scarcity of registries and to the presence of numerous biases: reported maternal death rates range between 1% and 25%, maternal cerebral hemorrhage between 1.5% and 40%, perinatal death between 7.4% and 34%, intrauterine growth restriction between 38% and 61%, and premature delivery accounts for 70% of cases (15% before 28 gestational weeks). Rats expressing trophoblast-derived circulating antiangiogenic factors, soluble fms-like tyrosine kinase-1 (sFlt1), and soluble endoglin (sEng), alone or in combination, developed, only in the sFlt1 + sEng group, nephrotic-range proteinuria, severe hypertension, and biochemical evidence of complete HELLP syndrome. These experimental data definitely link HELLP syndrome to the two placental mediators, acting in concert to induce severe PE[83] and the related severe endothelial dysfunction. Liver biopsy, which is risky and nonnecessary for diagnosis, shows pronounced changes with extensive fibrinous thrombi along hepatic sinusoids and hemorrhages into the Disse space. HELLP syndrome is thus placenta-mediated and cured by delivery.

Patients manifesting the HELLP syndrome are usually seen in the late third trimester, before term (<36 weeks of gestation, frequently ≥34 weeks). But early-onset HELLP cases are seen before 34 weeks, with some rare cases being described as early as the second trimester, as well as after delivery. Most patients, but not all, will have concomitant signs and symptoms of PE (hypertension, proteinuria, and edema). Bleeding due to thrombocytopenia is a very uncommon mode of presentation. The complaints are often nonspecific,[84] such as malaise (90%), nausea and vomiting (50%), and sometimes flu-like symptoms. Jaundice is a presenting feature in fewer than 5% of cases. Epigastric or right upper quadrant pain (90%) may be severe, radiations to the neck or shoulder evoking complications such as the development of hepatic hematoma or hepatic rupture.

Three standardized diagnostic laboratory criteria have been proposed by Sibai[84]: MAHA with characteristic peripheral blood smear and serum lactate dehydrogenase LDH levels ≥600 IU/L; serum aspartate aminotransferase (ASAT) levels ≥70 IU/L, and platelet count <100 G/L. Partial HELLP syndromes are defined by one or two but not all the three features. The incidences of complete and partial HELLP syndromes were described similar among severe PE patients (21% vs. 22%, respectively, among 178 severe PE women).[85] Comparison of complete HELLP

syndrome versus partial syndrome versus severe PE showed more frequent gastrointestinal symptoms (nausea, vomiting, epigastric or right upper quadrant pain), lower gestational age at delivery, higher incidence of C-section and DIC, and need for transfusion in women with complete HELLP syndrome.[85] Strictly defined HELLP syndrome is usually considered as an indication for delivery in ≤48 hours whereas conservative management is frequently used in PE women. The platelet nadir impacts on the rapidity of postpartum disease recovery.[86] In women with platelet nadir bellow 50 G/L (class 1 HELLP syndrome of the Mississippi classification[86]), as long as 11 days are generally necessary to achieve 100 G/L. In women with platelet nadir between 50 and 100 G/L (class 2 HELLP syndrome), recovery is generally reached by the 6th postpartum day.

Differential diagnoses are acute viral hepatitis, gastroenteritis, appendicitis, cholecystitis, ITP, lupus, antiphospholipid syndrome, TTP/HUS, and acute fatty liver of pregnancy (AFLP). AFLP may be confused with HELLP syndrome, both occurring in late pregnancy and having similar presentations. Liver dysfunction is usually more severe in AFLP, more often associated with liver failure-induced coagulopathy, hypoglycemia, and renal failure whereas coagulopathy in HELLP syndrome develops as a part of DIC syndrome. Thrombocytopenia in TTP/HUS is usually not associated with DIC syndrome, reduced antithrombin levels suggesting HELLP syndrome.[87] Hepatic ultrasound, computed tomography and MRI can routinely confirm the diagnosis, most frequently finding significant intra-abdominal hemorrhage or subcapsular hepatic hematoma. The main maternal complications of HELLP syndrome are DIC (30%), abruptio placentae (16%), and acute renal failure (8%). Cerebral hemorrhage (1.2%), retinal detachment (0.9%), and subcapsular hepatic hematoma/hepatic rupture (0.9%), which often requires surgical management, occur less frequently. Hepatic rupture usually presents with hypovolemic shock, vaginal bleeding, abdominal signs, and pain.

Initial management is stabilization then rapid delivery, which is indicated in case of severe maternal complications, signs of fetal distress, or gestation term >34 weeks. Before 34 weeks, the mother–fetus couple being stable, intravenous steroids are given for fetal lung maturity acceleration. Retrospective and small randomized studies suggested that the use of high-dose dexamethasone (10 mg every 12 hours) reduced maternal morbidity and induced more rapid improvement of the platelet counts.[88] A systematic review, however, shows no clear evidence of any effect of corticosteroids on substantive clinical outcomes.[89] This review shows that steroids improve platelet counts, with a greater efficiency of dexamethasone than of betamethasone: they may be justified in some clinical situations in which increased rate of recovery in platelet count is considered clinically worthwhile.[89]

Acute Fatty Liver of Pregnancy

This is a rare and potentially fatal condition, the reported incidence varying between 1 in 7,270 and 1 in 13,000 of deliveries, with no ethnic or regional variability.[90] It is more common in primiparous women and may reoccur. AFLP onset occurs more commonly between 30 and 38 weeks of gestation, but cases as early as 26 weeks have been reported. Maternal mortality ranges between 3% and 12.5%, and perinatal mortality is 66%. The relationship between AFLP and PE is not clearly defined, but case reports suggest that most women with AFLP have some degree of PE, with significant overlap of clinical and laboratory features with the HELLP syndrome, both conditions presenting in the later terms of pregnancy in patients with background PE.[90] Histopathology is, however, different in these two processes. Extensive hepatocellular necrosis is virtually nonexistent in AFLP, but is a common feature of HELLP syndrome. HELLP syndrome is characterized by periportal hemorrhages and fibrin deposits, whereas AFLP is characterized by microvesicular fatty infiltration. A mixed histologic picture can however be observed in 5% of the cases. Deficiencies in the enzymes of mitochondrial β-oxidation of fatty acids (FAO) may play a role in the development of this condition, but the precise mechanism is unknown. The hypothesis is that a heterozygous mother carrying a homozygous fetus for the FAO enzyme deficiency develops AFLP as a result of an impaired maternal ability to handle an increased load of fatty acids due to pregnancy-associated increase in lipolysis.[90]

AFLP has been associated with laboratory evidence of persisting DIC and profoundly depressed antithrombin plasma levels,[91,92] interpreted as the consequence of secondary consumption coagulopathy and liver function worsening.

Women usually present in the late term with nonspecific signs (malaise, nausea, vomiting), jaundice, upper quadrant pain, and epigastric pain being common. Some patients may present with frank liver failure and acute bleeding due to liver failure–induced coagulopathy, these symptoms being more commonly seen 1 to 2 weeks later. Concomitant PE is seen in 50% of the patients. A DIC coagulation pattern is frequent, from 55% to 75% of the cases according to some studies.[93] Thrombocytopenia is common when DIC is present: otherwise it is unusual. A moderate elevation of transaminase is usually found, although widely variable values have been described, the degree of transaminase elevation being unrelated to the severity of liver function worsening. Emerging acute renal failure is reflected by creatinine levels. Hypoglycemia and elevated ammonia develop when liver function worsens. Liver biopsy is helpful early in the disease, or in mid cases. Hypoglycemia and prolonged prothrombin time are suggestive of AFLP; frank hepatic failure is rare in HELLP. Imaging is of little value in diagnosis of ALFP but is useful to exclude other processes (e.g., hepatic hematoma/rupture or Budd-Chiari syndrome). Patients with severe PE associated liver disease and HELLP have more profound hematologic abnormalities, associating severe thrombocytopenia and MAHA, hemolytic syndrome being negative in AFLP.

Timely delivery after initial stabilization is a cure, together with supportive treatment in ICU or hepatic failure units. Maternal stabilization includes airway management, treatment of hypertension, and correction of hypoglycemia, electrolyte, and coagulation abnormalities. Due to potential bleeding complications around delivery, stabilization of the coagulation system is most important. This usually leads to an early and aggressive substitution of fibrinogen and platelets in addition to red packed cells and fresh frozen plasma (FFP).[93–97] In the absence of specific evaluations in AFLP, the substitution of antithrombin remains questionable since there is no general benefit on mortality in critically ill patients, but an increase in bleeding risk.[98] In case of uncontrollable bleeding, the use of recombinant activated factor VIIa has been described in AFLP.[99] Careful management of intravenous fluids and blood products, as well as frequent maternal signs assessment, and evaluation of changes in mental status are all crucial. Therefore, collaboration among different specialties such as intensive care, gastroenterology, perinatology, and hematology are essential.

Depending on the presence of the complications, complete recovery from AFLP may take anywhere from days to weeks, prothrombin time improvement being the first sign of recovery of hepatic function. In general, there are no hepatic sequelae postpartum after AFLP, and liver transplantation is rarely needed. Neonatal testing for enzymatic defects of β-oxidation of fatty acids is warranted; neonatal 3-hydroxyacyl-CoA dehydrogenase deficiency presents with Reye-like syndrome and is potentially lethal by 6 months of age if left untreated, dietary intervention being lifesaving.

Inherited Disorders Affecting Platelet Function and Platelet Production

Congenital disorders associated with platelet dysfunction, without/with impaired platelet production, are rare entities, the estimated incidence of the most severe subtypes being lower than 1 in 1 million although this is likely to be an underestimate due to misdiagnosis and underreporting, some subtypes striking <1,000 people worldwide. They induce bleeding syndromes of varying intensity.[100] Spontaneous bleeding is mostly mucocutaneous in nature, and a feature of milder forms is excessive trauma-related bleeding. Invasive procedures and delivery are thus critical events in pregnant women carrying such defects, the clinical risk being variable according to the type of disorder.

Women with severe disorders will need platelet transfusion during childbirth and sometimes during a week postpartum, early hospital discharge being therefore rarely possible. High forceps and ventouse extraction are contraindicated. Fetal scalp electrode monitoring and fetal blood sampling should be avoided. Epidural anesthetics are contraindicated in women with severe bleeding disorders and this must be discussed with the woman during antenatal assessments.[101]

Platelet transfusion can generate multiple risks, including alloimmunization to human leukocyte antigens (HLAs) or missing surface glycoproteins in Bernard-Soulier syndrome (BSS) or Glanzmann thrombasthenia (GT) as well as transmitted infection and allergic reactions. Single donor apheresis platelet collections reduce receiver exposure. Delivery in BSS and in GT can be complicated by maternal peripartum or late PPH, which may occasionally occur 2 to 4 weeks after delivery. The risk of antiglycoprotein alloantibodies-mediated platelet transfusion refractoriness is higher in GT than in BSS.[102] Reported treatment strategies in pregnant women with GT complicated by anti-GpIIbIIIa alloantibodies include plasmapheresis[103] and steroids.[104] Despite the introduction of leukocyte depleted blood components, which limits the incidence of HLA alloimmunization, a review by the United Kingdom Haemophilia Doctor's Organization recommend to transfuse HLA-matched platelets in those patients.[105] Antifibrinolytics are generally used as a useful adjunct in preventing and controlling bleeding, despite the absence of available documented medical evidence in the management of delivery in thrombocytopathic women, in conjunction with platelet transfusion. The dose of tranexamic acid is 15 to 25 mg/kg three times daily orally and, in case of intravenous route, 10 to 15 mg/kg three times daily, up to four times daily in selected cases. Desmopressin (DDAVP) is of limited or no value in platelet receptor deficiencies,[101] but patients with storage pool deficiency may respond.[106] Activated recombinant factor VII (rFVIIa) is licensed for use in GT patients with anti-GpIIbIIIa and/or HLA antibodies, which demonstrated refractoriness to platelet transfusion: the recommended dose is 90 μg/kg intravenously every 2 hours if required for three doses or until bleeding stops,[107] and was successfully used in PPH.[108,109] The PFA100 device is sensitive in detecting major platelet function defects such as BSS and GT but is frequently falsely negative for milder platelet defects such as storage pool disease and Hermansky-Pudlak syndrome; there is no evidence in its ability to reflect the hemorrhagic risk during pregnancy and around delivery in BSS and GT, and no evidence that it can reflect the clinical response to treatments.

Transplacental passage of anti-GpIbIX alloantibodies in BSS women and of anti-GpIIbIIIa alloantibodies in GT women has been described in some neonates, with fetal/neonatal thrombocytopenia and sometimes antenatal ICH.[110–112] Despite the rarity of pregnancy in type 1 GT, although successful outcomes have been reported in neonates born to GT women with GpIIbIIIa antibodies, fetal/neonatal thrombocytopenia and, specifically, intracranial hemorrhage are clearly a concern. The presence of GpIbIX antibodies resulting in neonatal thrombocytopenia was seen in five neonates from GT mothers, one of whom developed antenatal ICH.

COAGULATION DISORDERS

Most coagulation factors increase during pregnancy so pregnant women with mild to moderate deficiencies tend to have no bleeding.[113] However, even in case of mild deficiencies, the peripartum and postpartum periods may represent a difficult time and bleeding may occur. The situation is different when severe deficiencies are present, bleeding may occur not only during the peri- and postpartum but also during pregnancy. Moreover, for severe fibrinogen and FXIII deficiencies, spontaneous abortions are the rule without factor substitution.

von Willebrand Diseases and Platelet-Type von Willebrand

von Willebrand Diseases

There is an increase of von Willebrand factor (vWF) level during pregnancy; consequently many women with type 1 deficiency (partial deficiency of both functional and protein vWF as well as FVIII) reach normal levels at term. This explains the low incidence of bleeding before delivery. An improvement will not occur in women with type 3 (severe defect in vWF) and with type 2 (mainly qualitative defects) since even if vWF increases in type 2, the functional defect remains. Depending on their bleeding history women with types 2 and 3 may require substitution during pregnancy.[32,114,115]

The main problem for all types of von Willebrand disease (vWD) is the peripartum management. For type 1, if vWF and FVIII are below 50 IU/dL, DDAVP can be administered. DDAVP does not cross the placenta. It is advised to maintain vWF > 50 IU/dL during labor, delivery, and up to 5 days postpartum. For type 3, vWF concentrates will be given with the same aim, a vWF and FVIII > 50 IU/dL during labor, delivery and, up to 5 days postpartum with a continued close follow-up next since PPH may occur more than the first 5 days after delivery. In type 2, concentrates are usually required.[116]

Platelet transfusions around delivery are usually not indicated, being reserved to rare uncontrolled hemorrhages after adequate substitution. Women should receive a purified vWF

concentrate for procedures or bleeding in the antenatal period. They usually require peripartum factor prophylaxis for up to 7 days after cesarean section and 4 to 5 days after vaginal delivery.

Type 2B vWD presents a special challenge. Indeed, it may present as thrombocytopenia during pregnancy.[117,118] Platelet counts progressively decrease, to levels that can reach 20 G/L, with reversal to normal or minimally reduced platelet counts in the early post gestational period. Moreover, conditions such as infections or inflammation associated with enhanced vWF secretion favor thrombocytopenia, which is due to *in vivo* platelet aggregation induced by the gain-of-function of vWF variants and in some rare mutation cases due to abnormal megakaryopoiesis with circulating macrothrombocytopenia.[119] The risk of bleeding is higher in patients with thrombocytopenia with an adjusted hazard ratio of 4.57 (95% confidence interval 1.17 to 17.90) as shown in a cohort study of 67 patients with vWD type 2B.[120] The UK Haemophilia Centre Doctor's of obstetric management of women with type 2B vWD does not provide any specific recommendations for a target platelet count, which should be maintained.[121] In a recent small case series, successful management without bleeding complications was obtained by maintaining platelet counts of at least 50 G/L.[122]

Platelet-Type vWD

Platelet-type vWD can be revealed by severe bleeding during pregnancy associated with apparently acquired severe thrombocytopenia.[123] Platelets are hypersensitive when interacting with vWF, due to gain of function mutations in GPIBA gene coding for platelet GpIbα leading to spontaneous binding of circulating native normal vWF. vWF multimers are cleared from the blood to various degrees, some patients having a sustained secondary vWF:RCo reduction to levels reaching 15% of control values despite vWF:Ag concentrations around 100%.[123] While not giant as typically seen in blood smears from BSS or in some families with type 2B Willebrand disease, platelet size may be increased and associated with a moderate chronic thrombocytopenia, which can worsen in situations associated with enhanced vWF secretion. Diagnosis is based on enhanced ristocetin-induced platelet agglutination in platelet-rich plasma, which is no more evidenced in platelet-poor plasma mixed with control platelets.

vWF concentrates were used to control bleeding during C-section–mediated second delivery (platelets: 58 G/L) but bleeding reoccurred several days after[123]; first C-section (platelets 30 G/L) had been complicated by bleeding persisting over several days despite platelet transfusions. In platelet-type vWD women, substitution with vWF concentrates induces an accelerated platelet clearance, which maintains bleeding tendency. Substitution with normal platelets, from donors without the gain of function receptor, is necessary to break this vicious circle, thus allowing hemostasis. Increasing thrombocytopenia has been reported with the use of vWF/FVIII concentrates.[124] Similarly, desmopressin is contraindicated because the release of large vWF multimers from endothelial cells could induce *in vivo* agglutination of platelets and worsen thrombocytopenia. Patients with platelet-type vWD usually require platelet transfusions to treat hemorrhage.[125]

Hemophilia A and B Carriers

Carriers of hemophilia usually do not require factor replacement.[32] However, factor VIII or IX levels need to be monitored during the third trimester and at the time of delivery because for some women with particularly low factor levels, the risk of bleeding exists. Hemophilia B carriers are more prone to PPH than hemophilia A carriers because factor IX levels do not rise during pregnancy. Factor concentrates are useful in case of acute bleeding whereas DDAVP, more useful as a preventive measure, can only be used in case of hemophilia A. The effects of DDAVP are likely explained by a direct action on certain endothelial cells, via activation of endothelial vasopressin V2R receptor and cAMP-mediated signaling: this leads to exocytosis from Weibel Palade bodies where vWF is stored, with a secondary FVIII release. Factor IX is synthesized in hepatocytes: its secretion cannot be induced by DDAVP.

Fibrinogen Deficiency

Diseases affecting fibrinogen may be both acquired or inherited.[126] Fibrinogen disorders affect either the quantity (hypofibrinogenemia, characterized by fibrinogen levels lower than 1.5 G/L and afibrinogenemia, characterized by the complete deficiency of fibrinogen) or the quality of the circulating fibrinogen (dysfibrinogenemia) or both (hypodysfibrinogenemia).

Women with congenital afibrinogenemia are able to conceive and embryonic implantation is normal, but the pregnancy usually results in spontaneous abortion at 5 to 8 weeks of gestation unless factor replacement is given.[114,127] The importance of fibrinogen in pregnancy is demonstrated in studies with fibrinogen knock-out mice where gestation cannot be maintained to term.[128,129] Women with afibrinogenemia may also have placenta abruption as well as antepartum and PPH (they are at particularly high risk of PPH).

Hypofibrinogenemic women are usually asymptomatic with fibrinogen levels around 1.0 G/L, levels that are in theory high enough to protect against bleeding and maintaining pregnancy. However, some hypofibrinogenemic women may also have problems with pregnancy, those with the lower fibrinogen levels having the higher risk of miscarriage and therefore a need for fibrinogen substitution.[130,131]

Like hypofibrinogenemic women who have fibrinogen levels high enough for hemostasis (including normal platelet function), women with inherited dysfibrinogenemia are frequently asymptomatic; however, some patients suffer from bleeding, thromboembolic complications, or both. Patients with dysfibrinogenemia associated with hemorrhage bleed most often after trauma, surgery, or in the postpartum. Thrombosis may also occur in the postpartum. Problems of pregnancy vary depending on the type of dysfibrinogenemia, therefore individualized treatment is the rule.[127] Women with dysfibrinogenemia and a history of recurrent poor pregnancy outcome should be treated with fibrinogen concentrate replacement as soon as pregnancy is confirmed, using a target trough fibrinogen level of 1 G/L.[132]

During pregnancy Kobayashi et al.[133] recommend keeping the fibrinogen level >0.6 G/L and if possible >1.0 G/L. Lower fibrinogen concentrations (<0.4 G/L) have proven adequate to maintain pregnancy but not to avoid hemorrhagic complications. Fibrinogen clearance increases as the pregnancy progresses and thus the amount of fibrinogen required increases with advancing gestation. Continuous infusion of fibrinogen concentrates during labor to maintain fibrinogen higher than 1.5 G/L or ideally >2.0 G/L is recommended. However, recommendations for high-level replacement during pregnancy should be moderated

by the fact that thromboembolic events can occur,[132] particularly with the use of cryoprecipitates that contain appreciable quantities of FVIII and vWF in addition to fibrinogen.

Factor XIII Deficiency

Like in case of afibrinogenemia, women with severe factor XIII (FXIII) deficiency are able to conceive and embryonic implantation is normal, but the pregnancy usually results in spontaneous abortion unless factor replacement is given.[113,134,135,136] Therefore, FXIII should also be given as early as possible and during the whole pregnancy to avoid miscarriage and bleeding. Regular therapy should be commenced as early as possible in pregnancy to prevent bleeding and pregnancy loss. A level >10% during pregnancy should be considered.[137] The treatment should also be continued during labor and delivery to minimize the risk of bleeding complications. Indeed, PPH is a frequent complication if insufficient FXIII levels are reached. FXIII concentrates are the treatment of choice.

Other Factor Deficiencies

In addition to fibrinogen and FXIII deficiencies, other rare bleeding disorders (RBDs) include the inherited deficiencies of FII, FV, FV+, VIII, FVII, FX, and FXI and combined deficiency of vitamin K–dependent factors.[113,136] Recent issues of *Haemophilia* (November 2008) and *Seminars of Thrombosis and Hemostasis* (June 2009) have covered the main available treatments for RBDs. Concentrates are the treatment of choice because they are safer than FFP or cryoprecipitate (decreased risk of bloodborne pathogen transmission), there is no fluid overload and more precise dosing can be accomplished. In case of pregnancy and severe RBD, the UK guidelines on therapeutic products for coagulation disorders and a recent review by Kadir et al. provide some recommendations about the best treatment options.[113,114]

Pregnancy in women with severe hypothrombinemia may be complicated by bleeding and miscarriages.[138] Although probable, it is not really demonstrated whether prophylactic therapy is required during pregnancy. These women are also potentially at risk of PPH, so a prothrombin level >25 IU/dL during labor and delivery has been suggested.[113,114]

Women with low FV levels usually do not suffer from miscarriages[139] but from PPH. Therefore, careful monitoring of labor and immediate postpartum period is necessary. Substitution therapy with FFP (no FV concentrates are available) to maintain FV levels around 20 IU/dL is recommended.

As for many factor deficiencies (particularly true for FXI) there is a relatively poor correlation between absolute FVII level and bleeding tendency. Therefore, a decision for a substitution should be individualized, based particularly on the personal bleeding history.[113,114] Available data do not seem to indicate an increased risk of miscarriage during pregnancy.[140] Women with severe FVII deficiency are at risk of PPH; therefore, prophylactic treatment is proposed at term and in the postpartum. Current therapeutic options include rFVIIa, plasma-derived FVII, prothrombin-complex concentrates, and FFP. Although there is a poor correlation between factor levels and bleeding complications, a level above 20% should be reached in women with severe factor deficiency and a significant bleeding history.[113]

Successful term pregnancies as well as failures have been described in women with severe FX deficiencies, so prophylactic administration of FX (with prothrombin complex concentrates or FFP) is controversial. Women with a history of adverse pregnancy outcomes should receive FX replacement and should also receive replacement therapy to cover labor and delivery.[113]

Contrary to most coagulation factors, FXI level does not increase during pregnancy. FXI deficiency has not been reported to be associated with an increased risk of miscarriage, but in case of miscarriage or termination of pregnancy, excessive bleeding may occur. Clear prophylactic measures are difficult to state since it is well known that the bleeding tendency is only partially related to factor levels.[141] When there is a bleeding history, tranexamic acid,[142] FXI concentrates (careful with thrombotic complications and inhibitor formation), or FFP (if FXI concentrates are not available) should be discussed and adapted to FXI level and mode of delivery. Recombinant FVIIa is an alternative option in case of inhibitor. To prevent PPH, tranexamic acid should be considered for most women up to 2 weeks post delivery, especially for those with a bleeding history.

There is very little literature on combined FV and FVIII deficiency or inherited deficiency vitamin K–dependent clotting factors and pregnancy.[113] Concerning FV and FVIII deficiency, since FV does not increase during pregnancy any bleeding is likely to be dependent on the FV level and not on FVIII, which increases during pregnancy. Vitamin K is the treatment of choice in case of inherited deficiency vitamin K–dependent clotting factors, and replacement therapy with vitamin K clotting factors could be considered in case of severe deficiency to cover delivery.[143]

Factor VIII Inhibitors

When a woman bleeds without obvious explanation in the postpartum period, the diagnosis of acquired hemophilia should be suspected. This condition may occur already during the puerperium but is observed also several months postpartum. Finding of a prolonged activated partial thromboplastin time, not corrected by incubating patient's plasma with a normal plasma, should lead to measure FVIII. The diagnosis of an inhibitor can be confirmed with the Nijmegen assay.[144] Management of women with acquired hemophilia depends on bleeding manifestations and on the titer of the inhibitor.[145] When bleeding is not severe, DDAVP and/or FVIII concentrates can be used. In case of acute bleeding, rFVIIa or activated prothrombin complex concentrates are more effective. Spontaneous remission is common when a FVIII inhibitor occurs after pregnancy, which is not the case in the aging population where immunosuppressive agents are often required.

DISSEMINATED INTRAVASCULAR COAGULATION

DIC is a characteristic complication of obstetric dramas, including amniotic fluid embolism, severe PE/eclampsia/HELLP syndrome, placental abruption, *placenta praevia*, intrauterine fetal demise, septic abortion and intrauterine infection, PPH, and AFLP.[146]

Placenta is a key actor of most of the obstetrics-related DIC features, explaining obstetrical DIC management and an overall favorable clinical prognosis despite impressive acute coagulopathies. The vascular bed of the placenta contains fetal trophoblast cells, which can regulate hemostasis in the intervillous space. It

expresses high tissue factor and low TFPI levels[147] thus activating coagulation. It also produces PAI-2 and gradually increasing PAI-1 amounts, whereas t-PA levels are unchanged, thus impairing the fibrinolytic system. The spillover of localized coagulation activation from the placenta into the systemic circulation can disseminate thrombin generation, induce consumption coagulopathy, and precipitate organ damage. Acute placenta and vasculoplacental disorders/dramas, with their characteristic sudden onset, are thus prone to orientate the hemostatic system toward an enhanced activation state, which can become uncontrolled after inhibitors exhaustion. This generally leads to frank DIC features, in which the consumption coagulopathy predominates, with hemorrhagic symptoms and marked laboratory abnormalities. Accurate obstetrical intervention usually prevents DIC, even in its more severe forms, a particularity of most of the obstetrics-related DICs.

The clinical association between intrauterine fetal death and DIC has to be discussed. The incidence of undiagnosed intrauterine fetal demise is likely to be rare in a well-resourced obstetric environment: spontaneous delivery would usually ensue within the first 2 weeks whilst marked coagulation disturbances only tend to occur after a month of fetal demise.[148] The fetus being retained longer, up to 25% of cases develop a coagulopathy, which is mediated by the release of tissue factor from dead products of conception. This can be a slow process, which may correct spontaneously before evacuation. Even when pregnancies are complicated by death of one fetus and survival of another, marked coagulation disturbances are rare.[149]

It is necessary to recognize DIC early to facilitate timely intervention. Diagnosis of obstetrics-related overt DIC is possible through a scoring system as proposed by the International Society on Thrombosis and Haemostasis[150] (see Chapter 98). After checking for clinical conditions that may be associated with overt DIC, obstetric calamities being one of them, useful tests are platelet count, fibrinogen, prothrombin time, and a fibrin generation-related maker, practically D-dimers or soluble fibrin monomers, serial testing being recommended to increase diagnostic precision. Management involves delivery of the placenta and supportive treatment, including blood product substitution.

One peculiar feature is DIC complicating severe PPH. PPH is generally not due to an underlying intrinsic coagulation disorder, being mainly secondary to uterine atony, lower genital tract lacerations, and retained placental. PPH coagulopathy can be a complex disorder mixing consumption, dilution due to resuscitation, and effects of substitution. Fibrinogen levels assayed in PPH patients requiring uterotonic prostaglandin E2 infusion can anticipate the risk of severe bleeding if ≤2 G/L.[151] Normal but low fibrinogen, FXI, or vWF levels assayed in nonpregnant women are retrospectively not associated with the risk PPH, but to the risk of severe PPH, thus acting as risk factor for a poor outcome.[152]

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Full Reference Lists can be found online

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CHAPTER

119 Developmental Hemostasis

PAUL MONAGLE, VERA IGNJATOVIC, AND LESLIE ROY BERRY

Hemostasis is a complex homeostatic system that is critical to maintaining healthy life. Disturbances of the hemostatic system account for a large proportion of the noninfectious morbidity and mortality in westernized societies and are integral to many disease processes including cardiovascular disease, stroke, venous thrombosis, and even cancer. In some respects, nothing has changed since the mid-1800s when the German doctor, Rudolf Virchow, described the critical components of hemostasis as being the blood vessel wall, blood composition, and blood flow (Virchow triad). In considering the nonflow aspects of this equation, hemostasis can be considered as an interaction between the blood vessel wall, coagulation proteins within the plasma, and blood cellular components, predominantly platelets. Clearly over recent years, however, there has been an explosion in our understanding of the interactions within this system, and in particular, of the roles of individual plasma proteins. The hemostatic system interacts with other physiologic systems such as wound repair, inflammation, and angiogenesis in a complex and as yet incompletely understood manner.

Hemostasis is a dynamic, evolving process that is age dependent and begins *in utero*. The evolution of the hemostatic system continues throughout life, as evidenced by studies of the coagulation system in centenarians.[1] However, the changes are most marked during childhood and hence are of most clinical relevance during this time. Although evolving, the hemostatic system in healthy fetuses, infants, and children must be considered physiologic. The term "Developmental Hemostasis" was coined in the late 1980s by Dr. Maureen Andrew to describe this phenomenon. Dr. Andrew demonstrated in a single large cohort study of Canadian children that the concentrations of the majority of coagulation proteins, as measured by functional assays, changed significantly with age. The landmark papers, published in *Blood* in 1987, 1988, and 1992, not only changed the approach to research related to the coagulation system in children, but also the clinical interpretation of coagulation assays used by many hemostasis laboratories around the world since that time.[2–4]

This chapter outlines our current understanding of developmental hemostasis, focusing on the plasma proteins. While there is evidence of age-related developmental changes in platelets[5,6,7,8–10] and the blood vessel wall,[6,7,8–11] these changes are beyond the scope of this chapter. The implications of developmental hemostasis are discussed, in particular the clinical implications, the evidence that the age-related changes in the coagulation system influence the age-related epidemiology of thromboembolic disease, and the potential implications of developmental hemostasis for integrated physiologic systems. The impact of development hemostasis on the pharmacology and effectiveness of anticoagulant drugs are discussed in Chapter 121. There remain many gaps in our knowledge, and much work is required in this area. An understanding of developmental hemostasis in the broadest sense optimizes the prevention, diagnosis, and treatment of hemostatic problems during childhood and undoubtedly provides new insights into the pathophysiology of hemorrhagic and thrombotic complications for all ages.

THE HEMOSTATIC SYSTEM

The fundamental principle underlying developmental hemostasis is that the functional levels of coagulation proteins change in a predictable way with age.[12] While the absolute values of these changes are reagent and analyzer dependent, the trends in changes observed are consistent across a number of studies.[13] These changes in functional protein levels are reflected in the results of global tests of coagulation such as the activated partial thromboplastin time (APTT). Other global measures of hemostasis may be more or less sensitive to age-related changes in hemostatic proteins. For example, there is little difference in normal thromboelastography (TEG) with age.[14] Studies in this field have predominantly involved functional assays of the coagulation proteins due to the fact that these assays are commonly used in clinical practice. In fact, differences in immunologic levels of the coagulation proteins have not been reported to date. Table 119.1 lists major studies that have described age-related changes in coagulation proteins and routine coagulation assays. Tables 119.2–119.8 list values for these parameters as reported in a more recent publication.

Early evidence suggests that these age-related changes in protein concentration are not isolated to the coagulation system, but are in fact evident across multiple protein systems within the plasma proteome. Variation in the human plasma proteome with age has been examined using plasma samples collected from healthy neonates (day 1 and day 3), children (<1 year, 1 to 5, 6 to 10, and 11 to 16 years), and adults. The study reported significant changes in number and abundance of up to 100 proteins across various age groups tested.[26] FIGURE 119.1 shows a two-dimensional (2-D) DIGE gel image demonstrating differences in plasma protein abundance between a day 1 neonate and an adult. Table 119.9 lists the plasma proteins that were shown in this initial study to significantly change in abundance with age. Further work is required to identify the many more proteins that likely change consistently with age. The implications of these age-related changes are yet to be understood.

The possibilities for how plasma levels of coagulation proteins are different in children compared to adults include regulation at the gene level, posttranslational modifications (PTMs) that affect protein function, delivery or release of proteins, as well as differences in protein clearance. A recent study

Table 119.1

Studies reporting age-related differences in coagulation assays or proteins during childhood

Author	Year	Assays/Proteins Reported	Age Groups	N
Perlman[15]	1975	PT, TT, APTT, fibrinogen, FDP, platelet count, hematocrit, FV, FVIII, plasminogen, hemoglobin	Healthy infants Small-for-dates infants Postmature infants	*N* = 35 *N* = 26 *N* = 30
Beverley et al.[16]	1984	APTT, FII-VII-X, fibrinogen, α_2-antiplasmin, platelet count, MPV, megathrombocyte index, plasminogen	Cord blood Newborns (48 h)	*N* = 80
Andrew et al.[3]	1987	PT, APTT, TCT, fibrinogen, FII, FV, FVII, FVIII, vWF, FIX, FX, FXI, FXII, PK, HMW-K, FXIIIa, FXIIIb, plasminogen, AT, α_2-M, α_2-AP, C_1E-INH, α_1-AT, HCII, protein C, protein S	Day 1 newborn Day 5 newborn Day 30 newborn Day 90 newborn Day 180 newborn Adult	28–75 samples per age group
Andrew et al.[2]	1988	PT, APTT, TCT, fibrinogen, FII, FV, FVII, FVIII, vWF, FIX, FX, FXI, FXII, PK, HMW-K, FXIIIa, FXIIIb, plasminogen, AT, α_2-M, α_2-AP, C_1E-INH, α_1-AT, HCII, protein C, protein S	Premature newborns (30–36 wk gestation) Day 1 Day 5 Day 30 Day 90 Day 180	23–67 samples per age group
Andrew et al.[4]	1992	PT/INR, APTT, bleeding time, fibrinogen, FII, FV, FVII, FVIII, vWF, FIX, FX, FXI, FXII, PK, HMW-K, FXIIIa, FXIIIs, plasminogen, TPA, PAI, AT, α_2-M, α_2-AP, C_1E-INH, α_1-AT, HCII, protein C, protein S (total and free)	1–5 y 6–10 y 11–16 y Adults	20–50 samples per age group
Reverdiau-Moalic et al.[17]	1996	PT/INR, APTT, TCT, FI, FII, FVII, FVII, FIX, FX, FV, FVIII, FXI, FXII, PK, HMWK, AT, HCII, TFPI, protein C (Ag, Act), protein S (free and total), C4b-BP	Fetuses 19–23 wk gestation 24–29 wk gestation 30–38 wk gestation Newborns (immediately after delivery) Adults	*N* = 20 *N* = 22 *N* = 22 *N* = 60 *N* = 40
Carcao et al.[18]	1998	PFA100 Hb Platelet count	Neonates Children Adults	*N* = 17 *N* = 57 *N* = 31
Salonvaara et al.[19]	2003	FII, FV, FVII, FX, APTT, PT/INR, platelet count	Premature infants 24–27 wk 28–20 wk 31–33 wk 34–36 wk	*N* = 21 *N* = 25 *N* = 34 *N* = 45
Flanders et al.[20]	2004	PT, APTT, FVIII, FIX, FXI, AT, RCF, vWF, protein C, protein S	7–9 y 10–11 y 12–13 y 14–15 y 16–17 y Adults	124 per age group

Table 119.1

Studies reporting age-related differences in coagulation assays or proteins during childhood *(Continued)*

Author	Year	Assays/Proteins Reported	Age Groups	N
Monagle et al.[13]	2006	APTT (four reagents), PT/INR, fibrinogen, TCT, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, AT, protein C, protein S, D-dimers, TFPI (free and total), endogenous thrombin potential	Day 1 Day3 <1 y 1–5 y 6–10 y 11–16 y Adults	Minimum 20 samples per age group
Chan et al.[14]	2007	TEG [R. K. α, MA, LY30]	<1 years 1–5 y 6–10 y 11–16 y Adults	*N* = 24 *N* = 24 *N* = 26 *N* = 26 *N* = 25
Sosothikul et al.[21]	2007	PT, APTT, fibrinogen, TAT, PC:Ac, TF, FVIIa, sTM, vWF (Ag and RCo), D-dimer, tPA, PAI-1, TAFI	1–5 y 6–10 y 11–18 y Adults	*N* = 19 *N* = 26 *N* = 25 *N* = 26
Mitsiakos et al.[22]	2009	INR, PT, APTT, fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI, FXII, AT, protein C, protein S, APCr, tPA, PAI-1, vWF	Small-for-growth newborns Appropriate for growth newborns	*N* = 90 *N* = 98
Newall et al.[23]	2008	PF4 and vitronectin	<1 y 1–5 y 6–10 y 11–16 y Adults	15 per age group
Ries et al.[24]	1997	TAT, F1+2, PAP, D-dimer	1–6 y 7–12 y 13–18 y Adults	20 per age group
Boos et al.[25]	1989	PIVKA II, FVII, FII, FII:Ag	Day 1, 2, 3 neonates	*N* = 57 total

demonstrated that even with a transplanted adult liver *in situ*, children maintain plasma levels of certain coagulation proteins at their expected age-specific levels.[27] Thus the liver, despite being the site of production for most of the coagulation proteins, is not the regulator of plasma levels. The authors hypothesized hormonal control, vascular endothelial control via an as yet unidentified mechanism, or control via variable clearance. However, whatever the mechanism, the vascular endothelium seems the most likely candidate as the primary regulator.[28] The endothelium is intimately involved with the function of the coagulation proteins.[29] Vascular endothelial dysfunction, as observed in disseminated intravascular coagulation, is usually measured by the degree of disturbance in coagulation proteins, even though it is not a primary disorder of coagulation.[30]

To date, efforts to understand developmental differences in the hemostatic system have mostly focused on *quantitative differences* in coagulation proteins based on functional assays.[3,4,12] However, coagulation proteins have complex tertiary structures, which in general bestow multiple functions.[31,32] PTMs affecting the structure of hemostatic proteins are known to occur and likely have significant impact on the function of these proteins.[31,32] As an example, fibrinogen has previously been demonstrated to exist in a "fetal" form, in cord blood of term infants.[32] This "fetal" form of fibrinogen has increased sialic acid content compared to adult fibrinogen, a direct result of PTM. The phosphorus content of fetal fibrinogen is increased up to fourfold compared to the adult form of this protein.[33,34] In addition, thrombin clotting times are prolonged in newborns, suggesting differences in polymerization of fibrin from "fetal" fibrinogen,[35] an observation that has led to claims that fibrinogen in infants is "dysfunctional."[36] The importance of differences in sialic acid content of fibrinogen is in the fact that sialic residues of fibrinogen directly bind Ca^{2+},[37] leading to a decrease in the intermolecular repulsion between fibrinogen chains and thereby facilitating fibrin polymerization.[37]

A recent study investigating differences in fibrinogen purified from neonates and children to that from adults determined that the molecular weight of the Aα fibrinogen chain

Table 119.2

APTT reference values for neonates and children using four different reagents compared to APTT results from Andrew et al.[3,4]

	Age						
APTT Results (s)	**Day 1**	**Day 3**	**1 Month–1 Year**	**1–5 Years**	**6–10 Years**	**11–16 Years**	**Adults**
PTT-A	**38.7[a] (34.3–44.8)** *N* = 21 (10F/11M)	**36.3[a] (29.5–42.2)** *N* = 25 (13F/12M)	**39.3[a] (35.1–46.3)** *N* = 35 (3F/30M)	**37.7[a] (33.6–43.8)** *N* = 56 (26F/30M)	**37.3[a] (31.8–43.7)** *N* = 71 (27F/44M)	**39.5[a] (33.9–46.1)** *N* = 54 (12F/42M)	**33.2 (28.6–38.2)** *N* = 42
CK PREST	**Not available**	**Not available**	**34.4[a] (31.1–36.6)** *N* = 20 (3F/17M)	**32.3[a] (29.8–35.0)** *N* = 22 (11F/11M)	**32.9[a] (30.8–34.8)** *N* = 22 (12F/10M)	**34.1[a] (29.4–40.4)** *N* = 39 (8F/31M)	**29.1 (25.7–31.5)** *N* = 40
Actin FSL	**Not available**	**Not available**	**37.4[a] (33.4–41.4)** *N* = 20 (3F/17M)	**36.7[a] (31.8–42.8)** *N* = 20 (10F/10M)	**35.4[a] (30.1–40.4)** *N* = 21 (12F/9M)	**38.1[a] (32.2–42.2)** *N* = 39 (9F/30M)	**30.8 (27.1–34.3)** *N* = 40
Platelin L	**Not available**	**Not available**	**36.5[a] (33.6–40.4)** *N* = 20 (3F/17M)	**37.3[a] (32.5–43.8)** *N* = 21 (11F/10M)	**35[a] (31.0–39.3)** *N* = 22 (12F/10M)	**39.4[a] (32.6–49.2)** *N* = 35 (7F/28M)	**31.3 (27.2–35.4)** *N* = 38
Andrew et al.[3,4]	*42.9[b] (31.3–54.5)*	*42.6[b] (25.4–59.8)*	*35.5 (28.1–42.9)*	*30 (24–36)*	*31 (26–36)*	*32 (26–37)*	*33 (27–40)*

For each reagent, the first row shows the mean and boundaries including 95% of the population. The second row shows the number of individual samples and the ratio of males to females for each group.
[a]Values that are significantly different from adult values ($P < 0.05$).
[b]Values that are significantly different from adult values for data from Andrew et al.[3,4]
M, males; F, females.
NOTE: Andrew et al.[3,4] results for day 3 are actually day 5 results.
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Table 119.3

TCT, PT, INR, and fibrinogen reference values for neonates and children compared to results from Andrew et al.[3,4]

	Age						
Coagulation Tests	**Day 1**	**Day 3**	**1 Month–1 Year**	**1–5 Years**	**6–10 Years**	**11–16 Years**	**Adults**
Thrombin clotting time (TCT) (s)	**Not available**	**Not available**	**17.1[a] (16.3–17.6)** *N* = 20 (10F/10M)	**17.5[a] (16.5–18.2)** *N* = 21 (11F/10M)	**17.1 (16.1–18.5)** *N* = 21 (11F/10M)	**16.9 (16.2–17.6)** *N* = 22 (11F/11M)	**16.6 (16.2–17.2)** *N* = 20
TCT Andrew et al.[3,4]	*23.5 (19.0–28.3)*	*23.1 (18.0–29.2)*	*24.3 (19.4–29.2)*	*Not available*	*Not available*	*Not available*	*Not available*
Prothrombin time (PT) (s)	**15.6[a] (14.4–16.4)** *N* = 21 (10F/11M)	**14.9[a] (13.5–16.4)** *N* = 25 (13F/12M)	**13.1 (11.5–15.3)** *N* = 35 (8F/27M)	**13.3[a] (12.1–14.5)** *N* = 43 (23F/20M)	**13.4[a] (11.7–15.1)** *N* = 53 (22F/31M)	**13.8[a] (12.7–16.1)** *N* = 23 (7F/16M)	**13.0 (11.5–14.5)** *N* = 51
PT Andrew et al.[3,4]	*13 (11.6–14.43)*	*12.4 (10.5–13.86)*	*12.3 (10.7–13.9)*	*11 (10.6–11.4)*	*11.1 (10.1–12.1)*	*11.2 (10.2–12.0)*	*12.0 (11.0–14.0)*
International Normalised Ratio (INR)	**1.26[a] (1.15–1.35)** *N* = 21 (10F/11M)	**1.20[a] (1.05–1.35)** *N* = 25 (13F/12M)	**1.00 (0.86–1.22)** *N* = 35 (8F/27M)	**1.03[a] (0.92–1.14)** *N* = 43 (23F/20M)	**1.04[a] (0.87–1.20)** *N* = 53 (22F/31M)	**1.08[a] (0.97–1.30)** *N* = 23 (7F/16M)	**1.00 (0.80–1.20)** *N* = 51 (43F/8M)
INR Andrew et al.[3,4]	*1[b] (0.53–1.62)*	*0.91[b] (0.53–1.48)*	*0.88[b] (0.61–1.17)*	*1 (0.96–1.04)*	*1.01 (0.91–1.11)*	*1.02 (0.93–1.10)*	*1.10 (1.0–1.3)*
Fibrinogen (g/L)	**2.80 (1.92–3.74)** *N* = 22 (10F/12M)	**3.30 (2.83–4.01)** *N* = 21 (10F /11M)	**2.42[a] (0.82–3.83)** *N* = 34 (7F/27M)	**2.82[a] (1.62–4.01)** *N* = 43 (23F /2OM)	**3.04 (1.99–4.09)** *N* = 52 (22F/30M)	**3.15 (2.12–4.33)** *N* = 21 (7F/14M)	**3.1 (1.9–4.3)** *N* = 55 (47F/8M)
Fibrinogen Andrew et al.[3,4]	*2.83 (2.25–3.41)*	*3.12 (2.37–3.87)*	*2.51 (1.5–3.87)*	*2.76 (1.70–4.05)*	*2.75 (1.57–4.0)*	*3 (1.54–4.48)*	*2.78 (1.56–4.0)*

[a]Values that are significantly different from adult values ($P < 0.05$).
[b]Values that are significantly different from adult values for data from Andrew et al.[3,4]
M, males; F, females.
NOTE: Andrew et al.[3,4] results for day 3 are actually day 5 results.
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Table 119.4

Coagulation factor reference values for neonates and children compared to results from Andrew et al.[3,4]

Coagulation Factors (%)	Age						
	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
II	**54[a] (41–69)** *N* = 23 (13F/10M)	**62[a] (50–73)** *N* = 22 (11F/11M)	**90[a] (62–103)** *N* = 22 (7F/15M)	**89[a] (70–109)** *N* = 67 (26F/41M)	**89[a] (67–110)** *N* = 64 (23F/41M)	**90[a] (61–107)** *N* = 23 (6F/17M)	**110 (78–138)** *N* = 44
II *Andrew et al.*[3,4]	*48[b] (37–59)*	*63[b] (48–78)*	*88[b] (60–116)*	*94[b] (71–116)*	*88 (67–107)*	*83[b] (61–104)*	*108 (70–146)*
V	**81[a] (64–103)** *N* = 22 (13F/9M)	**122 (92–154)** *N* = 22 (11F/11M)	**113 (94–141)** *N* = 20 (6F/14M)	**97[a] (67–127)** *N* = 75 (26F/41M)	**99[a] (56–141)** *N* = 64 (23F/41M)	**89[a] (67–141)** *N* = 20 (5F/15M)	**118 (78–152)** *N* = 44
V *Andrew et al.*[3,4]	*72[b] (54–90)*	*95[b] (70–120)*	*91[b] (55–127)*	*103 (79–127)*	*90[b] (63–116)*	*77[b] (55–99)*	*106 (62–150)*
VII	**70[a] (52–88)** *N* = 22 (12F/10M)	**86[a] (67–107)** *N* = 22 (11F/11M)	**128 (83–160)** *N* = 20 (6F/14M)	**111[a] (72–150)** *N* = 66 (25F/41M)	**113[a] (70–156)** *N* = 64 (23F/41M)	**118 (69–200)** *N* = 22 (6F/16M)	**129 (61–199)** *N* = 44
VII *Andrew et al.*[3,4]	*66[b] (47–85)*	*89[b] (62–116)*	*87[b] (47–127)*	*82[b] (55–116)*	*85 (52–120)*	*83[b] (58–115)*	*105 (67–143)*
VIII	**182 (105–329)** *N* = 20 (9F/11M)	**159 (83–274)** *N* = 25 (12F/13M)	**94[a] (54–145)** *N* = 21 (6F/15M)	**110[a] (36–185)** *N* = 45 (26F/19M)	**117[a] (52–182)** *N* = 52 (20F/32M)	**120[a] (59–200)** *N* = 24 (6F/18M)	**160 (52–290)** *N* = 44
VIII *Andrew et al.*[3,4]	*100 (61–139)*	*88 (55–121)*	*73[b] (50–109)*	*90 (59–142)*	*95 (58–132)*	*92 (53–131)*	*99 (50–149)*
IX	**48[a] (35–56)** *N* = 24 (11F/13M)	**72[a] (44–97)** *N* = 23 (11F /12M)	**71[a] (43–121)** *N* = 21 (5F/16M)	**85[a] (44–127)** *N* = 44 (25F/19M)	**96[a] (48–145)** *N* = 51 (19F/32M)	**111[a] (64–216)** *N* = 25 (6F/19M)	**130 (59–254)** *N* = 44
IX Andrew et al.[3,4]	*53[b] (34–72)*	*53[b] (34–72)*	*86[b] (36–136)*	*73[b] (47–104)*	*75[b] (63–89)*	*82[b] (59–122)*	*109 (55–163)*
X	**55[a] (46–67)** *N* = 22 (12F/10M)	**60[a] (46–75)** *N* = 22 (11F/11M)	**95[a] (77–122)** *N* = 21 (6F/15M)	**98[a] (72–125)** *N* = 66 (25F/41M)	**97[a] (68–125)** *N* = 49 (20F/29M)	**91[a] (53–122)** *N* = 24 (7F/17M)	**124 (96–171)** *N* = 44
X Andrew et al.[3,4]	*40[b] (26–54)*	*49[b] (34–64)*	*78[b] (38–118)*	*88[b] (58–116)*	*75[b] (55–101)*	*79[b] (50–117)*	*106 (70–152)*
XI	**30[a] (7–41)** *N* = 20 (10F/10M)	**57[a] (24–79)** *N* = 22 (11F /11M)	**89[a] (62–125)** *N* = 22 (6F/16M)	**113 (65–162)** *N* = 41 (24F/17M)	**113 (65–162)** *N* = 50 (18F/32M)	**111 (65–139)** *N* = 24 (5F/19M)	**112 (67–196)** *N* = 44

XI *Andrew et al.*[3,4]	*38[b] (24–52)*	*55[b] (39–71)*	*86[b] (49–134)*	*97 (56–150)*	*86 (52–120)*	*74 (50–97)*	*97 (67–127)*
XII	**58[a] (43–80)** *N* = 20 (9F/11M)	**53[a] (14–80)** *N* = 21 (11F/10M)	**79[a] (20–135)** *N* = 21 (7F/14M)	**85[a] (36–135)** *N* = 39 (20F/19M)	**81[a] (26–137)** *N* = 45 (17F/28M)	**75[a] (14–117)** *N* = 22 (7F/15M)	**115 (35–207)** *N* = 44
XII *Andrew et al.*[3,4]	*53[b] (33–73)*	*47[b] (29–65)*	*77[b] (39–115)*	*93 (64–129)*	*92 (60–140)*	*81[b] (34–137)*	*108 (52–164)*

[a]Values that are significantly different from adult values ($P < 0.05$).
[b]Values that are significantly different from adult values for data from Andrew et al.[3,4]
M, males; F, females.
NOTE: Andrew et al.[3,4] results for day 3 are actually day 5 results.
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Table 119.5

Coagulation inhibitor reference values for neonates and children compared to results from Andrew et al.[3,4]

Coagulation Inhibitors (%)	Age						
	Day 1	**Day 3**	**1 Month–1 Year**	**1–5 Years**	**6–10 Years**	**11–16 Years**	**Adults**
AT	**76[a] (58–90)** *N* = 18 (9F/12M)	**74[a] (60–89)** *N* = 22 (10F/12M)	**109[a] (72–134)** *N* = 41 (8F/33M)	**116[a] (101–131)** *N* = 49 (26F/23M)	**114[a] (95–134)** *N* = 59 (25F/34M)	**111[a] (96–126)** *N* = 26 (8F/18M)	**96 (66–124)** *N* = 43
Antithrombin (AT) *Andrew et al.*[3,4]	*63[b] (51–75)*	*67[b] (54–80)*	*104 (84–124)*	*111 (82–139)*	*111 (90–131)*	*105 (77–132)*	*100 (74–126)*
Protein C chromogenic	**36[a] (24–44)** *N* = 22 (9F/13M)	**44[a] (28–54)** *N* = 21 (10F/11M)	**71[a] (31–112)** *N* = 25 (5F/20M)	**96[a] (65–127)** *N* = 42 (21F/21M)	**100 (71–129)** *N* = 53 (21F/32M)	**94[a] (66–118)** *N* = 25 (8F/17M)	**104 (74–164)** *N* = 42
C chromogen *Andrew et al.*[3,4]	*35[b] (26–44)*	*42[b] (31–53)*	*59[b] (37–81)*	*66[b] (40–92)*	*69[b] (45–93)*	*83[b] (55–111)*	*96 (64–128)*
Protein C clotting	**32[a] (24–40)** *N* = 20 (9F/11M)	**33[a] (24–51)** *N* = 22 (11F/11M)	**77[a] (28–124)** *N* = 24 (4F/20M)	**94[a] (50–134)** *N* = 39 (16F/23M)	**94[a] (64–125)** *N* = 50 (17F/33M)	**88[a] (59–112)** *N* = 20 (6F/14M)	**103 (54–166)** *N* = 44
C clotting *Andrew et al.*[3,4]	*Not available*	*Not available*	*Not available*	*Not available*	*Not available*	*Not available*	*Not available*
Protein S clotting	**36[a] (28–47)** *N* = 22 (13F/9M)	**49[a] (33–67)** *N* = 24 (11F/13M)	**102[a] (29–162)** *N* = 41 (8F/33M)	**101[a] (67–136)** *N* = 49 (26F/23M)	**109[a] (64–154)** *N* = 59 (25F/34M)	**103[a] (65–140)** *N* = 27 (9F/18M)	**75 (54–103)** *N* = 44
Protein S *Andrew et al.*[3,4]	*36[b] (24–48)*	*50[b] (36–64)*	*87 (55–119)*	*86 (54–118)*	*78 (41–114)*	*72 (52–92)*	*81 (60–113)*

[a]Values that are significantly different from adult values ($P < 0.05$).
[b]Values that are significantly different from adult values for data from Andrew et al.[3,4]
M, males; F, females.
NOTE: Andrew et al.[3,4] results for day 3 are actually day 5 results.
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Table 119.6
D-dimer reference values for neonates and children

	Age						
	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
D-dimers (μg/mL)	1.47[a] (0.41–2.47) *N* = 20 (10F/10M)	1.34[a] (0.58–2.74) *N* = 23 (12F/11M)	0.22 (0.11–0.42) *N* = 20 (7F/13M)	0.25[a] (0.09–0.53) *N* = 40 (19F/21M)	0.26[a] (0.10–0.56) *N* = 39 (12F/27M)	0.27[a] (0.16–0.39) *N* = 21 (6F/15M)	0.18 (0.05–0.42) *N* = 32 (19F/13M)

[a]Values that are significantly different from adult values ($P < 0.05$).
Reproduced from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost* 2006;95(2):362–372, with permission.

Table 119.7
Tissue Factor Pathway Inhibitor (TFPI) Reference values for children

	Age						
	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
TFPI free (ng/mL)	Not available	Not available	7.13[a] (5.63–8.44)	10.16 (5.06–9.05)	6.69[a] (4.29–9.31)	7.66[a] (5.15–8.74)	10.70 (6.12–12.34)
TFPI total (ng/mL)	Not available	Not available	77.49 (69.42–85.58)	76.33 (61.27–89.80)	73.99[a] (59.13–88.02)	74.09 (61.63–87.36)	87.49 (63.64–104.38)

[a]Values that are significantly different from adult values ($P < 0.05$).
Reproduced from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost* 2006;95(2):362–372, with permission.

Table 119.8
Endogenous Thrombin Potential (ETP) reference values for children

	Age						
	Day 1	Day 3	1 Month–1 Year	1–5 Years	6–10 Years	11–16 Years	Adults
ETP (Pm/min)	Not available	Not available	4,865[a] (2,653–7,162) *N* = 20 (10F/10M)	4,429[a] (2,537–6,084) *N* = 22 (11F/11M)	5,363[a] (2,719–8,938) *N* = 21 (11F/10M)	7,593 (3,373–7,516) *N* = 21 (11F/11M)	8,475 (7,043–10,205) *N* = 20

[a]Values that are significantly different from adult values ($P < 0.05$).
Reproduced from Monagle P, Barnes C, Ignjatovic V, et al. Developmental haemostasis. Impact for clinical haemostasis laboratories. *Thromb Haemost* 2006;95(2):362–372, with permission.

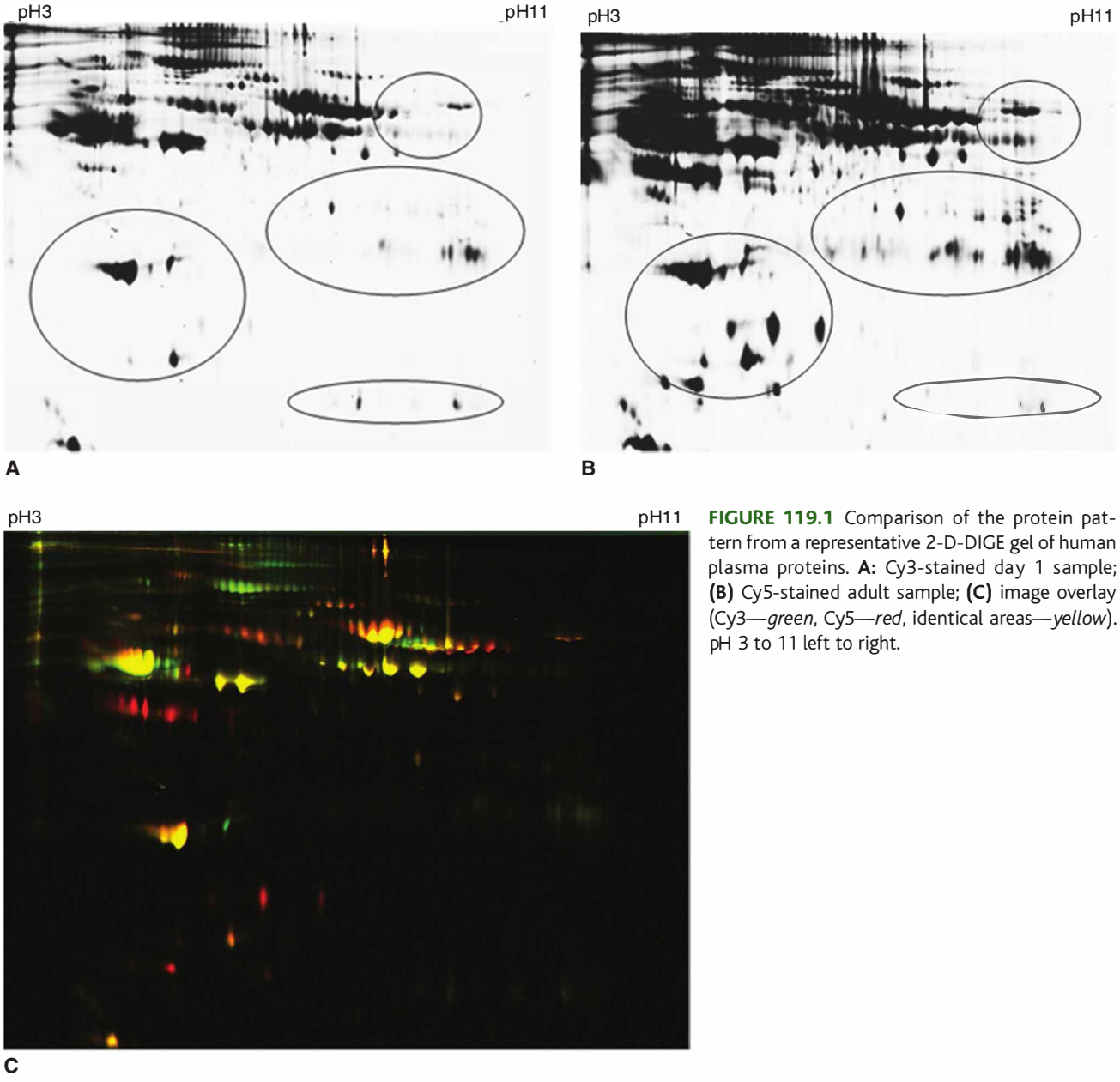

FIGURE 119.1 Comparison of the protein pattern from a representative 2-D-DIGE gel of human plasma proteins. **A:** Cy3-stained day 1 sample; **(B)** Cy5-stained adult sample; **(C)** image overlay (Cy3—*green*, Cy5—*red*, identical areas—*yellow*). pH 3 to 11 left to right.

was consistently higher by up to 1,500 Da in neonates and children compared to adults.[38] This trend toward a higher molecular weight of fibrinogen chains in younger age groups was also consistent for the Bβ and γ chains with differences of up to 400 and 500 Da, respectively. These differences in fibrinogen chains could represent multiple additional sialic acid or residues associated with increased glycan branching[39] in the neonatal and pediatric fibrinogen compared to the adult form of this protein. The same study also demonstrated significant differences in the chromatogram profile (area under the peak and peak height) in neonates and children compared to adults, suggesting differences in the interaction of the fibrinogen molecule from each age group with the chromatography column used, which also implies structural differences for fibrinogen in these age groups.[38]

There has been very little research examining whether age-related PTMs are relevant in other coagulation proteins, and none in humans. In addition to fibrinogen, a "neonatal" form of protein C has been detected in the ovine fetus.[40] Compared to the adult two-chain molecule, the "neonatal" protein C has an increased proportion of single-chain molecules. Preliminary reports have also indicated that forms of protein C with increased glycosylation are more prevalent in newborns than in adults. Differences have also been demonstrated in mRNA levels of antithrombin (AT) in fetal compared to adult sheep, suggesting age-specific differential translation for this protein.[41]

Despite changes in the concentration of individual coagulation proteins as well as in global tests of coagulation, the hemostatic system in neonates and children does not seem to be "disadvantaged" compared to the "normal" coagulation system

Table 119.9

Examples of plasma proteins known to change in abundance with age

Proteins that Increase in Abundance with Age	Proteins that Decrease in Abundance with Age
Clusterin	A2M
Haptoglobin	Complement C3
Alpha-1-antitrypsin	Complement factor B
Hemopexin precursor	Fibrinogen
Kininogen 1 variant	Vitamin D–binding protein
Chain A, human factor H	Fibronectin 1 variant
	Heparin cofactor II
	Alpha-1-antichymotrypsin
	Bikunin

Modified from Ignjatovic V, Lai C, Summerhayes R, et al. Age-related differences in plasma proteins: how plasma proteins change from neonates to adults. *PLoS One* 2011;6(2):e17213.

of adults. There is no data to support either an increased bleeding or thrombotic risk during infancy and childhood for any given stimulus, and on the contrary, one could argue that the hemostatic system in neonates and children protects against bleeding and thrombotic complications compared to adults. This is despite the fact that when considering individual proteins, many proteins exist at levels during stages of infancy that would be associated with disease in adults. For example, AT levels during the first 3 months of life are decreased compared to those observed in many adults with heterozygous AT deficiency and recurrent thrombosis.[32] Similarly, levels of factor IX in children may be below 30% of adult levels in early life and yet those children have no evidence of a bleeding phenotype. Prolongation of global tests of coagulation, such as the APTT, are also not associated with an increase in bleeding, despite the APTT being considerably prolonged compared to that in many adults with clinically relevant von Willebrand disease.[32] The absolute levels of hemostatic proteins as measured by the currently available functional assays are clearly not the key factor in determining clinical phenotype, and there remains much research to be performed to better understand the relationships between the concentration of hemostatic proteins and functional outcomes of the hemostatic system.

IMPLICATIONS OF DEVELOPMENTAL HEMOSTASIS

Clinical Implications

Defining Reference Ranges

As previously stated, a number of studies have confirmed that age-related reference ranges for coagulation assays in the healthy population are analyzer and reagent dependent.[13] Despite this, very few clinical laboratories have adequately determined age-related reference ranges specific to their analyzer–reagent combination, mostly for cost and ethical difficulties. Collecting blood from otherwise healthy children to enable reference range determination is not easy. However, the commonly used alternatives of either using adult reference ranges or published pediatric reference ranges are unsatisfactory in terms of the highest standard of clinical care of the patients.

A typical example would be a 4-year-old child who has an APTT performed using the PTT-A reagent (Diagnostica Stago, France) on an STA-compact analyzer (Diagnostica Stago, France). The 95% confidence interval for APTT results in this age group is 33 to 44 seconds (rounded to whole seconds).[13] On the other hand, if the reagent being used for the APTT assay is CK-PREST (Diagnostica Stago, France), even using the same analyzer, the reference range encompassing 95% of the population is reported to be 30 to 35 seconds.[32] Therefore, if one used the published reference range based on a CK-PREST analysis, but the actual laboratory used the PTT-A reagent, then over 30% of children (assuming normal distribution) in this age group would be labeled as having prolonged APTT when in fact they were perfectly healthy. The majority of APTT reagents currently in clinical use have no published age-specific references ranges, so one can only assume that such errors are likely to be frequent. As each new reagent is developed for clinical use, unless the appropriate normative data are available, then children will continue to be classified as either normal or abnormal erroneously.

Consider the other alternative strategy of using an adult-based reference range. For example, the adult reference range for an APTT using the PTT-A reagent (Diagnostica Stago, France) and an STA-Compact analyzer is 27 to 38 seconds (95% confidence intervals rounded to whole seconds). Once again approximately 30% of children aged 1 to 5 years would be classified as abnormal if they were compared to this adult-specific reference range, even if their samples were analyzed using the same reagent and analyzer combination.

The clinical implications of this type of error are significant. Considering the most common clinical indications for performing an APTT assay in nonhospitalized children, then the true cost of this misclassification is startling. Children commonly have coagulation studies ordered because they are preoperative and the surgeon is concerned about the past history ("my child bruises easily, or has blood noses"), or because of a positive family history. Erroneous interpretation of an APTT as prolonged, due to the use of an inappropriate reference range, leads to cancellation of surgery, multiple investigations, referral to specialist hematologists, and often overtreatment of the child during the procedure. Similarly, children often have coagulation studies because of a positive family history of a bleeding disorder. Misclassification of the child due to nonspecific reference ranges can lead to a child being mislabeled as having a bleeding or thrombotic disorder when in reality they do not. This can have significant lifestyle and other implications for the child and their family which can be problematic for many years to come. Reversing an already made "diagnosis" is never easy. Finally, children thought to be at risk from physical or mental abuse will often have coagulation studies as part of their workup if they present with unusual bruising or injuries. Forensic medicine requires exact and explainable answers if justice is going to be achieved and "at-risk" children protected.

All laboratories that report coagulation studies in neonates and children must ensure that they are using appropriate age-related reference ranges that are specific for their analyzer/reagent combination.

Diagnosing Disease

Understanding the true age-related 95th centiles of a hemostatic parameter is of scientific value. However, this does not necessarily help us in defining disease. For example, if the lower limit of the reference range for factor VIII in a child is 59% based on a population study, and a patient of appropriate age presents who, when using the same analyzer and reagent, has a factor VIII of 50%, then it is unlikely that the patient has even mild hemophilia. This is because by definition, we define hemophilia by arbitrary plasma levels (<30% for mild hemophilia), or at least plasma levels that are thought to be associated with increased risk of bleeding. In the absence of genetic studies, definition of heterozygote states remains difficult. The dilemma is that for the laboratory tests to be clinically useful, we need to understand the level at which results define clinically relevant disease probably more so than the levels at which the results are outside the 95% confidence limits of the healthy population. To date, there have been no specific studies in children which address this issue. Correlation of phenotype and genotype, as well as longitudinal studies mapping the hemostatic protein changes in a cohort of patients in conjunction with their clinical outcomes, would be most helpful. The issue of results being outside the reference range versus being indicative of clinical disease is most problematic when considering the procoagulant proteins. One must always remember that a positive diagnosis of a hemostatic disorder should include the presence of a family history, a positive clinical phenotype, and reproducible abnormal laboratory results. The problem is that the clinical phenotype is much more difficult to define given the infrequency of thrombotic events; the family history is often equivocal in terms of the role of clinical precipitants of thrombosis, and yet the labeling of children as having a thrombophilic condition can have significant impact. Frequently, clinicians are asked to advise on the need for thromboprophylaxis related to clinical interventions, oral contraceptive pill use, or prolonged travel in children in whom the presence or absence of a true thrombophilic condition seems almost impossible to determine.

The Risk of Thromboembolic Disease in Infants and Children

The epidemiology of thromboembolism in pediatric patients is vastly different from that observed in adults.[42,43–54] The evidence that infants and children are protected from thrombosis comes from a number of different perspectives. Patients with congenital deficiencies of AT, protein C, or protein S or with activated protein C resistance (APCR)[55–59] usually do not present with thrombosis until the late teenage years or even later. Similarly, thrombosis secondary to acquired risk factors occurs considerably less frequently in children compared to adults. For example, thrombosis in children with nephrotic syndrome occurs in only 2% of children compared with approximately 20% of adults.[60–65] Children routinely undergo major abdominal and lower limb orthopedic surgery and do not require anticoagulant prophylaxis because secondary thrombosis is so rare. In contrast, adults undergoing similar procedures have a high risk of thrombosis and benefit from prophylactic anticoagulant therapy.[66]

Thus for the same risk factor, whether inherited or acquired, the thrombosis risk is substantially reduced in children compared to adults, which suggests there are protective mechanisms in play. This may be secondary to age-related differences in hemostatic proteins, platelets, blood vessel wall, or a combination of all three.

Developmental Hemostasis and Integrated Physiologic Systems

The fundamental question remains as to why the plasma levels of coagulation proteins differ with age. Potentially, this has nothing to do with coagulation. Coagulation proteins are examples of broad-acting proteins such as serpins, and many have been shown to also have actions in multiple key biologic processes such as inflammation, wound repair, and angiogenesis.[30,67] Changing requirements of those systems could equally provide the rationale for developmental changes in the hemostatic system, which are therefore seen as necessary compensations allowing normal hemostatic function. Whether it is the requirements of one, two, or a mixture of these basic systems involved in multicellular organism survival that drive the plasma levels of these multifunctional proteins remains to be determined but is a most enticing theory. Evidence for this theory is mounting, and two important examples to consider are antithrombin and alpha-2 macroglobulin (A2M).

AT is a major plasma serine proteinase inhibitor (serpin) which inhibits thrombin and activated factor X generated upon activation of the hemostatic system.[68] The 3-D structure of AT was determined more than 15 years ago by two independent research groups.[69,70] AT inhibits thrombin and in the presence of unfractionated heparin (UFH), this ability to inhibit thrombin is increased by 1,000-fold.[71] AT is described as occurring in two distinct isoforms, native AT (NAT) or latent AT (LAT). Recent studies have demonstrated that LAT is associated with severe and sudden onset of thrombosis, has potent antiangiogenic properties, specifically downregulates several proangiogenic genes, and upregulates a number of antiangiogenic genes.[72]

Preliminary experiments demonstrate the concentration of the LAT isoform in the healthy population increases significantly with age, such that day 1 to 3 neonates, <1-year-old children, and 1- to 5-year-olds have 30%, 50%, and 80% LAT compared to adult levels, respectively (Unpublished data Vera Ignjatovic). In addition to NAT and LAT, AT isolated from adults has been shown to circulate as two glycoforms[73]; α-AT has four identical sialylated complex oligosaccharides attached to asparagines, which account for 15% of the 58 kDa total mass, while β-AT is only glycosylated on three of the potential four asparagine sites.[74] In adults, α-AT constitutes 90% to 95% of circulating AT, compared to only 5% to 10% β-AT, yet β-AT has higher affinity for UFH, endothelial surfaces and is a more effective thrombin inhibitor.[74–76] This suggests physiologically and clinically specialized functions for the AT glycoforms, where β-AT may be vital for controlling thrombogenic events arising from vessel wall injury, while α-AT may be largely responsible for inhibition of fluid-phase thrombin. The location of the carbohydrate side chain at Asn-135 in α-AT (absent in β-AT) interferes with the binding of UFH due to its proximity to the heparin-binding site.[77] This demonstrates that glycosylation or deglycosylation of a single amino acid, particularly in close proximity to the heparin-binding site of AT, has significant functional implications for the function of the AT molecule. Despite the clinical significance of the ratio of α-AT to β-AT in terms of their interaction

with UFH, whether this ratio changes with age or health state has not been investigated previously.

Recently, AT has been shown to have potent antiangiogenic properties as well as being an important anticoagulant.[72] AT has been shown to downregulate several proangiogenic genes and upregulate a number of antiangiogenic genes. The antiangiogenic forms of AT are known to include heparin-binding sites, and heparin is shown to potentiate this effect.[72] This is one of the postulated mechanisms in the positive effect of heparinoids on cancer survival which is independent of the antithrombotic effect.[78–80] AT levels are naturally reduced in newborns to <50% of the levels observed in adults and then increase to approach adult levels by approximately 6 months of age.[13] Early evidence suggests that there is a difference in the balance of isoforms of AT in newborns compared to adults. One postulated reason for the decreased levels of AT (and the altered balance of isoforms) observed in neonates is related to the role of this protein in angiogenesis.[41] Fetal and early neonatal life is a time of prolific angiogenesis, much more so than any later stage of life, and given the known antiangiogenic properties of AT, the low levels of this protein are likely beneficial for healthy development. Hence, AT replacement therapy during neonatal life may well be deleterious by altering the normal balance of angiogenesis. In the only published randomized trial of AT replacement therapy in newborn infants (as a treatment for lung disease of prematurity), there were seven (11.5%) deaths in the AT-treated group and three (4.9%) deaths in the placebo (no treatment) group.[81] A similar trial (reported only in abstract format) also reported a similar trend in the setting of a clinical trial; however, the mechanism for this finding was not established.[81]

Thus in summary, preliminary evidence indicates that age-related changes in the quantity, structure, and function of AT are important in normal physiologic development, with implications much broader than the coagulation system alone.

Another coagulation protein that is important in this context is A2M, a major natural inhibitor of thrombin that is present in two- to threefold higher concentrations during childhood compared to adults and remains elevated until adulthood.[3] As a major plasma protease inhibitor, A2M represents 2% to 4% of the total protein content in adult plasma[82,83] and plays a role in multiple processes including hemostasis, innate immunity, and inflammation. To date, there has been no evidence for a case of a total deficiency or homozygosity of A2M, which could be evidence of an absolute requirement of A2M for fetal survival *in utero*. In the hemostatic system, A2M exhibits anticoagulant, procoagulant, and antifibrinolytic activity. A2M has also been linked to the development of Alzheimer disease.[84] In addition, A2M has a function in the immune system, as a major inhibitor of metalloproteinases, assisting in their clearance from tissue fluids.[85] Recently, A2M has been demonstrated to also function as a molecular chaperone, where native A2M binds to plasma proteins that are partially unfolded or inactivated by heat or oxidative stress. Specifically, the A2M–protein complex is still able to interact with and inhibit proteases and subsequently the A2M–protein–protease complex is able to be rapidly cleared via endocytosis from the circulation.[86]

A2M levels are increased in healthy infants and children and only approach adult levels in late teenage years as confirmed in a number of studies.[2–4,82] A2M has been shown to be influential in the regulation of the hemostatic system in neonates, where A2M functions as a major inhibitor of coagulation.[87,88] Up to 64% of thrombin in neonates is bound and inhibited by A2M, while the corresponding inhibition of thrombin in adults accounts for 7% of thrombin.[89] In fact, in neonates and children, A2M plays a more important role in thrombin inhibition compared to AT, which is the predominant inhibitor in adults.[7] In AT-deficient children, thrombin inhibition by A2M was demonstrated to be as effective as in plasma from children and adults without the AT deficiency.[87] Hence, A2M has a compensatory role for thrombin inhibition in children with an AT deficiency.

Interestingly, decreased A2M levels have been reported in neonates with chronic heart disease compared to otherwise healthy neonates.[90] In contrast, asymptomatic dyslipidemia in children has been associated with increased levels of A2M (as well as plasminogen and fibrinogen) compared to control subjects.[91]

In summary, it seems highly likely that A2M may in fact have potential functions in the regulation of multiple growth processes. However, the biologic basis for the significantly elevated plasma levels of A2M in infancy and childhood has not been clearly defined to date, and much further research is clearly required.

The two examples discussed previously represent the two proteins for which there is the most available data at this time. Once the focus on research in this area increases, the list of coagulation proteins for which there are multifunctional reasons for age-related changes will also undoubtedly increase. A better understanding of the roles of each of the coagulation proteins in normal growth and development, and a better understanding of how these complex physiologic systems integrate and function in unison, is clearly important. Such knowledge would help in terms of understanding the effect of treating children with plasma products or anticoagulants, which directly impact on the plasma levels of such proteins, and likely change the balance of isoforms in circulation. Further, such knowledge may provide critical clues to the processes that underlie so much of the disease burden within Western society which is clearly associated with aging.

CONCLUSION

The fact that the hemostatic system changes with age is indisputable. However, fundamental questions remain as to why these changes occur, what the relative influence is of quantitative versus qualitative protein changes, and how in fact these changes are mediated. The implications of altering the time course or progress of these age-related changes are unknown, but may potentially be either advantageous or deleterious to long-term survival. There remain many unanswered questions about the hemostatic system in adults, and its role in health and disease, further complicating our ability to answer the unknown questions related to developmental hemostasis. Of specific and immediate interest is what these changes mean for anticoagulation and other hemostatic therapies in children. Given it is only 20 years since the pioneering work of Andrew et al. initiated the concept of developmental hemostasis, our lack of current knowledge is not surprising. However, we require a concerted research effort to ensure that 20 years from now we have a much greater understanding of developmental hemostasis.

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CHAPTER

120 Bleeding in Neonates and Children

ANTHONY K. C. CHAN, ELIZABETH A. CHALMERS, SAM SCHULMAN, AND ALAN D. MICHELSON

Neonates and children may be born with a congenital bleeding disorder or they may acquire a bleeding disorder as a consequence of a disease process. Correct diagnosis is crucial to management of the disease and to reduce morbidity and mortality associated with any bleeding diathesis. The natural history of diseases in children can differ substantially from that observed in adult patients due to developmental differences in their hemostatic system amongst other causes. Thus, treatment and management of children with bleeding disorders is often age specific and cannot simply be extrapolated from adult data. The following chapter highlights some common bleeding disorders in neonates and children and where available, treatment recommendations are summarized.

NEONATAL THROMBOCYTOPENIA

Platelet production begins in fetal life and progressively increases to adult levels by the end of the first trimester (≥22 weeks gestational age). The normal range for adult platelet count of 150 to 450 × 10^9/L is reached in the healthy fetus by 22 weeks of gestation. Therefore, neonates with platelet counts <150 × 10^9/L are considered to have neonatal thrombocytopenia. Otherwise, healthy neonates commonly have platelet counts in the range of 100 to 150 × 10^9/L, but these are mostly transient thrombocytopenias and need no further investigation unless the clinical condition worsens.[1] Developmental differences in the size of the megakaryocytes between neonates and adults, such as smaller megakaryocytes, might predispose neonates to thrombocytopenia.[2]

The overall incidence of neonatal thrombocytopenia has been estimated to be 0.7% to 0.9%. However, the incidence is considerably higher in neonates admitted into the neonatal intensive care unit (NICU), where it is estimated to be 22% to 35%.[1] Neonatal thrombocytopenia can be caused by increased platelet consumption, decreased platelet production, hypersplenism, or a combination of any of these. Underlying causes include immune deficiency, infections, placental insufficiency, disseminated intravascular coagulation (DIC), genetic disorders, and medication-induced thrombocytopenia (Table 120.1).

In general, the underlying cause may be determined by the timing of the onset of thrombocytopenia as different conditions lead to either early-onset (within 72 hours of life) or late-onset (>72 hours) thrombocytopenia.[1] Although there can be significant overlap between pathologies underlying neonatal thrombocytopenia, specific diseases associated with early-onset type include alloimmune disease, parasitic infections, placental insufficiency, chromosomal defects (notably trisomies 13, 18, and 21), and familial thrombocytopenias.[1] Late-onset thrombocytopenias are usually associated with fungal infections, medication use (some antibiotics, heparin, anticonvulsants), and necrotizing enterocolitis (NEC).[1]

As stated, approximately three-quarters of all neonates with thrombocytopenia are transient or mild in nature and do not usually require further intervention. However, the remaining cases (20% to 25% or 2% to 9% of NICU admissions) require platelet transfusions in order to treat or decrease the risk of bleeding.[1] Although neonates with active major bleeds (intracranial, pulmonary, gastrointestinal [GI], hepatic or renal) with platelet counts <100 × 10^9/L receive platelet transfusions, the majority of platelet transfusions are given to thrombocytopenic neonates without bleeding events and with platelet counts <50 × 10^9/L.[2–4] There is only one randomized controlled study[5] investigating the effectiveness of platelet transfusions on reducing the incidence/severity of intracranial hemorrhage (ICH) in sick preterm infants with thrombocytopenia. The authors found no significant difference in the frequency or severity of ICH in the treatment group (platelet transfusions for platelet count <150 × 10^9/L) compared to the control group (platelet transfusions only for clinical indications or for a platelet count of <50 × 10^9/L). Although, platelet transfusion increased platelet counts and shortened bleeding times, it did not have an effect on the incidence or severity of ICH.[5]

There is a lack of solid evidence for guiding platelet transfusions in neonates, but several guidelines have been published,[1,3,6–10] providing a starting point of treatment options for thrombocytopenic neonates. Although there is a range of transfusion practices, since the early 1990s, there is a trend toward accepting a lower threshold of platelet count before starting platelet transfusions in nonbleeding sick and stable preterm infants (from platelet count <100 × 10^9/L to <30 × 10^9/L before initiating platelet transfusion).[1]

Neonatal Alloimmune Thrombocytopenia

Neonatal alloimmune thrombocytopenia (NAIT) occurs when there is an incompatibility of fetomaternal human platelet antigens (HPAs). The incidence of NAIT has been estimated to be 1 in 5,000, but the incidence was higher (1 in 1,000) in screening studies using only HPA-1a incompatibility criteria.[11–13] Severe NAIT can occur even in the first pregnancy (~50% of the cases). In the most severe cases, there is massive ICH, but the presentation can in other cases be mild bleeding that is easily managed. The diagnosis is established by verification of incompatibility between the platelets of the mother and the baby. It is important to provide effective treatment quickly as NAIT is associated with 11% to 21% risk of ICH.[14] Screening studies have indicated that

Table 120.1

Neonatal thrombocytopenias and their typical time of presentation

Etiology	Examples	Onset
Alloimmune	Maternal antibodies against HPA-1a or -5b	Fetal—neonatal <72 h
Congenital infections	Cytomegalovirus, rubella, toxoplasma, HIV	Fetal—late neonatal
Aneuploidy	Trisomy 18/13/21; triploidy	Fetal
Autoimmune	ITP, systemic lupus	Fetal—neonatal <72 h
Severe Rhesus disease		Fetal
Congenital–inherited	*With abnormal platelet function* Wiskott-Aldrich, Bernard-Soulier, X-linked thrombocytopenia, or Chediak-Higashi syndrome, Quebec platelet disorder, giant platelet syndromes *Without abnormal platelet function* TAR, CAMT, Fanconi anemia, May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, Epstein syndrome, autosomal dominant thrombocytopenia	Fetal—late neonatal
Placental insufficiency	Intrauterine growth retardation, preeclampsia, diabetes	Neonatal <72 h
Perinatal asphyxia		Neonatal <72 h
Perinatal infection	*Escherichia coli*, group B *Streptococcus*, *Haemophilus influenza*	Neonatal <72 h
DIC		Neonatal <72 h
Thrombosis	Arterial or venous	Neonatal <72 h
Congenital leukemia		Neonatal <72 h
Kasabach-Merritt syndrome		Early–late neonatal
Metabolic disease	Propionic acidemia, methylmalonic acidemia	Early–late neonatal
Late-onset sepsis	NEC	Neonatal >72 h

CAMT, congenital amegakaryocytic thrombocytopenia; HIV, human immunodeficiency virus; ITP, immune thrombocytopenic purpura; NEC, necrotizing enterocolitis; TAR, thrombocytopenia absent radius syndrome.

neonatal platelet count $<50 \times 10^9$/L identified 90% of patients with NAIT.[15–17]

Recommended platelet transfusion thresholds include neonates with severe thrombocytopenia (platelet count $<30 \times 10^9$/L), neonates with minor bleeding events and a platelet count of $<50 \times 10^9$/L or those with a sibling who previously had ICH, or neonates with severe bleeding with a platelet count of $<100 \times 10^9$/L.[1] The standard platelet dose is 10 mL/kg. However, dosing can be increased or repeated if response is insufficient.[18] The majority of antibodies are against HPA-1a and HPA-5b platelet antigens (79% and 9%, respectively).[19] Thus, platelet transfusion from HPA-1a/HPA-5b negative donors should be effective in increasing platelet counts in most cases of suspected or proven NAIT.[19,20] When compatible platelets are unavailable, high-dose intravenous immunoglobulin (IVIG) and/or a trial with random donor platelets may be a viable alternative. The recommended dose for IVIG is 1 to 2 g/kg administered as 0.4 g/kg daily for 3 to 5 days or 1 g/kg daily for 1 or 2 days.[21] Neonates with NAIT will respond to incompatible platelet transfusions;[22,23] thus random platelet transfusions can be used as first-line therapy, with close monitoring, for neonates with severe thrombocytopenia regardless of etiology.[18] Head sonography should be performed on thrombocytopenic neonates in order to determine the presence of ICH and to set appropriate platelet threshold for the treatment of the neonate. NAIT often resolves within 2 to 4 weeks, but platelet count should be followed until normal levels are attained off treatment to exclude inherited or other forms of thrombocytopenia.[18]

The recurrence of thrombocytopenia is very high and usually results in increased severity in subsequent pregnancies. Most of the current information is mainly derived from retrospective data. The highest risk of NAIT-related complications in subsequent pregnancies is among infants whose sibling had antenatal ICH.[24] Current management of subsequent pregnancies of women with a history of a child diagnosed with NAIT is focused on the prevention of ICH in the fetus during pregnancy and

delivery.[25] Antenatal diagnosis can be made with genotyping on chorionic villi or on amniotic cells. The titer of the maternal anti-HPA-1a antibody seems to be correlated with severe events to the fetus. The fetal development is followed with ultrasonography to detect ICH, preferably at tertiary care centers. The therapeutic options are weekly maternal injections with high-dose immunoglobulin, possibly with the addition of corticosteroids or, if that fails, weekly intrauterine transfusions with antigen negative platelets. A severely affected fetus should be delivered by cesarean section.

Neonatal purpura or hematoma as well as more serious bleeding complications is investigated with complete blood count and coagulation screening. The thrombocytopenia is severe in case of HPA-1a or -3a immunization, whereas the mother has a normal platelet count. The platelet phenotype and genotype of both parents and antibodies should be analyzed to verify the diagnosis. However, already with a strong suspicion of NAIT and a platelet count below 30×10^9/L, the baby should be transfused with washed and irradiated maternal platelets or with HPA-1b/1b platelets. Babies with higher platelet count should be followed closely and transfused in case of a sudden drop in platelet count. This monitoring is necessary for 1 to 2 weeks until the platelet count normalizes. ICH should be excluded with ultrasonography or magnetic resonance imaging. However, optimal management of high-risk pregnancies is still under debate. High-risk obstetric and adult hematologist with appropriate experience should be consulted.

Immune Thrombocytopenic Purpura

Immune thrombocytopenic purpura (ITP) is the result of platelet destruction by platelet autoantibody production coupled with impaired platelet production. Previously, it was thought that only macrophages in the reticuloendothelial system were involved in this process,[26,27] but emerging evidence now supports the involvement of both the cellular immune (T-cell abnormalities) and humoral immune (antibody secretion) systems.[28–30] ITP is one of the most common acquired bleeding disorders in children with complete resolution of thrombocytopenia in the majority of affected individuals within weeks or months of onset, regardless of whether therapy was instituted. It is estimated that 20% to 30% of these children will go on to have persistent thrombocytopenia (more than 6 months duration since diagnosis) but over 25% of these with persistent thrombocytopenia will have disease resolution by 12 months.[31]

Clinical Characteristics of ITP

ITP presentation can be at any age in childhood, with approximately 70% presenting between 1 and 10 years of age, peaking at age 5 to 6 years.[32] The majority of children do not have any documented infection prior to ITP presentation. However, in some children, it is preceded by a presumed viral illness or live-virus vaccination such as the measles, mumps, and rubella vaccine.[33,34]

Before ITP diagnosis, other causes of thrombocytopenia should be excluded by careful consideration of history, physical examination, and peripheral blood smear assessment.[35] Presentation symptoms in children include mucocutaneous bleeding, bruising, petechiae, purpura, epistaxis, hematuria, and menorrhagia (teenage girls). Physical findings and complete blood count are otherwise normal except for thrombocytopenia with large platelets. ITP with atypical clinical or laboratory findings warrant further investigations.[36] Even with severe thrombocytopenia (platelet count $<20 \times 10^9$/L) at the onset of ITP, the incidence of severe or life-threatening bleed is minimal.[32,37,38] The long-term outcome for children with ITP is favorable.[39–41]

Treatment of ITP

Many children with mild-to-moderate thrombocytopenia and minimal bleeding events require only close observation, although some with severe thrombocytopenia with overt bleeding will require therapy. The aim is to increase platelet counts to prevent hemorrhage while minimizing therapy side effects. Currently, there is no standard of care for children with symptomatic, acute, or chronic ITP. Thus, each case should be considered on an individual basis with benefits and risks of each treatment assessed when creating a management plan. Table 120.2 outlines some of the available therapies used for children with ITP.[36] These treatments do not cure or alter the disease course but are only helpful in the management of symptomatic ITP.

An alternative therapy for children with chronic ITP is splenectomy, but this should be reserved for children with severe thrombocytopenia in which conservative management is unsuccessful. There are many publications to support the effectiveness of splenectomy in children with ITP, citing successful response rates up to 85%.[35,51–55] However, long-term follow-up studies of children with splenectomy to assess failure/infection rates are lacking, and sepsis remains a significant risk. The recommendation from the American Society of Hematology group regarding consideration of splenectomy for children with chronic ITP include the following criteria: chronic thrombocytopenia >12 months duration, bleeding symptoms with severe thrombocytopenia, or thrombocytopenia that is unresponsive to "standard" therapy of corticosteroids, IVIG, and anti-D immune globulin.[35] Laparoscopic surgery is recommended for elective splenectomy with the patient immunized against *Neisseria meningitides*, *Streptococcus pneumoniae*, and *Haemophilus influenza* type B prior to surgery. Children must undergo lifelong monitoring to prevent infection and should receive daily prophylaxis against pneumococcal infection with antibiotics up to the age of 16 and in case of continued high risk also thereafter.[36]

Bleeding ITP patients who are refractory to steroids, IVIG, and anti-D immune globulin may be candidates for immunomodulatory therapy or thrombopoietin mimetics. Another second-line therapy in refractory patients with significant hemorrhagic problems may include rituximab. Standard doses of rituximab induce B-cell depletion for 3 to 6 months and is well tolerated in children, although the long-term effects of using this drug in children with immature immune systems is not known.[56] There are no randomized controlled studies of rituximab in children with ITP, but a review of cohort studies and case reports indicate that rituximab treatment results in a complete response (defined as platelet count $>50 \times 10^9$/L) in approximately 50% of the children with few adverse effects.[57] Prognostic indicators of response could not be identified.

INHERITED PLATELET DISORDERS

Hereditary disorders of platelet function are likely to be detected only when clinically relevant bleeding occurs. Many patients will have more than one hemostatic defect and will most likely already be undergoing evaluation for bleeding disorder so platelet dysfunction should be included as part of the investigative

Table 120.2

"Standard" therapies for children with chronic ITP

Type of Therapy	Dose	Side Effects
Corticosteroids	Various dose and regimens Short-course, high-dose corticosteroids without a taper[42,43] Suggested high-dose corticosteroid of ~4 mg/kg/d of prednisone or equivalent, orally or parenterally (for acute ITP) until cessation of bleeding or platelet count >20 × 10^9/L[42] Low-dose prednisone at 0.1–0.2 mg/kg/d orally[44]	High dose not recommended for long-term therapy due to adverse side effects (immunosuppression, hirsutism, weight gain, growth delay, osteoporosis, and cataracts)
IVIG	0.8–1.0 g/kg IVIG × 1–2 doses, slow infusion[42,45] Repeated doses can be given every 2–6 wk for symptomatic thrombocytopenia[44]	Higher doses are not recommended as it is associated with headaches[46] Infusion-related side effects include flushing, fever, headaches, allergic reactions, aseptic meningitis Minimal risk of pathogen transmission due to IVIG derived from pooled donor plasma
Anti-D immune globulin	75 μg/kg anti-D (used in children with ITP who are Rhesus+ with intact spleen)[47,48] Given as a short infusion, though s.c. route is well tolerated and effective[49]	Infusion-related headaches, chills, fatigue Mild anemia (Hb fall of 0.8–1.4 g/dL)[47] Severe hemolytic anemia with DIC and acute renal failure is rare (1 in 20,000–30,000 infusions)[50] Minimal risk of pathogen transmission due to anti-D derived from pooled donor plasma

DIC, disseminated intravascular coagulation; Hb, hemoglobin; ITP, immune thrombocytopenia purpura; IVIG, intravenous immunoglobulin; s.c., subcutaneous.

process. Typical bleeding manifestations indicative of platelet function defects can include inexplicable or extensive bruising, prolonged epistaxis (>30 minutes, causing anemia or hospital admission), menorrhagia, oral cavity bleeding, or bleeding after dental extraction or invasive procedures.[58] Severe platelet function disorder can lead to ICH and subdural hemorrhage or excessive bleeding in the umbilical stump in the neonatal period. Milder forms of platelet function disorders are more likely to present later in life. The lack of well-defined testing system for platelet function disorder makes diagnosis more complicated for the clinician.[59,60] There are guidelines and recommendations published by International Society on Thrombosis and Haemostasis (ISTH) subcommittees, Permanent Paediatric Group of the German Thrombosis and Haemostasis Research Society, and other scientific panels for the diagnosis of patients with inherited platelet disorders.[59,61,62]

Disorders of Platelet Numbers

Inherited thrombocytopenias are a group of heterogeneous conditions that consequently present as early-onset thrombocytopenia. Although rare, the clinical symptoms, including bleeding, can be severe.

MYH-9 Disorders

MYH-9–related thrombocytopenias are rare autosomal dominant disorders defined by the presence of deleterious mutations within the *MYH*-9 gene, coding for the nonmuscle myosin heavy chain IIa, that influences a component of the contractile cytoskeleton in megakaryocytes, platelets, and other tissues.[63–65] Defective megakaryopoiesis is responsible for the thrombocytopenia. This disorder encompasses other previously defined disorders including May-Hegglin anomaly, Sebastian syndrome, Fechtner syndrome, and Epstein syndrome.[63,64] Bleeding severity is usually mild, and life-threatening bleeds are rare. Other phenotypic manifestations can include hearing loss, glomerulonephritis, and cataract.[66,67] These patients usually have platelet counts in the range of 20 to 130 × 10^9/L, with most showing elevated mean platelet volume and a noticeable population of very large platelets with variable platelet morphology.[61,63]

A specific diagnostic strategy has been suggested by the Working Group on Standardisation in Perinatal and Pediatric Hemostasis for the group of children and neonates with inherited thrombcytopenias.[68] Differential diagnosis should include inspections of peripheral blood smear and platelet ultrastructural features as symptoms are similar to ITP or other causes of thrombocytopenia such as Bernard-Soulier syndrome.[69–71] Treatment options for inherited platelet function disorders include antifibrinolytic agents such as tranexamic acid to treat mucous membrane bleeding, desmopressin (DDAVP) for storage pool disorders or for mild bleeding events where tranexamic acid alone was unsuccessful, platelet transfusion for severe disorders but there is a risk of alloantibodies to HLA antigens, and recombinant FVIIa (rFVIIa) that has only been approved for use in patients with Glanzmann thrombasthenia.[68] It is stressed that treatment strategies should be discussed on a case-by-case basis, taking into account personal and family history, to determine the risk–benefit ratio for each patient.

Congenital Amegakaryocytic Thrombocytopenia

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare, recessive disorder caused by mutations in the MPL gene affecting the cytokine thrombopoietin receptor c-mpl that regulates megakaryopoiesis and platelet production.[72,73] CAMT usually presents in the neonatal period with bleeding symptoms as a result of severe thrombocytopenia associated with absent or markedly decreased megakaryocyte numbers in the bone marrow. Disease progression leads to hematopoietic failure and the development of severe aplastic anemia.[74] Physical abnormalities were also noted including cardiac defects, growth anomalies, and retardation of psychomotor development.[75] Molecular analysis of *MPL* gene will give definitive confirmation of CAMT. Management of CAMT initially includes platelet transfusion, but patients will eventually require hematopoietic stem cell transplantation due to the progression of the disease to severe aplasia.[58] Є-aminocaproic acid (EACA) used for bleeding episodes in adolescent and adult patients with amegakaryocytic thrombocytopenia decreases the need for platelet transfusions.[76]

Amegakaryocytic Thrombocytopenia with Radioulnar Synostosis

Amegakaryocytic thrombocytopenia with radioulnar synostosis seems to be dominantly inherited with mutations in the *HOXA11* gene,[77] which is thought to be involved in the regulation of megakaryocytic differentiation.[78] Platelet morphology is normal, but megakaryocytes are markedly decreased or absent in the bone marrow. This disease is similar to CAMT presenting with severe thrombocytopenia and bleeding symptoms early in the neonatal period but is associated with radioulnar synostosis, and some may present with other skeletal abnormalities.[79] Amegakaryocytic thrombocytopenia with radioulnar synostosis will lead to haematopoietic failure. Management of this disease includes platelet support, and some may need haematopoietic stem cell transplantation.[58]

Thrombocytopenia Absent Radius Syndrome

Thrombocytopenia absent radius (TAR) syndrome is characterized by severe isolated thrombocytopenia at birth that spontaneously improves after the 1st year of life. Physical abnormalities include the bilateral absence of radii with the retention of normal thumbs and may be associated with other defects including GI, renal, cardiac, and facial development.[80,81] Genetic basis of the disorder and mode of inheritance remain unclear. TAR syndrome is associated with megakaryocyte deficiencies in number and maturation.[58] Although there is progressive improvement in the severity of thrombocytopenia, the majority of morbidity occurs before 14 months of age according to a review of 100 cases.[81] Causes of death have been attributed to bleeding events resulting from thrombocytopenia (intracranial, visceral, and pulmonary hemorrhage), sepsis, and congestive heart failure (in a patient with teratology of Fallot).[81] Management of TAR syndrome includes platelet transfusions to prevent significant hemorrhage until platelet counts improve after infancy.[58]

X-Linked Thrombocytopenia with Dyserythropoiesis

This is an X-linked disorder as result of the mutations in the *GATA1* gene, which encodes for megakaryocyte and erythroid transcription factor affecting platelet and erythrocyte morphology.[82,83] Clinical presentations include abnormal hematopoiesis including severe macrothrombocytopenia with and without β-thalassemia and dyserythropoiesis with or without anemia.[82] Bleeding diathesis can occur at birth and continue onward. Confirmation of the diagnosis is by genetic finding of a mutation in the *GATA1* gene.[58] Patients with this syndrome may be treated with platelet transfusions for thrombocytopenia or DDAVP for mild-to-moderate bleeding.[84] Other forms of treatment include erythrocyte transfusions for anemia, parenteral antibiotics for confirmed neutropenia, and bone marrow transplantation may be considered as a cure for severe cases but it is associated with significant risks and morbidities.[84]

Disorders of Platelet Function

Wiskott-Aldrich Syndrome

Wiskott-Aldrich syndrome (WAS) is a rare X-linked recessive disease with multiple symptoms including thrombocytopenia with small platelets, recurrent otitis media, eczema, infections, and malignancies[85] with an incidence estimated to be 4 per million live births.[58] The genetic defect is in the *WASP* gene affecting signal transduction and maintenance of the cytoskeleton.[86,87] In the neonatal period, clinical presentation often involves bruising and purpura, GI bleeds, and prolonged bleeding after circumcision, and there is an increased risk of ICH. Eczema, usually occurring in the 1st year of life, is varied in its severity. Infection, usually in the first 6 months of life, can be bacterial such as otitis media and respiratory tract infection. Also, autoimmune disorders become more prevalent with increasing age, with hemolytic anemia and vasculitis being the most prominent.[85] Management of WAS should involve a multidisciplinary team to address bleeding manifestation, recurrent infections, eczema, autoimmune disease, and risk of malignancy. Stem cell transplantation should be considered if an HLA identical donor is available.

Glanzmann Thrombasthenia

Glanzmann thrombasthenia is a rare autosomal recessive disorder, which affects platelet aggregation resulting in a bleeding diathesis. The genes *ITGA2B* and *ITGB3*, encoding for platelet glycoproteins GPIIb and GPIIIa, respectively, are affected.[88,89] The most common presenting features include purpura, epistaxis, gingival bleeding, and menorrhagia, with the majority presenting before the age of 5 years.[90] Major bleeding, such as ICH, in the neonatal period is rare.[91] Bleeding severity is unpredictable, and spontaneous bleeding is uncommon as most episodes occur posttrauma or postsurgery.[90] Diagnostic criteria for Glanzmann thrombasthenia include prolonged bleeding time, normal platelet count, and morphology but absent or diminished clot retraction and platelet aggregation defects. Definitive diagnosis can be determined by the absence of GPIIb and GPIIIa on platelets using antibodies CD41 and CD61, respectively, using flow cytometry.[58] Glanzmann thrombasthenia can be managed by supportive care. Platelet transfusion can be used to treat bleeding episodes.[90] However, use of random donor platelets in Glanzmann thrombasthenia is associated with a very high incidence of platelet alloimmunization. This is a significant complication of treatment and makes it difficult to provide optimal platelet support for these patients. Use of EACA, tranexamic acid, and DDAVP[92] has also been reported to be successful in the management of bleeding in thrombasthenia

patients.[93] rFVIIa has been reported to be successful in preventing bleeds in patients with thrombasthenia undergoing invasive dental procedures.[94,95] Bone marrow transplants can cure patients with severe bleeding episodes,[96] but risk/benefit ratio should be considered for each patient.

Bernard-Soulier Syndrome

Bernard-Soulier syndrome is an autosomal recessive bleeding disorder, with an absence or decreased expression of GPIb/IX/V complex on the platelet surface resulting in platelet adhesion defects.[97] It is characterized by thrombocytopenia, large platelets, and prolonged bleeding time. It is distinguished from other syndromes by the failure of platelets to aggregate with ristocetin and is not corrected by the addition of normal plasma.[58] Although symptoms may present in childhood, some may be misdiagnosed or confused with ITP or NAIT, leading to inappropriate treatment.[98,99] The incidence has been estimated to be <1 in a million with common clinical symptoms of frequent epistaxis, gingival and cutaneous bleeding, and hemorrhage after trauma.[100] rFVIIa has also been reported for use in the management of Bernard-Soulier syndrome patients with severe bleeding episodes.[101]

VITAMIN K DEFICIENCY BLEEDING IN EARLY INFANCY

Vitamin K (VK) is an important factor for normal function of several proteins in the coagulation system. Research on developmental hemostasis has shown that neonates have almost half the normal adult values of plasma concentrations of VK-dependent coagulation factors.[102] These values gradually rise and reach adult levels by 6 months of age.[102,103] VK-dependent procoagulant proteins include prothrombin (FII), FVII, FIX, and FX. Protein C and protein S are also VK-dependent proteins that play a role in the negative feedback control of the hemostatic system. Taken together, failure to produce these functional hemostatic proteins will eventually lead to bleeding diathesis known as vitamin K deficiency bleeding (VKDB; previously known as hemorrhagic disease of the newborn). Thus, it has been standard practice in developing countries to give VK prophylaxis to newborns, especially those that will be exclusively breast-fed, as breast milk is low in VK.

Vitamin K Assessment

The ISTH Pediatric/Perinatal Subcommittee's definition of VKDB is "bleeding due to inadequate activity of VK-dependent coagulation factors (II, VII, IX, and X), correctable by VK replacement."[104] Early recognition of the signs of predisposing conditions such as prolonged jaundice and failure to thrive can reduce the incidence of intracranial VKDB.[104] The diagnostic criteria of VKDB in a bleeding infant include prolonged prothrombin time (PT) International Normalized Ratio >3.5, coupled with a normal fibrinogen level and platelet count. Diagnosis is confirmed if VK administration corrects PT and/or bleeding is stopped.[104]

When there is a deficiency of VK, FII, FVII, FIX, and FX are undercarboxylated and circulate as proteins induced in vitamin K absence (PIVKA) form. The PIVKA-II assay that detects undercarboxylated prothrombin has been used to identify subclinical VK deficiency in infants,[105,106] or enzyme immunoassays utilizing antibodies to PIVKA-II can also be used.[107,108]

Classification of VKDB

VKDB can be of three types, based on the time of presentation: early (first 24 hours), classical (days 1 to 7), and late (≥day 8, peaking at 3 to 8 weeks) (Table 120.3).[104,116] This helps to differentiate between idiopathic and secondary VKDB, where the latter has late onset and is associated with an undiagnosed disease such as biliary atresia, α_1-antitrypsin deficiency, cystic fibrosis, or drug effects on the mother or fetus.[116]

Early VKDB

Early VKDB presents within the first 24 hours after birth, and although rare, it is often life threatening. Common bleeding sites include scalp, intracranial, intrathoracic, intra-abdominal, or GI tract.[116] Its etiology may be due to maternal use of prescribed drugs, which may interfere with VK activity or metabolism.[110] The drugs include anticonvulsants (carbamazepine, phenytoin, and barbiturates, but not valproic acid), antibiotics (cephalosporins), tuberculostatic agents (rifampicin and isoniazid), and VK antagonists (phenprocoumon and warfarin).[104]

Classical VKDB

Clinical symptoms of classical VKDB present between days 1 and 7 (i.e., excluding the first 24 hours) with common bleeding sites in GI, umbilical, skin, nose, and circumcision sites. It is mainly idiopathic, but poor milk intake during breast-feeding may be a factor.[110,117,118] Prolonged bleeding after circumcision can reveal VK deficiency,[109] and although rare, ICH can lead to significant morbidity and death.

Late VKDB

Late VKDB occurs between day 8 and 6 months of age, incidence peaking at 3 to 8 weeks[104] often with ICH as the first presenting sign. In a pooled analysis of 131 published cases from 14 countries (1969 to 1992), 63% presented with ICH with a mortality rate of 14%.[119] Another study of 14 infants with late VKDB, in which 10 did not receive VK prophylaxis, indicated that the predominant clinical manifestation was ICH (78%), with a mortality rate of 57% and another 36% had neurological sequelae.[120] Late VKDB is mostly confined to exclusively breast-fed babies[119,121] and is more prevalent in boys than girls.[122] An underlying disease such as cholestasis or malabsorption problems is usually associated with infants with late VKDB, to whom VK prophylaxis has been given. Clinical manifestations may emerge earlier with mild bruises, nose bleeds, or umbilical oozing that can be "warning bleeds" of VKDB and may escalate to ICH.[104]

Incidence of VKDB

The incidence of VKDB is difficult to estimate as there is very little data available in industrialized countries and even more difficult to assess in underdeveloped countries. The incidence rate is also affected by the type of study whereby hospital-based surveys may have missed infants with mild bleeding symptoms that were not referred to the hospital or did not make it altogether which would result in a lower incidence report (Table 120.3). With the adaptation of prophylaxis with VK, the incidence of VKDB has decreased dramatically.[110]

Table 120.3

Characteristics of VK-deficient bleeding

	VK-Deficient Bleeding		
	Early	**Classical**	**Late**
Time of Presentation	**First 24 h**	**Days 1–7 (Excluding the First 24 h)**	**≥Day 8 up to 6 mo (peaking at 3–8 wk)**
Common bleeding sites	Scalp subperiosteal or skin Intracranial Intrathoracic Intra-abdominal GI tract	GI tract Umbilical Skin Nose Circumcision sites	Intracranial Skin GI tract
Etiological factors	Maternal drugs	Mainly idiopathic: breast-feeding (low milk intake)	Idiopathic or secondary: breast-feeding, undiagnosed cholestasis. Secondary cases from VK malabsorption (liver or bowel disease)
Incidence (publication date)		Cincinnati, USA (1967): 1.7% among breast-fed infant with no VK prophylaxis[109] British Isles (1991): 5.4 per 100,000 births[111] Ethiopia (1993): 0.9% for early and classic VKDB (80% had classic syndrome)[113] Malaysia (1994): 25 per 100,000 births (hospital-based study)[115] Thailand (1998): 89 per 100,000 births and 54 per 100,000 births (two longitudinal field studies)[114]	British Isles, Sweden, Switzerland, Germany (1980s): 4–7 per 100,000[110] Thailand (1988): 35 per 100,000 births (hospital-based study)[112] Thailand (1998): 72 per 100,000 births (regional field study)[114] Malaysia (1994): 13 per 100,000 births (hospital-based study)[115]

GI, gastrointestinal; VK, vitamin K; VKDB, vitamin K deficiency bleeding.

Prophylaxis for Neonates at High Risk for VKDB

The ISTH Pediatric/Perinatal Subcommittee published recommendations on VK prophylaxis, suggesting that for early VKDB, cessation or replacement of maternal medication causing VK deficiency be implemented if possible, or to initiate VK prophylaxis to the mother during pregnancy.[104] Infants born to mothers taking VK-interfering medication or those who will be exclusively breast-fed should receive a single dose of 1 mg VK intramuscularly very shortly after birth.[123] Oral or intramuscular VK (one dose of 1 mg) is suggested for classical VKDB, but intramuscular administration may provide better protection against late VKDB compared to a single oral dose.[104] Oral administration of VK prophylaxis should be followed by either weekly administration of 1 mg till 12 weeks or repeating 2 mg at weeks 1 and 4.[123] Pichler and Pichler[103] have published a mini-review outlining regimens of VK prophylaxis in different countries. Small preterm and sick full-term babies should be given 0.3 mg/kg VK (maximum 1 mg) immediately after birth via intramuscular or intravenous injection. The daily VK supply should be 5 to 10 mg/kg, and all babies should be given 2 mg VK orally at 4 weeks (or at discharge from the hospital, if earlier).[103]

Treatment for VKDB

In infants with suspected VKDB, VK should be given via intravenous or subcutaneous administration, while awaiting test results since VKDB is associated with a high risk of ICH. Intramuscular injection should be avoided due to the risk of severe intramuscular bleeding.[104]

Infants with non–life-threatening bleeds should be treated with vitamin K_1 given subcutaneously or slowly by intravenous route. A single IV dose of 250 to 300 μg/kg body weight is recommended. For infants up to 6 months of age, the dose range of 1 to 2 mg is sufficient to correct VK deficiency.[110] For severe bleeding in infants, fresh-frozen plasma (FFP) may be given at a dose of 10 to 15 mL/kg body weight.[110] For life-threatening bleeds, prothrombin complex concentrate (PCC) containing all four VK-dependent factors may be a better option with the added benefit of decreased volume load. There are no published guidelines for the use of PCC in neonates with VKDB, but extrapolation from adult data suggests a dose of 50 units/kg may

be sufficient.[110] In two reports of infants presenting with severe ICH due to VKDB, treatment with rFVIIa allowed for surgical intervention with little blood loss, but this therapeutic strategy cannot be assessed for its full benefits or risks based on so few cases.[124,125]

INHERITED COAGULOPATHIES

Hemophilia

The most common severe, inherited coagulopathy is hemophilia, with an incidence ratio between hemophilia A (deficiency of factor VIII) and hemophilia B (deficiency of factor IX) of 5:1 and in the population of 1 in 10,000 individuals but almost exclusively affecting males due to the X-linked inheritance. *De novo* mutations are the basis for a substantial minority of the cases, resulting in unexpected bleeding in the child. The first manifestation can be as ICH after a complicated vaginal delivery, as a muscle hematoma after injection of VK, as hemarthrosis when the child starts walking—and falling—or, for milder forms of hemophilia, only after surgical procedures or major trauma. Chapter 51 is devoted to the description of hemophilia.

von Willebrand Disease

In the severe form (type 3), von Willebrand disease (vWD) results in not only a severe lack of von Willebrand factor (vWF) but also of factor VIII. The manifestations can therefore be identical to hemophilia, whereas in milder forms with mainly a reduced level without or with a qualitative defect of the vWF (type 1 and type 2, respectively) but with normal factor VIII, there is a preponderance of mucocutaneous bleeding. The inheritance is autosomal, thus affecting both sexes. Neonatal bleeding occurs mainly in children with vWD type 3, which has a prevalence of 0.1 to 3 per million, depending on the geographical region. The type 1 and type 2 vWD forms are much more common. Chapter 52 provides a thorough description of vWD.

RARE COAGULOPATHIES

Inherited deficiency of factors V, VII, X, XI, XIII, prothrombin, and fibrinogen can, when they occur in the severe form, cause neonatal bleeding. This can present as ICH, as prolonged bleeding from the umbilicus, or after circumcision in the neonatal period. With the development of the Rare Bleeding Disorder Registries,[126] a better understanding of these rare conditions should be facilitated. The deficiencies are extensively described in Chapter 53 (*Rare Inherited Bleeding Disorders*) and in Chapter 54 (*Inherited Fibrinogen Disorders*).

INHERITED DISORDERS OF FIBRINOGEN

Inherited disorders of fibrinogen, although rare, could manifest quantitatively in the form of afibrinogenemia and hypofibrinogenemia (defect in synthesis or processing) or qualitatively in the form of dysfibrinogenemia (abnormal polymerization, cross-linking, or assembly in the fibrinolytic system). Clinical symptoms vary from asymptomatic to life-threatening bleeding or thromboembolic events.

Afibrinogenemia

The prevalence of afibrinogenemia is approximately 1 in 1,000,000, but the incidence is higher in countries where consanguineous unions are more common.[127,128] Fibrinogen levels are <0.1 g/L in plasma, and laboratory testing will show that PT, activated partial thromboplastin time (aPTT), and thrombin time (TT) are indefinitely prolonged and both functional (Clauss), and antigenic assays are unable to detect fibrinogen.[127] Clinical manifestation can present in early infancy with umbilical bleeds[129,130] or ICH.[131] Prophylaxis with cryoprecipitate or with 1 g of fibrinogen concentrate every 3 days to maintain trough levels of 1 g/L seems effective against recurrent bleeding.[131] Although extremely rare, afibrinogenemia has been associated with thrombotic events considered to be caused by thrombin-induced platelet aggregation due to the absence of the thrombin-neutralizing property of fibrin.[130]

Hypofibrinogenemia

This is a quantitative deficiency of fibrinogen with milder bleeding patterns, mainly after surgical procedures or major trauma. Fibrinogen levels are 0.1 to 2 g/L, aPTT is normal, and TT is mildly prolonged.[127] The incidence is lower than that of afibrinogenemia.

Dysfibrinogenemia

Dysfibrinogenemia is a mutation that affects the functional properties of fibrinogen. It is commonly diagnosed in adulthood (see Chapter 54).[127,132,133]

FXIII Deficiency

Congenital FXIII deficiency has an estimated incidence of 1 case per 2 to 5 million,[134,135] and generally higher in countries where consanguineous marriages are common. It is an autosomal recessive disease[135] that can present with umbilical bleeding in the neonatal period, soft tissue bruising, mucosal bleeding, and ICH. It is estimated that up to 30% of patients with this disorder have spontaneous ICH.[136] The first report of FXIII deficiency was in 1960 from Duckert et al.[137] who described a case of a 7-year-old Swiss boy with a history of bleeding. Bleeding episodes were delayed occurring 24 to 36 hours after injury and impaired wound healing. It was reported that the patient and his siblings had a similar bleeding tendency and his parents had a consanguineous marriage.[137] An observational case series of 10 children with FXIII deficiency in Pakistan (from 1996 to 2006) indicated that 9/10 had a history of prolonged umbilical bleeding, bruises and prolonged bleeding after trauma, and a positive family history of bleeding, and consanguineous marriage was present in 80% of the families.[138]

Standard laboratory testing of PT, aPTT, fibrinogen level, platelet count, and bleeding time, are all within normal parameters in FXIII-deficient patients[139] due to FXIII being involved in the final stages of coagulation. Quantitative FXIII activity and antigen assays are recommended over clot solubility testing, where the latter gives semiquantitative FXIII determination.[136] Interpretation of very low FXIII activity values, as measured by quantitative assays, are difficult and could complicate the start of prophylactic treatment.

A retrospective study of seven FXIII-deficient patients given purified, pasteurized concentrate of FXIII called Fibrogammin P (CSL Behring GmbH, Marburg, Germany) as prophylaxis showed a decreased incidence of bleeding episodes from 2.5 events per year to 0.2 events per year, with no reports of severe bleeds on prophylaxis.[140] Due to the long half-life of FXIII concentrate (7 to 12 days), administration for prophylactic treatment is scheduled once every 3 to 4 weeks with 10 to 30 units/kg given to prevent bleeding episodes. Fibrogammin P is derived from pooled human plasma screened for common viruses and has undergone extensive viral inactivation to minimize the chance of viral disease transmission.[140]

Additional commercially available products for treatment of FXIII deficiency are FXIII-BLP (Bio-Product Laboratory, Elstree, United Kingdom) and rFXIII-A2 (Novo Nordisk, Bagsvaerd, Denmark).[141] Other prophylactic treatment options include FFP (10 mL/kg every 4 to 6 weeks) and cryoprecipitate (1 bag per 10 to 20 kg every 3 to 4 weeks).[139,141] The prognosis is excellent for FXIII-deficient patients, despite the lifelong risk of bleeding.

FIBRINOLYTIC DISORDERS

α_2-Antiplasmin Deficiency

The deficiency of α_2-antiplasmin (α_2-AP) can be due to alterations in protein synthesis (low or no production of protein) or the synthesis of an abnormal protein (nonfunctional protein). Clinical manifestations of congenital α_2-AP deficiency include umbilical bleeding, central nervous system hemorrhage, epistaxis, hemarthrosis, and excessive blood loss during surgery or delayed bleeding after minor trauma.

There have been several publications of α_2-AP deficiency in children.[142–146] Many of the affected patients had shortened whole-blood clot lysis time (3 to 4 hours; normal >24 hour) and shortened euglobulin lysis time (~1.5 hours; normal >2 hours), but routine coagulation screening was normal (bleeding time, platelet count, PT, aPTT, fibrinogen, and FXIII levels).

Treatment of bleeding symptoms is usually with antiplasminic/antifibrinolytic agents such as tranexamic acid or EACA, as described in several case reports of patients with hetero- or homozygous deficiency of α_2-AP. Intraoral bleeding was treated with oral administration of 25 to 50 mg/kg per day of tranexamic acid and for tooth extractions 7.5 to 10 mg/kg was given every 6 hours for up to 7 to 10 days without any postoperative bleeding.[142,144,146]

Plasminogen Activator Inhibitor-1 Deficiency

Reports in adults have linked lifelong history of severe bleeding after surgery or trauma with defective functioning of plasminogen activator inhibitor-1 (PAI-1)[147,148] or undetectable PAI-1 antigen and activity.[149] Hemostatic screening is normal, but euglobulin lysis time is shortened. Also, the currently available diagnostic assays make it difficult to assess PAI-1 deficiency since accuracy in the lowest range of PAI-1 activity assays is lacking.[150–152] It is suggested that PAI-1 antigen and activity levels be assessed as well as taking a history to identify the presence of delayed posttraumatic or postsurgical bleeding before considering a diagnosis of PAI-1 deficiency.

Only a few cases have been reported, and the true incidence of PAI-1 deficiency is unknown. There is usually a history of bleeding episodes in early childhood. Bleeding symptoms are usually mild to moderate including epistaxis, easy bruising, and hematomas. Symptoms can be more significant such as ICH or menorrhagia in young women. Genetic analysis identified 7 individuals from 2 families that were homozygous for PAI-1 deficiency; all had bleeding tendencies after trauma or surgery, whereas, 19 other heterozygous carriers had no abnormal bleeding even after trauma or surgery.[153] Fay et al.[154] described a 9-year-old female child with a molecular defect that resulted in a complete loss of PAI-1 expression in both plasma and platelets. After genetic analysis, it was determined that PAI-1 deficiency was a heritable, autosomal recessive bleeding disorder. All bleeding episodes occurred after major hemostatic challenges such as trauma or surgery. A follow-up of this patient after 6 years showed significant improvement in her condition after management with tranexamic acid, taken orally for 3 to 5 days after a bleeding episode or prior to planned surgical procedures.[153] Menorrhagia was managed with low doses of tranexamic acid (25 mg/kg) one to two times daily, increasing to three times daily for the first few days of menstruation.[153]

A report of the youngest documented person with PAI-1 deficiency was that of a 4-month-old child who is homozygous for the mutant allele. A minor head bump on a table led to persistent head swelling and a large epidural hematoma that needed surgical drainage. Prior to surgery, 10 mg/kg of EACA was administered and no significant postsurgical bleeding was observed.[153] Other case reports confirm the efficacy of tranexamic acid for various bleeding manifestations, including hemarthrosis.[155,156]

SUMMARY

Bleeding disorders in children are complicated due to their developing hemostatic system that sets them apart from adults. There are many disease processes that are associated with bleeding diathesis and can lead to devastating consequences. Diagnosis and management of these disorders are challenging in the pediatric population, but research is ongoing and more pediatric patients are being successfully managed.

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Full Reference Lists can be found online

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CHAPTER

121 Venous and Arterial Thromboembolism in Children

PAUL MONAGLE, M. PATRICIA MASSICOTTE, AND ANTHONY K. CHAN

INTRODUCTION

Thromboembolic disease in infancy and childhood has been described as a new epidemic of tertiary pediatric care. Evidence supports the concept that the frequency of thromboembolism (TE), in all forms including venous, arterial, and intracardiac thromboembolic phenomenon, is increasing in neonates and children. The reasons for this are likely related to the increased survival of children with severe underlying medical and surgical conditions; the increased intensity and invasiveness of supportive care, in particular the increased use of vascular access devices; heightened awareness of this condition amongst pediatricians leading to more regular and appropriate investigations; and potentially the ever lowering age at which traditionally "adult" therapies such as, for example, the oral contraceptive pill are being used en masse. Similarly, the increased prevalence of previously "adult" risk factors such as obesity may also contribute to increased thrombotic risk in children.

The diagnosis of thrombosis in children is often difficult for practical and theoretical reasons. Invasive and even noninvasive imaging of children often requires general anesthesia which not only increases the risks involved, especially in sick children, but pragmatically often makes timely diagnosis difficult to organize. Further the algorithms for interpretation of diagnostic vascular imaging have not been validated in children.[1] There are multiple reasons including reduced vessel size, lower pulse pressures, and blood flow rates, and potential other causes of bias that raise questions about whether the same algorithms and strategies used in adults are valid in children.

The natural history of TE in neonates and children remains largely unknown. This is problematic when clinicians are trying to balance the risks of antithrombotic therapy versus the risks of untreated disease. This dilemma is most evident when considering asymptomatic TE, which is often diagnosed on routine imaging or postprocedural examination. The aim of pediatrics in general is not only to ensure long-term survival of children, but to do so in a manner that enables the child to lead a productive and full adult life. Therefore, any impact of antithrombotic therapy on normal development needs to be considered, especially in situations that long-term anticoagulation therapy is being considered. Currently, the ability of clinicians to give parents and children good information about their longer term health and support requirements subsequent to a thromboembolic episode is very limited.

The use of antithrombotic drugs in pediatric patients is different from adults for a variety of reasons.[2] First, the epidemiology of TE in pediatric patients is vastly different from that seen in adults.[3–14,15] Second, the hemostatic system is a dynamic evolving entity that not only likely affects the frequency and natural history of TEs in children, but also the response to therapeutic agents.[16–18] Third, the distribution, binding, and clearance of antithrombotic drugs are age dependent.[19–21] Fourth, the frequency and type of intercurrent illnesses and concurrent medications varies with age. Fifth, limited vascular access reduces the ability to effectively deliver some antithrombotic therapies and can influence the choice of antithrombotic agent. Often, the only vascular access available is used for drug delivery; consequently accurate monitoring of blood anticoagulant levels is difficult. Sixth, specific pediatric formulations of antithrombotic drugs are not available, making accurate, reproducible dosing difficult. This is especially the case for vitamin K antagonists (VKAs) (no suspension/liquid preparation) and low molecular weight heparin (LMWH) (available most readily in predosed syringes based on adult weights). Seventh, dietary differences make the use of oral VKAs particularly difficult. This is especially true in neonates, as breast milk and infant formulas have very different vitamin K levels. Finally, compliance issues are vastly different in, for example, small infants who cannot understand the need for therapy, in adolescents who intellectually comprehend but emotionally are unable to cooperate, and in children who suffer the effects of inadequate parenting. The social, ethical, and legal implications of these issues frequently interfere with the ability to provide the "best" treatment for individual neonates and children.

This chapter discusses the epidemiology of TE in neonates and children, and then focuses on issues of clinical presentation, diagnosis, and management that are specific for children compared to adults. There remain many unanswered questions, and much research is required to improve the current standards of care for children with thromboembolic disease.

EPIDEMIOLOGY OF THROMBOEMBOLISM IN NEONATES AND CHILDREN

Venous Thromboembolism and Pulmonary Embolism

There are a number of published national or international registries that have collected data on venous thromboembolism (VTE) and other thrombotic events in neonates and children (Table 121.1).[4,11–13,22] Two neonatal registries from Canada and Germany have prospectively collected data on neonatal thrombosis, while a third from the Netherlands includes data from both neonates and older children.[11–13] Two registries (from Canada and the United Kingdom) have collected data on older children.[4,22] Inclusion criteria have varied between registries, particularly with regard to the registration of arterial and central nervous system (CNS) events, and the inclusion or not of asymptomatic events.

Table 121.1

National and international large pediatric thrombosis registries

Registry	Dates of Case Collection	Age Criteria for Inclusion	Number of Children Reported	Types of Thrombosis Included	Inclusion of CNS Thrombosis	Incidence of Venous Thrombosis
Canadian & International-Neonatal[12]	1990–1993	1st mo	*N* = 97	VTE Arterial	No	2.4/1,000 NICU[b] admissions
German-Neonatal[11]	1992–1994	1st mo	*N* = 79	VTE Arterial	Yes	0.51/10,000 births
Canadian-Children[22]	1990–1992	1 mo to 16 y	*N* = 137	DVT/PE	No	0.07/10,000 children
DPSU[13]	1997–1999	Birth to 18 y	*N* = 115	VTE[a]	Yes	0.14/10,000 children
BPSU[4]	2001–2003	1 mo to 16 y	*N* = 200	VTE Arterial	No	0.07/10,000 children
United States[15]	2001–2007	Birth to 18 y	*N* = 11 337	VTE	No	34–58/10,000 pediatric hospital admissions

[a]Includes asymptomatic neonatal events.
[b]Neonatal intensive care unit.

The incidence of VTE is dramatically influenced by age in adults such that the estimated incidence of first time deep vein thrombosis (DVT) in those aged 25 to 30 years is around 30 cases per 100,000 persons, which increases exponentially to 300 to 500 cases per 100,000 persons in those aged 70 to 79 years.[14] The incidence of DVT and pulmonary embolism (PE) in children in Canada, using data collected from 15 tertiary care centers, was estimated to be 0.07 per 10,000 children and 5.3 per 10,000 hospital admissions.[22] However, more recently, Raffini et al.[15] reported that the annual incidence of VTE in children discharged from pediatric hospitals in the United States increased from 34 per 10,000 admissions in 2001 to 58 per 10,000 admissions in 2007, representing an increase of 70% in 7 years. The Dutch registry collected data via the Dutch Paediatric Surveillance Unit (DPSU) using a monthly mailing system sent to all pediatricians in primary and secondary care and to specific contact persons in tertiary centres.[13] The overall incidence of venous thrombosis was 0.14 per 10,000 children aged 0 to 18 years and 0.05 per 10,000 children after excluding neonates and nonextremity VTE.[13] The British Paediatric Surveillance unit (BPSU) used a similar methodology,[4] and the estimated incidence of all events was 0.07 per 10,000 children. The first registry of neonatal thrombosis was an international registry involving 64 centers in Canada, the United States, and Europe. Based on data reported from Canada, which was considered to be complete, the incidence of clinically apparent thrombosis was 2.4 per 1,000 admissions to the neonatal intensive care unit (ICU).[12] In contrast, the German neonatal registry reported the incidence of symptomatic events was 0.51 per 10,000 births.[11] The Dutch study reported the incidence of neonatal venous thrombosis as 0.07 per 10,000 children.[13]

Many other studies have examined the rates of VTE in specific high-risk groups such as children and neonates with central venous access.[23,24,25] Children with malignancy, with or without central venous access, are often highlighted as a particular high-risk group, with rates as high as 30% to 50%, although there are many causative factors involved, including insertion technique, site of central line placement type of cancer involved, treatment protocol, and the diagnostic techniques applied.[7,10,26–28] Children with short-term central venous access whilst in pediatric intensive care units have thrombosis in 18.3% to 26% of cases.[29,30] Children undergoing solid organ transplant have frequent thromboembolic complications,[31–35] but perhaps the highest risk group in children is in those receiving long-term home parenteral nutrition, with cross-sectional studies reporting thrombosis in up to 66%.[36,37,38] With respect to PE, there is limited data on epidemiology. General population retrospective autopsy studies report PE in children at 0.05% to 4%[39–42] (Table 121.2).

Arterial Thrombosis

There are few studies that report the incidence of arterial thrombosis in children. The majority of arterial thrombosis in children are iatrogenic, related to invasive vascular access,[43–59,60,61–63] less commonly associated with solid organ transplantation and Kawasaki disease. Spontaneous arterial thrombosis is rare in children.[64–76] One prospective registry conducted between 1999 and 2002 reported arterial thrombosis at 8.5 per 10,000 hospital admissions (102 thromboses in 119,487 admissions),[77] suggesting that arterial thrombosis is as common as venous thrombosis in tertiary institutions.

Intracardiac Thrombosis

Right atrial and intracardiac thrombosis are most commonly diagnosed in children who have central venous access devices extending into the right atrium.[78,79] Children also may develop intracardiac thrombosis following cardiac surgery, including

Table 121.2

Incidence of pulmonary embolus in children with selected underlying clinical conditions

Author	Year	Study Design	N	Clinical Condition	Diagnostic Method	Incidence (%)
Hoyer[343]	1986	Retrospective	26	Asymptomatic NS	V/Q	27
Desai[344]	1989	Retrospective	178	Fatal burns	Autopsy	1.7
Hsu[345]	1991	Cross-sectional	62	Preheart transplantation	V/Q, PA	9.7
Marraro[346]	1991	Prospective	205	Leukemia	Perfusion scan, PA	3.4
Uderzo[347]	1993	Retrospective	67	BMT for leukemia	V/Q, PA	4.5
Uderzo[348]	1993	Prospective	452	Leukemia and respiratory failure	Perfusion scan, PA	2.7
McBride[323]	1994	Retrospective	28,692	Trauma	Clinical	0.0069
Dollery[37]	1994	Cross-sectional	34	Long-term total parenteral nutrition (TPN)	V/Q, CRX	32
Andrew[22]	1994	Prospective	137	Venous thrombotic event	V/Q	16
Nuss[349]	1995	Retrospective	61	Thrombotic event	V/Q	20
Derish[350]	1995	Retrospective	21	Intensive care unit deaths	Autopsy	24
Massicotte[351]	1998	Prospective	244	Catheter-related thrombosis	V/Q	16
Huang[352]	2000	Prospective	20	NS and elevated D-dimer	V/Q, CRX	40
Monagle[8]	2000	Prospective	405	Venous thrombosis	V/Q	17
Van Ommen[13]	2001	Prospective	100	Venous thrombosis	V/Q	10
Levy[353]	2003	Retrospective	24	SLE with positive LAC	V/Q	8.3

Adapted from Van Ommen CH, Peters M. Acute pulmonary embolism in childhood. *Thromb Res* 2006;118(1):13–25.

after Glenn,[80–85] Fontan,[86,87] Norwood procedures,[88,89] and prosthetic valve replacement.[90–106]

These complex surgical procedures are common in children with congenital heart disease, and the underlying surgical procedures may have specific implications for the potential consequences of intracardiac thrombosis.[107] A few specific examples are described below.

Blalock-Taussig (B-T) shunts are commonly performed in the neonatal period to increase pulmonary blood flow. B-T shunts may be performed as a single palliative procedure with more definitive surgery planned later or as part of a more complex surgical intervention (e.g., Norwood procedure). B-T shunts are performed as either classic or modified B-T shunts. In classic B-T shunts, the subclavian artery is anastomosed directly to the ipsilateral pulmonary artery. The shunt is usually performed on the right side unless there is a right-sided aortic arch, in which case, the shunt is performed on the left. In contrast, modified B-T shunts involve a Gore-Tex tube graft being placed between the subclavian artery and the ipsilateral pulmonary artery. The Gore-Tex tube may be as small as 3 mm in diameter, depending on the size of the infant, but is usually 3.5 or 4 mm in diameter. Blockage of the B-T shunt will compromise pulmonary blood flow and is often a major clinical event requiring immediate surgery. Patients with B-T shunts may have reduced peripheral pulses in the ipsilateral arm and may have measurably reduced growth of the arm. This can be of significance when assessing for postthrombotic syndrome, which may occur in the upper limbs secondary to central venous thrombosis.

The Norwood procedure is the initial surgery performed in infants with hypoplastic left heart syndrome (HLHS). The surgery is performed early in the neonatal period. HLHS usually includes severe hypoplasia of the left ventricle, hypoplasia of the ascending aorta and aortic arch, and critical stenosis of aortic or mitral valves.

The first stage involves the following:

1. Division of the main pulmonary artery and closure of the distal stump with a patch.
2. Ligation and division of the patent ductus arteriosus.
3. Using an aortic or pulmonary artery allograft, the proximal pulmonary artery is anastomosed to the hypoplastic aortic arch, which itself is enlarged to create a large arterial trunk.

4. The atrial septum is excised to allow adequate interatrial mixing.
5. A modified B-T shunt (right-sided, Gore-Tex tube, usually 3.5 to 4 mm) from the right innominate to the right pulmonary artery. This will be the only source of pulmonary blood flow.

This procedure results in systemic venous and pulmonary venous return entering into a common atrium. Blood then enters a common ventricle and leaves the heart via a common outflow tract, providing systemic blood flow via the aortic arch and pulmonary blood flow via the B-T shunt.

The implications of this procedure with respect to thrombosis are as follows:

1. Venous flow has direct access to systemic arterial flow, so deep venous thrombosis, whether central venous line (CVL) related or otherwise, readily causes systemic emboli and potentially arterial ischemic stroke.
2. A clotted B-T shunt causes loss of entire pulmonary blood flow.
3. There are substantial intravascular suture lines as potential sources of thrombosis.
4. The patients will need a subsequent Glenn procedure and eventually completion to a full Fontan procedure. The development of central thrombosis with associated collateral branches can significantly hinder these subsequent surgical interventions. Therefore, increased aggressiveness of any antithrombotic therapy may be appropriate. Even in the absence of symptoms, thrombolysis or surgical thrombectomy may be considered if a central venous thrombosis was thought to be impairing the hemodynamics required for future surgery.

The bidirectional cavopulmonary shunt (BCPS) or bidirectional Glenn procedure is an end (superior vena cava [SVC]) to side (right pulmonary artery) venous shunt. This is a palliative procedure that increases oxygen saturation, but without increasing left ventricular work, and is usually performed in children with univentricular cardiac physiology as the first stage of the two-stage Fontan procedure. The inferior vena cava (IVC) is unchanged after a Glenn procedure.

The implications with respect to thrombosis are that, following a Glenn procedure, the SVC blood flows directly to the lungs without any assistance from the heart. Hence, any reduction in SVC flow, owing to thrombus occlusion, will dramatically reduce pulmonary blood flow. Pulmonary emboli are of specific concern in that if the pulmonary vascular resistance is increased, the patient may become unsuitable for a Fontan completion, which limits long-term survival significantly in the absence of cardiac transplantation. Blood flow from the IVC still bypasses the lungs, meaning that any thrombus in the lower venous system can give rise to paradoxical emboli. Collateral branches around central venous thrombosis may interfere with the ability to complete the Fontan surgery. The threat of being unable to complete the Fontan may be justification for more aggressive antithrombotic therapy (thrombolysis or surgery) than might otherwise be indicated.

There are numerous modifications of the final Fontan procedure, but the basic principles remain the same. Both the SVC (usually done in a first-stage BCPS) and the IVC are anastomosed to the pulmonary arteries. Hence, the pulmonary blood flow is totally passive, depending on venous flow mechanisms without any cardiac pump assistance. The univentricle is then able to function as a left ventricle, providing systemic blood flow. Postoperatively, many patients have deliberate, limited, right to left interatrial shunting via a "fenestration." The blood flow in such cases increases the risk of paradoxical emboli.

The implications with respect to thrombosis following Fontan surgery are significant. First, the presence of right to left communication continues the potential for paradoxical emboli. Second, any pulmonary emboli that increase pulmonary vascular resistance have an accentuated impact on pulmonary blood flow because the pulmonary pressure cannot be increased to overcome a pressure gradient. The Fontan circuit makes the diagnosis of pulmonary emboli particularly difficult. Depending on the attachment of the SVC and IVC to the pulmonary arteries, isotope may need to be injected into both an arm and a leg to achieve full lung perfusion scanning. As for patients with severe pulmonary hypertension having ventilation–perfusion scans, the transient obstruction of pulmonary capillaries by the macroaggregated isotope-labeled albumin may rarely precipitate a hypoxic episode. Finally, depending on the exact type of Fontan surgery, the risk of late (many years later) thrombosis associated with dysrhythmias may be increased.

CLINICAL PRESENTATION OF THROMBOSIS IN NEONATES AND CHILDREN

Venous Thrombosis

The clinical symptoms/complications of VTE can be classified as acute or long term and vary depending on site and severity. A high proportion of venous thrombosis in children occur in the upper venous system (defined as subclavian, brachiocephalic, internal jugular veins, and SVC), as distinct from the lower venous system (defined as the popliteal, femoral, iliac veins, and the IVC), which is more commonly affected in adults with VTE. These upper system VTEs are commonly associated with CVL placement. The acute clinical symptoms, besides loss of CVL patency, include swelling, pain, and limb discolouration,[22] swelling of the face and head with SVC syndrome,[108] and respiratory compromise with PE.[8] Renal vein thrombosis (RVT) classically presents with a flank mass, hematuria, proteinuria, thrombocytopenia, and nonfunction of the involved kidney. Any venous thrombosis may present with a paradoxical embolus causing a stroke, notably in neonates with a patent foramen ovale.

Children often do not present with acute symptoms (FIGURES 121.1 and 121.2). Long-term clinical symptoms and/or complications include prominent cutaneous collateral circulation, loss of CVL patency requiring local thrombolytic treatment, CVL replacement, loss of venous access, CVL-related sepsis, chylothorax,[109,110] chylopericardium,[111] recurrent VTE necessitating long-term anticoagulation, and postthrombotic syndrome (PTS).[112] Specific long-term sequelae of umbilical vein catheter (UVC)-related VTE include portal hypertension,[113] splenomegaly,[114] gastric and esophageal varices, variceal bleeding, and hypertension.[115]

Arterial Thrombosis

Arterial thrombosis is most frequently iatrogenic, and loss of vascular access is a common presenting symptom.[77] Acute symptoms will depend on the site of arterial thrombosis and whether there is adequate collateral flow to prevent tissue ischemia. Peripheral limb ischemia is characterized by diminished

FIGURE 121.1 This boy presented at 10 years of age with prominent abdominal collaterals. He had gastroschisis repaired during the neonatal period and had a lower system venous access device placed during this period. There were no acute symptoms of thrombosis documented; however on current scans he has chronic thrombosis with no flow to the IVC below the renal veins.

or absent pulses, a prolonged capillary refill time, and a cool, pale limb. Thrombotic occlusion of the radial artery produces more severe symptoms with accompanying ulnar artery occlusion. Chronic symptoms may develop even in the absence of acute symptoms and include limb hypoplasia and claudication (FIGURE 121.3). The clinical presentation of umbilical artery catheter (UAC)-related thromboembolic events varies, the majority of newborns being asymptomatic or only minimally affected, but a small proportion having major symptoms of severe leg ischemia and organ dysfunction, including necrotizing enterocolitis secondary to mesenteric artery occlusion, hypertension with or without renal failure secondary to renal artery thrombosis, and limb compromise secondary to embolic events.

DIAGNOSIS OF THROMBOEMBOLISM IN NEONATES AND CHILDREN

Venous Thromboembolism

There are no studies comparing the validity of the commonly used diagnostic tests for DVT in neonates, and little is known about the precision and accuracy of noninvasive imaging techniques. The low pulse pressure in premature newborns likely makes ultrasound (US) difficult to interpret, and the presence of a CVL makes compressibility difficult to assess, reducing sensitivity of the test. In neonates with UVCs, Doppler US is inferior to contrast venography for detecting asymptomatic thrombi.[116]

By contrast, US is the gold standard for diagnosing RVT, because of its sensitivity to an enlarged kidney, which is echogenic with prominent echo-poor medullary pyramids. Renal swelling subsequently decreases, with loss of corticomedullary differentiation, and ultimately, depending on the degree of recovery, US may demonstrate focal scarring or atrophy. Magnetic resonance imaging (MRI) and computed tomography (CT) have also been used to diagnose RVT, but have no apparent advantages over color Doppler, which may show absent renal venous flow in the early stages of RVT. In neonates with suspected VTE, venography is the gold standard, but clinicians must use clinical assessment and suboptimal imaging to make decisions, because the gold standard is practically unachievable.

In older children, US is poorly sensitive (20%) for upper system CVL-related thrombosis, compared to venography. In a comparison of venography versus US for the diagnosis of asymptomatic upper venous system CVL-related VTE, US had a sensitivity of only 20% for intrathoracic thrombosis, but detected jugular vein thrombi that were missed by venography.[24] Shankar et al studied 25 children with multiple CVL insertions, who were suspected of having major central venous thrombosis. The authors found: catheter contrast studies consistently underestimated the extend of thrombosis; US identified jugular thrombosis, but failed to identify more central thrombosis, in fact detecting only 7 of 18 thrombosis noted by magnetic resonance venography (MRV), and underestimated the extent of the thrombosis in 4 of the 7. (ref 116) Further, MRV identified a patent vein for reinsertion of CVL in 22 of 25 children, subsequently confirmed in 20 of 22 patients (91%).[116] Further, MRV identified a patent vein for reinsertion of CVL in 22 of 25 children, as confirmed in 20 of 22 patients (91%). There are no reported studies that determine the sensitivity and specificity of diagnostic testing for lower venous system CVL-related thrombosis in children.

In summary, for children with suspected upper system thrombosis, a combination of US (jugular veins) and bilateral upper limb venography (subclavians and central veins) is recommended, although MRV may be a viable alternative to formal venography depending upon local expertise. US is a reasonable alternative for DVT distal to the groin, based on adult experience, but for more proximal veins, venography or MRV should be considered.

Pulmonary Embolus

There are no published studies determining the sensitivity and specificity of diagnostic testing for PE in children. However, literature would support that PE is significantly underdiagnosed in children, especially in the ICU setting. There are difficulties with interpreting ventilation/perfusion (V/Q) scans in children in at-risk children, particularly following Fontan cardiac surgery, during which total pulmonary blood flow cannot be assessed by upper limb isotope injection. There are concerns as well regarding the safety of perfusion scanning in children with significant right to left cardiac shunts, as likely significant amounts of macroaggregated albumin may lodge in the cerebral circulation.[117] Ventilation perfusion scanning remains the recommended

A B

FIGURE 121.2 This girl presented during her teenage years with a rash on her legs that was demonstrated to be severe postthrombotic syndrome **(A)**. She developed venous ulceration 6 months later **(B)**. Imaging confirmed femoral vein occlusion with chronic collateral circulation. She had had a right femoral vein cardiac catheter when 3 years of age and had no immediate symptoms of her thrombosis.

FIGURE 121.3 This girl presented with right leg length shortening and atrophy. The femoral artery was occluded as a result of thrombosis following catheterization 10 years previously. There were no acute symptoms at the time of the catheter procedure.

investigation for PE in neonates and children, although pulmonary angiography is the gold standard, and clinicians may need to make a presumptive diagnosis based on clinical findings and the presence or absence of source thrombosis. CT pulmonary angiography may miss small peripheral pulmonary emboli, and repeat studies involve significant radiation exposure, notably to breast tissue in young female patients.[118] Clinical prediction rules, such as the Well clinical probability score, have been validated in adults presenting with symptoms of PE and provide a useful measure of pretest probability.[119]

The combination of a negative D-dimer and low clinical probability score can safely rule out PE, negating the need for diagnostic imaging.[120] However, no such clinical probability score has been validated in a pediatric cohort, and evidence suggests that the algorithms are not valid.[121] Further, a negative D-dimer test did not rule out PE in 4 of 30 children tested and was negative in 36% and 40% of children with PE in two other studies,[122] demonstrating a poor negative predictive value of this assay. Use of highly sensitive D-dimer tests would probably lead to more false-positive results. As symptoms and signs of DVT may be masked by the underlying diagnosis, the roles of clinical probability scores and D-dimer assays are currently unknown in children and should not be utilized.[121,123]

Arterial Thrombosis

There is little specific information related to diagnostic strategies in neonates or children,[77] although contrast angiography remains the "gold standard." Peripheral arterial thrombosis is usually diagnosed clinically, and US is the most commonly used diagnostic test.[72,124–126] Aortic thrombosis, either secondary to

UAC, or spontaneous, requires radiologic diagnosis. US cardiac echocardiography is of value if the obstruction is in the aortic arch.[68]

Contrast angiography is rarely feasible in critically ill newborns. In one comparative study, US failed to visualize aortic thrombosis in four patients, three of whom had complete aortic obstruction by contrast angiography. Thus, clinicians often use clinical findings and suboptimal imaging to make clinical decisions. In older children, arterial thrombosis is usually diagnosed on clinical grounds alone. False-negative US is reported in spontaneous femoral artery thrombosis in children, and false-positive MRA has been reported for chronic femoral artery obstruction, in comparison with angiography.[127] In suspected peripheral artery thrombosis, clinicians should consider the possibility of intramural or external hematoma causing arterial compression in the differential diagnosis, and for peripheral arteries, US may be sufficiently sensitive to exclude this phenomenon.[128] For hepatic artery thrombosis after liver transplant, serial testing with pulsed Doppler combined with real-time US of the liver parenchyma has a sensitivity of approximately 70%. As false-positive and -negative results occur, angiography is usually required to confirm the diagnosis, but CT may be useful in equivocal cases.[129] Spiral CT is sensitive and specific in adults, but the value of MRI is yet to be fully determined.

Intracardiac Thrombosis

Three studies have compared transthoracic echocardiography (TTE) to transesophageal echocardiography (TEE) in the diagnosis of intracardiac thrombosis following Fontan surgery.[130–132] Stumper et al. found three intracardiac thromboses in 18 patients using TEE, of which only one was detected by TTE, Fyfe et al. found six thrombi in four pediatric patients using TEE, of which only one was detected by TTE, and Balling et al. found 17 of 52 patients (33%) with intracardiac thrombosis after Fontan surgery by TEE, of which only one was identified on TTE. Thus, TTE is likely insufficient to exclude intracardiac thrombosis in children after Fontan surgery. Right atrial thrombosis, especially CVL-related thrombosis, is commonly diagnosed using TTE.[79]

ANTITHROMBOTIC THERAPY IN PEDIATRIC PATIENTS

The evidence supporting most recommendations for antithrombotic therapy in neonates and children remains weak.[133] Studies addressing appropriate drug target ranges and monitoring requirements are urgently required in addition to site- and clinical situation-specific thrombosis management strategies.[133] While there is some data to guide treatment decisions in neonates, almost none of the data reported separates premature from term infants, despite the obvious differences in risk/benefit considerations. Similarly adolescents are often managed according to data based on adults, but at what point an adolescent should transition from recommendations based on data in children to that in adults and whether age alone should be the determining factor for that transition is unknown.[133] The remainder of this chapter discusses the principles of antithrombotic therapy applicable to neonates and children. For specific treatment recommendations, the reader is referred to the most recent guidelines published by the American College of Chest Physicians.[133]

Heparin in Neonates and Children

Unfractionated heparin (UFH) remains a commonly used anticoagulant in pediatric patients. In tertiary pediatric hospitals, approximately 15% of inpatients are exposed to UFH each day.[134] There are a number of specific factors that may alter the effect of UFH in children. For example, UFH acts via antithrombin (AT)-mediated inhibition of thrombin and factor Xa, but children have reduced levels of AT and prothrombin,[17,135–137] reduced capacity to generate thrombin,[3,136,138] an age-related different ratio of anti-Xa: anti-IIa activity,[139–141] alterations in plasma binding of UFH,[19–21,139] and age-related differences in tissue factor pathway inhibitor release.[139] The clinical implications of these changes on dosing, monitoring, and the effectiveness/safety profile of UFH in children remain to be fully elucidated.

There have been no reported clinical outcome studies to determine the therapeutic range for UFH in neonates or children, so the therapeutic range for all indications is extrapolated from those used in adults. This equates to an activated partial thromboplastin time (APTT) that reflects a heparin level by protamine titration of 0.2 to 0.4 U/mL or an antifactor Xa level of 0.35 to 0.7 U/mL.[142] Extrapolating the APTT range from adults to pediatric patients may well be invalid. For example, baseline APTTs in pediatric patients, especially neonates, are often increased compared to adults, so a therapeutic range represents a reduced increment in APTT values in pediatric patients receiving adult-equivalent heparin therapy.[17,18] Schmidt reported that routine assays underestimate UFH concentration especially in neonates.[143] Recent *in vitro* and *in vivo* data also indicate that the APTT range that correlates to an antifactor Xa level of 0.35 to 0.7 U/mL varies significantly with age and heparin dose.[19,138–141,144] Further, protamine titration results vary with age[140] and different commercial anti-Xa kits produce substantial variations in results on the same samples.[138] These differences are magnified in infants whose endogenous AT levels are reduced.[145]

Bolus doses of 75 to 100 U/kg result in therapeutic APTT values in 90% of children at 4 to 6 hours postbolus. Maintenance UFH doses are age dependent, with infants (up to 2 months) having the highest requirements (average 28 U/kg/h) and children over 1 year of age having lower requirements (average 20 U/kg/h).[146] The doses of UFH required for older children are similar to the weight-adjusted requirements in adults (18 U/kg/h).[147] However, boluses of 75 to 100 U/kg in children receiving cardiac catheter prophylaxis result in excessive prolongation of APTT for over 100 minutes, implying that the recommendations may need to be reexamined.[140] Clearance of UFH is faster in newborns than in older children due to a larger volume of distribution.[148,149] Further, the pharmacokinetics of UFH in children fits a first-order model and is a function of weight rather than age.[140] Methodologically correct pharmacodynamic studies of UFH in children have not been performed.

Monitoring of UFH therapy is current standard practice, but there are difficulties with interpreting the monitoring assays, related to a lack of correlation between the anti-Xa, APTT, and thrombin clotting time,[138,140,148] as well as in making dosage adjustments.[147,150] There are no published studies in children that establish the ideal frequency of UFH monitoring.[151]

One cohort study reported bleeding in 1.5% (95% CI 0.0% to 8.3%) of children treated with UFH for DVT.[22] However, many children were treated with subtherapeutic doses of UFH in this study,[146] and a single-centre cohort study reported major bleeding in 24% of children in the pediatric ICU receiving UFH.[152] Further studies are required to accurately determine the frequency of UFH-induced bleeding in optimally treated children. Probably the most common cause of fatal bleeding secondary to UFH relates to accidental overdose, especially in neonates. While rarely reported in the medical literature, the number of cases reported in the popular press appears to be increasing.[18,153–158] In addition, anaphylaxis has been reported related to contamination of heparinoid drugs.[159–174] There are only three case reports of pediatric UFH-induced osteoporosis, two of whom received concurrent steroid therapy,[175,176] the third receiving high-dose IV UFH for a prolonged period.[176] Given the convincing relationship between UFH and osteoporosis in adults, clinicians should avoid long-term (weeks to months) use of UFH in children, especially considering the increased risk for heparin-induced osteoporosis in children compared to adults.[177,178]

Accurate diagnosis of heparin-induced thrombocytopenia (HIT) in children remains problematic. Overdiagnosis may occur in adults,[179] especially in the postcardiac surgical setting.[180] The frequency of HIT in children varies, likely related to criteria of patient inclusion and laboratory technique,[134,181–185] rates varying from almost nonexistent in unselected heparinized children[134] to 2.3% in children in the pediatric ICU.[183] Presumed HIT is a frequent reason for children to receive novel anticoagulant drugs,[186] including danaparoid, hirudin, and argatroban.[187–215]

Low Molecular Weight Heparin in Neonates and Children

LMWH has become the anticoagulant of choice in many pediatric patients, both for primary prophylaxis and for treatment of TE.[216] The potential advantages of LMWH for pediatric patients include reduced monitoring requirements compared to VKAs, lack of interference by other drugs or diet, reduced risk of HIT, and probable reduced risk of osteoporosis with long-term use. However, the predictability of the anticoagulant affect with weight-adjusted doses is less than in adults,[217] presumably due to differences in binding to plasma proteins.[21,218] Most clinical data of LMWH in pediatric patients utilized enoxaparin.[216,217,219–223,224,225–228] At 0.25 U LMWH/mL, thrombin generation is delayed and reduced by 50% in newborns compared to adults,[229] although no difference was observed in the anti-Xa activity of enoxaparin *in vitro* between plasma of children and adults.[230]

Therapeutic ranges for LMWH are extrapolated from results in adults and are based on anti-Xa levels, the guideline for subcutaneous administration twice daily being 0.50 to 1.0 U anti-Xa/mL at 2 to 6 hours following injection. Most studies in children have used this therapeutic range, although one study used a lower maximal level (0.8 U/mL) with good efficacy and safety outcomes.[228] For children dosed once daily, a target peak level of 0.5 to 0.8 U/mL or a trough level of 0.1 U/mL may be used.[224,228] Continuous infusion of intravenous LMWH has used the same target range of 0.5 to 1.0 U/mL.[231]

Table 121.3

Results of pharmacokinetic studies for LMWH in children

LMWH	Number of Patients	Maintenance Dose	Age or Weight
Enoxaparin[233]	19	1.6 mg/kg BD 1.0 mg/kg BD	<2 mo >2 mo
Enoxaparin[354]	12	1.0 mg/kg BD	>2 mo
Reviparin[355]	12	100 U/kg BD	>5 kg
Nadroparin[356]	154	146 U/kg BD	15 d to 8 y
Tinzaparin[357]	10	280 U/kg daily	0–2 mo
	8	245 U/kg daily	>2–12 mo
	6	240 U/kg daily	1 to >5 y
	4	200 U/kg daily	5 to >10 y
	7	177 U/kg daily	10 to >16 y

A two-compartment model describes enoxaparin kinetics in children, body weight being the most predictive covariate for clearance (15 mL/kg/h), central volume of distribution (169 mL/kg), intercompartmental clearance (58 mL/h), peripheral volume of distribution, and absorption rate. Interindividual variability is 54% for clearance and 42% for volume of distribution.[228] The subcutaneous doses of LMWH required in pediatric patients to achieve adult therapeutic peak anti-Xa levels have been assessed for enoxaparin, reviparin, dalteparin, tinzaparin, and nadroparin; however only enoxaparin, reviparin, tinzaparin, and nadroparin have been subjected to formal pharmacokinetic studies (Table 121.3).[224]

While initial doses most likely attain the therapeutic range, considerable interpatient dose differences exist, suggesting that routine monitoring of anti-Xa levels in children and neonates remains necessary.[232] Initial reports indicated that neonates required higher per kg doses than older children,[233,234] but recent studies of nadroparin[235] and enoxaparin[218] demonstrate a difference in dose requirements across age groups well outside of the newborn period. Whether clinical effectiveness will be altered by having multiple age-related initial and maintenance dose recommendations is unclear. Reviews of clinical reports of final dose requirements for neonates have suggested that an even higher initial dose schedule is more appropriate,[236] especially in preterm infants.[227,237,238]

A recent review of enoxaparin compiled data from multiple studies to describe a total of 240 neonates and reported that minor side effects were common and that major bleeding occurred in 12 (5%), although individual study results for major bleeding varied from 0% to 19%.[238] There were 10 premature neonates included in this review, with no major bleeds,[238] but the data are not sufficient to draw definitive conclusions. Risk factors for major bleeding are the use of subcutaneous catheters in newborns with little subcutaneous tissue and the presence of preexisting CNS abnormalities. For older children, a review of 308 children treated with therapeutic LMWH for VTE showed 9 children (1.2%) with major bleeding and 72 (23.4%) with minor bleeding.[224] This review included 133 children treated

with prophylactic doses of LMWH, of which 1 (0.8%) had major bleeding and 4 (3%) had minor bleeding. There are no data on the frequency of osteoporosis, HIT, or other hypersensitivity reactions in children exposed to LMWH; two cases of temporary hair loss are published.[239,240]

Vitamin K Antagonists in Neonates and Children

VKAs are problematic in newborns for several reasons. First, the plasma levels of the vitamin K–dependent coagulation factors are physiologically decreased in comparison with adult levels. Second, infant formula is supplemented with vitamin K to prevent hemorrhagic disease, which makes formula-fed infants resistant to VKAs. In contrast, breast milk has low concentrations of vitamin K, making breast-fed infants sensitive to VKAs,[241,242] a situation that can be compensated for by feeding 30 to 60 mL of formula each day. Third, VKAs are available only in tablet form in most countries, thus being unsuitable for newborns even if suspended in water. Fourth, VKA therapy requires monitoring, because of rapidly changing physiologic values of the vitamin K–dependent coagulation proteins, and changes in diet.[243] Finally, there is little efficacy or safety information specific to VKA use in neonates.

Warfarin is the most commonly used and studied VKA worldwide.[244–248] Acenocoumarol is administered with high frequency in some European and South American countries,[249,250] and phenprocoumon is the preferred VKA in some parts of Europe, although data about doses of phenprocoumon in pediatric patients have been reported. The current therapeutic international normalized ratio (INR) ranges for children are extrapolated from recommendations for adult patients, because there are no clinical trials that have assessed the optimal INR range for children. For most indications, the therapeutic target INR is 2.5 (range 2.0 to 3.0). The therapeutic range for prosthetic valves is also extrapolated from adult data and varies according to the type and position of the prosthetic valves.

The capacity of plasma from children receiving VKAs to generate thrombin is delayed and decreased by 25% compared to plasma from adults with a similar INR.[6] This raises the issue of whether the optimal INR therapeutic range for children should be lower than that for adults. This hypothesis is further supported by the observation that plasma concentrations of a marker of endogenous thrombin generation, prothrombin fragment 1.2, is significantly lower in children compared to adults at similar INR values.[6]

The published age-specific and weight-adjusted doses for children vary due to different study designs, patient populations, and varied results due to small sample size of the studied populations of children. The largest cohort study ($n = 319$) found that infants required an average of 0.33 mg/kg and teenagers 0.09 mg/kg warfarin to maintain an INR of 2.0 to 3.0.[248] For adults, weight-adjusted doses for VKA are not precisely known, but are in the range of 0.04 to 0.08 mg/kg to achieve an INR of 2.0 to 3.0.[251]

Monitoring oral anticoagulant therapy in children is difficult and requires close supervision with frequent dose adjustments.[244,248,252] Only 10% to 20% of children are safely monitored monthly.[244] Studies in children comparing point-of-care monitors to venepuncture INR, including comparison to WHO reference thromboplastins in the laboratory method, confirm their accuracy and reliability.[253,254] The major advantages of point-of-care devices include reduced trauma of venepunctures, minimal interruption of school and work, ease of operation, and portability.[254–260]

Bleeding is the main complication of VKA therapy, the risk for serious bleeding for treatment of mechanical prosthetic valves being <3.2% per patient year.[261] In one large cohort of 391 patient years, with variable target INR ranges, the bleeding rate was 0.5% per patient year.[255] In a randomized trial of 41 subjects, target INR range was 2.0 to 3.0 for 3 months and bleeding occurred in 12.2% (95% CI 4.1 to 26.2).[262] A single-center study with a nurse-coordinated anticoagulant service reported a bleeding rate of 0.05% per patient year.[263] Adequate patient and family education protocols are major factors in reducing bleeding events in children on warfarin therapy.[264,265] Nonhemorrhagic complications of VKA, such as tracheal calcification or hair loss, have been described on rare occasions in young children.[266,267] Two uncontrolled cohort studies described reduced bone density in children on warfarin for >1 year.[268,269]

Alternative Thrombin Inhibitors

Reports have documented pediatric use of argatroban,[195,197,200,202,270,271] bivalirudin,[194,272] lepirudin,[198,199,203,205,209,273–275] dabigatran,[276,277] danaparoid,[191,193,208,212,278–285] and fondaparinux,[271,286–290] most often used in children diagnosed with HIT. Two recent reviews have summarized the clinical indications, doses used, monitoring schedules, and clinical outcomes.[186,291] There is a wide range of dosing regimens reported, and most are empirical with relatively few formal dose-finding studies published. The clinical outcomes in children receiving these agents are often poor, and their use is difficult to recommend except in exceptional circumstances.[186]

Thrombolysis in Neonates and Children

At birth, plasma concentrations of plasminogen are approximately 50% of adult values (21 mg/100 mL),[292–294] slowing plasmin generation[295] and reducing the potential thrombolytic effect of streptokinase (SK), urokinase (UK), and tissue plasminogen activator (tPA) in *in vitro* fibrin clot systems.[137] Supplementation of plasma with plasminogen increases the thrombolytic effect of the agents.[137,296]

No study has compared the efficacy, safety, or cost of different thrombolytic agents in children. However, tPA has become the agent of choice in pediatric patients,[137,296,297,298–300] this preference being the result of a Federal Drug Administration (USA) warning regarding UK, improved clot lysis *in vitro* compared to UK and SK, fibrin specificity, and low immunogenicity.[301,302] However, tPA is considerably more expensive than SK or UK, and the increased *in vitro* clot lysis by tPA has not been demonstrated in clinical trials in children. There is minimal experience with other thrombolytic agents in children.[297,299,303] A recent survey has shown no consensus in indications for thrombolysis, dose, mode of delivery, or duration of therapy,[300] reflecting the lack of good quality studies available to guide clinicians in this area.

Success rates for thrombolysis in pediatric patients vary. Albisetti[297] reviewed the pediatric literature and described complete resolution of target thrombosis in 64% of 413 reported children receiving SK, UK, and tPA (53%, 43%, and 69%, respectively), but 21% had no response. Nowak Gottl reviewed

thrombolysis in neonates and calculated an overall thrombolytic patency rate of 39% for children with aortic thrombosis to 86.5% with intracardiac thrombosis. The collected data suggested no difference in efficacy between SK, UK, and tPA.[298] Differences in success rates have also been reported for thrombolysis in venous versus arterial thrombosis in children.[304] Most studies that have reported success or failure of thrombolysis have used thrombus resolution as an outcome measure, with methods of detection ranging from clinical assessment to radiologic assessment. One series of nine patients with DVT reported the postthrombotic syndrome as an outcome measure.[305] Despite a number of case series that have reported using thrombolysis for treatment of a wide range of venous and arterial thrombosis in children,[58,302,304,306–310] the likelihood of reporting bias, and the lack of control groups, who did not receive thrombolytics, and the use of inconsistent outcome measures makes the interpretation of data difficult.[299]

Manco-Johnson et al.[311] described specific contraindications for children, including prematurity (<32 weeks gestation). However, thrombolytics have been successfully given to premature babies,[307,312–316] including one with preexisting intraventricular haemorrhage,[317] but the number of studied patients was small.

At this time, there is no evidence to suggest that there is an advantage of local over systemic thrombolytic therapy in children with thrombotic complications.[297,322–324] There are more recent small case series reporting catheter-directed thrombolysis in children.[305,318,319]

Thrombolytics may not inhibit clot propagation; hence thrombin inhibition is required as adjunctive therapy. Concurrent low-dose heparin followed by therapeutic heparinization has since been described in single-centre studies.[304,305,307,310] While concurrent LMWH has also been reported,[310] easily reversible anticoagulation seems more appropriate.

Thrombolytic therapy has significant bleeding complications in children, requiring packed red blood cell transfusion in approximately 20% of patients.[296] A large single institution study reported bleeding in 68% of patients, requiring transfusion in 39%.[302] Multiple other studies have reported varying results.[58,304,306,316] Albisetti[297] calculated hemorrhage with the use of tPA as 26% minor and 17% major events.

In summary, there remains considerable variation in the risk of bleeding associated with thrombolysis use in children. The lack of uniform treatment regimen and lack of controlled data make the true adverse bleeding event rates difficult to estimate. However, it does appear that the bleeding risk from thrombolysis in children is at least an order of magnitude higher than the bleeding risk of anticoagulation alone. This risk needs to be weighed against the potential benefits of therapy in any child considered for thrombolysis.

Vena Caval Interruption

In addition to pharmacologic therapy, IVC are used for specific clinical indications in adults, primarily to reduce the risk of PE in patients with proven thrombosis in whom anticoagulation is contraindicated or who have failed anticoagulation therapy. There are reports on the use of IVC filters in children as young as 6 years of age.[320–325] Increasingly, temporary filters are used so they can be retrieved once the risk of pulmonary embolus is reduced, or the contraindication to anticoagulation has resolved. Temporary filters have been left *in situ* for over 140 days.[326] Raffini et al.[327] reported an institutional program that resulted in 5% of children presenting with DVT having IVC filters inserted, with minimal adverse outcomes. Chaudry et al.[328] reported the use of filters in the internal jugular veins as well as in the IVC. There are no specific guidelines for the use of filters in children and the risk/benefit ratio needs to be considered individually in each case. We strongly support, when required, the exclusive use of temporary filters in children.

Surgical Therapy

Surgical thrombectomy is rarely used in children and is restricted to situations such as IVC thrombosis in association with intravascular extension of Wilms' tumor, acute thrombosis of B-T shunts, life-threatening intracardiac thrombosis after complex cardiac surgery, prosthetic valve thrombosis, septic thrombosis, and peripheral arterial thrombosis secondary to vascular access.[79,128,329–339] There are no controlled data to compare the value of conservative therapy, and it is unlikely such data will become available. In most cases, concurrent or subsequent anticoagulation therapy is used.

CONCLUSION

Venous and arterial thrombosis in children are common clinical problems, especially in tertiary care facilities. There are significant differences to many key aspects of the epidemiology, pathogenesis, and coexisting conditions of thrombosis in children when compared to adults. There remains little information about the natural history of TE, and the potential for long-term consequences is significant. When considering treating thromboembolic events in children, clinicians must be aware of important differences in pharmacokinetics and pharmacodynamics of anticoagulant drugs from values in adults, as well as significant difficulties in performing and interpreting monitoring tests. The use of anticoagulants in children of all ages requires urgent research to improve the safety and efficacy of these drugs. Recent reviews and specific consensus guidelines are helpful for clinicians in this rapidly changing field.[151,186,340–342]

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CHAPTER

122 Thrombotic Complications of Sickle Cell Disease

KENNETH I. ATAGA AND ELLIOTT P. VICHINSKY

In addition to its well described vaso-occlusive and hemolytic complications, patients with sickle cell disease (SCD) are at risk for a variety of thrombotic complications. Patients with SCD have an increased risk of stroke, pulmonary hypertension, avascular necrosis of large joints, pregnancy-related complications, and leg ulcers, all of which may be due, at least in part, to thrombotic vascular occlusion.[1] A retrospective study based on reported discharge diagnoses showed that SCD patients were more likely to be diagnosed with pulmonary embolism compared with race-matched controls without SCD (0.44% vs. 0.12%), although interestingly, the prevalence of deep vein thrombosis was similar between the two groups.[2] SCD also appears to be a significant risk factor for pregnancy-related venous thromboembolism, with an odds ratio of 6.7 (95% confidence interval [CI]: 4.4 to 10.1).[3,4] Finally, although distinct from SCD in its clinical manifestations, individuals with the sickle cell trait appear to be at increased risk of venous thromboembolism compared to race-matched controls, with an odds ratio of about two.[5,6] Sickle cell trait appears to be associated with pulmonary embolism, rather than deep vein thrombosis,[5] analogous to the situation in patients with SCD.[2]

THE RED BLOOD CELL AND HEMOGLOBIN S POLYMERIZATION

Sickle hemoglobin (HbS) occurs when the normal β^6 glutamic acid residue is replaced by valine (GAG to GTG mutation at codon β^6). The polymerization of HbS ($\alpha_2\beta_2^S$) following its deoxygenation is the primary event in the pathophysiology of SCD and results in damage to red blood cells (RBCs), tissues, and organs.[7] The rate and extent of HbS polymer formation depend on the degree of deoxygenation of HbS, the intraerythrocytic HbS concentration, and the intracellular concentration of fetal hemoglobin (HbF).[7] The lag period required for the formation of HbS polymer is referred to as the delay time. As the range of transit times in the microcirculation is short relative to the range of delay times of sickle RBCs, polymers do not form in most of the cells.[8] HbF inhibits the polymerization of HbS, primarily owing to the glutamine residue at codon γ87, which prevents a critical lateral contact in the double strand of the sickle fiber.[7]

Patients with SCD exhibit an unusually broad density distribution of sickle RBCs due mainly to the high number of reticulocytes, with a relatively low intracellular hemoglobin concentration, and the presence of a high number of very dense cells. Dense cells occur due to enhanced cellular dehydration following polymerization-induced damage to the cell membrane.[7] As HbS polymerization is strongly dependent on the intracellular hemoglobin concentration,[9] dense sickle RBCs are more likely to polymerize and contribute to the hemolytic and vaso-occlusive complications of SCD than less dehydrated cells.

LEUKOCYTES

Leukocytes play an important role in the pathogenesis of SCD. Increased white blood cell count is a risk for early death,[10] acute chest syndrome,[11] and hemorrhagic stroke[12] in patients with sickle cell anemia. In addition, severe episodes of vaso-occlusive crisis and acute chest syndrome have occurred following the administration of granulocyte-colony stimulating factor (G-CSF) to patients with SCD in their steady state.[13,14] Leukocytes interact with sickle RBC and vascular endothelium and are stimulated to release injurious cytokines.[15,16] In addition to the adhesion to vascular endothelium, leukocytes interact with erythrocytes and platelets to form cell aggregates, stabilized via CD36-TSP, CD31-CD31, and CD62L-CD162 bonds.[17,18] Using intravital microscopy in mice expressing human HbS, sickle RBCs were observed to bind to adherent leukocytes in inflamed venules, producing vaso-occlusion of cremasteric venules.[17] However, both the adhesion of leukocytes to endothelium and the interaction of leukocytes with sickle RBCs were substantially reduced in sickle mice that were deficient in P- and E-selectins, suggesting that adhesion to endothelial selectins may enhance the ability of adherent leukocytes to bind circulating sickle RBCs.

Monocytes are activated in sickle cell anemia and can enhance vaso-occlusion through an endothelial inflammatory response promoted by the nuclear factor-κB (NFκB)-mediated upregulation of adhesion molecules and tissue factor (TF).[19] The activation of endothelial cells by sickle monocytes appears to be mediated by tumor necrosis factor-α and interleukin-1β, both markers of monocyte activation. The activated endothelial cells increase their expression of ligands for adhesion molecules on leukocytes and RBCs, thereby promoting vaso-occlusion.

ENDOTHELIUM

As the potential for sickle RBCs to initiate a vaso-occlusive event is dependent on whether the rate of polymer formation is within the range of the capillary transit time,[20] any factor that slows the transit of these cells through the microcirculation could be expected to have an effect on the pathogenesis of vaso-occlusion. Multiple studies demonstrate that sickle RBCs attach more readily to cultured endothelial cells than do normal RBCs.[21–24] Indeed, the degree of adherence of sickle RBCs to vascular endothelium strongly correlates with disease severity.[21]

In addition to WBC adhesion to endothelium, adhesion reactions are mediated by interactions between receptors on sickle RBCs and endothelial cells or subendothelial matrix proteins. The plasma ligand, thrombospondin (TSP) provides a bridge between the RBC receptor CD36 and several constitutively expressed endothelial receptors,[25,26] including the vitronectin receptor ($\alpha_v\beta_3$), the transmembrane glycoprotein CD36, and endothelial cell surface heparan sulfate proteoglycans. Sickle RBCs also interact with immobilized TSP via the integrin-associated protein, CD47.[27] Interactions occur between the integrin complex, $\alpha_4\beta_1$ (VLA-4), expressed on reticulocytes, and both endothelial vascular-cell adhesion molecule 1 (VCAM-1)[28–31] (typically expressed on the surface of endothelial cells following activation by inflammatory cytokines and hypoxia) and fibronectin.[32] High molecular weight multimers of von Willebrand factor (vWF)[33,34] promote RBC adhesion to endothelial vitronectin receptor, $\alpha_v\beta_3$ and the GPIb-IX-V complex. Interactions also occur between sickle RBC and subendothelial immobilized matrix proteins, including laminin via the erythrocyte basal cell adhesion molecule-Lutheran protein receptor, the protein that carries Lutheran blood-group antigens,[35] as well as TSP, vWF, and fibronectin, proteins that are also present in plasma in a soluble form.[36] Nonreceptor-mediated adhesive mechanisms include a role for RBC sulfated glycolipids and phosphatidylserine (PS).[37–39]

INFLAMMATION

SCD is often referred to as a chronic inflammatory state due to the presence of a chronic elevation in leukocyte counts, abnormal activation of cellular elements including neutrophils and monocytes,[40,41] circulating endothelial cells,[42] and platelets[43,44] as well as activation of the coagulation system.[1] In addition, patients exhibit elevated levels of inflammatory mediators (such as interleukin-6, tumor necrosis factor α, interleukin-1, placenta growth factor [PlGF]), acute phase reactants (such as C-reactive protein, secretory phospholipase A2, and G-CSF), and markers of endothelial cell injury (such as soluble VCAM)[40,45,46] even in the non-crisis, "steady state." The inflammatory biology in SCD patients may result from infection, as well as iron replete (or overloaded) states, sickle RBC adhesion to endothelium, and the reperfusion-injury physiology observed in these patients.[40]

COAGULATION ACTIVATION

As nearly every component of hemostasis, including platelet function, and the procoagulant, anticoagulant, and fibrinolytic systems, is altered in the direction of a procoagulant phenotype, SCD is frequently referred to as a "hypercoagulable state[1] (FIGURE 122.1)." Patients exhibit a loss of normal membrane phospholipid asymmetry in a subpopulation of RBCs, occurring in mature cells, RNA-containing reticulocytes, and transferrin receptor–positive "stress" erythrocytes.[47,48] PS is normally found in the inner monolayer of the plasma cell membrane, whereas choline-containing phospholipids such as phosphatidylcholine and sphingomyelin are located in the outer monolayer.[49] RBC membrane phospholipid asymmetry is usually maintained by the action of an ATP-dependent aminophospholipid translocase (or flipase), that transports PS and phosphatidylethanolamine from the outer to the inner membrane surface.[49] In addition, movement of all phospholipids in both directions is produced by scramblase, following activation by an influx of extracellular calcium, resulting in rapid PS exposure.[50] The abnormal PS exposure in sickle RBCs may occur due to repeated cycles of sickling and unsickling linked to polymerization and depolymerization of HbS, which results in the production of terminal spicules or microvesicles with exposed PS.[51] The abnormal exposure of PS may also result from reduced flipase activity in RBCs due to oxidative stress and sulfhydryl modification.[49,52,53] Abnormal PS exposure functions as a recognition signal for cell removal during apoptosis of nucleated cells,[54] as well as a docking site for enzymatic complexes involved in coagulation and anticoagulation pathways.[50] The external exposure of PS alters the adhesive properties of sickle RBCs[55] and has been reported to be involved in the hemostatic changes observed in SCD.[56,57] Several data show evidence for a relationship between RBC PS exposure and the coagulation activation in SCD. Plasma levels of prothrombin fragment 1.2 (F1.2) are associated with the number of circulating PS-positive RBCs.[57] However, although the number of PS-positive sickle RBCs is significantly correlated with plasma F1.2, D-dimer, and plasmin–antiplasmin (PAP) complexes, no correlation is found between PS-positive platelets and any of these markers of coagulation activation,[56] suggesting that sickle RBCs, and not platelets, are responsible for the coagulation activation observed in SCD. Finally, sickle RBCs containing high amounts of HbF are less likely to result in microparticle (MP) formation, PS exposure, and thrombin generation,[57] suggesting a protective effect of HbF on membrane bilayer flip-flop and PS exposure.

Measures of hemolysis are associated with plasma markers of coagulation activation such as thrombin–antithrombin complexes (TAT), F1.2, and D-dimers in patients with SCD.[45,58] Heme, an inflammatory mediator and a product of intravascular hemolysis, has been reported to induce TF expression on the surface of both macrovascular and microvascular endothelial cells in a concentration-dependent manner.[59] In addition, heme resulted in an upregulation of the expression of TF mRNA, TF protein, and TF procoagulant activity in endothelial cells in a time-dependent manner, effects that may be mediated, at least in part, by the transcription factor, NFκB. Heme-induced endothelial TF expression may provide a pathophysiologic link between hemolysis and the coagulation activation observed in patients with SCD.

The inflammatory state in SCD may also contribute to coagulation activation. Studies in transgenic SCD mice demonstrate that exposure of the mild sickle cell phenotype, NY1DD mice, to a hypoxic environment for 3 hours followed by a return to ambient air for 18 hours resulted in increased TF expression in the pulmonary veins. Thus, ischemia-reperfusion injury in patients with SCD may play a pathophysiologic role in the activation of coagulation.[60] The endothelial TF expression in the NY1DD mouse appears to be highly dependent on NFκB (p50) in peripheral blood mononuclear cells but not in the vessel wall.[61] Furthermore, a study of NY1DD animals bred to overexpress endothelial nitric oxide synthase (eNOS) or to have an eNOS knockout state demonstrated that eNOS modulates endothelial TF expression *in vivo* by downregulating it, suggesting that the biodeficiency of NO, characteristic of SCD patients, may increase the risk of coagulation activation.[62]

Finally, increased levels of soluble CD40 ligand, a tumor necrosis factor family member, may contribute to coagulation activation in SCD. Plasma from patients with sickle cell anemia

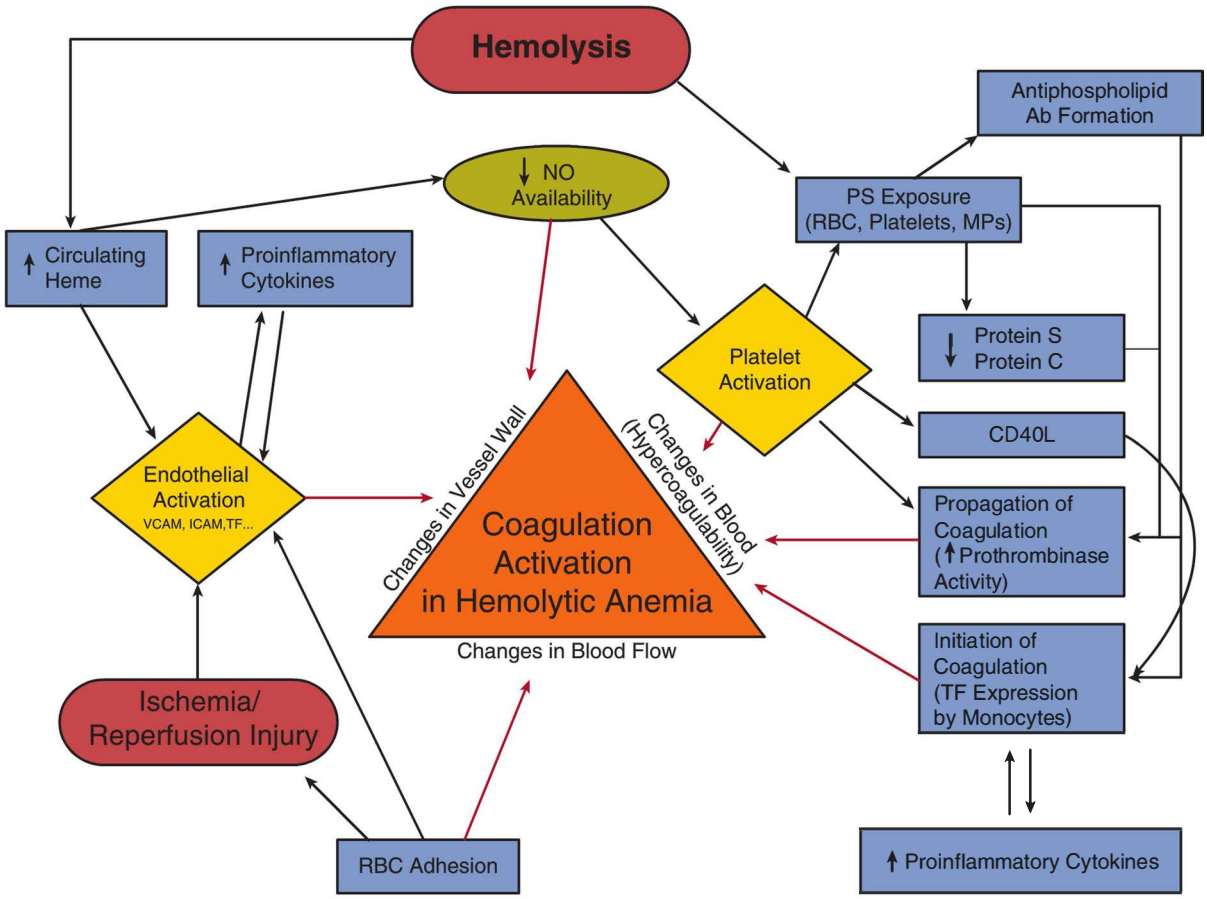

FIGURE 122.1 Schematic representation of pathophysiologic mechanisms (described or postulated) leading to activation of coagulation in SCD. Based on Virchow triad, the illustrated pathways contribute to activation of coagulation (and possibly eventual thrombosis) by one of three broad mechanisms, that is, (1) changes in the vessel wall; (2) changes in blood flow; and/or (3) changes in the composition of blood components ("hypercoagulability"). NO, nitric oxide; PS, phosphatidylserine; RBC, red blood cell; MPs, microparticles; TF, tissue factor; SCD, sickle cell disease. (Modified from Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. *Hematology Am Soc Hematol Educ Program* 2007:91–96.)

induced a significant increase in TF production in monocytic THP-1 cells relative to plasma from HbAA individuals or media alone.[44] Furthermore, preincubation of the THP-1 cells with a function blocking anti-CD40 antibody prevented increased TF expression in five of eight individual cases, suggesting that the CD40:CD40L interaction is only partly responsible for the elevation in TF production.

INCREASED TISSUE FACTOR EXPRESSION

The TF-FVIIa complex is the physiologic initiator of hemostasis.[63] Patients with SCD exhibit abnormal expression of TF on circulating endothelial cells, and this TF expression is increased further during acute pain episodes.[64] In addition, both TF antigen and TF procoagulant activity are elevated in the circulation of SCD patients when compared with normal controls,[44,65,66] although no difference in whole blood TF procoagulant activity was observed in patients in the non-crisis, "steady state" compared to those with pain crises.[65]

While numerous plasma factors that could increase TF expression in hematopoietic and/or endothelial cells such as thrombin, tumor necrosis factor, interleukin-1, and endotoxin are elevated in SCD patients, multiple potential mechanisms for the increased TF expression in these patients have recently been described. As already described, these include hemolysis with increased levels of heme,[59] ischemia-reperfusion injury,[60] as well as increased plasma levels of soluble CD40 ligand.[44]

CHANGES IN MARKERS OF THROMBIN GENERATION AND ANTICOAGULANT PROTEINS

Patients with SCD have increased plasma levels of markers of thrombin generation in the non-crisis, "steady state," although there are conflicting data on the effect of acute pain episodes on the levels of these markers.[67,68] Plasma levels of F1.2 and TAT complexes, both markers of thrombin generation, are elevated in SCD patients in the noncrisis, steady state.[67,68] Plasma levels of D-dimers,[66,67,68] PAP complexes,[67] and fibrinopeptide A[68] are also observed to be elevated in SCD patients in the non-crisis, "steady state." The frequency of pain episodes correlates with the extent of fibrinolytic activity (assessed by D-dimer levels) in

the non-crisis, "steady state," suggesting that D-dimer levels may predict the frequency of pain crises.[67]

The levels of natural anticoagulant proteins, protein C and protein S, are also decreased[68,69] in the non-crisis, "steady state" and perhaps even more so during acute pain episodes.[68,70] The reduced levels of these regulatory proteins may be a consequence of (a) chronic consumption due to increased thrombin generation resulting from intravascular TF expression and RBC prothrombinase activity; (b) increased binding of protein S by sickle RBCs due to red cell membrane PS exposure[71]; and/or (c) inhibition of the binding of protein S to β_2-glycoprotein 1 by antiphospholipid antibodies,[72] resulting in inactivation of protein S by circulating C4b-binding protein. Significantly decreased levels of proteins C and S have been reported in SCD patients who developed thrombotic strokes compared to neurologically normal SCD children.[73] Plasma levels of the serine protease inhibitor (SERPIN), heparin cofactor II, are also reported to be decreased in SCD patients.[74]

Antiphospholipid antibodies are frequently detected in SCD patients.[68] In addition, levels of certain subtypes of antiphospholipid autoantibodies, particularly those directed against PS, are markedly elevated in patients with sickle cell anemia, whereas they are normal in patients with HbSC disease.[68] In sickle cell anemia patients, a strong correlation exists between antibodies against PS and plasma D-dimers, suggesting a significant role for anti-PS antibodies in coagulation activation in SCD.[68]

PLATELETS, ENDOTHELIAL CELLS AND MICROPARTICLES

There is ample evidence suggesting that circulating platelets are chronically activated in patients with SCD, a phenomenon that may contribute to the observed coagulation activation in SCD.[44] Although there are conflicting reports of platelet survival,[75,76] platelet aggregation responses do appear to be increased in adult patients in the non-crisis, "steady state."[77] This may be explained by an increased number of circulating young, metabolically active platelets, or increased plasma levels of platelet agonists such as thrombin, epinephrine, and adenosine diphosphate. There is increased expression of both CD62P (P-selectin) and CD40L in platelets of SCD patients.[67,78] In addition to elevated plasma levels of the α-granule constituents, TSP, platelet factor 4, and β-thromboglobulin,[67] platelet-derived plasma soluble CD40 ligand is elevated in the non-crisis, "steady state," compared to normal controls.[44] The pathogenesis of platelet activation in SCD appears to be due, at least in part, to hemolysis with subsequent decreased bioavailability of NO.[79]

Circulating platelet aggregates (including both platelet–erythrocyte and platelet–monocyte aggregates) are increased in SCD patients during the non-crisis, "steady state"[77,80,81] and appear to increase further during acute pain episodes.[82] Platelets have also been detected in the heterotypic interaction between the monocyte and reticulocyte in a P-selectin/P-selectin glycoprotein ligand-1–dependent interaction.[83] Decreases in platelet lifespan and platelet counts have been reported during acute pain episodes.[75,76] In addition, acute pain episodes appear to be associated with increased platelet activation compared to the non-crisis, "steady state".[44,68] These findings suggest that decreased platelet survival and increased consumption occur during acute pain episodes.

Increased numbers of circulating endothelial cells have been reported in SCD patients in the non-crisis, "steady state," with a further increase during acute pain episodes.[42] In addition to expressing adhesion markers, these circulating cells abnormally express TF antigen in the "steady state", and to a greater extent during acute pain episodes.[64] Furthermore, these endothelial cells are positive for TF mRNA, with excellent correlation between TF antigen and mRNA expression.[64]Circulating MPs, small membrane-derived vesicles released by cells following activation or apoptosis, may be derived from RBCs, platelets, endothelial cells, and monocytes.[41] Both the total number of MPs as well as the number of TF-positive MPs are increased in SCD patients, with further increases during acute pain episodes. These TF-positive MPs are derived from endothelial cells and monocytes, but not from RBCs and platelets.[84] Plasma markers of coagulation activation, such as D-dimer, TAT, and F1.2, appear to correlate with total MPs, total TF-positive MPs, monocyte-derived TF-positive MPs, and with RBC-derived MPs,[84] suggesting a role for MPs in the hypercoagulable state observed in patients with SCD. In a more recent study, the majority of MPs in SCD patients were reported to originate from platelets and erythrocytes, and the numbers of these MPs did not differ significantly between crisis and steady state.[85] No MPs originating from monocytes or endothelial cells were detectable and no MPs expressing TF were identified.[85] However, erythrocyte-derived MPs correlated strongly with plasma levels of hemolytic markers, as well as with vWF, D-dimer, and F1.2 levels.

THERAPEUTIC IMPLICATIONS

Despite the abundant laboratory evidence of both platelet and coagulation activation in SCD patients, the vast majority of studies using anticoagulants and antiplatelet agents have not provided any convincing benefit in the prevention or treatment of vaso-occlusive complications. As most of these studies have been small and/or poorly controlled, it still remains uncertain whether the observed platelet and coagulation activation observed in these patients contribute to the vascular occlusive episodes that characterize SCD.

Multiple studies of anticoagulants[86,87,88,89,90] (Table 122.1) and antiplatelet agents[91–96] (Table 122.2) have been conducted in SCD patients with varying results. Unfractionated heparin decreases the adhesion of sickle RBCs to endothelium under static conditions, as well as P-selectin–mediated flow adherence of sickle cells to thrombin-treated human vascular endothelial cells.[97] A randomized, double-blind, placebo-controlled study of SCD patients during acute pain episodes showed that treatment with the low molecular weight heparin, tinzaparin, resulted in a significant reduction in the overall duration of painful crisis, number of days with the most severe pain scores, and duration of hospitalization compared with placebo.[90] The glycoprotein IIb/IIIa inhibitor, eptifibatide, was reported to be safe in a pilot study of sickle cell anemia patients.[96] By inhibiting platelet aggregation and decreasing the levels of the inflammatory mediator, soluble CD40 ligand, eptifibatide may be beneficial in the treatment of certain SCD-related complications.[96] Table 122.3 shows the rationale for the use of anticoagulants or antiplatelet agents in selected SCD-related complications.

Hydroxyurea is the only drug approved by the U.S. Food and Drugs Administration specifically for treating SCD. It has been shown to reduce the frequency of hospitalizations, acute painful

Table 122.1

Published studies of anticoagulants in patients with SCD

Study	Genotype	Number of Subjects	Treatment	Randomized	Duration	Efficacy Outcome
Salvaggio (1963)	HbSS	12	Warfarin	No	12–34 mo	Modest ↓ frequency of VOE
Chaplin (1989)	HbSS	4	Heparin	No	2–6 y	↓ Frequency of VOE
Wolters (1995)	HbSS HbSC	6 1	Acenocoumarol	No	2 mo	↓ F1+2
Schnog (2001)	HbSS HbSC	14 8	Acenocoumarol vs. placebo	Yes	14 wk	↓ Markers of coagulation markers, no ↓ in VOE with active treatment
Qari (2007)	HbSS	253	Tinzaparin vs. placebo	Yes	Maximum (7 d)	↓ in days with most severe pain score, duration of overall pain crisis and hospitalization duration with active treatment

VOE, Vaso-occlusive episodes
Modified from Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. *Hematology Am Soc Hematol Educ Program* 2007:91–96.

Table 122.2

Published studies of antiplatelet agents in patients with SCD

Study	Genotype	Number of Subjects	Therapy	Randomized	Duration	Efficacy Outcome Measure and Results
Chaplin et al. (1980)	HbSS	3	Aspirin/ Dipyridamole	No	104 wk	Modest decrease in frequency of pain episodes, platelet count, and fibrinogen level
Osamo[a] (1981)	HbSS	100	Aspirin	Yes	6 wk	Increase in oxygen affinity, hemoglobin, and RBC life span
Greenberg (1983)	HbSS HbSC HbS-O Arab	40 8 1	Aspirin vs. placebo	Yes	21 mo	No decrease in frequency of pain episodes
Semple (1984)	HbSS HbS-β thalassemia	8 1	Ticlopidine vs. placebo	Yes	4 wk	No improvement in frequency of pain episodes or platelet survival, but decrease in platelet release products
Cabannes[b] (1984)	HbSS	140	Ticlopidine vs. placebo	Yes	6 mo	Reduction of frequency and duration of pain episodes
Zago (1984)	HbSS HbS-β thalassemia	25 4	Aspirin vs. placebo	Yes	5 mo	No differences in frequency of pain episodes, hemoglobin, reticulocyte count, irreversibly sickled cells and HbF level
Lee (2007)	HbSS	4	Eptifibatide	No	1 wk	Decrease in platelet aggregation, decrease in plasma level of soluble CD40 ligand, and decrease in MIP-1α, TNFα, and myoglobin

[a]Increase from baseline in hemoglobin (7.5 ± 0.35 to 10 ± 0.39 g/dL, $P < 0.01$), O_2 saturation (80 ± 3.6% to 94 ± 3.7%, $P < 0.01$), pO_2 (52 ± 2.7 to 68 ± 2.9 mm Hg, $P < 0.01$), and decrease in 2,3-diphosphoglycerate levels (7.2 ± 0.24 to 4.6 ± 0.20 μmol/mL, $P < 0.01$) in patients on aspirin after 6 wk of treatment. No data provided for patients on placebo.
[b]Decrease in number of crises (−81% vs. −47%, $P = 0.0001$) and duration of crises (2.862 ± 1.75 d vs. 3.952 ± 2.2 d, $P = 0.001$) in patients on ticlopidine vs. placebo.
Modified from Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. *Hematology Am Soc Hematol Educ Program* 2007:91–96.

Table 122.3

Rationale for use of anticoagulants and/or antiplatelet agents in SCD

Complication	Anticoagulant/ Antiplatelet Agent	Caveat
Retinopathy	No	This is no evidence to support the use of anticoagulants or antiplatelet agents in this setting. Furthermore, these agents may increase the risk of bleeding in patients with neovascular malformations.
Thrombotic stroke	? Antiplatelet agent	Chronic RBC transfusion remains the standard of care in the management of thrombotic stroke in children with SCD. Although thrombotic stroke occurs less commonly in adult patients, chronic transfusion is also the accepted treatment. With the finding of superimposed thrombosis in large cerebral vessels in these patients, antiplatelet agents may be useful, particularly in those patients in whom RBC transfusion is not feasible. However, controlled clinical studies are required. There is concern about the use of anticoagulants in this setting because of the risk of intracranial bleeding, particularly in those patients with Moyamoya.
Pulmonary hypertension	? Anticoagulant	Anticoagulants are reported to prolong survival in patients with pulmonary arterial hypertension. With the similar pathologic findings of *in situ* thrombosis in SCD patients with pulmonary hypertension, anticoagulants may be similarly beneficial. Controlled studies of anticoagulants in SCD-associated pulmonary hypertension are required to confirm their benefit in this setting.
Acute pain episodes	? Anticoagulant/ antiplatelet agent	Although not standard of care, full dose anticoagulation with the low molecular weight heparin, tinzaparin, has been shown to shorten the duration of acute pain episodes. In addition, as platelets exhibit increased activation during acute pain episodes compared to the "steady state," antiplatelet agents may also be useful in this setting.
Acute chest syndrome	No	While pulmonary fat embolism is a known cause of acute chest syndrome, there is no evidence that thrombotic embolism is associated with acute chest syndrome. As a result, therapeutic anticoagulation is not recommended. However, as for most SCD-related hospitalizations, prophylactic anticoagulation to decrease the risk of deep venous thrombosis is required, unless there is a contraindication.
Priapism	No	This is no evidence to support the use of anticoagulants or antiplatelet agents in this setting.
Deep venous thrombosis/ pulmonary embolism	Yes	Anticoagulation is indicated. Although SCD is referred to a hypercoagulable state, the duration of anticoagulation is uncertain as the risk of recurrence is not defined. Most patients are treated based on recommendations for patients without SCD.
Leg ulcers	No	This is no evidence to support the use of anticoagulants or antiplatelet agents in this setting.

episodes, acute chest syndrome, and blood transfusions in severe sickle cell anemia.[98] Furthermore, treatment with hydroxyurea is reported to reduce mortality in adults with severe SCD.[99,100,101] Treatment modalities frequently employed in SCD appear to affect the hemostatic system in these patients. Hydroxyurea and the hypomethylating agent, decitabine (that also increases levels of HbF), have been reported to decrease plasma levels of markers of thrombin generation.[102,103] Prophylactic RBC transfusion significantly reduces the risk of strokes in children with sickle cell anemia. It has been proposed that the beneficial effect may in part be related to normalization of the levels of F1.2,[104] although another study found no significant reduction

in thrombin generation when children with SCD undergoing chronic transfusion were compared with normal controls.[105]

STROKE

The Cooperative Study of Sickle Cell Disease (CSSCD) defined stroke as "an acute neurologic syndrome due to vascular occlusion or hemorrhage in which neurologic symptoms lasted more than 24 hours."[12] In addition, imaging studies may show infarction of the brain in the absence of a history of neurologic symptoms, a condition referred to as "silent infarction." Neurologic symptoms lasting <24 hours are considered to be the result of a transient ischemic attack (TIA). However, if the results of neuroimaging studies show the presence of infarcts or hemorrhage, the patients are usually classified as having had a stroke.[106]

Based on data from the CSSCD, a natural history study that followed approximately 4,000 patients for up to 10 years, the overall prevalence of stroke in SCD was 4%, with a prevalence of 5% in patients with sickle cell anemia.[12] The annual incidence of first stroke was approximately 0.6 per 100 patient-years in sickle cell anemia, with the highest incidence occurring in the first decade of life with rates of 1.02 per 100 patient-years in sickle cell anemia patients 2 to 5 years of age and 0.8 in those 6 to 9 years of age. The cumulative risk of first stroke in patients with sickle cell anemia was 11% by age 20 years, 15% by 30 years, and 24% by 45 years.[12] Infarctive strokes are relatively more common in children than in adults, and hemorrhagic strokes are more common in adults. In the CSSCD report, the risk of hemorrhagic stroke (0.44 per 100 patient-years) was highest in the 20 to 29 year age group.

Prior TIA, low steady-state hemoglobin level, systolic blood pressure, acute chest syndrome within 2 weeks of the stroke event, and the rate per year of acute chest syndrome were found to be significant risk factors for infarctive stroke in the CSSCD.[12] Low steady-state hemoglobin levels and high white blood cell counts were significant risk factors for hemorrhagic stroke. In addition, the protective effect of alpha thalassemia on the incidence of stroke in sickle cell anemia was due largely to the improvement in steady-state hemoglobin level. There was no association between the frequency of pain episodes and stroke. Furthermore, the level of HbF was not associated with the risk of stroke.[12] Nocturnal hypoxemia is also recognized as a risk factor for acute neurologic events (stroke, TIA, or seizures).[107] However, the best predictor of stroke risk at the present time is an elevated transcranial Doppler (TCD) velocity.[108]

Genetic factors may increase the risk for stroke in SCD patients. In a clinical epidemiology study, 42 sibships of patients with sickle cell anemia in which one sibling had a stroke showed a greater than expected number of families having two or more children with a stroke.[109] HLA Class I and II typing of sickle cell anemia patients showed differences in Class I HLA-B, Class II HLA-DRB1, and DQB1 between 22 patients with cerebral infarction detected by magnetic resonance imaging (MRI) and 31 with normal MRI examination.[110] Although there are conflicting reports on the association between stroke and β-globin haplotypes,[109,111,112] coinheritance of alpha thalassemia genes may be associated with diminished stroke risk.[12,113,114] Single nucleotide polymorphisms (SNPs) within the vascular cell adhesion molecule-1 gene may influence the risk of stroke, although no increased risk was noted with genes regulating immune function and inflammation.[115,116] Distinct allelic associations were found with stroke in children enrolled in the CSSCD. In this population, the TNF(-308)A, IL4R 503P, and ADRB2 27E polymorphisms were associated with the large vessel subtype of stroke,[115] a finding that has been replicated in an independent population of children with sickle cell anemia.[117] A Bayesian network, a multivariate dependency model that accounts for simultaneous associations and interactions among multiple genes and their interplay with clinical and physiologic factors, was used to analyze 108 SNPs in 39 candidate genes involved in vasoregulation, inflammation, cell adhesion, coagulation, hemostasis, cell proliferation, oxidative biology, and other functions using data and samples from 1,398 patients in the CSSCD.[118] Eleven genes whose variants had a direct effect modulated by levels of hemoglobin F and nine genes whose variants were indirectly associated with stroke were determined in 92 patients with overt stroke and the results were validated in a population of 114 individuals not included in the original study. The model predicted the correct outcome for all individuals with stroke and for 105/107 individuals without stroke, yielding an overall predictive accuracy of 98%. Finally, using blood outgrowth endothelial cells from patients shown to be either at risk or not at risk for ischemic stroke, gene expression profiling identified no significant single gene differences between the two groups, although an analysis of Biological Systems Scores, using gene sets that were predetermined to survey each of nine biologic systems, suggested that the pathobiology of circle of Willis disease in sickle cell anemia predominantly involves inflammation biology.[119]

The pathogenesis of cerebral infarction in children with SCD is not simply sickling of RBCs in the microcirculation. Large vessel arterial obstruction with superimposed thrombosis is commonly observed in SCD patients with ischemic stroke.[120] In response to chronic anemia and hypoxemia, cerebral blood flow is markedly increased in SCD.[121] When acute or chronic conditions lead to diminished availability of oxygen, the increased hypoxic stress combined with the inability of the cerebral vasculature to undergo further dilatation leads to ischemia.[110]

Endothelial injury is likely a major contributor to the pathophysiology of stroke in SCD. Chronic intravascular hemolysis is associated with a state of endothelial dysfunction characterized by reduced NO bioavailability and pro-oxidant and pro-inflammatory stress, resulting in a proliferative vasculopathy.[122] It has been proposed that pulmonary hypertension, priapism, leg ulceration, and stroke may be linked to the intensity of hemolysis.[122] A case series reported the association of obliterative central nervous system vasculopathy with pulmonary hypertension and a high rate of hemolysis in six patients with SCD.[123] Compared with 164 patients of similar age, those with newly diagnosed or clinically active cerebrovascular disease had significantly lower hemoglobin and higher lactate dehydrogenase levels.[123] Levels of plasma-free hemoglobin, lactate dehydrogenase, and bilirubin were found to be lower in patients receiving chronic transfusion as treatment for the secondary prevention of stroke.[124] A study of 74 adult SCD patients, 11 with a history of stroke, showed lower hemoglobin levels and higher levels of lactate dehydrogenase, soluble VCAM, and PlGF in patients with a history of stroke, although the difference in PlGF did not achieve statistical significance.[125]

The Stroke Prevention Trial in Sickle Cell Anemia (STOP) addressed the use of prophylactic RBC transfusion in sickle cell anemia patients aged between 2 and 16 years, at high risk for an initial stroke based on TCD ultrasonography.[126] The incidence

of stroke in patients randomized to receive prophylactic transfusions to reduce the hemoglobin S level below 30% (1 of 63) was significantly decreased compared to those patients who received standard care (11 of 67). The optimum duration of chronic transfusion necessary to provide protection from a cerebrovascular event remains uncertain. In the Optimizing Primary Stroke Prevention in Sickle Cell Anemia (STOP 2) trial, involving 79 patients with abnormal TCD before transfusion and who had received at least 30 months of RBC transfusion, the patients were randomized to stop or continue transfusions.[127] Of the 41 patients who were randomized to discontinue RBC transfusion, 16 reached the primary end-point events (14 patients reverted to high-risk TCD and 2 patients had ischemic strokes) compared to neither of these events in the 38 patients on continued RBC transfusions. Based on these results, discontinuation of chronic transfusion after 30 months cannot be recommended. There are, to date, no primary prevention trials of cerebrovascular events in older patients with SCD.

The recurrence rate of stroke in children with ischemic events not receiving subsequent treatment was found to be 66%.[128] Two case series demonstrated that long-term blood transfusion substantially reduced the risk of recurrence.[129,130] The current clinical practice is to begin a chronic transfusion program to maintain the HbS level below 30%. After 3 to 4 years a decrease in frequency of transfusion to allow the target value to rise to 50% may be considered.[131] Randomized clinical trials addressing the duration of transfusion for secondary stroke prevention have not been conducted. Children stopping transfusion after 5 to 12 years experienced a 50% recurrence rate.[132] The current standard of care is to continue a chronic transfusion program indefinitely. Recurrent events are observed for patients continuing on a chronic transfusion program. A retrospective multicenter study of 137 children with stroke maintained on chronic transfusion for at least 5 years reported a recurrence rate of 2.2 per 100 patient-years.[133]

Hydroxyurea therapy combined with phlebotomy has been evaluated in children as an alternative to chronic transfusion.[134] The stroke recurrence rate was 5.7 events per 100 patient-years with a mean duration of 3.5 years of follow-up. The protocol was modified to overlap hydroxyurea rather than abruptly discontinuing transfusions and resulted in a reduced recurrence rate of 3.6 events per 100 patient-years. A recent multicenter randomized controlled trial examining hydroxyurea and phlebotomy as an alternative to chronic RBC transfusion and iron chelation for the prevention of secondary stroke and reduction of transfusional iron overload was discontinued due to an increased rate of stroke in the hydroxyurea and phlebotomy arm and no differences in the level of iron overload.[135]

PULMONARY HYPERTENSION

Pulmonary hypertension is defined as a mean pulmonary artery pressure that exceeds 25 mm Hg at rest on right heart catheterization.[136] Multiple studies, based on echocardiographic assessment, suggest a high prevalence of pulmonary hypertension in adult patients with SCD, with prospective screening studies reporting a prevalence of approximately 30%.[137,138] More recently, a multicenter study found no evidence of pulmonary hypertension following right heart catheterization in 72 of 96 patients with elevated tricuspid regurgitant jet velocities on echocardiography, suggesting that Doppler echocardiography may overestimate the prevalence of this condition.[139] Although several studies report that children have a varying prevalence of elevated tricuspid regurgitant jet velocity on echocardiography,[140–143] the significance of this elevation remains uncertain.

Right heart catheterization of SCD patients with pulmonary hypertension shows evidence of a hyperdynamic state.[137,144] The mean pulmonary artery pressure in SCD patients with pulmonary hypertension is approximately 40 mm Hg, and pulmonary vascular resistance is approximately 250 dyn·sec·cm^{-5}.[145] The relatively low pulmonary vascular resistance is thought to be due to the high cardiac output that is characteristic of anemia. Up to 40% of patients have left ventricular end-diastolic pressures that are >15 mm Hg, indicating a component of left ventricular diastolic dysfunction.[146] Despite the relatively low pulmonary vascular resistance in SCD-associated pulmonary hypertension, these patients have an increased risk of death.[137,145,147,148] Follow-up from a prospective study of pulmonary hypertension in SCD[137] showed that patients with a tricuspid regurgitant jet velocity of 2.5 to 2.9 m/s had a rate ratio for death of 4.4 (95% CI, 1.6 to 12.2; $P < 0.001$) compared with those with a velocity of <2.5 m/s. Patients with a velocity of 3.0 m/s or more had a rate ratio of 10.6 (95% CI, 3.3 to 33.6; $P < 0.001$).[145] Furthermore, patients with both elevated tricuspid regurgitant jet velocity and echocardiographic evidence of diastolic dysfunction are at particularly high risk for death (relative risk ratio, 12.0; 95% CI, 3.8 to 38.1; $P < 0.001$).[149] It remains uncertain whether pulmonary hypertension, particularly at lower tricuspid regurgitant jet velocities, is causative of death or is simply a marker of a severe vasculopathy that predicts an increased risk of death.

The pathophysiology of pulmonary hypertension in SCD is likely multifactorial. The destruction of nitric oxide (NO) by cell-free plasma hemoglobin and reactive oxygen species may play a central role.[150–152] NO is normally produced by the endothelium and regulates basal vasodilator tone,[153,154] inhibits platelet and hemostatic activation,[155–157] inhibits transcriptional expression of NFκB–dependent adhesion molecules such as VCAM-1, intercellular adhesion molecule-1, and the selectins, and reduces superoxide levels through radical scavenging.[158] Furthermore, arginase, which converts L-arginine (the substrate for NO synthesis) to ornithine, is released following hemolysis,[159] and elevated arginase activity, with the resultant decrease in the arginine/ornithine ratio, is associated with pulmonary hypertension in SCD.[137,160] Recently, doubts have been raised about the role of NO depletion in the pathophysiology of pulmonary hypertension in SCD.[161] These authors argue that SCD-associated pulmonary hypertension is more likely due to pulmonary embolization or *in situ* thrombosis than by NO scavenging by elevated plasma hemoglobin. However, several mouse models of hemolysis, including the Berkeley sickle cell mouse[162] and the spherocytosis mouse,[163] have been shown to develop spontaneous pulmonary hypertension and right heart failure. In addition, pathologic evaluation in these models found no chronic thrombosis in the pulmonary vasculature, but rather a functional impairment in NO signaling driven by NO scavenging by plasma hemoglobin.[164] Increased levels of erythropoietin,[165] PlGF,[166–168] soluble fms-like tyrosine kinase-1,[169] platelet-derived growth factor-BB,[170] as well as interleukin-6 and possibly other cytokines[45,170] are associated with elevated tricuspid regurgitant jet velocity, while high vascular endothelial growth factor concentration has been reported to be associated with decreased odds of elevated tricuspid regurgitant velocity in SCD patients.[170]

Although pulmonary emboli and in situ thrombosis are often identified clinically or at autopsy in SCD patients with lung disease (171,172), large to medium sized pulmonary artery obstruction has been reported to be uncommon following ventilation-perfusion scintigraphy of SCD patients with elevated tricuspid regurgitant jet velocity.[173] Following three dimensional, contrast-enhanced, magnetic resonance angiography in six adult sickle cell anemia patients with an elevated tricuspid regurgitant jet velocity, five had no thrombi in their pulmonary arteries, with filling defects observed in only one patient with multiple hospitalizations for pain and acute chest syndrome.[174] Platelet activation marked by activated fibrinogen receptor is correlated with the tricuspid regurgitant jet velocity and may contribute to thrombosis and pulmonary hypertension in SCD.[79] Although several studies have shown correlations between the measures of hemolysis and the plasma markers of thrombin generation in SCD,[45,173,175] no significant differences in these markers have been identified in SCD patients with pulmonary hypertension diagnosed based on Doppler echocardiography compared with patients without this complication.[45,173] However, it is possible that plasma-based studies may not be sensitive enough to detect differences in hypercoagulability in these patients. Indeed, a recent study in patients with β thalassemia, a condition characterized by hypercoagulability,[176] much like SCD, showed evidence of hypercoagulability upon evaluation using whole blood thromboelastometry, while thrombin generation determined in platelet-poor plasma was not significantly different from that in healthy individuals.[177] There are, to date, no published studies evaluating hypercoagulability in SCD-associated pulmonary hypertension using thromboelastometry of whole blood. Multiple studies in SCD have shown an association between nephropathy and pulmonary hypertension diagnosed using Doppler echocardiography,[137,147,148] suggesting that both complications may share a similar pathophysiology. Soluble fms-like tyrosine kinase-1, a vascular endothelial growth factor receptor family member, by inducing endothelial dysfunction, may be a link between nephropathy and pulmonary hypertension in SCD.[178] Finally, although it is widely held that repeated episodes of the acute chest syndrome, with resulting chronic lung disease, causes pulmonary hypertension,[179] multiple studies show no association between pulmonary hypertension and history of acute chest syndrome.[137,147,148,180]

The symptoms of pulmonary hypertension in patients with SCD are nonspecific in the early stages.[181] With the association of pulmonary hypertension with increased mortality, many experts in the field recommend screening using Doppler echocardiography.[137,147] However, as Doppler echocardiography may overestimate the presence of pulmonary hypertension, a right heart catheterization is required to confirm the diagnosis and obtain additional hemodynamic measurements. The 6-minute walk test has been validated in SCD and correlates inversely with the tricuspid regurgitant jet velocity as well as the estimated pulmonary artery systolic pressure.[182] Evaluation and management of these patients should be performed by a hematologist and pulmonologist or cardiologist with expertise in pulmonary hypertension.

There is no consensus on the management of SCD-associated pulmonary hypertension. As hypoxia results in pulmonary vasoconstriction,[183] supplemental oxygen would be expected to be beneficial in hypoxic patients. Patients with breathing disorders during sleep should also be evaluated for nocturnal hypoxia. Although there are conflicting reports on the effect of hydroxyurea in this setting[137,147,165] and the role of blood transfusion remains uncertain, it is generally accepted that SCD treatment should be maximized. There are no studies of anticoagulation as treatment for pulmonary hypertension. However, as SCD is characterized by hypercoagulability, patients with pulmonary thromboembolic disease associated with pulmonary hypertension will likely benefit from anticoagulation. Chronic thromboembolic pulmonary hypertension in SCD has been treated surgically with success.[184] A short course of oral arginine given to 10 SCD patients with PHT produced a significant decline in the estimated pulmonary artery systolic pressures.[185] However, adequately powered, controlled studies are required to confirm the efficacy of arginine in these patients. Two randomized, double-blind, placebo-controlled studies of bosentan, a dual endothelin-1 receptor antagonist, in SCD patients with pulmonary arterial hypertension and pulmonary venous hypertension were discontinued for slow accrual.[186] More recently, a randomized, placebo-controlled, multicenter trial of sildenafil, a phosphodiesterase-5 inhibitor, as treatment for SCD-associated pulmonary hypertension was terminated prematurely due to an increased frequency of pain crises in the active treatment group compared with placebo.[187]

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CHAPTER

123 Thrombohemorrhagic Complications of Sepsis

MARCEL LEVI

Sepsis is a clinical syndrome that is caused by an infection, often associated with bacteremia and characterized by the presence of systemic signs and symptoms of inflammation.[1] When sepsis leads to organ failure, the term severe sepsis is used. The incidence of sepsis is estimated to be about 2.5 per 1,000 in the Western world and shows a rapid 8.7% annual increase over the last 20 years.[2] Total inhospital mortality of sepsis is around 20%, whereas severe sepsis is associated with mortality rates of 40% to 50%.[3] Treatment of sepsis is focused on adequate antibiotic therapy, source control, and appropriate supportive care and organ function replacement, if required.

Virtually all patients with sepsis have coagulation abnormalities. These abnormalities range from subtle activation of coagulation that can only be detected by sensitive markers for coagulation factor activation to somewhat stronger coagulation activation that may be detectable by a small decrease in platelet count and prolongation of global clotting times to fulminant disseminated intravascular coagulation (DIC), characterized by simultaneous widespread microvascular thrombosis and profuse bleeding.[4,5] Septic patients with severe DIC may present with manifest thromboembolic disease or clinically less apparent microvascular fibrin deposition and multiple organ dysfunction.[6,7,8] Alternatively, severe bleeding may be the leading symptom,[9] but quite often a patient with DIC has simultaneous thrombosis and bleeding. Bleeding is caused by consumption and subsequent exhaustion of coagulation proteins and platelets, due to the ongoing activation of the coagulation system.[10] In its most severe form, this combination may present as the Waterhouse-Friderichsen syndrome as seen during fulminant septicemia.[11]

INCIDENCE OF COAGULATION ABNORMALITIES IN SEPSIS

Clinically relevant coagulation abnormalities may occur in 50% to 70% of patients with sepsis, whereas about 35% of patients will meet the criteria for DIC.[1,12] In general, the incidence of thrombocytopenia (platelet count $<150 \times 10^9$/L) in critically ill medical patients is 35% to 50%.[13–15] Typically, the platelet count correlates with sepsis severity, decreasing during the first 4 days of illness.[16,17] The factors that contribute to thrombocytopenia are impaired platelet production and increased consumption, destruction, or sequestration. Although the high levels of platelet production-stimulating proinflammatory cytokines and thrombopoietin circulate,[18] marked hemophagocytosis by monocytes and macrophages[19] and thrombin-induced platelet activation and destruction at the endothelial cell surface explain the thrombocytopenia.[20] A prolonged global coagulation time (prothrombin time [PT] or activated partial thromboplastin time [aPTT]) occurs in 14% to 28% of patients.[21,22] Other coagulation abnormalities include high fibrin split products in virtually all patients with sepsis[23,24,25] and decreased levels of coagulation inhibitors, such as antithrombin-3 (AT3) and protein C in 90% of sepsis patients.[25,26]

RELEVANCE OF COAGULATION ABNORMALITIES IN PATIENTS WITH SEPSIS

There is strong evidence that activation of coagulation in concert with inflammatory activation can result in microvascular thrombosis, which contributes to multiple organ failure in patients with severe sepsis.[27] Autopsy findings include diffuse bleeding and microthrombi in small blood vessels and thrombi in midsize and larger arteries and veins, in association with ischemia and necrosis.[28–30] Further, experimental bacteremia or endotoxemia causes intra- and extravascular fibrin deposition in kidneys, lungs, liver, and brain, and interventions in these experimental models improve organ failure and, in some cases, mortality.[31–33,34,35] Last, clinical studies support the notion of coagulation as an important denominator of clinical outcome, DIC being an independent predictor of organ failure and mortality in patients with sepsis,[6,36] associated with mortality in 43%, as compared with 27% in patients without DIC.[37]

Thrombocytopenia in patients with sepsis is related to an increased risk of bleeding, those with a platelet count of $<50 \times 10^9$/L having a four- to fivefold higher risk than patients with a higher platelet count.[13,15] The overall risk of intracerebral bleeding in ICU patients with sepsis is relatively low (0.3% to 0.5%). However 88% of patients with intracranial bleeding have platelet counts $<100 \times 10^9$/L.[38] Regardless of the cause, thrombocytopenia is an independent predictor of ICU mortality in multivariate analyses, with a relative risk of 1.9 to 4.2.[13,15,39] In particular, a sustained thrombocytopenia for more than 4 days after ICU admission or a drop in platelet count of >50% during ICU stay is related to a four- to sixfold increase in mortality.[13,16] The platelet count is a stronger predictor for ICU mortality than composite scoring systems, such as the Acute Physiology and Chronic Health Evaluation (APACHE) II score or the Multiple Organ Dysfunction Score. Prolonged global coagulation tests to >1.5 times control also predict excessive bleeding and increased mortality.[21,22]

PATHOGENETIC PATHWAYS IN THE COAGULOPATHY OF SEPSIS

Various mechanisms at different sites in the hemostatic balance act simultaneously toward a procoagulant state (FIGURE 123.1), the most important mediators of which are cytokines.[40] There

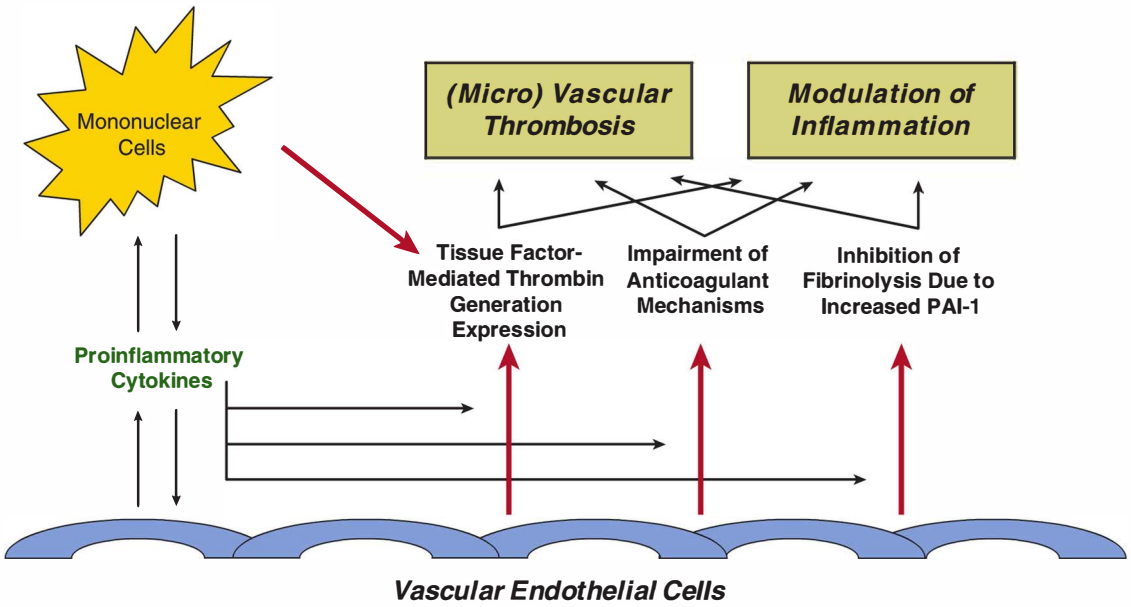

FIGURE 123.1 Schematic representation of pathogenetic pathways involved in the activation of coagulation in sepsis. During sepsis, both perturbed endothelial cells and activated mononuclear cells may produce proinflammatory cytokines that mediate coagulation activation. Activation of coagulation is initiated by TF expression on activated mononuclear cells and endothelial cells. In addition, downregulation of physiologic anticoagulant mechanisms and inhibition of fibrinolysis by endothelial cells will further promote intravascular fibrin deposition. PAI-1; plasminogen activator inhibitor type 1.

is extensive cross-talk between inflammation and coagulation,[41] and interestingly, systemic activation of coagulation and inflammation in sepsis can have some organ-specific manifestations.[42]

Initiation of Coagulation Activation

Systemic activation of coagulation in patients with sepsis has been considered to be a result of direct activation of the contact system by microorganisms or endotoxin.[43] However, it has become apparent that the principal initiator of thrombin generation in sepsis is tissue factor (TF), without significant change in markers reflecting activation of the contact system.[44,45] Furthermore, abrogation of the TF/factor VII(a) pathway by monoclonal antibodies completely inhibits thrombin generation in endotoxin-challenged chimpanzees and prevents DIC and mortality in baboons that are infused with *Escherichia coli*[33,46,47]

TF is a transmembrane 45 kDa protein, that is constitutively expressed on a number of cells throughout the body.[48] The majority of these cells are in tissues not in direct contact with blood, such as the adventitial layer of large blood vessels. However, TF gets into contact with blood upon disruption of the vascular integrity, or if cells present in the circulation are altered to express TF. In sepsis, circulating mononuclear cells, stimulated by proinflammatory cytokines, express TF, but other than in severe meningococcemia,[49] it is difficult to demonstrate *ex vivo* TF expression on monocytes of septic patients or experimental animals. However, low dose endotoxemia in healthy subjects results in a 125-fold increase in monocyte TF mRNA levels.[50] Another source of TF may be on polymorphonuclear cells,[51] although it is unlikely that these cells actually synthesize TF in substantial quantities.[52] Based on the transfer of TF from leukocytes to activated platelets in an *ex vivo* perfusion system, it is hypothesized that this "blood borne" TF is transferred between cells through microparticles derived from activated mononuclear cells.[53]

Platelets play a pivotal role in the pathogenesis of coagulation abnormalities in sepsis. Platelets can be activated directly by proinflammatory mediators, such as platelet activating factor.[54] Once thrombin forms, additional platelets are activated, which then accelerates further fibrin formation. The expression of P-selectin on the platelet membrane not only mediates the adherence of platelets to leukocytes and endothelial cells but also enhances the expression of TF on monocytes by nuclear factor kappa-B (NFκB) activation.[55] P-selectin is shed from the platelet membrane and soluble P-selectin levels are increased during systemic inflammation.[55]

Impairment of the Antithrombin, Protein C, and TFPI Anticoagulant Pathways in Sepsis

Activation of coagulation is regulated by three major anticoagulant pathways, namely, AT3, the protein C system, and TF pathway inhibitor (TFPI). During sepsis-induced activation of coagulation, the function of all three pathways can be impaired (FIGURE 123.2).

AT3 is a serine protease inhibitor and the main inhibitor of thrombin and factor Xa. During severe inflammatory responses, AT3 levels are markedly decreased due to consumption (as a result of ongoing thrombin generation), impaired synthesis (as a result of a negative acute phase response), and degradation by elastase from activated neutrophils.[56,57] A reduction in

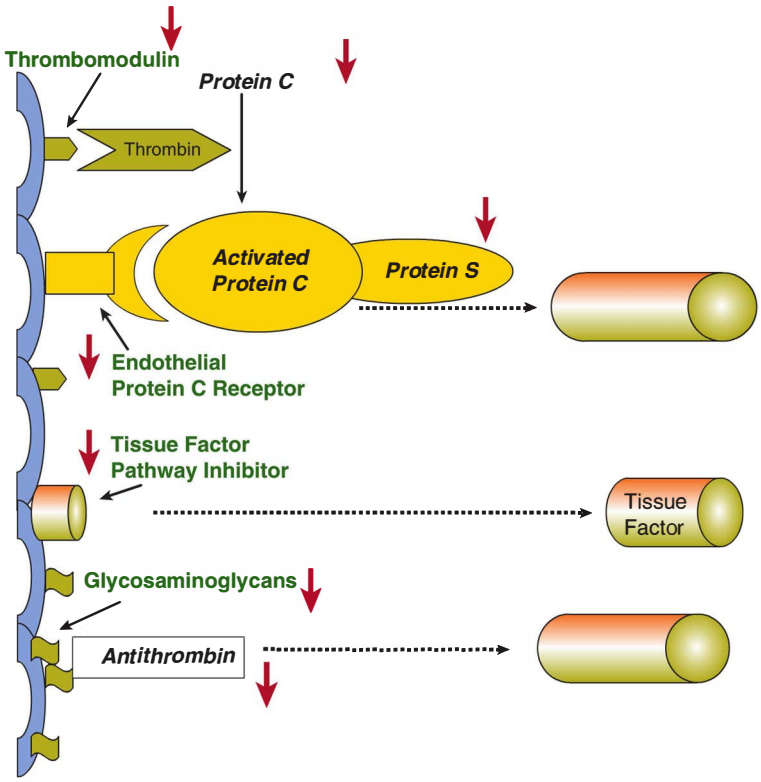

FIGURE 123.2 Schematic representation of the three important physiologic anticoagulant mechanisms and their point of impact in the coagulation system. In sepsis, these mechanisms are impaired by various mechanisms (*red arrows*). The protein C system is dysfunctional due to low levels of zymogen protein C, downregulation of TM and the EPCR, and low levels of free protein S due to acute phase-induced high levels of its binding protein, that is, C4b-binding protein. There is a relative insufficiency of the endothelial cell-associated TFPI. The antithrombin system is defective due to low levels of antithrombin and impaired glycosaminoglycan expression on perturbed endothelial cells.

glycosaminoglycan availability at the endothelial surface (due to the influence of proinflammatory cytokines on endothelial synthesis) also contributes to reduced AT3 function, since glycosaminoglycans act as physiologic heparin-like cofactors of AT3. Binding of glycosaminoglycans to AT3 induces a conformational change at the reactive center of the molecule, thereby rendering this protease inhibitor from a slow to a very efficient inhibitor.[58] Prospective clinical studies in patients at high risk for sepsis show that a marked decrease in levels of AT3 precedes the clinical manifestation of the infection, which may indicate that AT3 is involved in the early stages of coagulation activation during sepsis.[59]

Endothelial dysfunction is even more important in the impairment of the protein C system during inflammation. Under physiologic conditions, protein C is activated by thrombin bound to the endothelial cell membrane–associated thrombomodulin (TM). TM is a membrane protein with several domains, including a lectin-like domain, six epidermal growth factor (EGF)-like repeats, a transmembrane domain and a short cytoplasmatic tail.[60] The binding of thrombin to TM occurs at the site of the EGF-repeats.[61] This binding not only results in a 100-fold increase in the activation of protein C, but also blocks thrombin-mediated conversion of fibrinogen to fibrin and inhibits thrombin binding to other receptors on platelets and inflammatory cells. TM accelerates the activation of the plasma carboxypeptidase thrombin-activatable fibrinolysis inhibitor (TAFI), an important inhibitor of fibrinolysis.[62] Activated protein C (APC) regulates coagulation activation by proteolytic cleavage of the essential cofactors Va and VIIIa. Binding of protein C to the endothelial protein C receptor (EPCR) results in a fivefold augmentation of protein C activation by the TM–thrombin complex.[63] However, during sepsis, in addition to low levels of protein C due to impaired synthesis[56] and degradation by neutrophil elastase,[64] the protein C system is defective due to downregulation of TM at the endothelial surface, mediated by the proinflammatory cytokines tumor necrosis factor-alpha (TNF-α) and IL-1β.[65]

Observations in patients with severe Gram-negative septicemia confirm the downregulation of TM in vessels with and without thrombosis and impaired activation of protein C *in vivo*.[66] Low levels of free protein S (the cofactor of APC) may further compromise function of the protein C system. In plasma, 60% of protein S is complexed to a complement regulatory protein, C4b binding protein (C4bBP). Increased plasma levels of C4bBP as a consequence of the acute phase reaction in inflammatory diseases may result in a relative free protein S deficiency. Although the β-chain of C4bBP (which mainly governs the binding to protein S) is not much affected during the acute phase response,[67] C4bBP infusion increases organ dysfunction and mortality in septic baboons.[68] Animal experiments of severe inflammation-induced coagulation activation convincingly show that compromising the protein C system results in increased morbidity and mortality, whereas restoring an adequate function of APC improves survival and organ failure.[69] Interestingly, experiments in mice with a one-allele targeted deletion of the protein C gene (resulting in heterozygous protein C deficiency) have more severe DIC and organ dysfunction and a higher mortality than wild type littermates.[70]

A third inhibitory mechanism of thrombin generation involves TFPI, the main inhibitor of the TF–factor VIIa complex. TFPI is a complex multidomain Kunitz-type protease

inhibitor that binds to the TF–factor VIIa complex and factor Xa.[71] The TFPI–factor Xa complex binds to negatively charged membrane surfaces, which may increase the local concentration of TFPI at cellular sites and facilitate inhibition of membrane-bound TF–factor VIIa complex. The role of TFPI in the regulation of inflammation-induced coagulation activation is not completely clear. Administration of recombinant TFPI (r-TFPI) (and thereby achieving higher than physiologic plasma concentrations of TFPI) blocks inflammation-induced thrombin generation in humans, and pharmacologic doses of TFPI prevent mortality during systemic infection and inflammation.[31,72] However, the endogenous concentration of TFPI is presumably insufficient to regulate coagulation activation and downstream consequences during systemic inflammation, as confirmed in patients with sepsis.[73,74]

Inhibition of Endogenous Fibrinolysis in Sepsis

Experimental models indicate that at the time of maximal activation of coagulation in sepsis, the fibrinolytic system is largely shut off. The acute fibrinolytic response to inflammation is the release of plasminogen activators, in particular tissue-type plasminogen activator and urokinase-type plasminogen activator, from storage sites in vascular endothelial cells. However, this increase in plasminogen activation and subsequent plasmin generation is counteracted by a delayed but sustained increase in plasminogen activator inhibitor type 1 (PAI-1).[75,76] The resulting effect on fibrinolysis is complete inhibition and, as a consequence, inadequate fibrin removal, thereby contributing to microvascular thrombosis. Experiments in mice with targeted disruptions of genes encoding components of the plasminogen–plasmin system confirm that fibrinolysis plays a major role in inflammation-induced coagulation. Mice with a deficiency of plasminogen activators have more extensive fibrin deposition in organs when challenged with endotoxin, whereas PAI-1 knockout mice, in contrast to wild-type controls, have no microvascular thrombosis upon endotoxin.[77,78] A functional mutation in the PAI-1 gene, the 4G/5G polymorphism, not only influences the plasma levels of PAI-1, but also is linked to significantly higher plasma PAI-1 concentrations and an increased risk of death.[79] The PAI-1 polymorphism does not influence the risk of contracting meningitis, but probably increases the likelihood of developing septic shock from meningococcal infection.[80]

Regulatory Role of Cytokines in the Coagulopathy of Sepsis

Most proinflammatory cytokines activate coagulation *in vitro*. In patients with sepsis high levels of cytokines are detectable in the circulation, and experimental bacteremia or endotoxemia results in the transient enhancement of serum cytokine levels.[81] TNF is detectable first, followed by an increase in levels of interleukin-6 (IL-6) and IL-1 (Table 123.1). The hypothesis that TNF plays an important role in coagulation activation *in vivo* is strengthened by studies in which cancer patients or healthy human volunteers injected with purified recombinant TNF showed coagulation activation that is virtually identical to endotoxin-induced effects on coagulation.[45,82] However, blocking TNF activity completely abolished endotoxin-induced increase in TNF but coagulation activation was unaffected.[83,84]

Table 123.1

Most important cytokines governing the interaction between inflammation and coagulation

Proinflammatory Cytokines	
TNF-α	First cytokine to appear after endotoxin challenge, major regulator of impairment of physiologic anticoagulant pathways (e.g., downregulation of TM) and antifibrinolytic effect (upregulation of PAI-1)
IL-6	Most important driver of TF expression on mononuclear cells and endothelial cells
IL-1	Important effect on coagulation propagation and downregulation of coagulation inhibitors and fibrinolysis
Anti-Inflammatory Cytokines	
Soluble TNF receptors 1 and 2	Physiologic inhibitors of TNF-α and downstream effects
IL-10	Main anti-inflammatory cytokine, blocker of TNF-α and IL-6 effects

Also, in baboons infused with a lethal dose of *E. coli*, treatment with an anti-TNF antibody had little or no effect on fibrinogen consumption.[85] Moreover, clinical studies in septic patients with an anti-TNF monoclonal antibody did not show a beneficial effect of this treatment.[86]

These observations forced a reconsideration of the role of TNF as principal mediator of endotoxin-induced activation of coagulation. Subsequent studies of IL-6 showed that infusion of a monoclonal anti–IL-6 antibody resulted in the complete abrogation of endotoxin-induced activation of coagulation in chimpanzees.[87] In addition, studies in cancer patients receiving recombinant IL-6 indicated that thrombin is generated following the injection of this cytokine.[88] These data suggest that IL-6 rather than TNF is the relevant mediator for the procoagulant response in DIC.

While IL-1 is a potent agonist of TF expression *in vitro*, its role has not been clarified *in vivo*. Administration of an IL-1 receptor antagonist partly blocks the procoagulant response in a baboon sepsis model and treatment of patients with an IL-1 receptor inhibitor reduces thrombin generation.[89–91] However, most of the procoagulant changes after an endotoxin challenge occur well before IL-1 becomes detectable in the circulation, leaving a potential direct role of IL-1 in coagulation activation in sepsis an unresolved issue. Anti-inflammatory cytokines, such as IL-10, may modulate coagulation activation. Infusion of recombinant human IL-10 completely blocks the endotoxin-induced changes in coagulation and fibrinolysis in human volunteers,[92] but its relevance in the pathogenesis of the sepsis-associated coagulopathy remains to be established.

Cross-Talk Between Coagulation and Inflammation in Sepsis

Coagulation proteases and protease inhibitors not only interact with coagulation protein zymogens, but also with specific cell receptors to induce signaling pathways. In particular, protease interactions that affect inflammatory processes may be important in sepsis. Coagulation of whole blood *in vitro* results in a detectable expression of IL-1β mRNA in blood cells,[93] and thrombin markedly enhances endotoxin-induced IL-1 activity in culture supernatants of guinea pig macrophages.[94] Similarly, clotting blood produces IL-8 *in vitro*.[95] Factor Xa, thrombin, and fibrin can also activate endothelial cells, eliciting the synthesis of IL-6 and/or IL-8.[96,97] The most important mechanisms by which coagulation proteases influence inflammation is by binding to the so-called protease-activated receptors (PARs), of which four types (PAR 1 to 4) have been identified, all belonging to the family of transmembrane domain, G-protein–coupled receptors.[98] A peculiar feature of PARs (in contrast to most other receptors of the superfamily) is that they serve as their own ligand. Proteolytic cleavage by an activated coagulation factor leads to exposure of a neoamino-terminus that activates the same receptor, initiating transmembrane signaling. PARs 1, 3, and 4 are thrombin receptors whereas PAR-2 (and PAR-1) can be activated by the TF–factor VIIa complex and factor Xa. PARs are localized in the vasculature on endothelial cells, mononuclear cells, platelets, fibroblasts, and smooth muscle cells.[98] Binding of thrombin and TF–factor VIIa to its receptor in macrophages affects neutrophil infiltration and proinflammatory cytokine (TNF-α, IL-1β) expression.[99,100] Administration of recombinant factor VIIa to healthy human subjects causes a three- to fourfold rise in plasma levels of IL-6 and IL-8.[101]

Fibrinogen and fibrin can directly stimulate expression of proinflammatory cytokines (such as TNF-α and IL-1β) on mononuclear cells and induce production of chemokines (including IL-8 and MCP-1) by endothelial cells and fibroblasts.[102] The effects of fibrin(ogen) on mononuclear cells are at least in part mediated by Toll-like receptor-4, which is also the receptor of endotoxin.[103] Fibrinogen-deficient mice indeed show inhibition of macrophage adhesion and less thrombin-mediated cytokine production *in vivo*.[102]

There is also considerable cross-talk between physiologic anticoagulant pathways and inflammatory mediators. AT3 can act as a mediator of inflammation by direct binding to neutrophils, thereby attenuating cytokine and chemokine receptor expression.[104] The protein C system has an important function in modulating inflammation.[60,105] APC inhibits endotoxin-induced production of TNF-α, IL-1β, IL-6, and IL-8 by cultured monocytes/macrophages,[106,107] abrogates endotoxin-induced cytokine release and leukocyte activation in rats *in vivo*,[108] and blocking the protein C pathway by monoclonal antibody in septic baboons exacerbates the inflammatory response, as evidenced by increased levels of proinflammatory cytokines.[109,110] Conversely, administration of APC ameliorates inflammatory activation, for example, APC improves organ function and survival in an experimental *E. coli* sepsis model in baboons, and improves organ function in endotoxin-induced shock and lung and kidney injury in rats.[69,105] Mice with a one-allele targeted disruption of the protein C gene (resulting in heterozygous protein C deficiency) not only have a more severe coagulation response to endotoxin but also demonstrate higher levels of circulating proinflammatory cytokines.[70] Binding of APC to the EPCR affects gene expression profiles of cells by inhibiting endotoxin-induced calcium fluxes, by blocking NFκB nuclear translocation, which is a prerequisite for increases in proinflammatory cytokines and adhesion molecules,[111,112] and by activation of PAR-1.[113] Like APC, EPCR itself may have anti-inflammatory properties. Soluble EPCR, the extracellular domain of the cell-associated EPCR shed from the cell surface by the action of an inducible metalloproteinase,[114] in complex with proteinase 3, binds to the adhesion integrin macrophage 1 antigen[115]; its inhibition with a monoclonal antibody aggravates both the coagulation and the inflammatory responses to *E. coli* infusion.[110] Lastly, binding of APC to the EPCR inhibits endothelial cell apoptosis.[116,117]

TM also exerts significant anti-inflammatory activity via TAFI activation.[62] TAFI is a primary enzyme responsible for inactivation of complement factor C5a[118] a process that likely protects against complement-mediated injury in the microvasculature. The lectin-like domain of TM has a function in inhibiting leucocyte adhesion to activated endothelium.[119]

DIAGNOSTIC APPROACH TO COAGULATION ABNORMALITIES IN SEPSIS

Apart from DIC there are several other reasons for coagulation abnormalities in patients with sepsis (Table 123.2). Although thrombocytopenia is common in patients with severe sepsis, this may also be caused by other (sometimes simultaneously occurring) diseases, such as immune thrombocytopenia, medication-induced bone marrow depression, heparin-induced thrombocytopenia, or thrombotic microangiopathies.[14,120] Laboratory tests can be helpful in differentiating the coagulopathy in sepsis from other hemostatic disorders, but since such conditions may occur simultaneously, this differentiation is not always simple.[121,122]

Tests for Intravascular Fibrin Formation and Fibrin Degradation Products

According to the current understanding of sepsis-associated coagulation abnormalities the determination of soluble fibrin in plasma appears to be crucial.[123–126] Indeed, initial clinical studies indicate that if the concentration of soluble fibrin has increased above a defined threshold, a diagnosis of DIC can be made.[23,123,125,127] Most of the clinical studies show a sensitivity of 90% to 100% for the diagnosis of DIC but a rather low specificity,[128] and there is a wide discordance among various assays for soluble fibrin.[129] Since soluble fibrin in plasma can only be generated intravascularly, this test will not be influenced by extravascular fibrin formation.

Fibrin degradation products (FDPs) may be detected by specific ELISA or by latex agglutination assays, allowing rapid and bedside determination in emergency cases.[130] None of the available assays for FDPs discriminates between degradation products of cross-linked fibrin and fibrinogen degradation, which may cause spuriously high results.[131,132] The specificity of high levels of FDPs is therefore limited and many other conditions, such as trauma, recent surgery, inflammation, or venous thromboembolism, are associated with elevated FDPs. Tests that detect

Table 123.2

Routine laboratory value abnormalities in patients with sepsis, due to DIC or other causes

Test	Abnormality	Causes Other than DIC Contributing to Test Result
Platelet count	Decreased	Sepsis, impaired production, major blood loss, hypersplenism hemophagocytosis, immune thrombocytopenia, microangiopathy, heparin-induced thrombocytopenia
PT	Prolonged	Vitamin K deficiency, liver failure, major blood loss
aPTT	Prolonged	Liver failure, heparin treatment, major blood loss
Coagulation factors II, X, IX	Decreased	Liver failure, vitamin K deficiency
Fibrinogen	Decreased or false	Acute phase response may positively affect normal levels and may mask ongoing consumption
FDPs	Elevated	Surgery, trauma, infection, hematoma
Antithrombin	Decreased	Liver failure, capillary leakage
Proteins C and S	Decreased	Liver failure, vitamin K deficiency
Plasminogen activators	Increased	May also be due to plasminogen activators complexed to PAI-1
PAI-1	Increased	Liver failure

DIC, disseminated intravascular coagulation; aPTT, activated partial thromboplastin time; PAI-1, plasminogen activator inhibitor type 1.

an epitope related to plasmin-degraded cross-linked γ-chain, resulting in fragment D-dimer, better differentiate degradation of cross-linked fibrin from fibrinogen or fibrinogen degradation products.[133] D-dimer levels are high in patients with DIC, but also poorly distinguish patients with DIC from patients with venous thromboembolism, recent surgery, or inflammatory conditions.[130,134]

Markers for Thrombin Generation and Coagulation Activation

Activation peptides that are released upon the conversion of a coagulation factor zymogen to an active protease are sensitive markers for coagulation activation. Examples of such markers are prothrombin activation fragment F1 + 2 (F1 + 2), and the activation peptides of factors IX and X.[135–137] Elevated plasma concentrations of thrombin–antithrombin complexes reflect the increased generation of thrombin, and thrombin-mediated conversion of fibrinogen to fibrin can be monitored by increased levels of fibrinopeptide-A.[138,139] All these markers are increased in most patients with sepsis and their high sensitivity may be helpful in detecting even low-grade activation of coagulation. The specificity of coagulation factor activation is limited, since many conditions may result in elevated plasma levels. The most important disadvantage of these tests may be that their use is limited to specialized coagulation laboratories, so their practical use in clinical medicine is limited.

Platelet Count and Coagulation Factors in Patients with Sepsis

The platelet count in sepsis is inversely correlated with markers of thrombin generation, since thrombin-induced platelet aggregation is for a large part responsible for platelet consumption.[120] A progressive drop in platelet count, determined at intervals of about 4 hours, may indicate the generation of thrombin causing intravascular platelet aggregation. Low levels of coagulation factors in patients with sepsis result from consumption, impaired synthesis, and loss via massive bleeding.[140] Although the accuracy of one-stage clotting assays in DIC may be influenced by activated coagulation factors in plasma, the level of coagulation factors correlates with DIC severity.[140] Plasma levels of factor VIII may be paradoxically increased in patients with DIC, due to massive release of von Willebrand factors from the endothelium in combination with an acute phase increase of factor VIII.[141] Measurement of fibrinogen has been widely advocated as a useful tool for the diagnosis of DIC but it is not useful for the diagnosis of DIC.[10,142] Fibrinogen is an acute-phase reactant and despite ongoing consumption, plasma levels can remain within the normal range. In a consecutive series of patients the sensitivity of a low fibrinogen level for the diagnosis of DIC was only 28%, and hypofibrinogenemia was detected in very severe cases of DIC only. Sequential measurements of fibrinogen might be more useful.

AT3 is the principal inhibitor of thrombin and may be exhausted during continuous thrombin generation; plasma levels of AT3 are strong predictors for survival in patients with sepsis and DIC.[59] In patients with meningococcal septicemia, very low plasma levels of protein C may play a pivotal role in the occurrence of purpura fulminans,[143,144] and the plasma level may be a strong predictor for outcome in DIC patients.[145]

Point of Care Tests

Thrombelastography (TEG) is a method that has been developed decades ago and provides an overall picture of *ex vivo* coagulation. Modern techniques, such as rotational thrombelastography, enable bedside performance of this test and is popular

in acute care settings.[146] The theoretical advantage of TEG over conventional coagulation assays is that it provides an idea of platelet function as well as fibrinolytic activity. Hyper- and hypocoagulability as demonstrated with TEG correlates with clinically relevant morbidity and mortality,[147,148] although its superiority over conventional tests has not unequivocally been established.[149] There are no systematic studies on the diagnostic accuracy of TEG for the diagnosis of DIC.

A new method that has proved sensitive and specific for hypercoagulability in critically ill patients is the partial thromboplastin test biphasic waveform analysis.[150,151] This test, which requires specific instrumentation, detects precipitates of very-low-density lipoprotein and C-reactive protein complexes that appear very early in DIC. When such complexes first appear in the plasma of individuals with diseases known to predispose to hypercoagulability, they confer a >90% sensitivity and specificity for subsequent development of DIC and fatal outcome.[150]

Composite Scoring Systems

A simple scoring system has been developed for the diagnosis of DIC (see Chapter 98), calculated with routinely-available laboratory tests.[152] Tentatively, a score of 5 or more is compatible with DIC, whereas a score of <5 may be indicative but is *not* affirmative for nonovert DIC. Prospective studies show that the sensitivity of the DIC score is 93%, and the specificity is 98%.[153,154] The severity of DIC according to this and other scoring systems is related to the mortality in patients with sepsis (FIGURE 123.3).[37,155] Linking prognostic determinants from critical care measurement scores such as APACHE-II to DIC scores is an important means to assess prognosis in critically ill patients. In a prospective study in 840 patients, continuation of coagulopathy during the first calendar day correlated with development of new organ failure and 28-day mortality in patients with severe sepsis.[156]

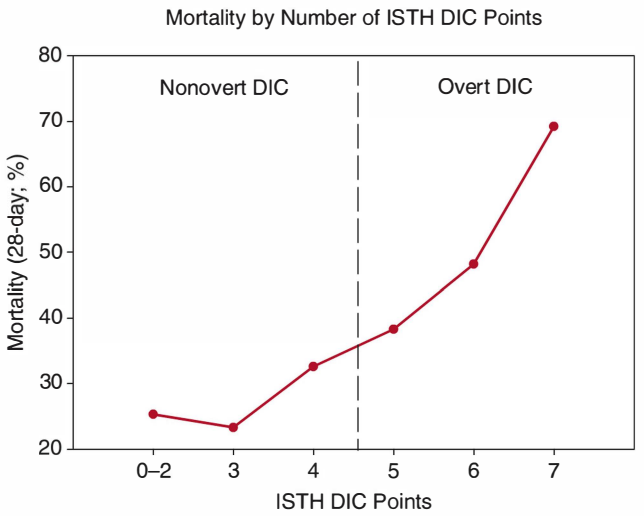

FIGURE 123.3 Number of points on the ISTH DIC score and 28-day mortality in patients with severe sepsis. Data were derived from the placebo group (n = 840) in the Prowess trial on the efficacy of APC in sepsis.

SUPPORTIVE TREATMENT OF COAGULATION ABNORMALITIES IN SEPSIS

The keystone of the treatment of hemostatic abnormalities in patients with sepsis is appropriate antibiotics to control the infectious source. However, in many cases, additional treatment aimed at circulatory and respiratory support and replacement of organ function is required, and coagulation abnormalities may proceed even after proper treatment has been initiated.

Plasma and Platelet Substitution Therapy

Plasma or platelet substitution therapy should not be instituted on the basis of laboratory results alone; it is indicated only in patients with active bleeding and in those requiring an invasive procedure or who are at risk for bleeding complications.[157] The suggestion that administration of blood components might "add fuel to the fire" has in fact never been proven in clinical or experimental studies. The presumed efficacy of treatment with plasma, fibrinogen, cryoprecipitate, or platelets is not based on randomized controlled trials but appears to be rational therapy in patients with active bleeding and who have significant depletion of hemostatic factors.[158]

It may be necessary to use large volumes of plasma to correct the coagulation defect. Coagulation factor concentrates, such as prothrombin complex concentrate, may overcome this obstacle, but these compounds may lack essential factors, such as factor V. Moreover, in older literature caution is advocated with the use of prothrombin complex concentrates in DIC, since it may worsen the coagulopathy due to small traces of activated factors in the concentrate. It is, however, not clear whether this is still relevant for the concentrates that are currently in use. Specific deficiencies in coagulation factors, such as fibrinogen, may be corrected by administration of purified coagulation factor concentrates.

Anticoagulants

Experimental studies have shown that heparin can at least partly inhibit the activation of coagulation in sepsis.[159] Uncontrolled case series in patients with sepsis and DIC have claimed to be successful. However, a beneficial effect of heparin on clinically important outcome events in patients with DIC has never been demonstrated in controlled clinical trials,[160] and the safety of heparin is debatable in DIC patients who are prone to bleeding. Therapeutic doses of heparin are indicated in patients with clinically overt thromboembolism or extensive fibrin deposition, like purpura fulminans or acral ischemia. Patients with sepsis may benefit from prophylaxis to prevent venous thromboembolism, which may not be achieved with standard low-dose subcutaneous heparin.[161] Theoretically, the most logical anticoagulant agent to use in DIC is directed against TF activity. Potential agents include r-TFPI, inactivated factor VIIa, and recombinant NAPc2, a potent and specific inhibitor of the ternary complex between TF/factor VIIa and factor Xa.[162] Phase II trials of r-TFPI in patients with sepsis showed promising results[163] but a recently completed phase III trial did not show an overall survival benefit in patients that were treated with r-TFPI.[164]

Restoration of Anticoagulant Pathways

In view of the deficient state of physiologic anticoagulant pathways in patients with sepsis, restoration of these inhibitors may be a rational approach.[165] Since AT3 is one of the most important

physiologic inhibitors of coagulation, AT3 concentrates have been studied intensively in patients with DIC. All trials show some beneficial effect in laboratory parameters, shortening of the duration of DIC, or even improvement in organ function,[10] and small trials show a modest (nonstatistically significant) reduction in mortality in AT3-treated patients.[166–168] A large-scale, multicenter, randomized controlled trial to directly address this issue showed no significant reduction in mortality of patients with sepsis who were treated with AT3 concentrate,[169] although *post hoc* subgroup analyses indicated some benefit in patients who did not receive concomitant heparin and in those patients with the most severe coagulopathy.[170]

Based on the notion that depression of the protein C system may contribute to the pathophysiology of DIC, supplementation of APC might be beneficial.[171] A beneficial effect of recombinant human APC was demonstrated in two randomized, controlled trials. In a dose-ranging clinical trial, 131 patients with sepsis[172] received APC by continuous infusion at doses of 12 to 30 μg/kg/h. Based on D-dimer plasma levels the optimal dose of recombinant human APC was 24 μg/kg/h, and there was a trend (not statistically significant) toward a 40% lower mortality in patients receiving higher doses of APC. A subsequent phase III trial of APC concentrate in patients with sepsis was prematurely stopped because of efficacy in reducing mortality.[25] All-cause mortality at 28 days was 24.7% in the APC group versus 30.8% in the control group (19.4% relative risk reduction). APC caused an amelioration of coagulation abnormalities and APC-treated patients had less organ failure.[173] Interestingly, a recent analysis demonstrated that patients who classify for having overt DIC (see Table 123.2) had a relatively greater benefit of APC treatment than patients who did not have overt DIC[37] (relative risk reduction in mortality 38% versus 18% in patients with sepsis who did not have DIC). Recombinant human-APC has been licensed for treatment of patients with severe sepsis and two or more organ failures. The most frequently encountered adverse effect of APC is bleeding, the incidence of major bleeding during the infusion period being 2.4% as compared with 1.0% in the control group ($p = 0.02$), and 3.5% in the APC group and 2.0% in the placebo group during the 28-day study period ($p = 0.06$).[25] Most bleeding episodes were procedure-related or occurred in patients with a severely deranged coagulation system (aPTT > 120 seconds or PT [INR] > 3.0), and spontaneous bleeding was rare. Of note, severe thrombocytopenia (i.e., platelet count $<50 \times 10^9$/L) was an exclusion criterion for the trial, but patients with lower platelet counts had relatively more benefit of APC than patients with higher platelet counts.

In an extensive analysis recombinant APC was beneficial in virtually all subgroups defined, including age, type, and site of infection.[174] Efficacy was most prominent in patients with a relatively high disease severity. To prospectively analyze this issue, the ADDRESS trial was performed in 2,640 patients with sepsis and a relatively low disease severity (defined as a single sepsis-associated organ failure or an APACHE II score below 25).[175] The initial sample size of this trial was 11,400 patients; however, the trial was prematurely stopped after a second interim analysis according to the predefined futility rules. The 28-day mortality rates were 18.5% in the APC group and 17.0% in the placebo group ($p = 0.34$). Disappointingly, in patients with two or more organ failures, the 28-day mortality rate was not lower in patients treated with APC as compared with those that received placebo (20.7% vs. 21.9%, not significant), although a direct comparison with PROWESS is not possible due to many differences in the patient population between the two studies.[176] Treatment with APC seems not to be indicated in patients with sepsis and a relatively low disease severity.

Patients treated with the combination of APC and heparin appeared to have a higher mortality, but as the decision to administer heparin was not random, this result may have been biased. The XPRESS trial enrolled 1,994 patients with severe sepsis (and two or more organ failures), who received APC and were subsequently randomized to unfractionated heparin, low molecular weight heparin, or placebo.[177] The results of the trial did not meet the noninferiority assumption, but showed a small survival advantage in the group of patients treated with APC and heparin (28.3% vs. 31.9% in the group treated with APC and placebo, $p = 0.08$). Interestingly, a subgroup analysis demonstrated that the difference between the two groups was mainly driven by the subset of patients that were already receiving heparin before entering the trial. The mechanism underlying this result is not entirely clear, however, it has been suggested that cessation of heparin is associated with a rebound hypercoagulability.[178] The conclusion from this trial was that adding prophylactic heparin to treatment with APC is not harmful, but that interruption of heparin in patients with severe sepsis should be done with caution or prevented.

There is debate on the role of recombinant APC in the treatment of sepsis. Clearly, the pivotal phase III clinical trial (PROWESS) showed a reduction in 28-day mortality and this study formed the basis for the approval of this therapy by regulatory agencies. Consequently, the use of APC in patients with severe sepsis and multiple organ failure, and in the absence of major risk factors for bleeding, has been advocated in guidelines for the treatment of sepsis.[179] However, meta-analyses of published literature conclude that the basis for treatment with APC, even in patients with a high disease severity, is not very strong or even insufficient.[180,181] The series of negative trials in specific populations of patients with severe sepsis has added to the skepticism regarding the use of APC. In view of these equivocal results many clinicians believe that one placebo-controlled trial is not enough to support the use of this agent in patients with severe sepsis, whereas—in contrast—others believe it is ethically not justified to withhold APC in patients with severe sepsis and multiple organ failure and to expose these patients to placebo. On top of that, there is uncertainty regarding the bleeding risk of APC in patients with severe sepsis. The bleeding rate in the clinical trials seems to be acceptable,[182] but it may be that in the "real world" the risk of bleeding, including intracranial bleeding, is higher.[183,184] Taken together, there is quite some uncertainty and doubt surrounding the exact place of APC in patients with severe sepsis and this equipoise has convinced the manufacturer of this agent to start a new placebo-controlled trial in patients with severe sepsis and septic shock.[185] Certainly, the result of this trial will be helpful to more precisely assess the effectiveness and safety of APC in the treatment of patients with severe sepsis.

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CHAPTER

124

Hemorrhagic Fevers: Epidemiology, Pathophysiology, and Clinical Features

L. GAYANI TILLEKERATNE, JENNIFER COHN, AND HILLARY DUNLEVY

HEMORRHAGIC FEVERS

Background

Hemorrhagic fevers (HF) refer to illnesses largely caused by several distinct classes of viruses. The syndrome is characterized by fevers, multisystem illness, vascular permeability, and coagulopathy, which results in hemorrhage that ranges from mild to life-threatening in severity.[1]

Four classes of viruses have been implicated in most cases of HF: the Arenaviruses, Filoviruses, Bunyaviruses, and Flaviviruses. These are all RNA viruses with a lipid envelope and are transmitted to humans either directly through animal hosts or through arthropod vectors.[1] The epidemiology of disease in humans is thus largely determined by the geographical areas of the animal reservoir and vectors (FIGURES 124.1 and 124.2).[2] Rodents and domesticated animals are the main reservoirs for viruses causing viral HF. Rarely, nonviral pathogens such as scrub typhus, caused by *Rickettsia tsutsugamushi*, can result in HF.[1]

Human cases of HF occur when vector arthropods bite humans or when humans come into contact with urine, blood, or other body excretions from infected vertebrates.[1] Ebola, Marburg, Lassa, and Crimean–Congo hemorrhagic fever viruses can be transmitted between humans.[2]

Burden of Disease and Emerging Infections

Although there are accounts of HF-like syndromes since at least 1,545, the epidemiology of many HF is changing and new viruses are emerging.[3] In 2009, heightened surveillance of acute febrile illness in China led to the identification of a novel Bunyavirus.[4] The incidence of several HF has recently increased dramatically and spread to novel areas due to changes in global climate, the increased incidence of global travel, and the rise of urbanization.[2] The past 50 years have seen a 30-fold increase in cases of dengue virus, and there has been a sharp increase in both the incidence of dengue fever and dengue hemorrhagic fever/dengue shock syndrome since 2000. This is largely attributed to the geographic expansion of the vector, *Aedes aegypti* mosquitoes, leading to increased co-circulation of all four serotypes of dengue.[5] Expansion of the vector and increasing urbanization with crowded housing and poor sanitation have also increased the risk for yellow fever (YF) outbreaks.[6]

Scope of Clinical Illness

The symptoms of HF vary by type of pathogen and the immune system of the host. General initial symptoms include fever, headache, myalgia, and malaise. Patients with HF may exhibit signs of mild hemorrhage including petechiae and gingival bleeding or more severe hemorrhage including gastrointestinal bleeding. Considering the differential diagnosis in the initial presentation of HF is important, as it includes treatable illnesses, including malaria, typhoid, rickettsial, and other bacterial infections.

In general, the viral HF has a high case fatality rate. However, even with severe hemorrhage, patients do not usually die from blood loss. Death is often related to shock from a capillary leak syndrome and nervous system malfunction including coma and seizures.[1] Laboratory findings often show thrombocytopenia, transaminitis, a coagulopathy, and/or evidence of disseminated intravascular coagulation (DIC).

Travel history and incubation period of particular HF must be considered when evaluating a patient in the United States with a febrile multisystem illness and a bleeding diathesis. An infectious disease consultant and hospital infection control team should be immediately notified of any suspected case of HF.

Diagnosis and Treatment

Given that many of these diseases occur in resource poor settings, diagnosis is often clinical. General lab tests may reveal leukopenia with predominant lymphopenia, thrombocytopenia, and elevated transaminase. During the early viremic period of the illness, PCR may be useful in detecting the virus.[7] Paired acute and convalescent serology is also used.[8,9] Treatment is generally supportive, and those with hemorrhagic complication deserve ICU level care.[10] For those without clinically significant bleeding, replacement of blood products (particularly platelets) and clotting factors is not indicated but should be pursued in those with significant bleeding complications. Ribavirin has been shown to have benefit in human or animal models, the Arenaviridae and Bunyaviridae.[11] Treatment with hyperimmune plasma has been shown to have some benefit in Argentinean and Bolivian HF in case studies but needs further study.[12]

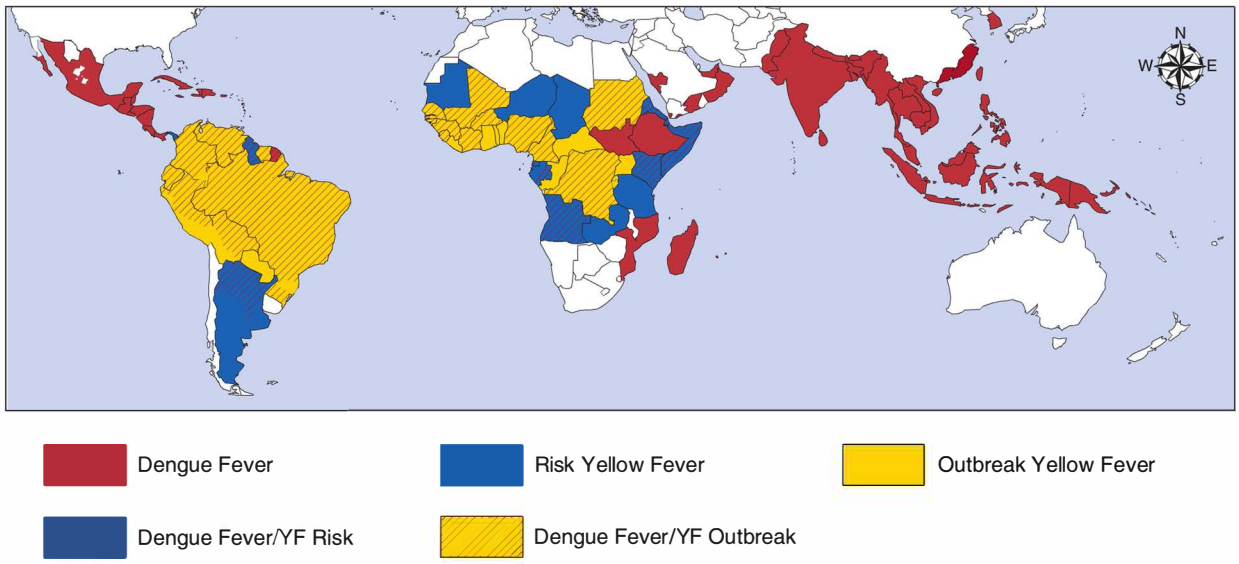

FIGURE 124.1 Distribution of yellow fever (YF) and dengue fever. (Courtesy of Colleen Kiley.)

Pathogenesis

The exact pathogenesis is incompletely understood. Initial local replication of virus in dendritic cells and macrophages progresses to viremia and widespread viral replication, which causes an intense cytokine response.[13,14] Cells, particularly macrophages, infected early in the course of disease produce an inflammatory response that is characterized by a variety of cytokines including IL-6, IL-8, reactive oxygen species, and nitrogen oxide. These cytokines may trigger immunosuppression by inducing lymphopenia through promotion of apoptosis as well as hypotension through increased vascular permeability and vasodilation.[15,16] In addition to vascular permeability and vasodilation, infection of and damage to the adrenal glands promote relative adrenal insufficiency and shock. Although

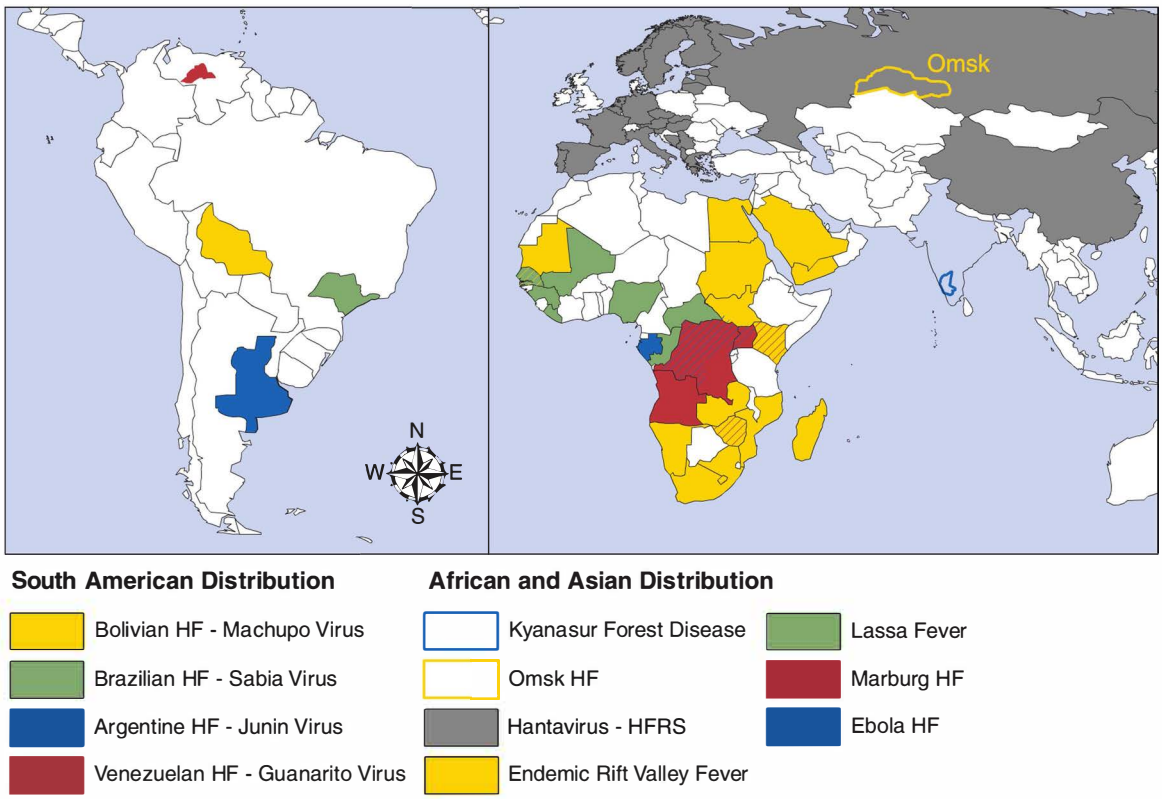

FIGURE 124.2 Distribution of other important hemorrhagic fevers (HF). (Courtesy of Colleen Kiley.)

cytokines may trigger widespread tissue damage, particularly of the liver, infection and direct injury to endothelial cells seem to be a late event in the disease.[17]

Several different mechanisms lead to hemorrhage including coagulopathy, vascular injury, vasodilation, and permeability of blood vessels.[18,19] Bleeding is frequently mucosal and includes gastrointestinal bleeding. Cytokines, in particular IL-6, are associated with coagulopathy that may be partially driven by increased expression of tissue factor.[12] Rapid decrease in protein C has also been noted in Argentine hemorrhagic fever. Additionally, most HF can cause liver damage through direct viral mechanisms or through cytokine-induced injury, with resulting alteration in coagulation factors. Thrombocytopenia and deranged platelet function may be related to maturation arrest of megakaryocytes.[12] DIC is most commonly noted in Rift Valley fever (RVF) and the Filoviruses but may be seen in late stages of any of the HF and represents a poor prognostic factor.[20]

Important Hemorrhagic Fever Viruses

Flaviviridae

The genus Flavivirus contains two of the most prevalent HF: YF and dengue. Omsk hemorrhagic fever virus and Kyasanur Forest virus can also cause severe disease in their endemic regions (Table 124.1).

Yellow Fever Virus

YF occurs in tropical and subtropical areas of Central and South America as well as sub-Saharan Africa, with the vast majority of cases occurring in Africa (see FIGURE 124.1).[21] YF is characterized by sporadic outbreaks, and annual incidence may vary from a few thousand to hundreds of thousands of cases each year. Mosquitoes transmit the virus either from an infected monkey or another human.

After an infective mosquito bite, the incubation period lasts 3 to 6 days, with viral replication at the site of inoculation spreading to the lymphatics, liver, and spleen.[22] The patient subsequently becomes viremic and infective for several days. Approximately 50% of patients are asymptomatic. For those who develop symptoms, viremia brings on a flu-like illness that may be associated with conjunctival injection, flushed skin, and relative bradycardia while febrile.

While most YF illness does not progress to the hemorrhagic form, in those that do, there is a 20% to 60% mortality.[23] After the initial flu-like illness, individuals may experience an afebrile period lasting 1 to 2 days after which the fever returns accompanied by myalgias, abdominal pain, vomiting, and somnolence followed by jaundice and bleeding diathesis leading to gastrointestinal bleeding and the coffee-ground emesis that typically accompanies hemorrhagic YF. The liver may undergo extensive noncoagulative necrosis leading to the loss of synthetic function. Lab tests proteinuria, transaminitis (AST greater than ALT), anemia, and leukopenia.

Isolation from mosquitoes is important to prevent vector transmission, especially during the viremic period. Although no specific treatment exists, several potential antiviral agents and YF-specific hyperimmune γ-globulin deserve further study.[24] Immunity is conferred either through natural infection or through the 17D live attenuated vaccine. YF vaccine is effective and leads to long-lasting (at least 10 years) immunity in over 95% of recipients.[25]

Dengue Virus

Dengue occurs throughout the world in tropical and subtropical regions (see FIGURE 124.1). The World Health Organization states that 2.5 billion people are now at risk for the disease and estimates the annual incidence to be 50 million people.[26] There are four virus species of dengue (dengue-1 to dengue-4) that have incomplete cross-immunity and an individual who has

Table 124.1

Other hemorrhagic fever viruses of importance

Disease	Virus	Principal Vectors	Geographic Area
Arenaviruses			
Argentine hemorrhagic fever	Junin virus	Drylands vesper mouse	Argentina
Bolivian hemorrhagic fever	Machupo virus	Large vesper mouse	Bolivia
Brazilian hemorrhagic fever	Sabia virus	Unknown	Brazil
Venezuelan hemorrhagic fever	Guanarito virus	Short-tailed cane mouse	Venezuela
Bunyaviruses			
Hanta fever	Hantaan, Dobrava, Puumala, and Seoul viruses	Rodents	Asia, Europe, North and South America
Flaviviruses			
Alkhurma hemorrhagic fever	Alkhurma virus	Possibly ticks	Saudi Arabia
Kyasanur Forest disease	Kyasanur Forest virus	Ticks	South Asia, China
Omsk hemorrhagic fever	Omsk hemorrhagic fever virus	Ticks	Forest-steppe regions of northern Russia

been infected with one species is at risk for subsequent dengue infections with other species.

After an infective bite from an *A. aegypti* mosquito, a 3 to 7 day incubation is usually followed by a self-limited, flu-like illness with prominent myalgias (hence the name breakbone fever), fatigue, diffuse and blanching rash, vomiting, and retro-orbital headache. Minor bleeding and few scattered petechiae may occur even with uncomplicated dengue. Mild-to-moderate hepatitis and cytopenias are common.[22]

Severe dengue is also referred to as dengue hemorrhagic fever–dengue shock syndrome (DHF–DSS). This severe form of dengue occurs almost exclusively in those who have had a previous dengue infection and acquire a secondary dengue infection. DHF–DSS is an example of antibody-dependent enhancement where nonneutralizing antibodies actually increase viral entry into cells, leading to increased infectivity and worse clinical outcomes. Returning US travelers who have not previously spent time in an endemic area are at low risk for severe dengue.[27]

The WHO defines DHS–DSS as a patient with a febrile illness suspected to be dengue who also has a bleeding diathesis, severe thrombocytopenia, a plasma leak syndrome leading to hemoconcentration, and, in more severe cases, hypovolemic shock. DHF–DSS is often accompanied by hepatosplenomegaly, anasarca, and petechiae. Mortality from DHF–DSS ranges from 1% to 50% depending on one's access to experienced health care.[22]

Filoviridae

The family *Filoviridae* members, Ebola and Marburg, cause a severe febrile illness in humans and primates that often results in death.[28] Transmission of the Filoviruses occurs during close contact with infected persons through bodily fluids, including sweat, and infection of health care providers has occurred. Airborne transmission in humans has not been documented but has been observed with nonhuman primates in a laboratory setting.[20] Though the reservoir has not been definitively identified, the fruit bat is suspected.[29–31]

Marburg virus has been seen in a few sporadic cases and was first discovered in 1967 when lab workers in Germany and Yugoslavia handled tissues from green monkeys and subsequently developed HF. The fatality rate for Marburg HF ranges from 25% to 90%.[32]

Ebola virus was first detected in 1976 with two almost simultaneous outbreaks in the Democratic Republic of Congo (DRC) and Southern Sudan with two different species of virus. Ebola became known as one of the most deadly viruses with fatality rates in the DRC of 90% and in Sudan 50%. Large outbreaks have occurred in 1995 in the DRC and in 2000 in Uganda.[33,34]

The Filoviridae target dendritic cells and macrophages and replicate to infect parenchymal cells of organs and endothelial cells. Suppression of innate cell response, lack of T-cell activation, apoptosis, and release of cytokines such as TNF-α, interferon-γ, and nitric oxide lead to vascular permeability and multiorgan failure. Specific organ effects include liver necrosis, interstitial pneumonitis, and cerebral infarcts. Cases resulting in death usually do not have an adequate antibody response.[14,20,30] In convalescent patients, the virus has been identified in semen weeks after recovery.[20]

Incubation of Marburg virus is 3 to 9 days and Ebola virus is 2 to 21 days with symptoms initially very similar to other viral HF, including fever, chills, myalgia, chest pain, and sore throat followed by a maculopapular rash, conjunctivitis, gastrointestinal symptoms, and hepatitis.[34,35] This leads to a more severe clinical picture of hemorrhage and shock.[30] No specific treatment is currently available for these viruses, but some success has been seen in animal studies with treatment with factor VIIa/tissue factor and with activated protein C.[36]

Arenaviridae

The Arenaviridae family of viruses includes Old World and New World. Lassa fever and Lujo virus are examples of the Old World viruses, while New World viruses include Junin, Machupo, Guanarito, Sabia, and Chapare viruses. Lassa fever virus is described in more detail below.[37]

Lassa Fever Virus

Lassa fever is found throughout West Africa, with approximately 300,000 cases each year and 5,000 deaths. The animal host is the multimammate rat, a common household pest.[28,38] After direct contact or inhalation of mouse excrement, incubation lasts 6 to 21 days. Lassa fever is mild in 80% of those infected with the virus with bleeding in only 15% to 30%. For those who are symptomatic, the clinical presentation is similar to other viral HF with fever, myalgia, cough, vomiting and diarrhea, and coagulopathy.[14] It differs in that it leads to hearing loss (up to 33% of patients), anasarca, and neurologic manifestations such as tremors.[38] Women in their third trimester are more susceptible to severe disease, death, and spontaneous abortion. Poor prognostic indicators are high levels of viremia, bleeding, AST >150 IU/L, encephalitis, edema, and being in the third trimester of pregnancy.[36,39,40]

Bunyaviridae

The family Bunyaviridae contains several genera, members of which include the Hanta virus group, Crimean–Congo hemorrhagic fever (CCHF) virus, and RVF virus. CCHF and RVF virus are described in further detail below.

Rift Valley Fever

RVF is transmitted by *Aedes* mosquitoes or through contact with fresh carcasses or blood of the animal reservoir, generally cattle and sheep. The primary range of RVF is sub-Saharan Africa.

RVF has an incubation period of 3 to 6 days and then presents as a febrile illness with mild hepatitis. Complications of the illness include a retinitis in about 10% of patients occurring 1 to 3 weeks after the initial illness, potentially leading to blindness. Approximately 1% of patients may also experience meningoencephalitis 1 to 4 weeks after initial symptoms. Less than 1% of patients go on to develop hemorrhagic complications. For these patients, after 3 to 6 days of fever, hepatitis and jaundice accompany spontaneous bleeding, myalgia, headaches, and joint pain. The overall case fatality rate for all manifestations of RVF is around 1% but is 20% to 50% in those with the hemorrhagic form.[41]

Crimean–Congo Hemorrhagic Fever Virus

CCHF may be acquired from the bite of an infected *Hyalomma* tick or through contact with blood from infected herd animals. The disease primarily occurs in the former Soviet Union states, Middle East, and Africa, especially during spring and fall, times of high tick activity.[9]

After an incubation period of 2 to 6 days, patients experience the sudden onset of fevers, headaches, photophobia, myalgias,

and abdominal pain. Several days into the illness, the patient may have episodes of somnolence and agitation. In the second week of the illness, liver failure, bleeding complications, and DIC become apparent and may progress to hepatorenal syndrome, multisystem organ failure, and death. Case fatality approaches 30%.[41]

PREVENTION AND CONTROL

Since vaccines have only been developed for a handful of HF viruses including YF and Argentine HF, and pharmacologic treatment options are minimal, preventive efforts are of great importance.[1] For mosquito-borne infections, vector control including insecticide fogging of affected areas and source control of larvae have been implemented by public health officials.[42] For tick-borne infections, personal preventative efforts including performance of frequent tick checks are useful. For HF viruses such as Ebola and Marburg, which can be transmitted from person to person, avoiding close contact with infected patients and their body fluids is of great importance.[43] These viruses can be destroyed with heat, cleaning detergents, and acidity but can persist for weeks at room temperature.

NEW RESEARCH AND FINDINGS

Given the rapidly rising number of dengue cases worldwide, the search for a dengue vaccine has become especially important. The development of an effective vaccine has been hampered by the need for providing complete protection against all four serotypes of dengue, as infection with one serotype provides immunity against that type but often enhances disease severity to infections with other serotypes. A live, attenuated, chimeric vaccine that uses the 17D YF virus vaccine as its backbone has recently been shown to induce 100% seroconversion against all four serotypes of virus, when given in 3 doses over 15 months.[5] Phase III efficacy trials are ongoing in several dengue-endemic countries, and licensure of a successful vaccines is expected as early as 2014.[44] Vaccines for several other HF are currently in development, and other novel control techniques, including the engineering of mosquitoes that have a short life span or that cannot transmit viruses, are under exploration.[45] Finally, researchers are exploring immunomodulation and therapies directed at interrupting signals that cause derangements of the clotting cascade.[12]

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CHAPTER

125 Cancer-Related Thrombosis

ANNA FALANGA, AGNES Y. Y. LEE, AND FREDERICK R. RICKLES

The close association between malignant disease and thrombosis is well known and was first described more than a century ago by Trousseau, a French doctor who first observed that unexplained episodes of migratory thrombophlebitis in patients with gastrointestinal symptoms might indicate an underlying, undiagnosed tumor. Subsequent postmortem studies showed an increased incidence of thromboembolic disease in cancer, particularly in patients who died of mucinous cancer of the pancreas, lung, and gastrointestinal tract. On the other hand, we know today that venous thromboembolism (VTE) may be indeed the first manifestation of an occult malignancy in an otherwise healthy individual, anticipating the clinical detection of tumor by months or even years. Unlike thrombosis in other settings, venous thrombosis in cancer can be migratory, may involve superficial as well as deep veins, may affect unusual sites such as the arms and chest, and may be relatively unresponsive to standard anticoagulant therapy. However, thromboembolism is not the only form of abnormal hemostasis found in patients with cancer. Also of importance is the bleeding diathesis that results from consumption of platelets and clotting factors as occurs in disseminated intravascular coagulation (DIC). Cancer patients commonly present with abnormalities in laboratory coagulation tests, indicating an ongoing subclinical hypercoagulable condition. The results of laboratory tests demonstrate that a process of fibrin formation and removal parallels the development of malignancy, which is of particular interest since fibrin and other clotting products are of importance for both thrombogenesis and tumor progression. Many factors can increase the thrombotic risk in cancer patients, including classical risk factors such as immobility, old age, surgery, and cancer-specific factors such as advanced disease stage, and cancer therapies. In addition, tumor cell–specific prothrombotic properties and the subsequent host cell inflammatory response dramatically enhance the risk for thrombosis in these patients. Cancer cells produce and release procoagulant and fibrinolytic proteins, as well as inflammatory cytokines. In addition, they are capable of directly adhering to host cells (i.e., endothelial cells [ECs], monocytes, platelets, and neutrophils), thereby stimulating additional prothrombotic properties of the host effector cells. Notably, most of these mechanisms, in addition to generating thrombosis, can also promote tumor growth and metastasis.

EPIDEMIOLOGY

VTE, including deep vein thrombosis (DVT) and pulmonary embolism (PE), is the most extensively studied thrombotic complication associated with malignant disease. However, arterial thromboembolism, migratory thrombophlebitis, thrombotic nonbacterial endocarditis, and DIC add to the spectrum of complications. The incidence of VTE in cancer populations has been estimated at approximately 1 out of 200 patients,[1] and active malignant disease is an independent risk factor for VTE.[2,3] Overall, 18% to 29% of all VTE events in the population are associated with cancer.[4–6] In a registry of 5,451 patients hospitalized with objectively confirmed DVT, cancer was reported in 39% of patients; of these 62% had ongoing cancer and 38% a history of cancer.[7] Several large retrospective analyses of hospital discharge data reported a 28% to 36% increase in VTE events over the period 1995 to 2003.[8,9] Hospitalization, anticancer surgery, and active treatment regimens, particularly chemotherapy, further increase the risk of thrombosis in cancer patients.[2,8,10]

The Risk of VTE Associated with Cancer

Large, population-based studies have shown that the risk of VTE is increased between four- and sevenfold in patients with malignancy,[2,11] and studies based on hospital discharge have reported an overall VTE incidence of 0.6% to 7.8% in patients with cancer, more than double that in patients without cancer.[8,9,12–14] Recently, a retrospective cohort study of nearly 2 million patient hospitalizations in the United States reported an overall VTE rate of 4.1% among 1,015,598 cancer patients.[8] Nevertheless, the incidence of VTE in patients with cancer is probably underestimated by retrospective, population-based studies that report only symptomatic or objectively confirmed VTE events. Routine-staging CT scans in cancer patients have reported a high prevalence of unsuspected PE,[15–17] for example, in 4% of inpatients and 0.9% of 589 outpatients.[15]

The increased risk of VTE associated with cancer portends an increased likelihood of VTE recurrence. For example, in a retrospective analysis of 1,303 patients with symptomatic VTE receiving oral anticoagulant therapy, patients with cancer ($n = 264$) experienced greater VTE recurrence compared with patients without malignancy (27.1 vs. 9 events per 100 patient-years, $P = 0.003$).[18] Likewise, a prospective follow-up study of 842 patients with confirmed DVT, with 181 patients with cancer and 661 without cancer, reported an annual VTE recurrence rate of 20.7% in cancer patients compared with 6.8% in patients without malignancy.[19] These studies also found that cancer patients are more likely to suffer bleeding complications during VTE treatment,[18,19] both bleeding and recurrence being correlated with the extent of cancer.[19]

Among cancer patients, those experiencing a VTE event have a worse survival outcome compared to thrombosis-free subjects.[8,9,20,21] A report from the Danish Cancer Registry highlighted a mortality ratio of 2.2 in cancer patients with symptomatic

VTE compared with matched cancer patients without VTE.[21] A retrospective analysis of 68,142 patients reported that VTE was a significant predictor of mortality within 1 year of colorectal cancer diagnosis among patients with local (hazard ratio [HR] 1.8, 95% confidence interval [CI] 1.4 to 2.3) and regional malignancies (HR 1.5, 95% CI 1.3 to 1.8),[22] and a necropsy review revealed that 21.8% of fatal PE events occurred in hospitalized medical cancer patients.[23]

These studies are strengthened by findings from the RIETE registry of 14,391 patients with symptomatic, acute VTE, in which the 2,945 patients with cancer had significantly higher rates of fatal PE (2.6% vs. 1.4%) and fatal bleeding (1.0% vs. 0.3%) than those without cancer.[24] Among 512 patients who presented with upper-arm DVT,[25] those with cancer had a higher rate of recurrent VTE (6.1% vs. 2.8%, odds ratio [OR] 2.2), major bleeding (4.1% vs. 0.9%, OR 4.4), and mortality (22% vs. 3.5%, OR 7.8) than did patients without cancer. The risk of VTE steadily accumulates with each additional patient-related thrombotic risk factor in cancer patients, for example, advanced age, prolonged immobility, prior history of VTE, and comorbid conditions of infection and cardiopulmonary disease.[2,9,19,26]

The Incidence of VTE According to Tumor Type and Stage

Cancer with the highest rates of VTE include those involving the pancreas, stomach, and kidney,[27] and studies based on hospital discharge data confirm autopsy data that link VTE with tumors of the brain, pancreas, ovary, lung, and uterus and leukemia[9,28] (FIGURE 125.1). Among the hematologic malignancies, lymphoma and myeloma have the highest rates of VTE.[8] A retrospective cohort study of 66,106 adult neutropenic cancer patients reported an overall rate of symptomatic VTE of 8%: among these, patients with lymphoma and leukemia represented a third of all cases of VTE and almost half of the arterial thrombotic events.[9] Advanced metastatic cancer is associated with an increased risk of VTE compared with localized tumors.[27] Data from the California Cancer Registry (1993 to 1995) highlighted metastatic disease as the strongest predictor of VTE complications.[20] Similarly, Blom et al.[28] reported that cancer patients with distant metastases had almost a twofold increased risk of VTE compared with those without metastases.

The Effect of Anticancer Treatments on VTE Incidence

Many anticancer treatments increase the risk of VTE, including surgical procedures, nonsurgical treatments, and use of a central venous catheter (CVC).[29]

Surgery

In 1975, V.V. Kakkar et al. analyzed 4,031 patients undergoing major abdominal surgery; half of these received low-dose unfractionated heparin (UFH) to prevent thromboembolism. Overall, 170 (4.2%) of patients died postoperatively. The autopsies, performed in about 70% of patients who died after surgery, demonstrated the great benefit of heparin prophylaxis. In particular, among the 23% of patients operated for malignant disease, the frequency of fatal PE was 1.6% in the control group and 0.4% in the heparin group.[30] Patients with cancer are at increased risk (i.e., three- to fivefold) for postoperative thrombosis compared to noncancer patients.[31] Without prophylaxis, the postoperative incidence of symptomatic or asymptomatic DVT in cancer patients is 40% to 80% and that of proximal DVT 10% to 20%.[30] Data from the @RISTOS registry identified VTE as the most common cause of death 30 days after surgery in cancer patients (46%); 40% of VTE events occurred >21 days after surgery.[10]

Nonsurgical Cancer Treatments

Nonsurgical cancer treatments are associated with a high incidence of VTE, including the use of chemotherapy, adjuvant chemotherapy, hormones, and antiangiogenic agents. In a population-based study of VTE risk factors among 625 patients, malignant disease increased the risk of VTE 4.1-fold, but with chemotherapy it increased by 6.5-fold.[2] By contrast, radiotherapy has not been proven to increase the risk of VTE.[28,32]

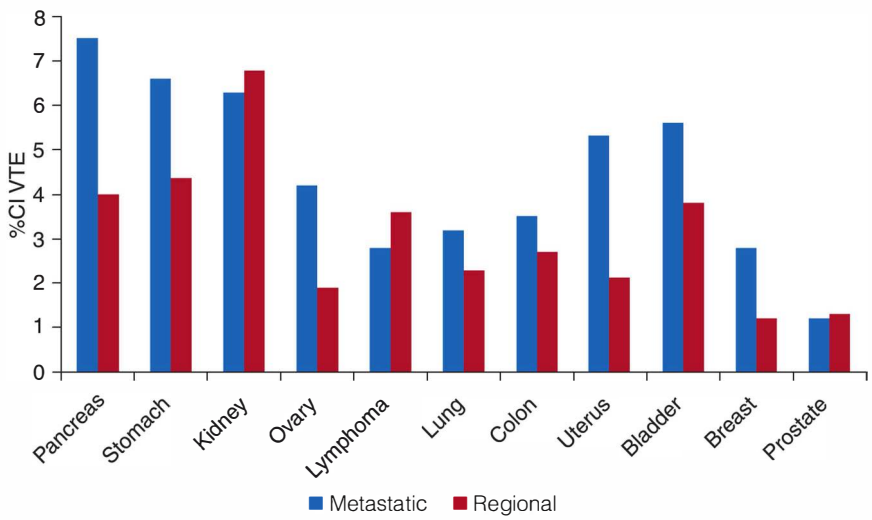

FIGURE 125.1 VTE according to type and stage of cancer. Cancer types associated with the highest incidence of VTE are pancreas, stomach, kidney, uterus, and bladder, whereas lymphoma, prostate, and breast have a much lower incidence. For the majority of cancers, VTE risk is further increased when the tumor is metastatic. CI, cumulative incidence. (Adapted from Wun T, White RH. Epidemiology of cancer-related venous thromboembolism. *Best Pract Res Clin Haematol* 2009;22(1):9–23.)

Chemotherapy and Hormonal Therapy

A prospective study of nearly 4,500 patients receiving outpatient chemotherapy reported a 2.7-fold increase in arterial thrombosis, and a 47-fold increase in the mortality rate from VTE compared with the general population.[33] At present, it is not known if specific chemotherapeutic agents carry a higher risk of thrombosis. An elevated prechemotherapy platelet count is associated with an increased risk of VTE.[32] There is a cause-and-effect relationship between L-asparaginase use and thrombosis,[34] and high-dose corticosteroids are associated with a 3.5-fold increased risk of VTE.[35] Women who receive the selective estrogen receptor modulator tamoxifen have a 1.5- to 7.1-fold increase in the risk of developing symptomatic VTE, compared with placebo or no treatment.[36]

Adjuvant Chemotherapy

The incidence of VTE in women with early-stage, node-negative, or node-positive breast cancer receiving chemotherapy treatment is 0.9% to 9.6%,[37] with the greatest risk seen in older postmenopausal women.[38] In 1988, Levine et al.[39] published the results of a randomized multicenter trial comparing chemotherapy with chemohormonal therapy in 205 women with stage II breast cancer. There were 14 objectively confirmed symptomatic VTE events distributed in both groups, and all occurred during active treatment. Anastrozole, a third-generation aromatase inhibitor used as an alternative agent for adjuvant treatment in women with early breast cancer and a low risk of recurrent tumors,[37] may be associated with a lower risk of VTE complications than tamoxifen.[40]

Antiangiogenic Agents

Targeted antiangiogenic agents, such as bevacizumab, the monoclonal antibody to vascular endothelial growth factor (VEGF), have shown efficacy in improving survival rates among patients with advanced malignant disease.[41–43] However, the addition of bevacizumab to chemotherapy regimens is associated with a high incidence of thrombotic events. A recent meta-analysis from 15 randomized controlled trials of 7,956 patients with advanced solid tumor indicated that bevacizumab was associated with an increased risk of VTE, with a relative risk of 1.33.[44] Similarly, treatment regimens involving thalidomide or lenalidomide are associated with an increased risk of VTE when used concomitantly with chemotherapy or corticosteroids in patients with multiple myeloma.[45,46]

Supportive Therapy

The use of recombinant human erythropoietin (EPO) and other hematopoietic growth factors as supportive therapy in cancer patients appears to increase the risk of VTE. A study in 147 patients with nonmetastatic cervical or vaginal cancer showed that EPO increases the risk of thrombosis by 15-fold.[47] The use of EPO and granulocyte growth factors is independently associated with VTE risk among patients on chemotherapy.[32] Red blood cell transfusion may increase the risk of VTE, one study reporting that 7.2% of patients receiving transfusions developed VTE compared with 3.7% of patients who did not receive transfusions; transfusions were also associated with an increased risk of in-hospital mortality.[48]

Central Venous Catheters

CVCs promote thrombosis through a number of mechanisms such as trauma to vessel wall, and by local high drug concentrations delivered by the CVC.[49] Retrospective studies indicate a risk of CVC-related thrombosis in malignancy of 12% to 66%, depending on the type of cancer and its treatment, and the type, position, and duration of catheter use.[50] However, recent randomized controlled trials in cancer patients report lower rates of CVC-related thrombosis, 3.1% to 4.0% symptomatic and 18.1% asymptomatic. The use of warfarin, dalteparin, or enoxaparin did not reduce the incidence of catheter-related DVT.[51–53] The RIETE registry revealed that among patients diagnosed with an objectively confirmed symptomatic DVT, upper-extremity DVT was more frequent in patients with cancer than in those without.[25] Upper-extremity DVT typically presents as symptomatic PE less often than lower-extremity DVT (9% vs. 29%), but is associated with a higher rate of recurrent VTE (6.1% vs. 2.8%) and overall mortality (22% vs. 3.5%).[25]

PATHOPHYSIOLOGY AND RISK ASSESSMENT

Patients with cancer display many types of hemostatic disorders,[39] including overt hemorrhage, thrombosis, and DIC, or only a finding of hemostatic abnormality recognized as the result of laboratory testing that reflects an *in vivo* hypercoagulable state.

The Hypercoagulable State

The underlying principle in patients with cancer is that the clotting system is systemically activated, coagulation factors are consumed, and fibrinolysis is activated. Approximately half of all patients with cancer, and as many as 90% of those with metastases, exhibit abnormalities in one or more routine coagulation parameters.[54–57] The development of novel laboratory tests with increased sensitivity has enabled the detection of subtle alterations in the hemostatic system. Levels of plasma markers of activation of coagulation, such as the thrombin–antithrombin (TAT) complex, prothrombin fragment 1+2 (F1+2), fibrinopeptide A (FPA), and D-dimer, tend to be universally elevated in patients with cancer,[58,59] indicating the presence of *in vivo* thrombin generation and fibrin formation and fibrinolysis. These abnormalities can worsen with the start of antitumor therapies.[60] For example, Falanga et al.[61] demonstrated that the plasma levels of F1+2 and plasminogen activator inhibitor type 1 (PAI-1) in patients with non-Hodgkin lymphoma and multiple myeloma were elevated at baseline and significantly increased after receiving high-dose cyclophosphamide, followed by granulocyte colony-stimulating factor (G-CSF) for hematopoietic stem cell mobilization (FIGURE 125.2).

Hemostatic Markers and Risk of VTE

Few prospective studies have evaluated the utility of serial measurements of hemostatic markers for predicting the occurrence of VTE (as confirmed by objective test) in cancer patients. One such study of patients undergoing surgery for abdominal cancer found that presurgical TAT complex levels were significant predictors of postoperative DVT.[62] Khorana et al. showed that a prechemotherapy platelet count >350,000/μL is predictive of subsequent thrombosis during chemotherapy,[32] and data from the Vienna Cancer and Thrombosis Study (CATS) show that a P-selectin level above the 75th percentile is predictive of thrombosis in cancer patients[63] and increased levels of both D-dimer and F1+2 characterize the highest risk of VTE in patients with cancer.[64]

Improvements of laboratory techniques allow the measure of plasma microparticles (MPs), the small vesicles shed by platelets,

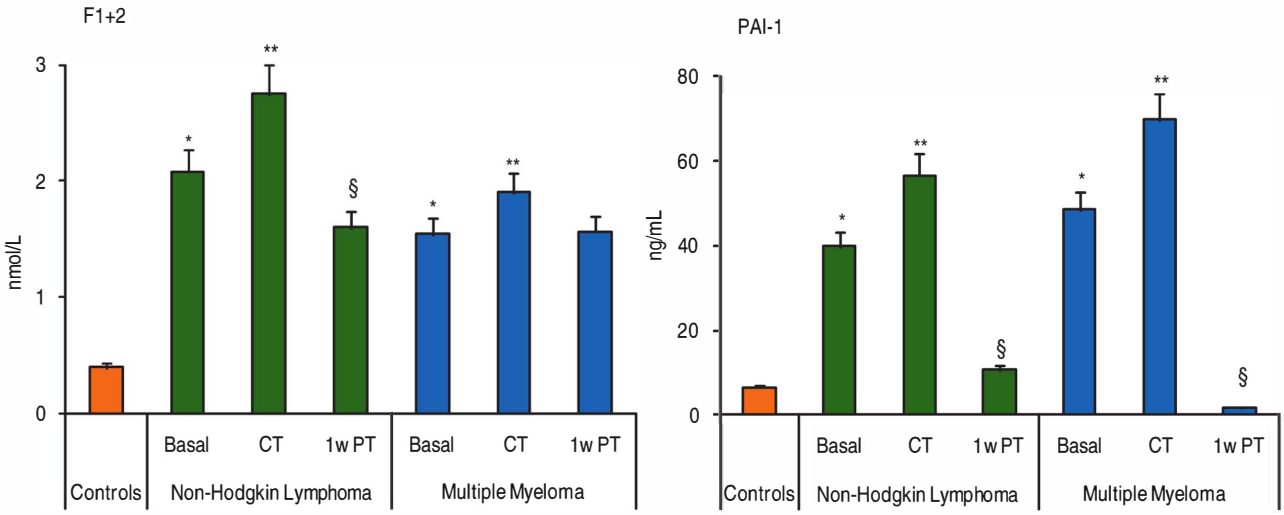

FIGURE 125.2 Hypercoagulation (F1+2) and endothelial activation (PAI-1) markers in patients with non-Hodgkin lymphoma or multiple myeloma. Patients received high-dose cyclophosphamide for autologous hematopoietic stem cell transplantation. Hemostatic markers were evaluated at different time points: basal, before chemotherapy; CT, after high-dose chemotherapy (before starting G-CSF); 1w PT, 1 week posttransplantation. F1+2 and PAI-1 levels increased under chemotherapy and returned toward normal after transplantation. Data are expressed as mean ± SE. $^{*}P < 0.05$ versus controls, $^{**}P < 0.05$ versus basal, §$P < 0.05$ versus CT. (Modified from Falanga A, Vignoli A, Marchetti M. Coagulation in hematological malignancies. *Cancer Invest* 2009;27(Suppl 1):17–27.)

leukocytes, and endothelium in response to cell activation. The MPs are characterized by a diameter of <1 μm, the presence on their surface of antigens of the parental cell, and by procoagulant properties. The procoagulant capacity is related to the presence of higher amount of the anionic plasma membrane phospholipid phosphatidylserine and of tissue factor (TF). Toth et al.[65] showed that levels of MP derived from platelets are elevated in patients with breast cancer, compared to patients with benign breast lesions, and Trappenburg et al.[66] showed that the levels of circulating platelet and EC MPs in patients with essential thrombocythemia increased with the number of risk factors for thrombosis. TF-bearing MPs are elevated in 60% of cancer patients with VTE and in 27% of those without VTE, thus predicting a significantly increased risk of VTE.[67,68] A large prospective study is currently assessing the predictive value of determining TF-rich MP levels, along with other factors, in patient populations with cancer at high risk for developing VTE (Clinicaltrials.gov NCT00876915). The MicroTEC study (Microparticles and Thromboprophylaxis with Enoxaparin in Cancer Study, Clinicaltrials.gov NCT00908960) is evaluating the benefit of primary thromboprophylaxis in patients with advanced colon, lung, or pancreatic cancer, administered on the basis of their level of TF-bearing MPs.[69]

Risk Stratification

The interaction and relative effects of the risk factors associated with VTE in cancer patients is highly complex, making the pretreatment assessment of VTE difficult. Several demographic, cancer-associated and treatment-related factors increase the risk of thrombosis in patients with cancer (FIGURE 125.3). A VTE risk assessment model for use in patients undergoing chemotherapy patients described in 2008 by Khorana et al. is based on five predictive variables, namely, cancer site, platelet count, hemoglobin level (or the use of erythropoiesis-stimulating agents), leukocyte count, and body mass index,[70] and is under evaluation in a prospective cohort of cancer patients initiating outpatient chemotherapy but not receiving thromboprophylaxis.[71] Preliminary results show that 27% of cancer outpatients deemed at high risk with this prediction rule developed VTE. Recently, Pabinger et al. expanded the Khorana model[70] by adding P-selectin and D-dimer quantification, allowing an even better stratification of thrombotic risk. In particular, the HR of patients with the highest score compared with those with the lowest score was 25.9.[72]

THE PATHOGENESIS OF THE HYPERCOAGULABLE STATE

The pathogenesis of the prothrombotic state of cancer patients is due at least in part to the ability of cancer cells to activate the coagulation system and interactions that may favor tumor progression (FIGURE 125.4).

The Activation of the Hemostatic System by Tumor Cells

Several complex and not fully recognized interactions between malignant cells and coagulation shift the hemostatic balance toward a procoagulant state in multiple ways.[73]

Tumoral Production of Hemostatic Factors

Procoagulant Proteins

TF is a transmembrane glycoprotein that is the primary initiator of hemostasis. TF forms a complex with activated factor VII (TF/FVIIa) to trigger coagulation by proteolytically activating

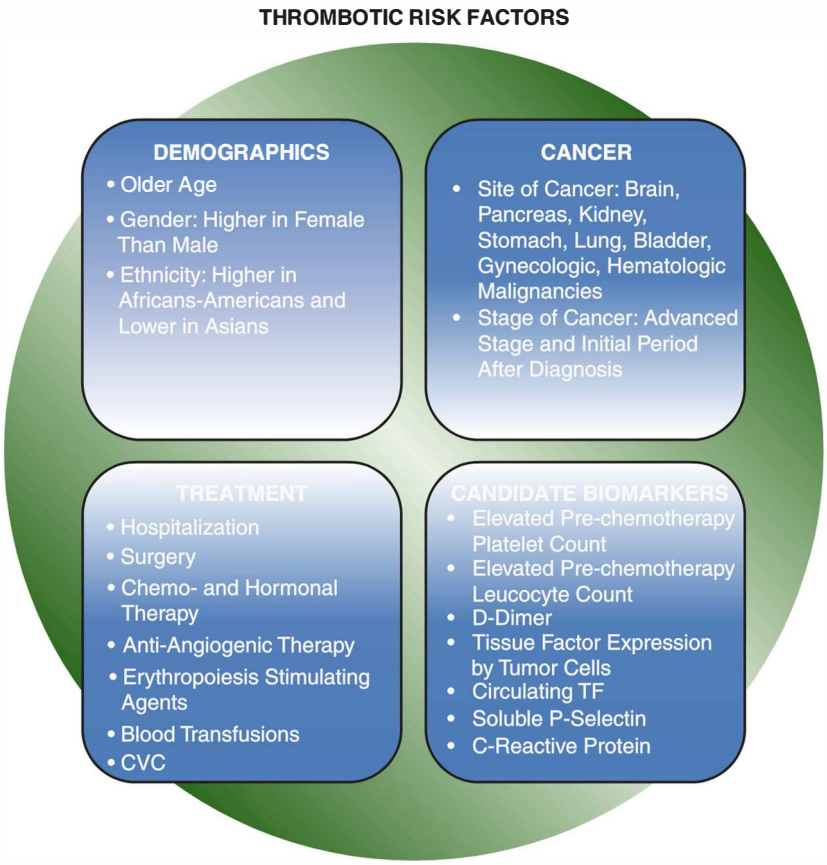

FIGURE 125.3 Prothrombotic risk factors in cancer patients. Several factors can contribute to thrombotic risk in cancer patients; these include their demographic characteristics, site and stage of cancer, anticancer therapies (including surgery), and hospitalization. CVC, central venous catheters; TF, tissue factor.

FIX and FX. ECs and monocyte–macrophages do not normally express TF, unless they are stimulated.[74] Differently, constitutive TF expression characterizes numerous malignant tumor tissues. In addition, TF expression can be induced on tumor-associated macrophages and EC either by inflammatory endogenous agonists, that is, tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), or by exogenous agonists, that is, bacterial endotoxin or tumor-specific antigens. In breast cancer, the expression of TF in tumor stroma correlates with progression to invasive cancer. Similarly, the expression of TF by

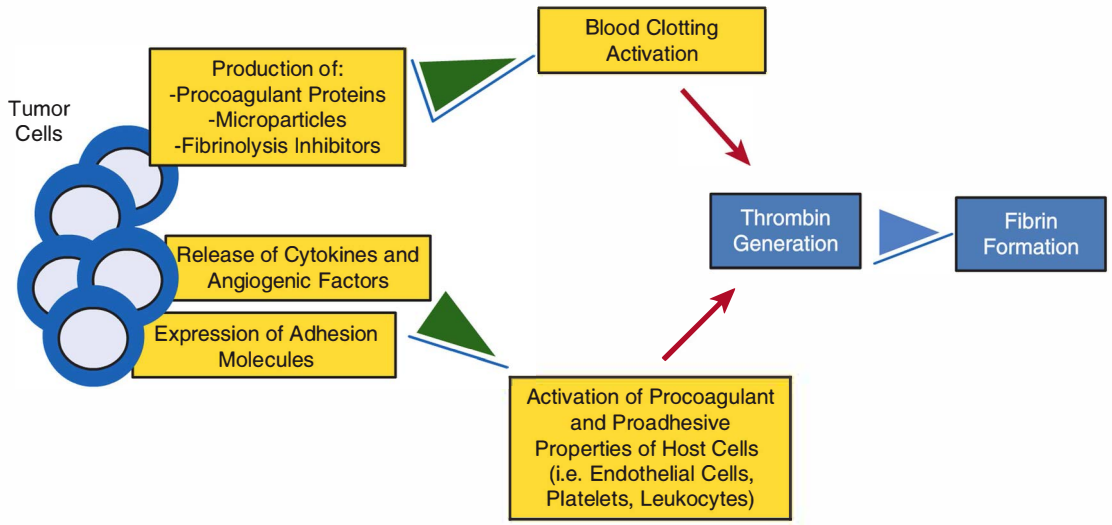

FIGURE 125.4 Principal prothrombotic mechanisms associated with cancer cells. Tumor cells can activate the hemostatic system by multiple mechanisms. The scheme summarizes the ways by which tumor cells activate the coagulation cascade and hemostatic cellular components, ultimately leading to thrombin generation and fibrin formation.

glioma cell lines and glioma surgical specimens correlates with the histologic grade of malignancy.

Cancer procoagulant (CP) is a cysteine protease that directly activates FX in the absence of FVIIa. CP is synthesized by malignant cells, and its activity has been found in extracts of different tumors.[75–77] The presence of CP antigen as well as of CP procoagulant activity (PCA) have been identified in cancer patients' sera. Although CP is found almost exclusively in malignant cells, its role as a tumor marker or as a factor predictive of clinical thrombosis has not been demonstrated. CP expression in acute promyelocytic leukemia blasts parallels the degree of malignant transformation and is lost upon cellular differentiation after therapy with all-transretinoic acid (ATRA). In contrast, cells resistant to ATRA maintain their malignant phenotype, including CP expression.[78] Similar observations have been reported for breast cancer.[79]

MPs released by tumor cells have been associated with the hypercoagulable state of cancer. The first demonstration that tumor-shed vesicles possess PCA was made by Dvorak et al.[80] in 1981, who showed that a mouse breast carcinoma cell line and a guinea pig hepatocarcinoma cell line released PCA in culture and *in vivo*, in association with small vesicles shed by the tumor cell surface. Circulating MPs have been described in the plasma of patients with various tumors, such as pancreas and breast adenocarcinoma, giant-cell lung carcinoma, colorectal cancer, and acute leukemia.[81–83] MPs contribute to intravascular thrombin generation by exposure of phosphatidylserine and procoagulant proteins such as TF[84] and may explain the increased TF level in the blood of cancer patients. Patients with colorectal cancer have twofold higher TF-positive MPs compared to healthy controls, and levels of MP correlate positively with the levels of D-dimer as a marker of clotting activation.[85] Elevated MP-associated TF activity significantly relates to the development of VTE in patients with disseminated mucinous carcinoma.[81]

Fibrinolytic Proteins

Tumor cells possess both the urokinase-type and the tissue-type plasminogen activators and can also produce PAI-1 and PAI-2. There are specific receptors on tumor cells that support the assembly of the fibrinolytic components, thus facilitating the activation of the fibrinolytic cascade.[86] In patients with leukemia, the expression of these activities by blast cells may play a role in bleeding symptoms. On the other hand, a defect in the generation of normal plasma fibrinolytic activity, for example, by increased production of PAI-1, has been found in patients with solid tumors and may represent another mechanism for these patients to develop VTE.[59]

Inflammatory Cytokines

Tumor cells synthesize and release a variety of cytokines including proinflammatory (TNF-α, IL-1β) and proangiogenic factors (VEGF, basic fibroblast growth factor [bFGF]). Cytokine overproduction also derives from inflammatory tissues as a part of the host-reactive process in response to the tumor. Most of these cytokines induce the expression of a procoagulant phenotype by monocytes and ECs. Monocyte activation occurs in malignant tissue both *in vitro* and *in vivo*, expressed, for example, as significantly more TF than in control cells.[59] Cytokines stimulate ECs to increase the production of PAI-1 and upregulate EC surface TF expression and downregulate expression of thrombomodulin,[87,88] a potent anticoagulant that forms a complex with thrombin to activate the natural anticoagulant protein C.[88]

Adhesion Molecules

The expression of tumor cell-surface adhesion molecules and/or their receptors allows the direct interaction of these cells with host cells, including EC, platelets, and leukocytes. The attachment of tumor cells to vascular ECs is relevant to promote localized clotting activation to the vessel wall and to start thrombus formation. The adhesion can occur directly or via the cooperation of granulocytes. The interaction between tumor cells and ECs is mediated by different pathways of adhesion molecules expressed by different tumor types.[89] In flow conditions, the interaction involves rolling and firm adhesion, depending on the respective adhesive properties of both ECs and tumor cells. Some tumor cell types roll and adhere using E-selectin, while others adhere without rolling using vascular cell-adhesion molecule-1.[90] Thus, a variety of molecules expressed on EC surface, including selectins, integrins, and immunoglobulins, are necessary for the adhesion of cancer cells and their migration to distant sites to form metastases. P-selectin (CD62P) is an important cell-adhesion molecule expressed by stimulated ECs and activated platelets, which mediates heterotypic aggregation of activated platelets to cancer cells and the adhesion of cancer cells to stimulated ECs. In addition, P-selectin mediates the homotypic aggregation of activated platelets and the transfer of procoagulant MPs between cells, which further contribute to fibrin deposition and microthrombus formation.[91]

Oncogene-Driven Activation of the Hemostatic System

In 2000, Rak and Klement[92] first postulated that "the procoagulant conversion of tumor cells is related to their oncogene status." In 2005, three seminal papers were published that provided support for this hypothesis,[93,94,95] the evidence for which has only grown stronger over the ensuing years.[96] In particular, Boccaccio et al.[94] developed a model for human liver carcinoma by targeting activated human MET oncogene to mouse liver with a lentiviral vector and liver-specific promoter. Genome-wide expression profiling of mouse hepatocytes expressing the MET oncogene demonstrated significant upregulation of the genes for PAI-1 and cyclooxygenase-2, along with two- to threefold increases in circulating levels of the proteins. Rong et al.[95] initially linked inactivation of the tumor suppressor gene PTEN in human astrocytoma cell lines with induction of TF gene expression, and Magnus et al.[97] provided further support for the importance of both PTEN loss and the expression of the oncogenic epidermal growth factor receptor and its mutant (EGFRvIII) in the aggressive cell behavior of glioblastoma multiforme. These models show that direct oncogene activation and/or tumor suppressor gene inactivation may result in the upregulation of blood clotting *in vivo*. Furthermore, these data imply that targeting clotting proteins might prove to be complementary to traditional chemotherapy, perhaps by reducing the risk for thrombosis, as well as by impairing tumor growth.[96]

The Role of the Coagulation System in Tumor Progression

TF induces angiogenesis via clotting-dependent mechanisms by downstream generation of thrombin and fibrin and by thrombin-induced platelet activation. In addition, the formed fibrin induces upregulation of TF on tumor cells and EC and

the secretion of the proangiogenic factor, interleukin-8, thus generating a vicious cycle of clot formation and tumor growth.[98] The function of TF in tumor angiogenesis can also be mediated through mechanisms independent of thrombin generation and fibrin deposition. These mechanisms are induced via the phosphorylation of the TF cytoplasmic tail and subsequent signal transduction cascades. In a protease-receptor pathway, the TF/FVIIa complex activates one or more protease-activated receptors (PARs), which, coupled to G proteins, impact multiple signaling pathways and support angiogenesis *in vivo*.[99] TF can also promote an increased expression of the angiogenic factor VEGF and decreased expression of the antiangiogenic peptide thrombospondin. Moreover, TF promotes tumor invasion and metastasis by (a) the capacity of TF–VIIa–Xa complex to promote local thrombin generation with subsequent proteolytic activation of platelet and endothelial PARs and fibrin deposition; (b) the capacity of the tumor-associated TF–VIIa–Xa to directly activate PAR-1 and PAR-2 signaling pathways; (c) the capacity of TF/FVIIa complex to cooperate with TF pathway inhibitor expressed by tumor vessels in inducing cell adhesion and migration by a thrombin-independent circle (FIGURE 125.5).[73]

Thrombin is an effective activator of angiogenesis via clotting-dependent mechanisms involving platelet activation and fibrin deposition. Furthermore, thrombin induces angiogenesis through clotting-independent mechanisms mediated by PAR activation and resulting signal transduction cascades. Thrombin activation of PARs leads to upregulation of many angiogenesis-related genes, including VEGF, VEGF receptors (VEGFR), TF, bFGF, and metalloproteinase (MMP)-2. In addition, thrombin stimulates the release of VEGF and angiopoietin from platelets, which induce tube formation by EC. Thrombin contributes significantly to a more malignant phenotype by inducing (a) platelet-tumor aggregation, (b) tumor adhesion to EC and extracellular matrix (ECM), and (c) tumor cell proliferation and metastasis. Important in this setting is the capacity of thrombin to trigger the release of growth factors, chemokines, and extracellular proteins that promote the proliferation and migration of tumor cells. The prometastatic activity of thrombin is demonstrated by experimental pulmonary metastasis models, in which lung metastases are increased by thrombin-treated tumor cells compared to untreated tumor cells.[100,101,102] The role of thrombin-activated platelets is shown by murine models, in which the elimination of circulating platelets results in a significant reduction in tumor metastasis formation. Indeed activated platelets facilitate tumor cell adhesion to EC and favor their migration through the vessel wall by the release of heparinase activity. Additionally, tumor cell–associated platelets may support metastasis formation by preventing interactions between tumor cells and innate immune cells, as shown by immunodepletion studies *in vivo*.[103] Finally, activated platelets support the angiogenic process by releasing VEGF and platelet-derived growth factor.[104]

Fibrin plays important roles in thrombogenesis as well as in tumor progression, and its deposition within the vascular endothelium of new tumor vessels, or attached around inflammatory cells or tumor cells, suggests that angiogenesis is facilitated. Local deposits of fibrin can stabilize the adhesion of tumor cells or tumor cell–associated emboli to the endothelium, particularly in a high-shear stress environment. In addition, fibrin matrix provides an excellent scaffold for the formation of new vessels.[105] Last, the fibrin matrix protects from proteolytic degradation the proangiogenic growth factors bFGF, VEGF, and insulin-like growth factor-1.[106]

Leucocytes, particularly polymorphonuclear (PMN) leukocytes, promote tumor growth and metastasis,[107–110] as the interaction of tumor cells with PMN or EC represents a crucial step in the early phase of extravasation and formation of new metastatic lesions.[111] In fact, PMN may adhere to tumor cells facilitating their migration across the endothelial barrier. In addition, PMNs release numerous MMP that hydrolyze ECM components and favor cancer cell dissemination. Furthermore, the selectins, including L-, E-, and P-selectin, mediate the initial interactions between circulating PMNs, activated ECs, and tumor cells. During hematogenous metastasis, tumor cells carrying selectin ligands enter the circulation and may encounter vascular selectins, thus

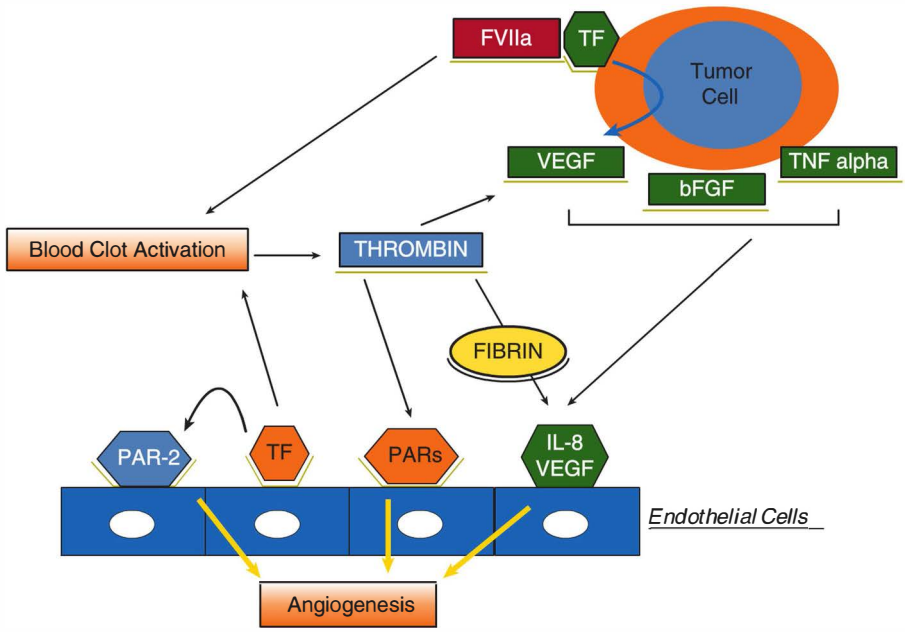

FIGURE 125.5 Role of coagulation system in tumor progression. In addition to their primary role in hemostasis and blood coagulation, hemostatic proteins play several roles in tumor angiogenesis, via clotting-dependent or -independent mechanisms. TF, tissue factor; IL-8, interleukin-8; PAR, protease-activated receptor; FVIIa, activated coagulation factor VII.

supporting cancer progression, as shown by different studies using mouse models and carcinoma cell lines.[111]

THROMBOPROPHYLAXIS IN CANCER PATIENTS

The prevention and treatment of VTE in cancer patients pose difficulties, in light of increased recurrence rates and bleeding complications.[18,19] Cancer patients undergoing surgery benefit from effective pharmacologic and mechanical prophylaxis,[112] as reflected in current guidelines that patients undergoing major cancer surgery should receive heparin-based prophylaxis for a minimum of 7 to 10 days, with supportive mechanical prophylaxis in patients at highest risk.[113,114]

Surgical Patients

Clinical trials have demonstrated the efficacy of subcutaneous UFH in preventing DVT and PE in patients undergoing major surgery, with or without a diagnosis of cancer.[115,116] In a meta-analysis of eight trials that included patients undergoing surgery for cancer, no differences in asymptomatic DVT, clinical PE, death, and major bleeding were detected between low molecular weight heparin (LMWH) and UFH.[117] In a subgroup analysis of over 6,000 patients with malignant disease receiving perioperative UFH or LMWH, compared to 17,000 patients without malignancy, fatal PE was three times as common in patients with cancer than in surgical patients who did not have cancer, regardless of the use of thromboprophylaxis.[118] In a study of over 2,000 patients undergoing laparotomy for cancer, increasing the dose of the LMWH dalteparin from 2,500 to 5,000 IU once daily was associated with a reduction in the frequency of postoperative DVT from 14.9% to 8.5%,[119] with no significant increase in bleeding complications. Although there was a reduction in the frequency of postoperative DVT with the higher dose of LMWH in patients without malignant disease, the increased dose was associated with an increase in perioperative bleeding complications.[119] A number of trials have shown that venographically documented DVT can be reduced with extended out-of-hospital prophylaxis with LMWH in patients undergoing major hip or knee replacement surgery.[120] In the ENOXACAN II study, patients undergoing surgery for abdominal malignancy received 1 week of enoxaparin and were randomized to enoxaparin or placebo for another 21 days.[113] Bilateral venography performed at the end of treatment showed a statistically significant reduction in DVT from 12% with placebo to 4.8% with extended prophylaxis; these results have been confirmed by a trial of patients undergoing general surgery who received routine postoperative or extended prophylaxis with LMWH dalteparin.[121] No benefit of extended prophylaxis has so far been demonstrated on symptomatic VTE.

Medical Cancer Patients

Hospitalized Cancer Patients

Patients with cancer who are bedridden, for example, in relation to pain crisis, infection, or hypercalcemia, are at risk for thrombosis. Clinical trials have demonstrated clear-cut benefit of VTE prophylaxis in hospitalized medical patients, including those with cancer. The first important study was the MEDENOX study,[122] in which a *post hoc* analysis demonstrated a 50% risk reduction of objectively confirmed VTE in cancer patients receiving LMWH enoxaparin compared with placebo.[123]

These results were substantially confirmed by the subsequent PREVENT trial, in which the incidence of VTE in acutely ill medical patients was reduced from 5.0% (73 of 1,473) in the placebo group to 2.8% (42 of 1,518) in the group treated with LMWH dalteparin.[124] A *post hoc* analysis revealed that in cancer patients, the VTE rate fell from 8.3% with placebo to 3% with LMWH, with a 63% risk reduction.[125]

Ambulatory Cancer Patients

Few data on the primary prevention of thrombosis in ambulatory patients with cancer are available. In a double-blind randomized trial, 311 patients with metastatic breast cancer were given either very low-dose warfarin (1 mg/day for 6 weeks followed by an adjusted dose to a target PT INR of 1.3 to 1.9) or placebo while receiving chemotherapy.[126] There was an 85% risk reduction in the rate of thromboembolism, which was statistically significant, without an increase in bleeding, but despite this study, oncologists do not routinely use prophylaxis in this situation. The CONKO-004 trial found a 87% risk reduction of VTE using LMWH enoxaparin at 1 mg/kg once daily for 3 months compared with no prophylaxis (9.9% vs. 1.3%, $P < 0.01$),[127,128] while the FRAGEM study reported a 61% risk reduction using the CLOT study therapeutic regimen of LMWH dalteparin (31% vs. 12%, $P = 0.02$).[129] However, results from CONKO-004 and FRAGEM trials are in contrast with other studies evaluating LMWH. In particular, the TOPIC-1 and TOPIC-2 trials in patients with advanced breast or non–small cell lung cancer did not show statistically significant improvement of VTE rate with the use of LMWH compared to placebo.[130] Similarly, in the PRODIGE study, the VTE rate was lower in patients with malignant glioma who received LMWH dalteparin (9%) compared to placebo (14.9%), but this difference did not reach statistical significance.[131]

This contrasting evidence suggests that standard prophylaxis doses of LMWH may be insufficient in patients with cancer to prevent thrombosis. However, another interpretation is that prophylaxis is beneficial in only certain tumor types. Recently, the PROTECHT study randomly assigned 1,150 patients with lung, gastrointestinal, pancreatic, breast, ovarian, or head and neck cancer to receive subcutaneous injections of LMWH nadroparin (3,850 anti-Xa UI/day) or placebo for the duration of chemotherapy or to a maximum of 4 months.[132] The results showed that 15/769 (2%) patients treated with nadroparin had a thromboembolic event versus 15/381 (3.9%) patients treated with placebo ($P = 0.02$). More recently, an ultra-LMWH, semuloparin 20 mg daily, was compared with placebo in patients with metastatic or locally advanced solid tumors starting on chemotherapy for a mean of 3.5 months.[133] There was a significant reduction of symptomatic or fatal VTE with semuloparin (1.2% vs. 3.4%, HR 0.36, 95% CI 0.21 to 0.60) without any increase in major bleeding.[134]

Overall, there is sound evidence that LMWH is effective in reducing clinically important VTE in selected outpatients receiving chemotherapy, but the optimal dose, duration, and specific patient populations have to be further defined.

Recommendations

Experts' guidelines and consensus statements have been published on the use of VTE prophylaxis for cancer patients, most notably by the American College of Chest Physicians,[134] the

International Union of Angiology,[135] the National Comprehensive Cancer Network (NCCN),[136] the Italian Association of Medical Oncology (AIOM),[137] French National Federation of the League of Centers Against Cancer (FNCLCC),[138] European Society of Medical Oncology (ESMO),[139] and the most recent guidelines from the American Society of Clinical Oncology (ASCO)[140] (Table 125.1). There is broad agreement among the scientific panels on the importance of thromboprophylaxis in hospitalized patients with cancer. Prophylaxis is not yet recommended for ambulatory patients with cancer (with exceptions) or for CVCs. All of the panels agree that LMWH is preferred for the long-term treatment of VTE in cancer.

Surgical Cancer

The AIOM, NCCN, ESMO, and ASCO guidelines recommend prophylactic anticoagulation in the surgical oncology setting. In particular, the ASCO guidelines recommend that patients undergoing laparotomy, laparoscopy, or thoracotomy lasting more than 30 minutes should receive pharmacologic thromboprophylaxis with either low-dose UFH or LMWH unless contraindicated because of a high risk for or active bleeding. Mechanical methods may be added to pharmacologic methods, but should not be used as monotherapy for VTE prevention unless pharmacologic methods are contraindicated. A combined regimen of pharmacologic and mechanical prophylaxis may improve efficacy, especially in the highest-risk patients. These recommendations are echoed by the NCCN and AIOM guidelines.

Since cancer surgery patients remain at elevated risk for VTE for an extended period of time following hospital discharge, the ASCO panel recommends that prolonged prophylaxis for up to 4 weeks be considered in patients undergoing major abdominal or pelvic cancer surgery with high-risk features, such as residual malignant disease postresection, obesity, or a previous history of VTE. The NCCN guidelines also recommend that all cancer patients with high risk (age ≥ 60 years, advanced cancer, operative times more than 2 hours, previous VTE, and >3 days of bed rest) undergoing major surgery should be considered for extended VTE prophylaxis. The AIOM and ESMO guidelines take an even stronger stance by recommending that prolonged prophylaxis be considered the standard of care for patients undergoing elective cancer surgery.

Hospitalized Medical Cancer Patient

ASCO guidelines recommend that hospitalized patients with cancer should be considered for thromboprophylaxis. NCCN guidelines recognize that multiple intrinsic and extrinsic factors play a role in the risk of VTE during hospitalization and ask that clinicians take into account these risk factors. The NCCN panel therefore recommends that all patients with cancer or suspected to have cancer should receive risk-stratified VTE prophylaxis on hospital admission. The AIOM and ESMO guidelines focus on immobility as a major risk factor and recommend prophylaxis only in immobilized hospitalized patients with cancer with an acute medical illness. These guidelines all recommend either low-dose UFH, LMWH, or fondaparinux as anticoagulants for thromboprophylaxis without expressing a preference for a particular agent or class of agents.

Ambulatory Patients

The guideline panels largely agree on not recommending routine thromboprophylaxis in this setting. Outpatient VTE prophylaxis is recommended by the AIOM, NCCN, ASCO, and ESMO panels only for multiple myeloma receiving highly thrombogenic thalidomide- or lenalidomide-based combination chemotherapy regimens. Recommendations in this setting may change in the near future with the availability of a validated thrombosis risk assessment tool.

Central Venous Catheters

Anticoagulant prophylaxis has yet to be proven effective in reducing the incidence of CVC-related DVT. Therefore, until effective regimens are identified, AIOM, NCCN, FNCLCC, and ESMO do not recommend routine prophylaxis for this purpose. However, AIOM and ESMO guidelines suggest tailoring prophylaxis based on individual risk profiles without specifying a method of risk assessment in this setting, while FNCLCC guidelines emphasize the importance of positioning the catheter tip at the superior vena cava–right atrial junction.

DIAGNOSIS AND TREATMENT OF CANCER-RELATED THROMBOSIS

Diagnosis of VTE

Deep Vein Thrombosis

Diagnosis of DVT in patients with cancer relies on the same principles and imaging criteria as in patients without cancer (see Chapter 83). Because cancer patients without DVT can have symptoms consistent with DVT, objective testing is of utmost importance to accurately confirm or exclude the

Table 125.1

Primary prophylaxis in the cancer patient: recommendations

Patients undergoing surgery	Prophylaxis with low-dose UFH or LMWH for at least 7–10 d Extended prophylaxis up to 4 wk after discharge in patients with high-risk features	If patient has contraindication to anticoagulation: consider mechanical methods alone
Hospitalized patients	VTE prophylaxis with anticoagulants	If patient has contraindication to anticoagulation
Ambulatory patients receiving chemotherapy	Only patients with multiple myeloma receiving thalidomide or lenalidomide prophylaxis with LMWH or adjusted-dose warfarin	Otherwise, no routine prophylaxis

presence of DVT. Compression ultrasonography is considered the first test of choice for the diagnosis of DVT, but it is sometimes not available and serial testing to detect extension of a distal DVT may be problematic. To reduce the need for ultrasonography, clinical prediction rules and D-dimer testing have been introduced and validated as reliable tools in excluding DVT.[141] Standardized management algorithms using a combination of these tests are used to maximize diagnostic accuracy and efficiency. However, it is important to note that these diagnostic tests and algorithms have not been evaluated rigorously in patients with cancer. Although many diagnostic studies included oncology patients, the results of this subgroup are often not reported separately. Definition of cancer may also vary from study to study, leaving some uncertainty as to whether the results are comparable across studies or generalizable to different cancer populations. Consequently, clinicians should be cautious in applying clinical prediction rules and D-dimer testing for excluding thrombosis in oncology patients and understand the limitations of these tools in this high-risk population.[142,143]

Clinical predictions rules, in general, have not included cancer-specific risk factors. Tumor type, stage of cancer, and timing of the cancer diagnosis are important determinants of the risk of thrombosis and should be considered when estimating a cancer patient's pretest clinical probability. For example, tumors of the pancreas and brain, advanced or metastatic disease, and the initial few months after a diagnosis of cancer are all associated with the highest risks of thrombosis.[11,22,28] In addition, alternative diagnoses that give rise to symptoms that mimic DVT may in fact increase the risk of thrombosis, rather than diminish it. For example, pelvic adenopathy can cause extrinsic venous compression, giving rise to leg swelling that mimic DVT, but it can also produce sufficient venous stasis to trigger thrombus formation. This alternative diagnosis creates a dilemma in deciding whether to reduce or increase the prediction rule score when estimating the pretest probability of thrombosis.

The use of D-dimer testing in cancer patients is also questionable. In patients without cancer, normal D-dimer levels have a high negative predictive value and, in combination with a low clinical probability or a normal leg ultrasound, can reliably exclude DVT.[141] In oncology patients, D-dimer levels are often elevated in the absence of thrombosis. This lowers the specificity of the test and limits its usefulness or efficiency in ruling out DVT (Table 125.2).[142–147] Also, the high prevalence of thrombosis can reduce the negative predictive value and increase the false-negative rate of moderately sensitive assays in this high-risk population.

A number of retrospective analyses have been done to critically evaluate the accuracy and usefulness of combining a clinical prediction rule and D-dimer testing in cancer patients. Using data from prospective cohort studies that applied the Wells rule and performed D-dimer testing at baseline using the SimpliRED whole-blood agglutination test, one study found that the prevalence of DVT in cancer patients was 9.7%, 27.1%, and 61.6% in the low, moderate, and high probability categories, respectively.[148] These are approximately twice as high as the corresponding values in patients without cancer. Similarly, the prevalence of DVT in the negative D-dimer group was 7.8% among cancer patients, versus 3.5% in patients without cancer. Overall, only 6% of cancer patients presenting with suspected DVT had a combination of a low clinical probability and a negative D-dimer result. Consequently, 94% of patients with cancer would require further investigations such as ultrasonography despite applying the prediction rule and performing D-dimer testing. This raises the question whether these tools are useful in oncology patients. Last, although the negative predictive value of the combination of low probability and negative D-dimer result was 100% (95% CI 69.8% to 100%) in this study, the lower bound of the CI suggests that up to 30% of patients with low probability and a negative D-dimer result may have a DVT. Similar findings were reported in a study using data derived also from prospective cohort studies.[149] In this study,

Table 125.2

Performance of D-dimer assays in patients with cancer

D-Dimer Assay	Cancer Patients (N)	VTE Incidence (%)	Sensitivity (%)	Specificity (%)	NPV (%) (95% CI)
SimpliRED[142]	121	49	86	48	7 (63%–90%)
SimpliRED[143]	217	40	98	48	97 (89–100)
MDA[144]	66	44	97	46	94 (73%–100%)
MDA[145]	50	34	100	45	100 (78%–100%)
Minutex[145]	47	62	100	22	100 (71%–100%)
Tinaquant[147]	72	26	100	21	100 (72%–100%)

NPV, negative predictive value.

the prevalence of thrombosis in cancer patients was 41%. The negative predictive value of a low clinical probability and negative D-dimer was also 100% (95% CI 85% to 100%), but only 9% of the cancer patients had this combination.

In summary, given the limited evidence available, it is uncertain if clinical prediction rules and D-dimer testing add much value to ultrasonography in diagnosing DVT in cancer patients. Further objective testing in those with a low clinical probability score and negative D-dimer result might be warranted, particularly if the physician's clinical suspicion is high or if a moderately sensitive D-dimer test was used.

Pulmonary Embolism

The diagnosis of PE has advanced in the past decade largely due to more sensitive and readily available imaging studies.[150] Computerized tomography pulmonary angiography (CTPA), or spiral CT, has become the first test of choice for most clinicians. This imaging technique enables physicians to accurately confirm or refute a diagnosis of PE in the majority of suspected cases. In contrast, ventilation–perfusion lung scanning has become less popular because of the lack of accessibility and perhaps lower sensitivity.[151] In patients with cancer, CTPA can also provide staging information and reveal an alternative diagnosis in many cases. Clinical prediction rules and D-dimer testing have been evaluated for diagnosis of PE.[152–155] Unfortunately, as in DVT, there is a paucity of evidence in support of these tools in cancer patients. Similar to clinical prediction rules used for diagnosing DVT, rules for PE also do not consider cancer-specific risk factors that are strong predictors of thrombosis. Many conditions give rise to the same symptoms as PE, making it difficult to decide on the significance of an alternative diagnosis. Studies have reported sensitivities and negative predictive values of 100% for D-dimer testing using enzyme-linked immunoassay methods[147,156] and that combining clinical probability and D-dimer testing appears to safely rule out PE.[157] But the numbers of patients with cancer in these studies were small, and so there is less confidence in applying these tools in oncology patients. The low prevalence of patients with a low clinical probability and negative D-dimer result at presentation also limits the usefulness of these tools, as fewer than 10% of patients would not require further testing.[157]

Incidental Thrombosis

Although advances in CT imaging have improved the accuracy in the diagnosis of PE, they have also led to the identification of an increasing number of incidental or unsuspected PE during routine cancer staging and treatment response assessment. The prevalence of incidental PE varies among different studies, ranging from 1.2% to 11%.[17,158,159] Up to 75% of these cases have proximal and multiple emboli. Furthermore, a retrospective study found that 75% of patients with unsuspected thrombosis in fact had symptoms of PE at the timing of scanning. This suggests that physicians often overlook these symptoms or attributed them to the underlying cancer or other conditions, such as anemia.

Staging CT scans of the abdomen and pelvis can also detect unsuspected DVT of the inferior vena cava (IVC) and iliofemoral veins. In a retrospective analysis of 435 consecutive staging CT scans performed at a single institution, an overall prevalence of DVT or PE observed was 6.3%.[159] The prevalence of unsuspected iliofemoral thrombosis was 6.8%, unsuspected common iliac thrombosis 1.2%, unsuspected IVC thrombosis 0.3%, and unsuspected PE in 3.3%. Thrombosis was more common in inpatients and in those with advanced disease. This finding is consistent with other reports.[160,161]

The clinical importance of these unsuspected thrombotic complications and whether treatment with anticoagulant therapy is necessary are uncertain. One report suggests that proximal PE (segmental level or higher) is associated with increased mortality while subsegmental PE does not have an impact on survival.[162] Because the majority of cases with unsuspected thrombosis were treated in studies, the "natural" history or outcome of these patients without treatment is unknown. Few studies have reported favorable outcomes in some patients who did not receive anticoagulation, but the numbers are small.[163] Given the bleeding risk and logistic burden associated with anticoagulant therapy is not insignificant, it should not be prescribed without careful discussion with each patient. On the other hand, proximal thrombosis and embolism are potentially life threatening, particularly in hypercoagulable cancer patients, and likely warrants standard treatment.

Treatment of VTE

LMWH is the preferred agent for monotherapy of cancer-associated thrombosis.[164,165] This replaces the traditional regimens for the treatment of acute VTE using initial therapy with UFH, LMWH, or fondaparinux followed by long-term therapy with a vitamin K antagonist (VKA). Novel oral anticoagulants that achieve rapid inhibition of activated factor X or thrombin may offer an easier solution than LMWH, but studies focusing on treatment of cancer-associated thrombosis with these agents are lacking. To date, some of these new agents have shown comparable efficacy and safety compared with traditional anticoagulants in randomized trials that included primarily patients without cancer.[166,167]

Initial Treatment

Multiple randomized trials and meta-analyses of these trials have confirmed that for initial therapy LMWH is at least as efficacious as UFH in reducing recurrent thrombosis and is associated with a lower risk of major bleeding.[168] Data for the cancer patient subgroup are much more limited but suggest a reduction in the 3-month mortality with LMWH use.[168,169] Fondaparinux also has similar efficacy and safety as the heparins for the initial treatment of VTE in the general population.[170,171] However, a recently published *post hoc*, subgroup analysis of the 477 cancer patients in the MATISSE DVT and PE trials suggests that fondaparinux may be less effective than LMWH but more effective than UFH.[172] Bleeding associated with these parenteral agents for initial therapy in cancer patients has not been reported.[173] Unfortunately, formal clinical trials comparing UFH, LMWH, and fondaparinux are unlikely to be conducted to define their relative risks and benefits in the oncology population.

Long-Term Treatment

In contrast, formal randomized trials have compared the effects of LMWHs with warfarin and other VKAs for long-term therapy in patients with cancer (Table 125.3).[174,175,176,177]

Table 125.3

Randomized studies comparing LMWH with VKA therapy for long-term treatment of cancer-associated thrombosis

Study	Patients (N)	Long-Term Treatment	Recurrent VTE (%)	Major Bleed (%)	Death (%)	P-Value
Meyer et al. 2002[174]	71	Warfarin	21.1		22.7	NS
	67	Enoxaparin 1.5 mg/kg	10.5		11.3	NS
Lee et al. 2003[175]	336	Warfarin	17	4	41	0.002
	336	Dalteparin 200/150 IU/kg	9	6	39	0.002
Deitcher et al. 2006[176]	30	Warfarin	10	2.9	8.8	NS
	29	Enoxaparin 1.0 mg/kg	6.9	6.5	6.5	NS
	32	Enoxaparin 1.5 mg/kg	6.3	11.1	19.4	NS
Hull et al. 2006[177]	100	Warfarin	10	7	19	NS
	100	Tinzaparin 175 IU/kg	6	7	20	NS

VTE, venous thromboembolism; NS, not significant.

Of these, the largest trial is the CLOT study.[175] In this trial, 676 cancer patients with proximal DVT, PE, or both were randomized to receive usual treatment with dalteparin followed by VKA therapy or dalteparin alone for 6 months. Patients in the dalteparin group received full therapeutic doses at 200 IU/kg once daily for the 1st month and then 75% to 80% of the full dose for the next 5 months. The cumulative risk of recurrent VTE at 6 months was reduced from 17% in the VKA group to 9% in the dalteparin group, resulting in a statistically significant risk reduction of 52% (log-rank $P = 0.002$). Overall, there were no differences in bleeding and mortality between the groups. The other trials differ in design from the CLOT study mainly in the duration of treatment (3 months) and the type and dose of LMWH used. Although none of these other trials demonstrated statistically significant differences between the LMWH and warfarin, there were strong trends favoring LMWH in all the studies. Based on published evidence, the use of LMWH alone for the treatment of DVT in patients with cancer is endorsed by international guidelines.[164,165] The dalteparin regimen used in the CLOT study is currently the only LMWH with regulatory approval for extended use in preventing recurrent VTE in cancer patients.

Novel Anticoagulants

Novel anticoagulants, including dabigatran, rivaroxaban, and apixaban, have limited data in patients with cancer. These are effective anticoagulants with no requirement for laboratory monitoring and have minimal drug interactions. They are administered orally once or twice a day, making them very attractive options for long-term use. The treatment trials to date vary in design, with one trial maintaining the two-phase approach starting with a 1-week course of heparin followed by either warfarin or dabigatran (RE-COVER),[166] while other studies initiated rivaroxaban (EINSTEIN DVT)[167] or apixaban (AMPLIFY) in the initial treatment phase. In the dabigatran trial, the risk of recurrent thrombosis and major bleeding were comparable between dabigatran and warfarin.[166] However, only 120 patients with cancer (5% of the total study sample) were included, and their results were not reported separately. In the EINSTEIN DVT trial, there was similarly a small number of oncology patients without separately reported results.[167]

One small phase II randomized feasibility trial has studied the use of apixaban in patients with advanced or metastatic cancer.[178] Patients were randomized to one of three doses of apixaban or placebo during the first 12 weeks of first- or second-line chemotherapy. With approximately 30 patients per group, the study found a very low bleeding rate in those treated with apixaban and a time and dose effect on F1+2 and other markers of coagulation.

Given the higher risk of recurrent thrombosis and bleeding in patients with cancer, it is important that further research is done to understand the antithrombotic impact of these new agents in cancer patients before accepting it for treatment of cancer-associated thrombosis.

Treatment of Recurrent Thrombosis

Up to 9% of patients with cancer-associated thrombosis treated with LMWH or 20% treated with warfarin can develop recurrent VTE. Studies have suggested that presence of metastasis, younger age, or a short interval between VTE and cancer diagnosis (>3 months) are predictors of recurrent thrombosis despite anticoagulation.[179,180] Whether the risk factors that increased the risk of a first episode of thrombosis also contribute to a higher risk of recurrent thrombosis is unknown.

Although randomized controlled trial data are lacking to guide optimal management in oncology patients with recurrent thrombosis, observational data and increasing clinical experience support the use of LMWH in this setting. In patients who developed a recurrence while on warfarin therapy, the recommended practice is to switch these patients to LMWH because it is more efficacious than warfarin. Raising the intensity of warfarin therapy is not recommended because of the potential for increasing bleeding without a benefit in reducing recurrent VTE. Patients with cancer have a high risk of bleeding as well as a high

risk of recurrent thrombosis despite achieving therapeutic and even higher INRs.[18,181]

For patients who developed a recurrence while on LMWH, dose escalation of LMWH is often effective. In a small cohort study of oncology patients with recurrent thrombosis while on LMWH or warfarin, escalating the dose of LMWH by 20% to 25% or switching to LMWH, respectively, was effective in preventing further thrombotic episodes.[181] During 3 months of follow-up, 6 of 70 (8.6%) of patients developed another recurrence while 1 patient had a major bleeding event and 2 had minor bleeding. The success of escalated doses of LMWH suggests that the standard weight-adjusted dose regimens are insufficient in some patients with cancer. This is not surprising given the heightened prothrombotic state of these patients. Anecdotal experience has found that doses twice that of the standard recommended LMWH dosing might be required to achieve therapeutic anti-Xa levels and are well tolerated in some oncology patients.

Inferior Vena Cava Filter

Insertion of a vena cava filter has been recommended for oncology patients with recurrent VTE despite adequate long-term LMWH therapy. However, adequate studies have not been done to evaluate or document the outcomes. Retrospective series have reported that up to 32% of oncology patients with filters inserted for thrombosis develop recurrent VTE.[182] The high recurrence rate is not surprising since filters do not treat the underlying hypercoagulable state in patients with cancer. The single randomized trial studying the efficacy of filters in patients who were also treated with anticoagulation showed a reduction in symptomatic PE but higher rates of recurrent DVT in the filter group.[183] Overall, the total VTE rates were the same and a difference in overall survival was not observed. Considering the cost and invasiveness of filters and the lack of proven efficacy, they should be used only in situations where anticoagulant therapy is contraindicated because of serious, active bleeding.

CONCLUSIONS

Patients with cancer are at high risk of developing VTE, and the risk of VTE varies according to the type of malignancy and its disease stage. The pathogenesis of cancer-associated VTE is multifactorial and includes demographic, cancer-associated, and treatment-related risk factors. Hospitalization, surgical, and nonsurgical cancer treatments (i.e., chemotherapy, hormonal, or immunosuppressive agents in conjunction with chemotherapy), or the presence of a CVC, increase the risk of VTE patients. Effective VTE prophylaxis for cancer patients at significant risk of thrombosis reduces the burden of VTE and improves outcomes. The current development of VTE predictive models, using the increasing knowledge of the clinical and biological markers of thrombosis in cancer, is important for identifying high-risk patients and reducing associated morbidity and mortality.

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CHAPTER

126 Hemostatic Challenges in Liver Disease

CHARLES S. EBY AND STEPHEN H. CALDWELL

The liver is the predominant site for synthesis and clearance of proteins involved in formation and lysis of fibrin, as well as its regulation, and the predominant source of thrombopoietin (TPO). Therefore, acute and chronic liver diseases cause major changes in multiple components of the hemostasis system. In the past, these changes have been viewed as shifting the balance toward bleeding based on traditional screening hemostasis tests and common bleeding problems in cirrhosis. However, the view is changing based on *in vitro* studies supporting suppression of both prothrombotic and regulatory pathways to produce a fragile, but rebalanced hemostasis state. The inability of traditional screening hemostasis tests to predict bleeding complications is perhaps most apparent in successful liver transplantation with minimal blood product replacement and minimal blood loss.[1] Multiple observations have also identified evidence of a hypercoagulable predisposition in chronic liver disease patients who are at risk for thrombotic consequences. General application of this new paradigm to patients with severe liver disease awaits development and validation of novel laboratory methods and therapies in order to evaluate alternative strategies for assessment and management of hemostatic dysfunctions in patients with advanced liver diseases.

At this time, it is not possible to provide detailed, evidence-based guidelines for management of important clinical situations including prophylaxis and treatment of bleeding complications. Recognition of these knowledge gaps continues to stimulate clinical and laboratory research of hemostasis challenges in liver disease, and it is likely that in the coming years there will be more definitive management recommendations.

ACUTE LIVER FAILURE

Acute liver failure (ALF), defined as new-onset encephalopathy and coagulopathy following the development of jaundice in patients without preexisting liver disease,[2] has an estimated incidence of approximately 2,000 cases/year[3] and a mortality rate of approximately 33% in the United States, primarily due to cerebral edema, sepsis, and bleeding.[2] The leading causes of ALF in the underdeveloped world are viral hepatitis A, B, and E, while in Europe and the United States, acetaminophen toxicity, idiosyncratic drug reactions, and indeterminate causes are the main etiologies.[4]

At presentation, most ALF patients have diminished procoagulant factors evident by international normalized ratio (INR) 1.5 to 5.0 in 81.1%, INR > 10 in 4.8%, platelet count <120,000/μL in 86.1%, and <30,000/μL in 2.6%, based on data from the Acute Liver Failure Study Group (ALFSG) prospective cohort study of 1,074 patients.[5] Decreased hepatic synthesis of procoagulant, anticoagulant, and fibrinolytic proteins and accelerated consumption of hemostasis factors and platelets are consistent features of ALF regardless of underlying cause.[6–9] Plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), and factor VIII are elevated, likely reflecting the endothelial response to inflammatory cytokines.[6–7,10] Despite considerable hemostatic derangements, spontaneous bleeding complications are uncommon (<10%) in ALF patients,[5] and prophylactic plasma and platelet transfusions are inappropriate since they increase vascular volume and may exacerbate cerebral hypertension.[11]

Assessment of coagulopathy plays a role in determining if ALF patients should be listed for liver transplantation. The King's College criteria for transplantation include INR > 6.5, while the Clichy-Villejuif criteria include factor V activity <20%,[4] and initial factor VII activity <20% is associated with increased mortality.[12] A high model of end-stage liver disease (MELD) score (in part dependent on INR, Table 126.1) appears to be a sensitive predictor of death without transplant, but requires validation.[13] Indications for liver biopsy (rarely done) and intracranial pressure monitoring, and blood component transfusions prior to these invasive procedures, are controversial.[11,14] "Safe" INR and platelet count targets are not evidence based, and aggressive transfusion practices may worsen intracranial pressure.[5,14] Based on favorable anecdotal reports, the ALFSG recommends that cautious treatment with fresh frozen plasma (FFP) or cryoprecipitate (avoiding potentially detrimental volume overload) followed by recombinant activated factor VIIa (rFVIIa) be limited to highly invasive procedures such as insertion of intracranial pressure monitors.[11] Notably, one recent study showed that ALF patients often maintain normal thromboelastography (TEG) in spite of prolonged INR consistent with the concept of a rebalanced system in liver disease.[15]

In bleeding patients who are also at high risk for thrombotic complications, plasma exchange could be an alternative therapy to improve hemostasis. A European randomized trial compared three daily plasma exchanges to routine management in 182 ALF patients. Fifty-nine percent of plasmapheresed patients survived to leave the hospital compared to 48% in the control group.[16] However, confirmation of these preliminary findings is necessary before considering plasma exchange to be a standard strategy in ALF.

CHRONIC LIVER DISEASE

Causes of chronic liver injury include hepatitis B and C; alcohol; nonalcoholic steatohepatitis; autoimmune, inherited disorders; and cryptogenic disease. Chronic liver disease has long been associated with disturbances in hemostasis, and

Table 126.1

Prognostic scoring systems in cirrhosis

Model of End-Stage Liver Disease (MELD) Score

9.57 × ln (creatinine mg/dL) + 3.78 × 3.78 × ln (bilirubin mg/dL) + 11.2 × ln (INR) + 6.43

Child-Pugh-Turcotte Score

Parameter	1 Point	2 Points	3 Points
Bilirubin (mg/dL)	<2.0	2.0–3.0	>3.0
Albumin (g/dL)	>3.5	2.8–3.5	<2.8
PT prolong (s)	<4.0	4.0–6.0	>6.0
Ascites	None	Easily controlled	Poorly controlled
Encephalopathy	None	Grades 1–2	Grades 3–4

Child A: 5–6 Points—well compensated
Child B: 7–9 Points—significant functional compromise
Child C: 10–15 Points—decompensated

both pro- and antithrombotic effects are recognized.[17] As liver disease progresses, conventional indices of the clotting cascade worsen and have been incorporated into liver disease prognostic scores including the classical Child-Pugh and the MELD scores (Table 126.1). The latter is now used to guide organ allocation in liver transplant candidates.[18] The relationship between abnormal coagulation indices and progressive liver fibrosis and cirrhosis is robust across various forms of chronic liver disease.

REBALANCED COAGULATION PATHWAYS IN STABLE CIRRHOSIS

The concept of a rebalanced but fragile hemostatic system in chronic liver disease has become established over the past few years (FIGURE 126.1).[1,19,20,21] In stable cirrhosis, the relative deficiency of hepatocyte-derived protein C and S, and antithrombin (AT), and increased factor VIII activity counterbalance the deficit in procoagulant factors. This concept is supported by *in vitro* methods that measure cumulative thrombin generation. Using small amounts of tissue factor and phospholipid to activate the extrinsic pathway, thrombin formation is monitored by measuring hydrolysis of a fluorescent-labeled peptide substrate to determine total thrombin generation or endogenous thrombin potential (ETP). Thrombin generation decreases as the severity of cirrhosis worsens.[20] However when endogenous protein C activation (aPC) is incorporated into the thrombin generation test, the ratio of (ETP + aPC)/(ETP unmodified) was significantly higher in the cirrhotic groups compared to healthy controls.[20,22,23] A higher ratio suggests a prothrombotic imbalance. Overall, the data suggest that protein C anticoagulant dysfunction is greater than the acquired coagulopathy in stable cirrhotic patients, producing a procoagulant state similar to congenital protein C deficiency or factor V Leiden (FVL) heterozygosity[24] despite prolonged prothrombin time (PT)/INR. Importantly, *in vitro* thrombin generation results have not yet been correlated with clinical outcomes so clinical recommendations cannot yet be formalized, although this work provides strong evidence for questioning long-standing practices such as INR cutoffs as indications for plasma transfusion.

Therefore, conventional coagulation tests such as PT/INR and activated partial thromboplastin time (aPTT) do not accurately reflect the bleeding risk in compensated cirrhosis patients. Reduced hepatic synthesis of AT accompanies progressive liver disease and may also contribute to a prothrombotic imbalance. However, while AT infusion prior to liver transplantation corrects the deficiency, it does not reduce thrombin generation.[25] Presently, there is no evidence to support a clinical benefit from AT replacement under any circumstance in patients with end-stage liver disease.

REBALANCED PRIMARY HEMOSTASIS IN STABLE CIRRHOSIS

Platelets serve as the phospholipid scaffold for the clotting cascade and undergo complex quantitative and qualitative changes in cirrhosis.[26] Prevalence estimates of thrombocytopenia in patients with chronic liver disease vary widely from 6% to 64%.[27] Severe thrombocytopenia requiring prophylactic platelet transfusions or corrective intervention is uncommon[27,28] and typically occurs in patients with decompensated liver disease (the term "decompensated" is a common way to describe cirrhotic patients requiring more aggressive care and often hospitalization for a variety of complications related to chronic liver failure and portal hypertension—*see also Decompensated Cirrhosis section*).

The major causes of thrombocytopenia in cirrhosis include splenic sequestration, decreased survival, and decreased production. However it can be challenging to identify and prioritize the likely causes for individual patients. Using radiolabeled platelets, investigators determined that thrombocytopenic, cirrhotic patients sequestered 50% to 90% of platelets in their spleen in an exchangeable pool while maintaining near-normal total body platelet mass and mild-to-moderate shortened platelet lifespans.[29,30] Splenic sequestration is a complex process, not simply dependent upon portal hypertension, as evidenced by

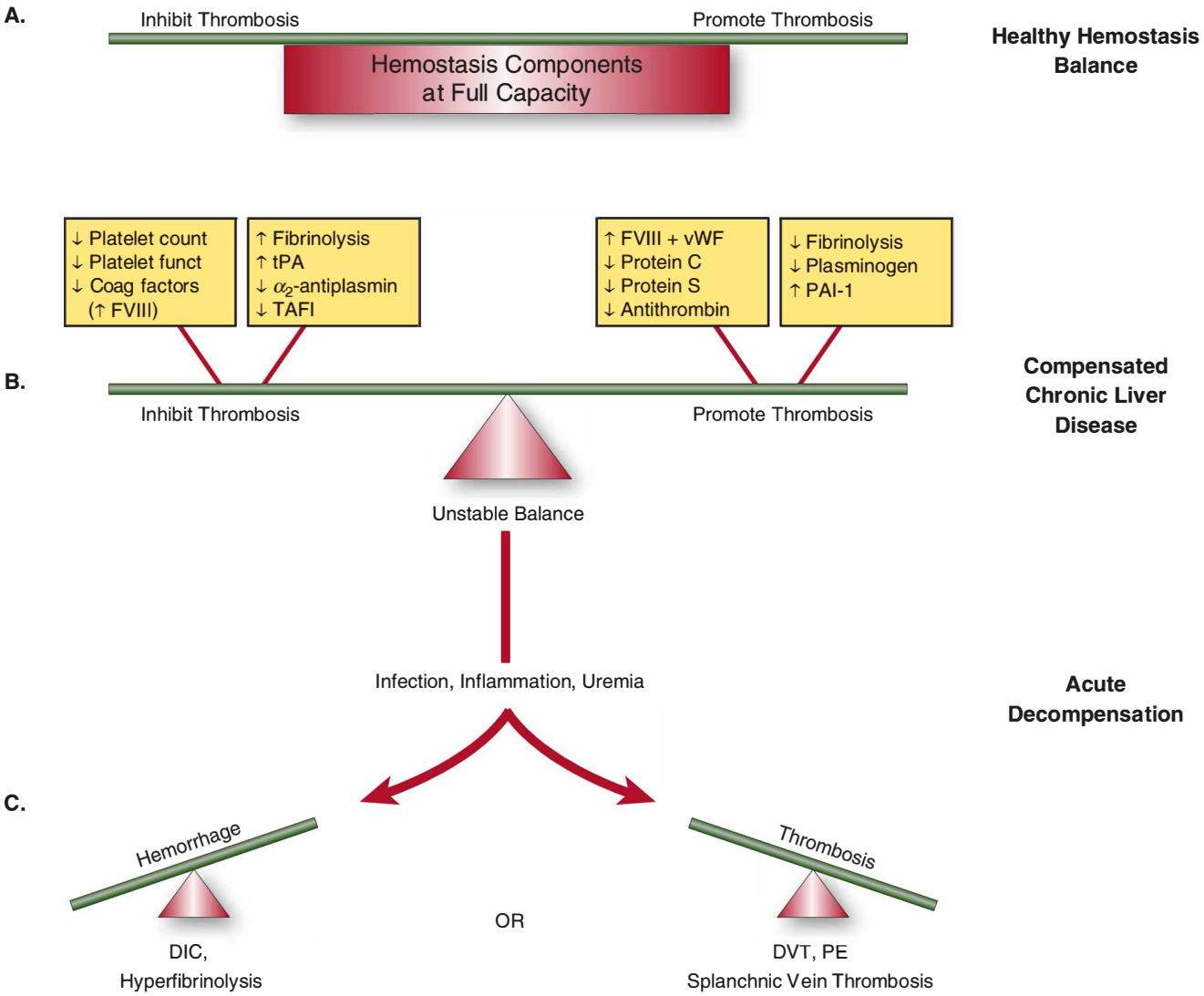

FIGURE 126.1 Changes in hemostasis balance with progressive liver disease. In healthy individuals **(1A)**, cellular and protein components of prohemostasis and antihemostasis pathways have adequate capacities to adapt to acute physiologic stresses. In patients with compensated liver disease **(1B)**, reductions in most coagulation/fibrinolysis factors and platelet number/function are offset by reductions in anticoagulant/antifibrinolysis factors and increased FVIII and vWF, resulting in a tenuous rebalancing of hemostasis. In patients with decompensated liver disease **(1C)**, comorbidities further deplete most pro- and antihemostasis functions producing a fragile and imbalanced state and increased risk of hemorrhage and thrombosis.

inconsistent improvement in platelet counts after transjugular intrahepatic portosystemic shunting or surgical decompression procedures.[31] Reduction of splenic tissue, by splenectomy[32] or partial splenic embolization,[33] results in higher platelet counts, albeit with potential morbidity. Shortened platelet survival due to immune-mediated platelet destruction may be a factor in patients with severe thrombocytopenia out of proportion to the stage of hepatic dysfunction, and in some cases, there have been responses to steroids or other immunosuppression therapies.[34,35] However, there is no value in routinely measuring platelet-associated immunoglobin G in thrombocytopenic patients with chronic liver disease due to a lack of specificity of the assay. Causes for decreased platelet production include suppression of megakaryopoiesis due to decreased hepatic production of TPO, or viral illness (HCV, HIV), alcohol, or medication (alpha interferon) toxicity.[36] Observations of platelet kinetics following orthotopic liver transplantation (OLT) indicate that TPO biology is disrupted in thrombocytopenic patients with advanced liver disease. A day after OLT, TPO concentrations increase followed by increased reticulated platelets and

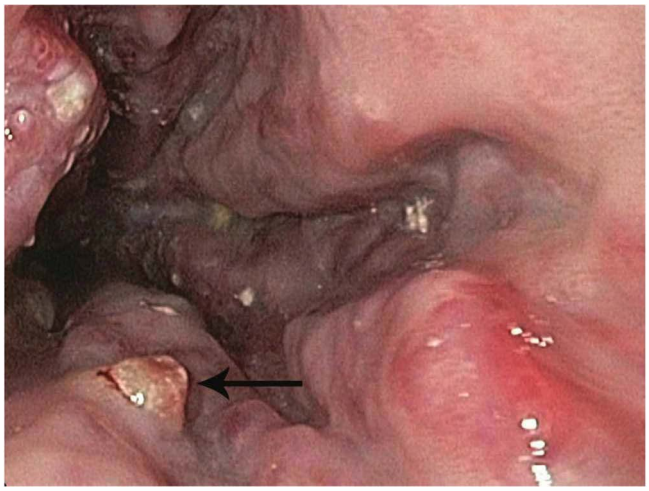

FIGURE 126.2 Gastric varices with platelet plug ("nipple") sign indicated by arrow. A scant amount of blood can be seen to be oozing from the base of the plug. (Image courtesy of Dr. Andrew Wang.)

resolution of thrombocytopenia.[37] In addition, pharmacologic interventions with recombinant TPO, TPO mimetics, and IL-11 increase platelet production.[28] However, TPO concentration in patients with chronic liver disease correlates poorly with platelet count,[28] partly explained by analytical problems for measuring TPO.[38] Therefore, it is of little value to measure TPO concentrations in thrombocytopenic patients with liver disease.

In vitro evidence supports thrombocytopenia as a contributor to defective hemostasis in patients with liver disease. Tripodi et al.[39] compared thrombin generation between healthy controls and patients with cirrhosis in the presence of soluble thrombomodulin and platelets. When platelet counts were normalized to 100,000/μL, ETP was similar in both groups. However, when platelet-rich plasmas were adjusted to patient and control whole-blood platelet counts, respectively, there was a positive correlation between platelet count and ETP.

Platelet dysfunction is especially important in mucosal and wound bleeding and possibly even in early stages of pressure-driven variceal bleeding, based on the well-known significance to endoscopists of the platelet plug sign (FIGURE 126.2).[40] There is ample experimental evidence of acquired platelet adhesion, activation, and aggregation defects in liver disease patients.[1,41,42] However *in vitro* model systems designed to assess platelet adhesion and aggregation in whole blood from liver disease patients under physiologic flow conditions suggest that primary hemostasis is not demonstrably defective, perhaps due to compensation from increased vWF activity.[26,43] Patients with advanced cholestatic liver diseases (primary biliary cirrhosis and primary sclerosing cholangitis) appear to have fewer bleeding complications and higher rates of portal vein thrombosis (PVT) compared to patients with hepatocellular injury.[42] Preliminary *in vitro* studies suggest enhanced platelet activity may be involved.[44,45]

FIBRINOLYSIS IN STABLE CIRRHOSIS

Multiple alterations to components of the fibrinolytic system occur with advanced liver disease[46,47] (Table 126.2). A lack of clinically validated functional tests to assess both fibrinolysis and its regulation hampers patient assessment.[48] Consequently, there is debate as to whether the dominant process is excess plasmin generation, due to increased tissue plasminogen activator (tPA) activity, decreased thrombin activatable fibrinolysis inhibitor (TAFI)[46,47] or diminished a_2-antiplasmin causing hyperfibrinolysis and elevated D-dimer, or decreased hepatic synthesis and clearance of fibrinolytic proteins and fibrin degradation products.[49] It is reasonable to propose that compensated cirrhotic patients have a fragile, poorly regulated fibrinolysis system, which can be tipped over into overt hyperfibrinolysis by increased thrombin generation due to activators from ascites, infection, malignancy, or hemorrhage.[21,47]

Clinically important secondary hyperfibrinolysis is estimated to occur in 5% to 10% of cirrhosis patients, typically during unstable, decompensated periods.[50,51,52–54] In one series of 86 consecutive admissions to an inpatient liver unit, 36% of cirrhotic patients had a shortened euglobulin lysis time (<120 minutes) and 22% had signs of mucocutaneous bleeding.[55] The condition should be suspected with spontaneous or delayed postprocedure bleeding and is accompanied by acute decreases in fibrinogen and platelet count, increased D-dimer, and prolonged PT and aPTT, or detected by TEG in the absence of significantly abnormal INR (personal observation). The clinical presentation and associated changes in hemostasis parameters are compatible with disseminated intravascular coagulopathy, although end-organ thrombotic injury is uncommon. Patients can present with spontaneous mucosal bleeding or multiple large hematomas due to primary hyperfibrinolysis and without evidence of an underlying precipitating event or multiorgan compromise. Typical laboratory results include acute hypofibrinogenemia and markedly shortened euglobulin lysis time, worsening anemia, but stable platelet count, PT, and aPTT.[56] Recognition of this clinical entity is important as more specific therapy is available (see Specific Agents section).

Table 126.2

Typical changes in activation and regulation proteins of fibrinolysis in patients with advanced hepatic dysfunction and cirrhosis

tPA	↑↑
PAI-1	↑
a_2-Antiplasmin	↓
TAFI	↓
Plasminogen	↓
Fibrin degradation products/D-dimer	↑

Sources: Colucci M, et al. Deficiency of thrombin activatable fibrinolysis inhibitor in cirrhosis is associated with increased plasma fibrinolysis. *Hepatology* 2003;38(1): 230–237; Lisman T, et al. Thrombin-activatable fibrinolysis inhibitor deficiency in cirrhosis is not associated with increased plasma fibrinolysis. *Gastroenterology* 2001;121(1):131–139; Tripodi A, Mannucci PM. The coagulopathy of chronic liver disease. *N Engl J Med* 2011;365(2):147–156.

DECOMPENSATED CIRRHOSIS

A number of complications that influence the hemostatic system balance can occur in stable cirrhosis patients. "Decompensation" in this situation refers to the development of overt problems associated with worsening portal hypertension and liver failure such as ascites and encephalopathy, often in the setting of jaundice, which commonly necessitate hospitalization—a process more recently referred to as "acute on chronic liver failure."[57] Bleeding is common and sometimes precipitates the decompensation. In this situation, bewildering arrays of complex processes are evident, but three principle conditions affect hemostatic balance and bleeding risk: *infection, renal failure, and vasomotor dysfunction*.[58–60]

Bacterial infection, for example, subacute bacterial peritonitis, urinary tract infection, or cellulitis, is a common cause of decompensation in cirrhosis, and more subtle bacterial effects such as changes in intestinal permeability and endotoxemia impact the hemostatic system.[61] One mechanism is release of endogenous heparinoids that inhibit factor Xa.[62,63] Renal failure, a common problem in decompensated cirrhosis (hepatorenal syndrome), contributes to platelet dysfunction.[64] In this setting, hemodialysis can be viewed as a hemostatic intervention by reversing uremic platelet dysfunction and reducing volume overload.[64,65] Cirrhotic endothelial dysfunction is most evident by the occurrence of "hyperdynamic circulatory dysfunction," an arteriolar vasodilated state characterized by stable but low mean arterial blood pressure and increased cardiac output that can progress to the hepatorenal syndrome.[66] Experimentally, the condition is associated with impaired vascular smooth muscle contractility and impaired vasoconstriction in cirrhosis.[67] Increased nitric oxide (NO) synthesis by activated or injured endothelial cells may have a role in cirrhosis-related altered vasodilatation since NO and vWF increase with progressive liver disease and elevations of both are associated with endotoxemia.[68,69] Other indirect signs of endothelial stimulation or damage in cirrhosis include progressive elevation of soluble thrombomodulin and tissue factor.[70]

LABORATORY ASSESSMENT OF BLEEDING RISK

Assessment of hemostatic status in patients with advanced liver disease is crucial for management decisions, including priority for liver transplantation, bleeding risk assessment, and monitoring response to treatment. However, most routine hemostasis tests are seriously flawed for lack of convincing evidence for prediction of bleeding tendency, incomplete interrogation of complex, regulated pathways, methods based on nonphysiologic conditions, and interlaboratory imprecision. This leaves clinicians in the difficult position of either using arbitrarily determined hemostasis test "cutoffs"[71] or relying on clinical assessment of bleeding risk when deciding whether or not to use potentially harmful hemostatic therapies.

The hemostasis system in most patients with compensated, advanced liver disease appears to be functionally, but precariously, rebalanced (see FIGURE 126.1). Much of the data correlating hemostasis test results with bleeding complications are derived from percutaneous liver biopsy (PLBx) experience in relatively stable patients. Major bleeding complications after PLBx have been consistently low,[72] with rates of 0.5% to 0.6% in large contemporary series.[73,74] Platelet count, occasionally a platelet function screening test, and INR thresholds are typically used to assess bleeding risk, and when exceeded, mandate alternative biopsy techniques or blood component transfusions. However, the supporting evidence for hemostasis parameter limits is insufficient.[72,75]

Retrospective case series have shown higher bleeding complication rates after PLBx with platelet counts <50,000/μL,[73,76,77] leading to widespread acceptance of a minimal platelet count.[73,74] Platelet function tests are limited in their utility to assess bleeding risk in patients with liver disease. Bleeding time (BT) may be prolonged in 40% of cirrhotic patients,[78] but a prolonged BT is a poor predictor of post PLBx bleeding, with the possible exceptions of bone marrow transplants.[78,79]

While a prolonged INR is an indicator of decreased hepatic synthetic capacity, it is a poor predictor of bleeding risk in patients with liver disease.[73,75,76,80] Therefore, an INR cutoff to determine bleeding risk prior to liver biopsies has limited value because the INR reflects the reduction in procoagulant factors, but not the decline in the protein C regulatory pathway. The thrombin generation test is an alternative method to assess changes in both procoagulant and regulatory functions in cirrhotic plasma, but it is neither standardized nor routinely available.

The INR is also limited because differences in reagents and instruments make it inappropriate to adopt specific INR thresholds derived from a single institution for general use, and conversion of a PT ratio to an INR is also problematic (FIGURE 126.3). INRs performed on plasma specimens from patients with liver disease demonstrate markedly poor interlaboratory agreement when using an ISI (International Sensitivity Index) exponent calculated from patients on oral vitamin K antagonists.[81,82] In addition to making uniform INR cutoffs for invasive procedures

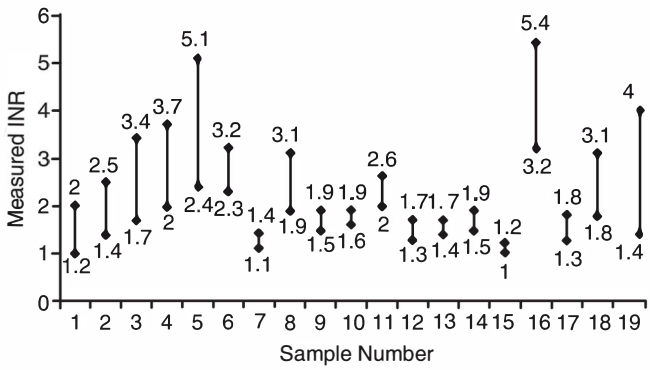

FIGURE 126.3 Interlaboratory variation of INRs performed on plasmas from patients with cirrhosis. For each sample (1 to 19 on *X*-axis), the table shows the high and low INR values (*Y*-axis) obtained from laboratories using different coagulation reagents and instruments. There is marked interlaboratory imprecision when reporting INRs on patients with cirrhosis using thromboplastins whose ISIs are derived from patients anticoagulated with vitamin K antagonists. (Reprinted from; Caldwell S, Northup PG. Bleeding complication with liver biopsy: is it predictable? *Clin Gastroenterol Hepatol* 2010;8:826–829, with permission from Elsevier.)

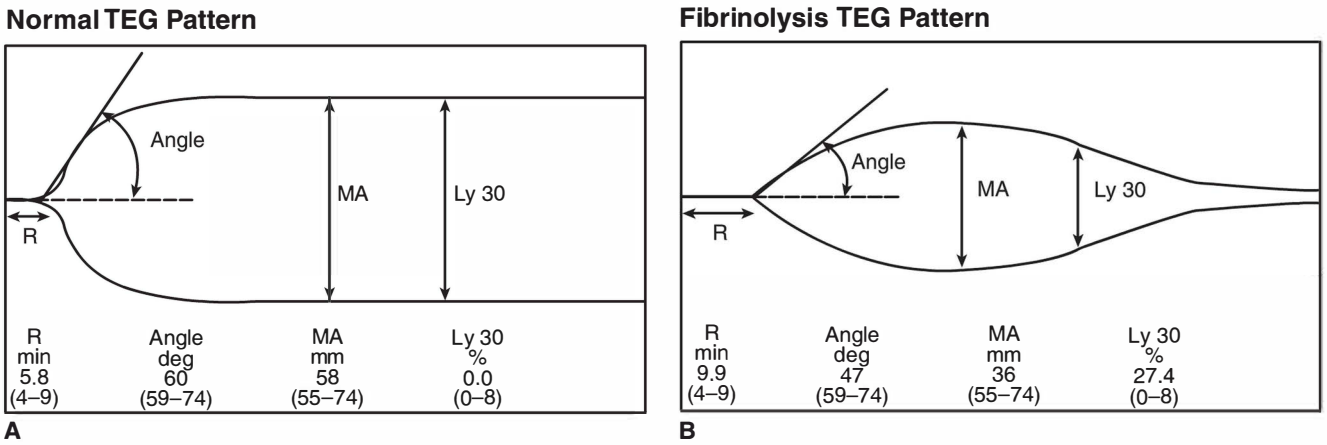

FIGURE 126.4 Thromboelastogram (TEG) patterns. Kaolin and calcium are added to a cup containing citrated blood. A pin attached to a torsion wire immersed in the cup records changes in whole-blood "shear elasticity" due to platelet aggregation and fibrin formation and lysis as the specimen cup rotates approximately 5 degrees clockwise and counterclockwise (*Y*-axis, mm) over time (*X*-axis, minutes). Measured parameters include: R-time to initial fibrin formation, α angle rate of increasing clot strength, MA (maximal amplitude)–clot strength, and Lys 30% change in clot strength due to fibrinolysis. **A**: TEG tracing from a healthy control. **B**: TEG tracing during OLT: Onset (R) and rate (α angle) of clot formation are delayed, and maximum clot strength (MA) is reduced, consistent with coagulation factor, fibrinogen, and platelet depletion. There is also evidence of hyperfibrinolysis indicated by 27.4% reduction of clot strength after 30 minutes (Lys 30). (TEG 5000, Haemonetics Corp. Braintree, MA, USA.)

untenable, INR variability affects the MELD score. If thromboplastin ISIs are calibrated with plasmas from patients with a prolonged PT due to liver disease (ISI_{liver}), INR and MELD score agreements are markedly improved,[81,82] but logistical challenges have prevented integration of the INR_{liver} into the MELD formula.[83]

TEG is a global hemostasis test sometimes used by anesthesiologists and surgeons to guide blood product utilization during OLT. A TEG test records changes in viscosity of whole blood over time as clot formation and fibrinolysis occur (FIGURE 126.4). Parameters are derived from the tracing, which correlate with routine hemostasis tests: PT and aPTT with TEG (R), and fibrinogen, factor VIII, and platelet count with TEG alpha (α) angle and maximum amplitude (MA).[84] TEG parameters are also sensitive indicators of hemostasis changes occurring when decompensated liver disease patients are infected,[61] but the utility of TEG to assess bleeding or clotting risks in patients with liver disease has not been adequately investigated.[85] In addition, TEG does not assess the protein C pathway. Nonetheless, clinical studies are underway to assess the clinical utility of various forms of TEG on the liver disease wards.

MANAGEMENT APPROACHES TO HEMOSTASIS IN LIVER DISEASE PATIENTS

Bleeding is common among liver disease patients and includes spontaneous hematomas, oozing from oropharyngeal mucosa, gastrointestinal (GI) hemorrhage, bleeding after dental extractions, skin puncture and biopsy sites, and rarely, intracranial bleeding. Approximately 50% of cirrhotic patients develop gastroesophageal varices, and one-third will experience variceal hemorrhage. With an in-hospital mortality rate of approximately 15% for each episode of acute bleeding,[86] variceal bleeding is the cause of death for approximately 6% of patients with cirrhosis.[87] Predictors of the first upper GI hemorrhage in cirrhotic patients include the presence[88] and endoscopic appearance of varices,[89] elevated variceal pressures,[90] and laboratory evidence of decreased hepatic synthesis, coagulopathy, and thrombocytopenia.[88] Scoring systems based on these parameters identify patients at high risk of GI bleeding.[88,91] Once bleeding has occurred, an elevated MELD score (≥17[92] or ≥18[93]), which includes INR, is the best predictor of early rebleeding and early mortality.

Prophylaxis versus Rescue Interventions

In general, the approach to bleeding in liver disease patients can be divided into prophylactic interventions, for example, prior to an invasive procedure, and rescue therapy for active bleeding.[94] Effective prophylaxis implies a valid means of risk stratification. However, recognition of the complex hemostatic changes in cirrhosis has eroded support for plasma administration to achieve empiric INR targets, resulting in a reevaluation of the risk/benefit of this practice. Although some societal guidelines continue to recommend INR targets to guide prophylactic FFP infusions,[95] others have recognized the limitations of this approach.[72] In contrast, a greater consensus exists for platelet administration in thrombocytopenic cirrhosis patients (threshold 50,000/μL) prior to moderate- or high-risk procedures.[39]

The rescue approach uses directed interventions for a bleeding complication and acknowledges the availability of emerging but nonestablished therapeutics.

General Supportive Measures

Several general measures should be considered to improve hemostasis in decompensated cirrhosis, including control of infection, therapy of renal failure, and avoidance of volume expansion to avoid effects on portal pressure and the collateral vascular bed including varices.[60] The recognition that volume expansion increases portal pressure and exacerbates bleeding in cirrhotic patients has led to the development of the "low-volume" approach to patients undergoing liver transplantation and to conservative recommendations for target hematocrit levels in variceal bleeding.[96,97] Treating the hyperdynamic state with octreotide (and/or terlipressin) to improve vascular tone may also diminish bleeding in collateral vessels other than varices for which these agents are commonly used.[98] For example, based on anecdotal experience, one of the authors (SC) recommends such agents prior to sphincterotomy in portal hypertensive patients to diminish mucosal vascular congestion.

Specific Agents

Vitamin K

Although replacement therapy is generally advised, in most decompensated cirrhosis patients, vitamin K replacement therapy for prolonged INR is ineffective. Vitamin K deficiency (causing impaired gamma-carboxylation of factors II, VII, IX, X, and proteins C and S)[99] is uncommon, unless there is coexisting cholestasis, antibiotic therapy, or recent malnutrition. If utilized, vitamin K should be administered parenterally, as oral absorption may be impaired in patients with liver failure. Intravenous vitamin K therapy carries a rare risk of anaphylaxis,[100] and absorption after subcutaneous administration may be reduced in patients with edema.

Plasma, Cryoprecipitate, Red Blood Cells, and Platelets

Plasma at common dosing (2 to 4 units or 10 mL/kg) has minimal effect on coagulopathy,[101] and only 10% of cirrhosis patients reach target reductions of INR.[102] INR imprecision in cirrhosis, the adverse effects on intravascular volume, and the risk of transfusion-associated acute lung injury (TRALI) have combined to temper enthusiasm for this approach.[103] Cryoprecipitate has the advantage of replacing fibrinogen if deficient without substantial volume expansion. In this regard, measurement of fibrinogen levels has been anecdotally recommended preprocedure with a target level of 120 to 150 mg/dL (using cryoprecipitate) to facilitate activity of rescue agents such as rFVIIa. However, there are no conclusive data to support a specific fibrinogen level to trigger replacement. Packed red blood cell replacement should also be tempered due to the effects on portal pressure.[97] Current recommendations indicate a target hemoglobin of 7 to 8 g/dL in variceal bleeding.[97] Platelet transfusion to target levels of 50,000/μL is reasonable based on *in vitro* studies discussed above but lacks a foundation in clinical outcomes research.

Desmopressin, 1-Deamino-8-Arginine Vasopressin

Intravenous or subcutaneous desmopressin, 1-deamino-8-arginine vasopressin (DDAVP) 0.3 to 0.5 μg/kg decreases BT possibly due to acute release of vWF from endothelial cells.[104,105] Intranasal DDAVP was as effective as FFP or platelet transfusions in cirrhosis patients with elevated INRs and/or platelet counts <50,000/μL undergoing dental extractions.[106] However, no benefit was seen in a study of variceal bleeding which compared terlipressin plus DDAVP to terlipressin alone.[107] Another randomized trial comparing DDAVP and placebo prior to partial hepatectomy in patients with hepatocellular cancer and cirrhosis showed no difference in blood loss or transfusion requirements.[108] The necessity of DDAVP prior to liver biopsies in patients with end-stage kidney disease was not supported by a review of 78 liver biopsies without DDAVP in hepatitis C patients on chronic dialysis that identified one minor bleeding complication.[109] While side effects from DDAVP appear low, and this agent is commonly used preprocedurally, clinical efficacy has not been established for most applications.

Antifibrinolytics

Antifibrinolytics may be useful when cirrhosis-related hyperfibrinolysis is strongly suspected or proven. The two agents are ε-aminocaproic acid (EACA) and tranexamic acid (TA), both of which inhibit binding of plasminogen or plasmin to fibrin, thus inhibiting fibrinolysis. EACA can be used topically (e.g., post-dental extraction), and both medications are available for oral or intravenous administration in the United States. Other than a handful of case reports, there is no convincing evidence associating thromboembolic complications with EACA and TA.[110] A reduction in red cell transfusions during OLT was confirmed in a meta-analysis of randomized control trial (RCT) comparing TA to placebo.[111] The efficacy of EACA during OLT is unproven due to only one underpowered RCT.[111] Results from single-institution case series indicate EACA is effective for controlling mucosal and subcutaneous bleeding in patients with shortened euglobulin clot lysis times[112] and TA reduces mortality in cirrhotic patients with GI bleeding,[113] but these observations have not been confirmed. There is a risk of ureteral occlusion with hematuria due to resistant clot lysis, and GI side effects, but overall the antifibrinolytic agents appear to be safe and well tolerated in decompensated cirrhotic patients.[55,112]

Recombinant Activated Factor VII

The development of rFVIIa to control hemorrhage in hemophilia A and B males with inhibitors led to application to cirrhosis-related hemostasis disorders. However, no controlled trial has demonstrated efficacy for prevention or control of hemorrhage compared to placebo[114–119] (Table 126.3). Arterial and venous thromboembolic (VTE) complication rates were low in cirrhotic patients exposed to rFVIIa and similar to rates in placebo groups.[120] However efficacy studies were not designed to detect thrombotic adverse events, and both safety and cost remain valid concerns.[119,121] Based on recent insights into cirrhotic hemostasis, it would be possible to determine if there is a subset of hypocoagulable cirrhotic patients, using thrombin generation or TEG testing, who would potentially benefit from rFVIIa.

Prothrombin Complex Concentrate

Prothrombin complex concentrate (PCC) contain clotting factors II, IX, X, and variable amounts of factor VII, protein C, and protein S, offering the possible advantage of restoring vitamin K–dependent coagulation factors with minimal volume and no risk of TRALI. PCCs are labeled with factor IX content, indicating the original indication to treat hemophilia B patients. PCCs are divided into two groups: four-factor PCCs (similar amounts

Table 126.3

rFVIIa clinical trials in chronic liver disease patients

Indication (Reference)	Design	rFVIIa Dosing	Frequency	Endpoint(s)	Outcome
Dental surgery (113)	RCT DB	Placebo 20 μg/kg 80 μg/kg	× 1	Hemostasis ≤15 min	No difference
Partial hepatectomy (114)	RCT DB	Placebo 50 μg/kg 100 μg/kg	Q 2 h to end of surgery	RBC treatment	No difference
OLT (115)	RCT DB	Placebo 20, 40, 80 μg/kg	×1 preop	RBC treatment	No difference
OLT (116)	RCT DB	Placebo 60 or 120 μg/kg	Repeat doses?	RBC treatment	No difference
UGI bleeding (117)	RCT DB	Placebo 100 μg/kg	8 doses/30 h	No bleeding ≤24 h Rebleed d 1–5 Death ≤d 5	No difference
UGI bleeding (118)	RCT DB	Placebo 300 μg/kg 600 μg/kg	5 doses/20 h	No bleeding ≤ 24 h Rebleed d 1–5 Death ≤d 5	No difference

OLT, orthotopic liver transplantation; RBC, red blood cell; RCT DB, randomized control trial, double blind; UGI, upper gastrointestinal bleeding.

of factors IX, X, VII, and II) or three-factor PCCs (contain minimal factor VII). In most countries, other than the United States, four-factor PCCs are available. PCCs are frequently used for "off-label" reversal of vitamin K antagonist therapy, but there is a paucity of clinical information regarding the efficacy and safety of PCC in liver disease patients. Twenty-two patients with advanced liver disease requiring immediate therapy for bleeding or invasive procedures received 26 IU FIX/kg of a four-factor PCC. Clinical response was very good in 76%, and there were no overt thrombotic complications, but FVIIa, F1.2, and thrombin–AT complex levels increased.[122] Infusing an activated PCC (factor VIII inhibitor bypass activity [FEIBA]) or a combination of a PCC or FEIBA with rFVIIa to control or prevent bleeding in hemophiliacs with inhibitors has generated a consumptive coagulopathy and thrombosis[123] and would be unsafe in patients with advanced or decompensated liver disease. Studies are underway in Europe to investigate appropriate PCC dosing, efficacy, and safety in cirrhosis patients.

Thrombopoietin Mimetics

Using TPO receptor agonists is an attractive approach to manage thrombocytopenia in patients with advanced liver disease. In a phase II trial of thrombocytopenic hepatitis C patients, most subjects (n = 53) randomized to daily oral eltrombopag (30, 50, or 75 mg/day) for 4 weeks reached target platelet counts of >100,000/μL, started peginterferon and ribavirin, and completed 12 weeks of antiviral therapy, while no subjects (n = 18) in the placebo arm reached a platelet count of 100,000/μL, and only 6% completed 12 weeks of antiviral therapy.[124] Side effects were mild, and there were no thrombotic complications. Another trial compared eltrombopag, 75 mg/day, to placebo for 14 days prior to an invasive procedure in 292 chronic liver disease patients with platelet counts <50,000/μL.[125] Average platelet counts on day 14 were 103,000/μL for the eltrombopag group and 39,000/μL for the placebo group. Avoidance of platelet transfusions occurred in 72% and 19% of eltrombopag and placebo patients respectively. However, the study was terminated early due to increased thrombotic events, primarily in the portal vein system, in the eltrombopag arm compared to the placebo arm. While awaiting results from additional ongoing clinical trials, off-label use of TPO receptor agonists is not recommended.

THROMBOTIC RISKS AND MANAGEMENT APPROACHES IN CHRONIC LIVER DISEASE

Epidemiologic, imaging, and histologic data support the conclusion that acquired coagulopathies and thrombocytopenia do not protect patients with chronic liver diseases from peripheral or visceral VTE complications. In a population-based case–control study of VTE in Danish citizens from 1985 to 2005, patients admitted with noncirrhotic liver disease or cirrhosis had increased relative risks of 1.74 (95% CI 1.54 to 1.95) and 1.87 (95% CI 1.73 to 2.03) for VTE compared to patients without liver disease.[126] Estimates of VTE frequency in hospitalized chronic liver disease patients, based on chart reviews or analysis of hospital discharge databases, range from 0.8% to 6.8%, reflecting differences in study design and patient populations.[127–134] Comparisons of VTE rates between hospitalized patients with and without liver disease either support an increased risk with cirrhosis,[132,134] or report a lower risk.[129] Variables associated with increased VTE risk in chronic liver disease patients include decreased albumin,[132,133] elevated aPTT,[132] and age <45.[134] Variables that did not correlate with VTE risk include MELD score,[133] INR quartile,[130] etiology,[132]

or decompensated cirrhosis.[129,132] Despite heterogeneous methods and results, these studies disprove the dogma that hospitalized patients with chronic liver disease and cirrhosis are "autoanticoagulated" and not at risk for VTEs. However, the risks and benefits of prophylactic anticoagulation of chronic liver disease patients during periods of additional acquired VTE risks have not been adequately investigated. Emerging literature suggests current prophylactic and therapeutic dosing guidelines for low molecular weight heparin (LMWH) may be subtherapeutic in patients with advanced cirrhosis due to decreased AT and larger volumes of distribution.[135]

Patients with chronic liver disease are also at increased risk for *visceral* venous thrombosis, particularly in the portal vein (PVT) and its tributaries. Risk factors for PVT include regional events (venous stasis, inflammatory, infectious, and malignant conditions), and possibly systemic acquired and inherited thrombophilias. As patients' cirrhosis progresses from stable to decompensated stage due to worsening portal hypertension, the prevalence of PVT increases, ranging from 6% to 17% and is as high as 44% in patients diagnosed with hepatocellular cancer.[136] Comparisons of the prevalence of inherited and acquired thrombophilias in cirrhotics with and without PVT do not consistently support an association of antiphospholipid antibodies,[137,138] or FVL and prothrombin gene 20,210 mutations,[137,139–142] with PVT. Assessment of inherited protein C, protein S, and AT deficiencies and PVT risk are complicated by acquired deficiencies of these natural inhibitors due to hepatic disease. In one prospective cohort study of serial ultrasound screening for PVT in 100 cirrhotic patients, the only independent variable was decreased portal vein blood velocity.[143] Published experience with anticoagulant therapy to prophylaxis against PVT is limited. A prospective trial randomized 51 patients with advanced cirrhosis without PVT to enoxaparin 40 mg/day or placebo for 12 months with periodic imaging. No patient in the treatment arm developed PVT or a bleeding complication, compared to 5 (20%) PVTs in the placebo group.[144]

While PVT can be an asymptomatic, incidental finding, complications include extension into the superior mesenteric vein causing small intestinal ischemia and portal hypertension leading to ascites, varices, and GI bleeding. In noncirrhotic patients with acute and chronic PVT, expert opinion supports searching for precipitating risk factors, and starting anticoagulant therapy, or occasionally thrombolytic therapy or thrombectomy in critical situations.[145] However, high-quality evidence on which to base recommendations is limited. Several small series employing LMWH or a vitamin K antagonist in cirrhotic patients with asymptomatic or symptomatic PVT report complete or partial recanalization rates of 29% to 59% and bleeding complications <3%.[146,147] Many issues require further investigation, including the long-term consequences of asymptomatic PVT, effects of anticoagulation therapy on portal hypertension, and variceal bleeding, in order to provide clinicians with practice guidelines for anticoagulation treatment of cirrhotic patients with PVT.

Clinical, histologic, and experimental evidence suggest that systemic and localized prothrombotic conditions may promote hepatic microvascular occlusions, tissue injury, and fibrosis in patients with chronic liver disease. The rate of hepatic fibrosis progression is faster in FVL-positive compared to FVL-negative hepatitis C patients.[148] In addition, investigators have observed hepatic and portal microvascular venous thrombi in human cirrhotic liver tissue.[149] Several mechanisms for intrahepatic venule thrombosis have been proposed. Liver inflammation may cause portal venule thrombosis, localized ischemia, hepatocyte death, and fibrosis, that is, parenchymal extinction.[149,150] Excess thrombin generation may also activate PAR-1 receptors on hepatic stellate cells with subsequent stimulation of fibrosis.[151] Platelets appear to play a role in experimental liver injury, and sequestration of activated platelets within the liver may contribute to parenchymal extinction as well.[152,153]

ADAMTS-13 activity decreases and vWF antigen concentration increases with progressive cirrhosis,[154] and some cirrhotic patients have circulating unusually large plasma vWF multimers,[155] suggesting a possible link between acquired ADAMTS-13 deficiency, thrombocytopenia, platelet sequestration within the hepatic microvascular, and ischemic liver damage.

CONCLUSION

Recent laboratory investigations are providing new insights into the hemostatic changes associated with acute and chronic liver disease. A reduction in both prothrombotic and antithrombotic components produces a fragile rebalancing that can be tipped in favor of either bleeding or clotting complications by additional acquired events including infection, renal failure, immobilization, inflammation, and trauma. While it is clear that screening coagulation tests such as PT/INR are imprecise and potentially misleading in the setting of advanced liver disease, alternative *in vitro* tests such as thrombin generation and TEG have not been sufficiently validated to be recommended as replacements. On the other hand, prophylactic use of plasma based on INR cutoffs is a questionable practice, and there are risks associated with its use. Avoidance of overvolume expansion, and its attendant effects on portal pressure and the collateral vascular beds, is supported by reports from liver transplant surgery. Both prophylactic and rescue management of bleeding complications remains empiric at this time, rests on experience, and must be individualized. Attention to treating infection, renal failure, and volume overload are supported by existing data. Antifibrinolytics also appear to have a role in liver disease patients with hyperfibrinolysis, but off-label use of hemostatic products such as rFVIIa, TPO receptor agonists, and PCCs is expensive and has potential thrombotic risks. While the future may bring better evidence, their use is not supported by sufficient clinical evidence at this time to make formal recommendations. Patients with advanced liver disease do develop VTEs. However, the risk/benefits of anticoagulant prophylaxis and treatment are unclear at this time. Clearly, there is a great need for clinical research to address these knowledge gaps.

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CHAPTER

127 Hemostatic Abnormalities in Renal Disease

MIRIAM GALBUSERA, GIUSEPPE REMUZZI, AND PATRIZIA ONDEI

Patients with renal disease suffer from complex hemostatic abnormalities leading to two opposite complications: bleeding tendency and thrombotic risk. Bleeding may be a serious complication of acute and chronic renal failure.[1] The clinical manifestations vary from ecchymoses, epistaxis, bleeding from gums, venipuncture sites, and sites of fistula access to overt gastrointestinal bleeding. Although the frequency of severe hemorrhage has been reduced by the advent of dialysis and the use of erythropoietin to correct anemia, a bleeding diathesis still represents a problem for uremic patients, particularly during surgery or invasive procedures such as biopsies.

Nonetheless, abnormalities of blood coagulation and fibrinolysis in chronic renal failure render uremic patients highly susceptible to the development of cardiovascular and thrombotic complications. In addition, contemporary patient populations are older and have a high prevalence of hypertension and atherosclerotic vascular disease.[2] The presence of these comorbidities renders uremic patients more likely to receive drugs such as antiplatelet agents and anticoagulants that may increase the risk of bleeding.

PATHOGENESIS OF UREMIC BLEEDING

In patients with kidney diseases, platelet defects and platelet–vessel wall abnormalities are the main determinants of hemorrhagic tendency, but other contributing factors including the abnormal production of nitric oxide (NO), uremic toxins, and anemia account for a multifactorial pathogenesis of bleeding (Table 127.1).

Platelet Abnormalities

Severe thrombocytopenia is very rare and is limited to renal failure associated with disseminated intravascular coagulation, hemolytic uremic syndrome (HUS)/thrombotic thrombocytopenic purpura (TTP), eclampsia, and renal allograft rejection. Thrombocytopenia may also be caused during hemodialysis by the anticoagulant heparin.[3]

The majority of studies on uremic platelet were reported during the 1980s and 1990s and identified numerous biochemical abnormalities (reviewed in ref.[4,5]) including a decreased capacity to synthesize thromboxane A_2 by the prostaglandin-forming enzyme cyclooxygenase,[6,7] a reduction in platelet granule ADP and serotonin content,[8] an increase in cyclic AMP (cAMP),[9] dysregulation of adenylate cyclase,[10] and impairment of Ca^{2+}-dependent platelet function.[11,12] A storage pool defect is supported by finding subnormal dense granule content,[8,13] impaired release of α-granule proteins and β-thromboglobulin,[14] and reduced release of platelet ATP in response to thrombin.[13] Moreover, platelet cytoskeletal dysfunction due to either a reduction in cytoskeletal proteins or a decrease in actin incorporation after thrombin stimulation has been reported.[15] All the above abnormalities and the defective platelet aggregation in response to various stimuli contribute to impaired platelet adhesion and aggregation in injured vessels.

Platelet adherence to foreign surfaces is significantly impaired in patients with end-stage renal disease.[16–18] Defective function of the platelet glycoprotein (GP)IIb–IIIa complex receptor may account for the decreased binding of von Willebrand factor (vWF) and fibrinogen to stimulated uremic platelets[19] and the reduced vWF-dependent adhesion and thrombus formation.[16–18] Dialysis improved the binding capacity of GPIIb–IIIa complex, suggesting a role of uremic toxins or receptor occupancy by fibrinogen fragments present in uremic blood.[19,20]

Although quantitative and qualitative vWF abnormalities have not been consistently reported in uremic patients,[17,21,22] a functional defect in the vWF-platelet interaction cannot be excluded based on evidence that cryoprecipitate (a plasma derivative rich in vWF) and desmopressin (a synthetic derivative of antidiuretic hormone that releases autologous vWF from storage sites) significantly shorten the bleeding time in these patients.[23,24] Increased levels of the platelet inhibitors prostacyclin (PGI_2) and NO have also been reported in uremia.[25,26] The beneficial effect of conjugated estrogens on the bleeding times of uremic patients might be mediated by changes in the NO synthesis pathway since the NO precursor L-arginine reversed the effect of estrogens on bleeding.[27] Dialysis partially correct platelet dysfunction, suggesting that uremic toxins such as the guanidinosuccinic acid involved in NO generation[26] may play a role in platelet abnormalities.

Role of Anemia

Anemia is a constant feature of acute and chronic renal failure and is the main determinant of the prolonged bleeding time in uremic patients. There is evidence that the bleeding time is inversely related to the hematocrit in uremia[28,29] as well as in other types of anemia. The cloning of the human erythropoietin gene and the production of recombinant human erythropoietin (rhEPO) has provided clinicians with a powerful tool to correct the anemia associated with renal failure, thereby eliminating dependency upon transfusion.

Dialysis

Dialysis improves platelet functional abnormalities and reduces, but does not eliminate, the risk of hemorrhage. Hemodialysis, because of the interaction between blood and artificial surfaces, may induce chronic platelet activation that

Table 127.1

Factors involved in uremic bleeding

- Abnormal Platelet Function
- Decreased capacity to form thromboxane A_2; reduction in serotonin and ADP content; increased intracellular cAMP; impaired Ca^{2+}-dependent functions; reduction in dense granule content; impaired release of the platelet α-granule protein and β-thromboglobulin; abnormalities in *ex vivo* platelet aggregation; impaired platelet adhesion and aggregation to injured vessel
- Enhanced Production of NO
- Uremic Toxins
- Anemia
- Altered blood rheology; defective platelet diffusivity; decreased release of ADP by erythrocytes; erythropoietin deficiency
- Medication Effects
- Anticoagulants; antiplatelet agents; NSAIDs; β-lactam antibiotics; third-generation cephalosporins

can lead to platelet exhaustion and dysfunction. It has been found that plasma levels of the potent NO inducers TNF-α and IL-1β increase during dialysis.[30,31] These cytokines are generated *in vivo* by monocytes during hemodialysis with complement-activating membranes. Thus, while dialysis removes uremic toxins, it also affects platelet activation and NO synthesis. Heparin, used to prevent filter clotting, can occasionally induce platelet activation and thrombocytopenia.[3]

Role of Medications

Antiplatelet agents such as aspirin, as well as anticoagulants, increase the frequency of major bleeding in individuals with chronic renal insufficiency. Aspirin, at a dose of 100 mg/day, has been shown to significantly prolong the bleeding time in uremic patients when compared to controls[32] and anticoagulant therapy increases rates of bleeding in patients with end-stage renal disease as compared to the general population.[33,34,35] A recent study[36] showed that the risk for major bleeding episodes per year of exposure was 0.8%, 3.1%, 4.4%, and 6.3% in patients undergoing hemodialysis while taking neither warfarin nor aspirin, taking warfarin alone, taking aspirin alone, or taking the combination of warfarin and aspirin, respectively.

Given this bleeding risk, randomized, controlled trials are necessary to evaluate the efficacy and the safety of these agents in the secondary prevention of cardiovascular events in this high-risk population. Other medications that may increase the risk of bleeding in uremia include β-lactam antibiotics that accumulate in uremic patients[37] and act by perturbing platelet membrane function and interfering with ADP receptors.[38] The prolonged bleeding time and the abnormal platelet aggregation are related to the dose and duration of treatment and are promptly reversible after drug discontinuation. Third-generation cephalosporins may also inhibit platelet function and lead to marked disturbance of blood coagulation.[39] Nonsteroidal antiinflammatory drugs (NSAIDs) such as indomethacin, ibuprofen, naproxen, phenylbutazone, and sulfinpyrazone also inhibit platelet cyclooxygenase and disturb platelet function, but, in contrast to aspirin, the inhibitory effect of these compounds is readily reversed as the blood concentration of the drugs falls upon cessation of administration.[40]

Clinical Manifestation

The clinical presentation of uremic bleeding ranges from mild and clinically unimportant bleeding (petechiae, purpura, and epistaxis) to severe hemorrhage (gastrointestinal bleeding, intracranial bleeding, and hemorrhagic pericarditis). Gastrointestinal bleeding occurs with the greatest frequency and has been observed in up to one-third of uremic patients. Low-grade gastrointestinal bleeding may be even common. Upper gastrointestinal bleeding accounts for 3% to 7% of all deaths in patients with end-stage renal disease.[41] A prospective study[42] found that acute gastrointestinal hemorrhage in patients with impaired renal function was associated with an increase in mortality and a 37% increase in length of hospital stay as compared to nonrenal patients.[42] The causes of bleeding are usually peptic ulcers, hemorrhagic esophagitis, gastritis, duodenitis, gastric telangiectasias, and diverticular disease.[43,44] Other bleeding complications reported in chronic uremia are subdural hematoma, spontaneous retroperitoneal bleeding, spontaneous subcapsular hematoma of the liver, intraocular hemorrhage, and although now rare, hemorrhagic pericarditis with cardiac tamponade.[1]

LABORATORY ASSESSMENT

To identify patients at risk for hemorrhagic complications several tests have been used to establish which abnormal laboratory findings in uremia correlate best with an increased likelihood of clinically significant bleeding. Coagulation screening tests such as activated partial thromboplastin time, prothrombin time, and thrombin time are generally normal in uremia,[45] and there is no good correlation between blood urea nitrogen or creatinine and clinical bleeding.[45] Diagnosis of uremic bleeding still relies on clinical symptoms, and the cutaneous bleeding time (normal values: 1 to 7 minutes)[45] remains the most useful test for bleeding caused by uremia.[46,47] The bleeding time is an index of the primary phase of hemostasis, that is, the interaction of the platelet with the blood vessel wall, and the formation of the platelet plug.

THERAPEUTIC STRATEGIES FOR UREMIC BLEEDING

The approach to uremic bleeding must be considered in two contexts: the treatment of patients with active bleeding and the prevention of bleeding in patients at high risk because of

invasive procedures or surgery. The strategy depends on the urgency of the situation, the severity of uremia, and the previous therapy employed (Table 127.2).

Dialysis

Bleeding in uremia has been easier to control since the introduction of dialysis.[28,48] Studies indicate that dialysis ameliorates clinical bleeding,[49] improves platelet aggregation[49,50] and prothrombin consumption index.[49] Dialysis removes uremic toxins, including urea, creatinine, phenol, phenolic acids, or guanidinosuccinic acid, and improves platelet functional abnormalities.[51–53] However, platelet activation induced by interaction of blood with the artificial surface and the use of systemic anticoagulation may contribute to bleeding tendency. The risk of bleeding may be minimized by using peritoneal dialysis or alternatives to routine heparinization to prevent clotting in the extracorporeal circulation during hemodialysis.

Peritoneal dialysis is more effective in correcting platelet abnormalities than hemodialysis.[54] The reasons for the superiority of peritoneal dialysis in ameliorating platelet function abnormalities are not completely understood, but are likely due in part to the avoidance of heparin and better clearance of the middle molecular weight molecules.

To minimize the risk of bleeding in hemodialysis and continuous renal replacement therapies several strategies (reviewed in ref.[55]) such as low-dose heparin, heparin-free dialysis, and regional anticoagulation with citrate can be used.

In patients with moderate risk of bleeding, the use of minimum dose of heparin reduces bleeding complications when compared with other methods such regional heparinization. The protocol usually involves a bolus of 500 U of heparin every 30 minutes or a continuous infusion of heparin to keep the activated clotting time >150 but <200 seconds. This technique has the advantage that no additional equipment is needed in the dialysis circuit, but some minimal degree of anticoagulation still occurs.[55]

Heparin-free dialysis is the method of choice in patients with active bleeding or at high risk of bleeding. To avoid clotting of dialyzer, extracorporeal blood flow is rapidly increased to 250 to 350 mL/min and maintained throughout the treatment and 250 to 300 mL saline flushes are administered every 15 to 30 minutes into the arterial limb (predialyzer) to reduce hemoconcentration and to wash fibrin strands. The volume of saline is removed during the dialysis session. Because of its simplicity and safety, heparin-free dialysis can be used in approximately 90% of intensive care patients.[55]

Low molecular weight heparin (LMWH) has been proposed to cause less bleeding and less thrombocytopenia than heparin, but is not different from heparin in terms of dialysis-related bleeding or other complications, and once a patient develops heparin-induced thrombocytopenia (HIT) LMWH cannot be used as a safe substitute due to the extensive cross-reactivity with heparin.

Regional citrate anticoagulation[56] is an alternative to heparin-free dialysis. Citrate binds to free plasma calcium; the fall in the plasma concentration of this ion (i.e., required for coagulation process) is responsible for the anticoagulant activity. Regional citrate anticoagulation involves the continuous infusion of isosmotic trisodium citrate solution into the arterial side of the dialyzer; the rate of infusion is adjusted to keep the activated clotting time above 200 seconds in the arterial limb. To prevent the return of blood with a very low ionized calcium concentration to the patient, the process is reversed by the infusion of 5% $CaCl_2$ into the venous return line. The rate of infusion is constantly adjusted according to frequent measurements of plasma calcium concentration to prevent hypo- or hypercalcemia. The advantages of regional citrate anticoagulation over heparin-free dialysis are blood flow rate not high and rare clotting. The major problems are hypocalcemia or hypercalcemia, hypernatremia, and metabolic alkalosis, but, if closely monitored, the complication rate is relatively low.[56]

Correction of Anemia

Platelet adhesion and aggregation in flowing systems[57,58] are greatly potentiated by red blood cells. Erythrocytes enhance platelet function by releasing ADP,[59] by inactivating PGI_2,[60] and by

Table 127.2

Guidelines for the prevention and treatment of bleeding in patients with uremia

In all patients with hemorrhagic complications or who are undergoing major surgery, the adequacy of dialysis should be reviewed
- The dialysis schedule—using alternatives to routine heparinization—should be changed for 1 or 2 months in patients who have experienced severe hemorrhage or who have undergone recent cardiovascular surgery
Patients with acute bleeding episodes may be treated with desmopressin at a dose of 0.3 μg/kg, administered intravenously (added to 50 mL of saline over 30 min) or subcutaneously
- Intranasal administration of this drug at a dose of 3 μg/kg is also effective and well tolerated
- The effect of desmopressin lasts only a few hours, and it tends to lose efficacy when repeatedly administered
In patients with persistent chronic bleeding, the treatment of choice should lead to a long-lasting hemostatic competence
- Bleeding in patients with renal failure and hematocrit <30% must be treated with erythropoietin
- Blood or red blood cell transfusions to improve hematocrit values should be administered to severely anemic patients. Hemostatic effect of red blood cell transfusion is achieved when the hematocrit rises above 30%
- Conjugated estrogens given by intravenous infusion in a cumulative dose of 3 mg/kg as daily divided doses (i.e., 0.6 mg/kg for 5 consecutive days) is the most appropriate treatment

increasing platelet–vessel wall contact by displacing platelets away from the axial flow and toward the vessel wall.[57] Since hemoglobin is a scavenger of NO,[61] the decrease of available hemoglobin in anemia may contribute to platelet dysfunction. These erythrocyte activities explain the successful managing of hemorrhagic tendency in uremia by raising the hematocrit value to above 30%.[29]

Treatment with rhEPO induces a progressive increase in hematocrit and is accompanied by a significant decrease in the bleeding time.[62,63] The hematocrit during rhEPO treatment should be controlled carefully because complete correction of anemia carries the risk of hypertension, encephalopathy, thrombosis, and hyperkalemia. A threshold of hematocrit between 27% and 32% has to be reached for the bleeding time to become normal, or nearly normal, indicating that a partial correction of renal anemia is sufficient for this purpose.[63] In patients treated with rhEPO and with hematocrit values of 36% to 39%, there is significant improvement of the quality of life, cardiac function, physical work capacity, cognitive function, and sexual function.[64] However, the benefits and the risk of complete correction of anemia (hematocrit, 38% to 42%) and the optimal target hematocrit have not yet been established. The safety of the long-term maintenance of a normal hematocrit has been questioned as a consequence, in part, of the early termination of the Normal Hematocrit Cardiac Trial, in which the patients randomly assigned to the normal hematocrit group had a higher mortality and a higher incidence of nonfatal myocardial infarction.[65]

Cryoprecipitate and Desmopressin

Cryoprecipitate is a plasma derivative, obtained when plasma is frozen and slowly thawed. Cryoprecipitate is rich in vWF, fibrinogen, and fibronectin and has traditionally been used in the treatment of hemophilia A, von Willebrand disease, hypofibrinogenemia, and dysfibrinogenemia. In the original report, the six uremic patients treated with cryoprecipitate had normalization or significant shortening of the bleeding time.[66] The maximal effects on the bleeding time are obtained 4 to 12 hours (average: 8 hours) after the infusion, and effects are no longer detected by 24 to 36 hours. *Further studies confirmed the original findings but also indicated that shortening of the bleeding time and the control of clinical bleeding may be quite variable in uremic patients* since as many as 50% of patients fail to respond to cryoprecipitate.[67–69] This therapy, which may also carry the risk of transmitting blood-borne diseases, has been largely replaced by other approaches. A therapeutic alternative to cryoprecipitate is desmopressin acetate (1-deamino-8-D-arginine vasopressin, DDAVP), a synthetic derivative of antidiuretic hormone. DDAVP induces the release of autologous vWF from storage sites into plasma and avoids the risk of transmitting serum hepatitis or other blood-borne diseases through the administration of blood products. DDAVP shortens the bleeding time in 1 hour and its effect can last for 4 to 8 hours.[23] The bleeding time returns to baseline values within 24 hours. Desmopressin loses its efficacy when repeatedly administered,[70] probably due to a progressive depletion of vWF stores in endothelial cells, but the response to DDAVP is usually restored when the drug is withheld for 3 to 4 days.[71] However, tachyphylaxis does not prevent the clinical use of DDAVP since most prophylaxis for minor surgical procedure and most bleeding episodes generally require only one or a few doses of drug.[71] The guidelines for DDAVP treatment are summarized in Table 127.2.

Although remarkably free of serious side effects, DDAVP is reported to cause a mild to moderate decrease in platelet count, facial flushing, mild transient headache, nausea, abdominal cramps, and mild tachycardia, water retention, and hyponatremia. Rarely, thrombotic events followed DDAVP administration, particularly in patients with underlying advanced cardiovascular disease. Nonetheless, desmopressin is useful in the treatment of bleeding, and it is used prophylactically in the prevention of bleeding during surgery or invasive procedures.[72]

Conjugated Estrogens

The anecdotal observation of diminished gastrointestinal bleeding in uremic patients treated with conjugated estrogens and the improved hemostasis in von Willebrand disease during pregnancy led to investigations of the effect of estrogens on the bleeding tendency in uremia.[22,73] One oral 25 mg dose of conjugated estrogens normalizes the bleeding time for 3 to 10 days with no apparent ill effects.[74] A controlled study showed that conjugated estrogens, given intravenously at the cumulative dose of 3 mg/kg divided over 5 consecutive days, produced a long-lasting reduction in the bleeding time in uremics. At least 0.6 mg/kg estrogen was needed to reduce bleeding time,[74] and four or five infusions spaced 24 hours apart were needed to reduce the bleeding time by at least 50%. The guidelines for estrogen treatment are summarized in Table 127.2. Low-dose transdermal estrogen (estradiol 50 to 100 μg/24 h) applied as a patch twice weekly was found to reduce recurrent gastrointestinal bleeding with parallel improvement of bleeding time and no side effects.[75] Estrogens are safe and well tolerated. Thus, estrogens may be a reasonable alternative to cryoprecipitate or desmopressin in the treatment of uremic bleeding, especially when a long-lasting effect is required.

PREVENTION OF UREMIC BLEEDING

It is safer to prevent uremic bleeding than to treat it, so patients with uremia who are to undergo elective surgery should be advised to avoid acetylsalicylic acid (ASA) and other NSAID for at least 1 week prior to the operation; patients receiving dialysis should have an adequate session before surgery, preferably without heparin, because patients who do not receive adequate dialysis tend to bleed more. It has been demonstrated increased intraoperative complications in uremic patients with preoperative hematocrits levels ranging from 20% to 26%.[76] Before surgery, a threshold hematocrit between 27% and 32% should be reached and iron stores should be checked and replaced if needed because patients who are receiving erythropoietin treatment and are maintaining relatively normal hematocrit levels usually do not have bleeding problems during the perioperative period.

ABNORMALITIES OF COAGULATION AND FIBRINOLYSIS

Despite a hemorrhagic tendency, activation of coagulation has been demonstrated in uremic patients. This finding is more prominent in patients treated by hemodialysis. Abnormalities of the coagulation cascade proteins such as increased plasma fibrinogen, FVIII:C and vWF, and decreased protein C anticoagulant activity and protein S have been found in uremic patients.[4,77] Thrombin is continuously formed, as demonstrated

by the increased levels of thrombin–antithrombin III levels,[78,79] D-dimers, and fibrinopeptide A,[79] and enhanced platelet aggregability has been reported. Increased expression of active tissue factor may be triggered by the anaphylatoxin C5a produced through complement activation induced by hemodialysis membranes.[80]

Contrasting results have been obtained regarding the fibrinolytic system.[81] First, a decrease of fibrinolytic activity has been found in uremia, either absolutely or relative to the extent of activation of the coagulation. This has been used as an explanation for the hypercoagulable state. On the other hand, later reports found activation of fibrinolysis in uremia, with an increase in plasmin–antiplasmin complexes,[78] and fibrinogen and fibrin degradation products,[78,79] together with a decrease of plasminogen activator inhibitor activity[78] after a hemodialysis session. Dialysis partially corrects the abnormalities of coagulation and fibrinolysis found in uremia.

THROMBOTIC COMPLICATIONS

The hemostatic abnormalities in chronic kidney disease may account for an increased risk of both atherothrombotic events and bleeding. The early stages of chronic kidney disease are dominated by derangements likely to result in an increased risk of cardiovascular events, while in advanced chronic kidney disease platelet dysfunction gives rise to an increased risk of hemorrhagic events. However, cardiovascular events related to thrombosis are a predominant cause of death and account for important morbidity in patients with end-stage renal disease who are either treated conservatively or when renal function is replaced by dialysis or transplantation. Cardiovascular disease accounts for about 40% of all deaths in patients undergoing dialysis.[82,83] Increased cardiovascular risk in uremic patients is also due to the higher prevalence of type 2 diabetes, obesity, hypertension, and older age.[84] Other potential factors affecting cardiovascular risk include malnutrition, endothelial dysfunction, arterial calcification, and vitamin D deficiency.[85] In addition, end-stage renal disease is associated with accelerated atherosclerosis due to oxidative stress, inflammation, and lipid disorders.[86]

Hemostatic abnormalities consistent with a hypercoagulable state have been widely described in patients with end-stage renal failure on hemodialysis. Risk factors, summarized in Table 127.3, include enhanced platelet aggregability, abnormalities of coagulation, impaired fibrinolytic activity, increased plasma lipoprotein(a), increased plasma concentrations of homocysteine, and the presence of lupus anticoagulants (reviewed in ref.[4]).

The association of the nephrotic syndrome with renal vein thrombosis has long been recognized.[87,88] In nephrotic syndrome, a hypercoagulable state caused by increased levels of coagulation factors, reduced levels of antithrombin, and an increased platelet response to various stimuli plays an important role in the development of renal vein thrombosis and other venous and arterial thromboembolic complications.[4,89]

Activity of the vWF-cleaving protease ADAMTS13 is reduced in uremia.[90] Complete loss of ADAMTS13 activity due to congenital or immune-mediated deficiency is the main cause of thrombotic occlusion of arterioles and capillaries in the majority of patients with TTP.[91] Undetectable ADAMTS13 activity has also been found in a subgroup of patients with an atypical form of hemolytic uremic syndrome.[91] Replacement of deficient ADAMTS13 activity with plasma is the treatment of choice for congenital cases of TTP, and clearance of the autoantibodies by plasma exchange, or inhibition of their production by immunosuppressive agents, is the key component of treatment in the immune-mediated form of the disease.

Table 127.3

Factors involved in thrombotic risk in patients with kidney disease

- Abnormalities of Coagulation
- Increased levels of fibrinogen, FVIII:C, and vWF
- Decreased protein C anticoagulant activity and protein S
- Decreased Fibrinolytic Activity
- Enhanced Platelet Aggregability
- Increased Levels of Homocysteine
- Accelerated Atherosclerosis
- Oxidative stress inflammation
- Lipid disorders
- Arterial Calcification
- High Prevalence in Patients With Chronic Kidney Disease of Type 2 Diabetes, Obesity, Hypertension, and Older age

PREVENTION AND TREATMENT OF THROMBOTIC COMPLICATIONS

Because platelet aggregation plays a major role in thrombus formation, antiplatelet agents have been used, with encouraging results. Aspirin, dipyridamole, ticlopidine, and sulfinpyrazone have proved useful in several studies. To prevent thromboembolic complications, systemic anticoagulation may be achieved with warfarin or acenocoumarol. Despite a significantly higher variability of coagulation parameters in hemodialyzed patients, oral anticoagulation therapy should not be considered automatically contraindicated in this patient group. Fibrinolytic agents, such as streptokinase or urokinase, as well as recombinant tissue plasminogen activator, have produced contrasting results. Generally, they can be effective in declotting recently occluded vascular hemodialysis accesses, whereas they can be useless in case of marked venous stenosis or sclerosis.[92] No data are available for systemic treatment of uremic patients with fibrinolytic agents.

For anticoagulants and/or antiplatelets eliminated through the kidneys, the doses used for treatment of thromboembolic or arterial events carry a higher risk of drug accumulation and bleeding in respect to the prophylactic doses. This implies that patients with chronic kidney disease should be strictly monitored and that an appropriate adjustment of the dosage is necessary.[93,94]

The current treatment recommendations for patients with chronic kidney disease are based on clinical trials conducted in the general population; thus further research is required to better understand the procoagulant state in patients with chronic kidney disease and to identify potential therapeutic interventions that could translate into improved cardiovascular outcomes.

Since a precarious balance of hemostasis is present in chronic renal failure, resulting both in bleeding and in thrombosis, therapeutical intervention must be carefully evaluated as it can shift the hemostatic equilibrium toward hemorrhage or thrombosis.

Vascular Access Thrombosis

As a consequence of the need for vascular access for hemodialysis, uremic patients are exposed to additional thrombotic complications. Percutaneous cannulation, arteriovenous shunts, native vein or prosthetic arteriovenous fistulas, and fistulas made of the artificial polymers used for chronic hemodialysis are particularly prone to thrombotic occlusion, accounting for 20% to 25% of hospital admission of dialysis patients.[95]

Access failure is primarily due to arteriovenous fistula thromboses that, when uncorrectable, accounts for 80% to 85% of arteriovenous access loss. The main predisposing factor is anatomic venous stenosis. Pathogenesis and risk factors are summarized in Table 127.4.

Strategies to prevent thrombosis, other than treatment of stenoses by percutaneous transluminal angioplasty, endovascular stent, or surgical revision, include antiplatelet agents, such as dipyridamole and low-dose aspirin with or without sulfinpyrazone.[96,97] Nowadays, the role of antiplatelet agents in preventing fistula thrombosis in patients with previous thrombosis is uncertain because of the high recurrence rate of access failure. Few data are available for the effectiveness of systemic anticoagulation to improve access patency. Warfarin can be used in patients with recurrent thrombosis but without anatomic stenosis.[98]

To prevent vascular access thrombosis in hemodialyzed patients, the use of clopidogrel, homocysteine-lowering agents, beta-hydroxybetamethyglutaryl coenzyme-A reductase inhibitors, heparinoids, eicosapentaenoic acid, renin–angiotensin system antagonists, calcium channel blockers, and alpha adrenergic antagonists has been proposed.[99–101] However, no randomized trials studying the effects of these agents are available in hemodialyzed patients.

Table 127.4

Vascular access thrombosis

- Pathogenesis
- Procoagulant environment
- Endothelial damage
- Inflammation
- Extrinsic deficiency of antithrombotic factors
- Mechanical obstruction associated with intimal hyperplasia
- Prothrombotic Risk Factors
- Diabetes; age > 65
- Antibodies: antiheparin-platelet factor 4; anticardiolipin IgM; antiprotein C; antiprotein S
- Increased levels of PAI-1, FVIII, prothrombin fragments 1+2; C reactive protein
- Hyperhomocysteinemia
- Hb between 10 and 12 g/dL
- Genetic risk factors
- Homozygous mutation of factor V Leiden
- 4G/5G PAI-1 polymorphic variant

In patients with unexplained recurrent access thrombosis, it should be useful to screen for lupus anticoagulant activity and anticardiolipin antibodies.

Therapeutic options to treat fistula thromboses include the use of thrombolytic agents, surgical thrombectomy, and mechanical dissolution. Thrombolysis in origin gave disappointing results, but now dosing adjustment improved the success rate and reduced the incidence of bleeding in patients with no contraindications for thrombolytic therapy (i.e., severe hypertension, recent bleeding episodes, or bleeding disorders).[102,103] The use of thrombolytic treatment with mechanical clot disruption yields access patency in the majority of cases with minimal complications.[102]

Heparin-Induced Thrombocytopenia

The use of anticoagulant heparin may cause HIT, a syndrome characterized by decrease in platelet count to <100,000 μL and/or thrombosis.[104] HIT, which usually present 5 to 14 days after the start of heparin therapy, is an immune-mediated disease due to the presence of IgG antibodies raised against the complex of platelet factor 4-heparin.[105] These antibodies possess the ability to activate platelets.

Thrombocytopenia, thrombosis, skin necrosis at heparin injection sites, venous limb gangrene, and acute systemic reaction that rises shortly after intravenous bolus of unfractionated heparin are events associated with HIT.[104]

In patients undergoing hemodialysis, the complications of HIT (reviewed in ref.[104]) range from no clinical sequelae despite positivity for HIT antibodies to thrombocytopenia, frequent clotting of extracorporeal circuit, and increase in arteriovenous fistula failure. Major complications include pulmonary embolism, deep venous thrombosis, myocardial infarction, and limb ischemia. A mortality of 30% has been reported in patients with HIT and thrombosis.[106]

In patients with thrombocytopenia developing during or shortly after heparin administration HIT should be suspected. Diagnostic criteria for HIT include normal platelet count before heparin treatment, presence of acute thrombotic event, no other known causes of thrombocytopenia, resolution of thrombocytopenia after heparin discontinuation, and HIT-antibody seroconversion.

After cessation of heparin, thrombocytopenia usually resolves in 1 to 2 weeks. Treatment (reviewed in ref.[104]) should be on the basis of clinical finding and not be delayed until laboratory confirmation is reached. In patients with high risk of HIT, alternative nonheparin anticoagulation should be sought and heparin discontinued. Platelet transfusion should be avoided. Platelet count normalization should be awaited before warfarin starting.

Systemic anticoagulation should be maintained in active HIT until platelet count is low to reduce the risk of life-threatening thrombosis. In patients with HIT, regional citrate anticoagulation can be employed together with alternative anticoagulation such as the direct thrombin inhibitors lepirudin and argatroban, and the factor Xa inhibitor danaparoid.

CONCLUSION

The association between a bleeding tendency and uremia has been demonstrated repeatedly. Although modern dialysis techniques and the use of erythropoietin to correct anemia

have reduced its frequency, bleeding is still a potentially life-threatening complication in uremic patients and limits surgery and invasive procedures. The pathogenesis of uremic bleeding is multifactorial and is not completely understood. However, it has been attributed mainly to abnormalities of primary hemostasis, particularly platelet dysfunction and impaired platelet–vessel wall interaction. Despite this hemorrhagic tendency, abnormalities of coagulation and fibrinolysis, as well as the presence of thrombotic risk factors, also predispose uremic patients to a hypercoagulable state and to thrombotic complications.

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CHAPTER

128 Multitrauma Derangements in Hemostasis and Thrombosis

DIANE A. SCHWARTZ, LUCY Z. KORNBLITH, JOHN B. HOLCOMB, AND MITCHELL JAY COHEN

Despite significant advances in prevention of traumatic incidents and focus on prehospital medicine, emergency medicine, and surgical and critical care, trauma remains the leading cause of death in adults until the age of 45.[1] Military trauma data and their application to the civilian population provide much of our recent knowledge regarding coagulopathy and hemostasis; these topics remain at the forefront of clinical research, the impact of which may affect the primary cause of death in the civilian pediatric and adult populations.

HISTORICAL PERSPECTIVE

Historically, trauma mortality has been defined by a trimodal temporal distribution.[2] The first phase comprises patients with nonsurvivable injury. These injuries result in uncontrollable and nearly instantaneous exsanguinations or catastrophic brain injury. The second phase on the spectrum encompasses the patients who succumb to early hospital death as a result of ongoing, uncorrected hemorrhagic shock or severe brain injury. The late phase of the model defines patients who have had reversible hemorrhage, either by surgical or interventional control, and manageable and correctable coagulopathy, but whose late course is complicated by inflammatory dysfunction and infection stemming from the initial traumatic insult.

The commonest contributor to immediate death after trauma, in both military and civilian settings, remains uncontrolled hemorrhage.[3] Seventy-five percent of early deaths, recognized above as phases one and two of the defined model, are due to uncontrolled hemorrhage, but late deaths tend to occur secondary to sepsis and related multiorgan failure to which early bleeding and coagulopathy contribute.[4,5,6]

Recent data have called the classic trimodal distribution into question because it seems that trauma deaths now fall largely within the first two phases.[7,8] The trimodal distribution is no longer valid because mortality rates are actually increased in the initial hours following the traumatic event, not evenly distributed among three phases. In reality what occurs is a large peak of early deaths, those seen in the first 6 to 12 hours, followed by a tail of later deaths.[4,9] Several centers even describe an earlier peak, one that occurs within the first 6 hours after injury. Large urban centers agree that few hemorrhagic deaths occur after 24 hours, and fewer patients die of multiorgan failure and sepsis than originally described in the trimodal model[10,11,12] (FIGURE 128.1).

Traditionally, bleeding after trauma was thought to be caused by surgical bleeding, which was then exacerbated by iatrogenic coagulopathy.[13] This mantra of iatrogenic coagulopathy has been altered and is no longer believed to be the singular cause of continued bleeding once surgical causes have been controlled.[14] Coagulopathy after trauma is now primarily caused by the injury and shock and can be compounded by the lethal combination of dilution, hypothermia, and acidosis and additional iatrogenic influences.[15,16]

In the past, trauma resuscitation included large volume crystalloid and cold blood product administration, which contributed to dilution and hypothermia. Surgery was expected to control hemorrhage but contributed further to the coagulopathy by worsening the hypothermia and perpetuating the acidosis. Considerable literature exists documenting the detrimental effects of hypothermia, acidosis, and dilution on coagulation and bleeding.[17] From this initial understanding, trauma and resuscitative efforts have evolved to combat these significant players of the downward spiral of coagulopathy and death.

Important to the evolution of the understanding of coagulopathy is the observation that trauma patients with life-threatening injuries who have not received large volume fluid resuscitation still arrive to the hospital coagulopathic. Several groups have explored these observations and coined the term, acute traumatic coagulopathy, or ATC.[14,18,19,20] ATC occurs in approximately 25% to 35% of trauma patients nearly immediately after severe trauma and shock, occurs prior to and independent of iatrogenic causes, and remains a primary determinant of poor outcome after severe injury.[18,19,21]

ACUTE TRAUMATIC COAGULOPATHY

While the traditional causes of the "lethal triad" of coagulopathy have been known and studied for some time, trauma practitioners have also described a coagulopathy that is independent of acidosis, dilution, and hypothermia. This coagulopathy, termed ATC, appears concurrently in works by Brohi et al.[19] and Macleod et al.[18] in 2003. Brohi et al. reported that one fourth of his study population demonstrated evidence of coagulopathy within 75 minutes of hospital arrival, without having received significant resuscitation volume or suffering acidosis or hypothermia. Patients afflicted with this newly described coagulopathy were four times more likely to die than those with a normal coagulation profile.[22] Macleod et al. also demonstrated the relationship of ATC to concurrent tissue injury and shock. ATC is clearly exacerbated and affected by hypothermia, acidosis, and dilutional causes, but it is equally clear that it is a separate entity.

In order to understand ATC, one must be well versed in the function of protein C, which is the main mediator of ATC.[22,23] Protein C is a serine protease. After minor tissue injury, it is

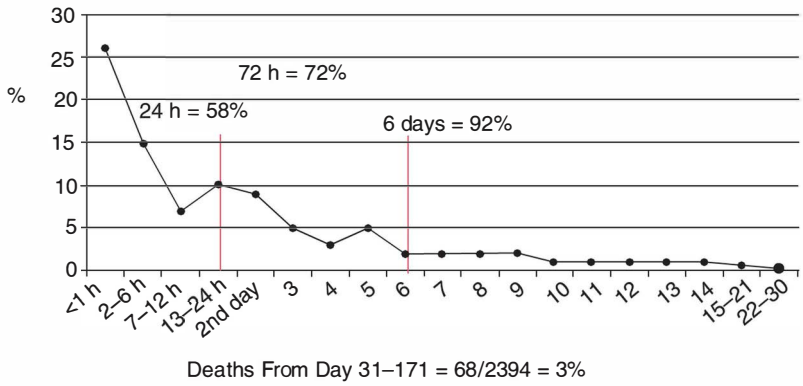

FIGURE 128.1 Trauma deaths experience at University of Texas Health Science Center-Houston (1999 to 2008). Of 26,028 trauma admissions, there were 2,394 deaths (6.6%), early deaths (at ≤24 hours) occurring in 1,398 (58%), and only 68 (3%) between days 31 and 171.

cleaved to its active form *via* the complexing of thrombin, thrombomodulin, and the endothelial protein C receptor (EPCR).[24–26] In patients without severe injury who are not coagulopathic, this pathway will downregulate itself from the inherent elevation in thrombin levels that occur after tissue injury. However, in patients with ATC, there is a pathologic response. Protein C is activated to the point of being acutely depleted. Thereafter, this event leads to global inactivation of factors V and VIII and yields an increase in tissue-type plasminogen activator and D-dimer.[22] Factors V and VIII are the main cofactors for thrombin and factor X activation, respectively. This derangement, stemming from the depletion of activated protein C (APC), which prohibits thrombin production, is the cornerstone of ATC.

ATC is characterized by prolonged prothrombin time (PT) and/or activated partial thromboplastin time, despite normal and appropriate thrombin production prior to injury.[27] As tissue injury progresses at the molecular level, there is a congruent reduction in thrombin in patients who have increasing levels of APC. In the face of tissue injury and hemorrhagic shock, the decrease in thrombin production and the subsequent resultant increase in fibrinolysis cause a proportional coagulopathy that defines ATC.[22,28] While thrombocytopenia and hypofibrinogenemia are clear contributors to hemorrhage and have been separately studied, ATC occurs even in patients with normal fibrinogen levels and platelet counts.[22]

It is not well understood why thrombin depletion is seen following severe traumatic injury and shock. It seems counterintuitive because mild tissue injury is known to cause an elevation in thrombin production, which is in contrast to the abrupt decrease seen in the severely injured patient.[29] It is believed that ATC is initiated with tissue injury, which exposes tissue factor, thereby inducing the coagulation cascade.[14] In patients who are not in shock, exposure of tissue factor leads to production of thrombin, fibrin deposition, and clot formation. However, in the severely injured patient, hypoperfusion secondary to hemorrhagic shock causes increased expression of thrombomodulin and EPCR on the endothelium while simultaneously activating and then depleting protein C.[30] Further augmentation of the coagulopathy of severe injury is the result of decreased activation of the thrombin-activatable fibrinolysis inhibitor, which depletes plasminogen activator inhibitor-1.[22,31] Thrombomodulin complexes with thrombin, diverting the normal cleavage of fibrinogen toward the APC and the consequent inhibitory effect on thrombin formation as described above.[32]

One protective mechanism to injury is the elaboration of thrombus formation at the site of injury. For reasons still being elucidated, patients in severe shock from trauma develop an exaggerated response of protein C, which prevents local thrombus formation. These complicated mechanistic relationships between injury, shock, and ATC are important avenues of research.

EFFECTS OF PROTEIN C DEPLETION IN ACUTE TRAUMATIC COAGULOPATHY

While there are numerous proteins involved in the generation of the traumatic response, protein C is one that is responsible for mediating the late cytoprotective response to injury.[33–35] Protein C activation is a primary instigator of ATC. Poorly regulated protein C activation, which occurs during the acute traumatic injury or hemorrhagic shock, will eventually deplete the natural baseline levels of protein C that the body otherwise, in physiologic normalcy, depends upon to fight infection and thrombotic complication. This is because, in addition to its anticoagulant effects, protein C has been shown to be cytoprotective and anti-inflammatory.[36,37] This mechanism is derived *via* its signaling through protease-activated receptor-1 and EPCR.[38] Cytoprotective effects of APC can be appreciated when observing their role in the pulmonary system. In patients with persistently low levels of protein C, such as those seen in the trauma population, there is a decrease in capillary endothelial barrier function. This decrease in barrier function, which is thought to be modulated in part by protein C, leads to higher rates of ventilator-associated pneumonia.[30,39,40] There is evidence that similar cytoprotective and anti-inflammatory effects of APC also exist in acute kidney injury.[41]

It seems that the phenomenon of early protein C activation, perpetuation of ATC, and eventual depletion of usable protein C may in fact represent one link of hemorrhagic shock to later inflammatory complications seen in the trauma population. The initial APC has an overall detrimental effect of contributing to coagulopathy. Additionally, while inflammatory modulation is helpful in combating the initial stress of injury and shock, the depletion of protein C is further detrimental because it occurs at a time when the patient requires an ongoing stress response and is at risk for thrombotic, inflammatory, and infectious sequelae.

This conundrum may drive investigation to further characterize and modulate these mechanisms in severely injured patients.[42]

ADDITIONAL CONTRIBUTORS TO ATC AND COAGULOPATHY

Additional factors that contribute to the ATC are reductions in natural anticoagulants, such as antithrombin and protein S.[43–46] Pharmaceutical hemostatic agents include recombinant factor VIIa (rFVIIa), prothrombin complex concentrate (PCC), and several antifibrinolytics. These hemostatic pharmaceutical adjuncts are used, at times off label, in patients with traumatic coagulopathy.

rFVIIa (NovoSeven; Novo Nordisk A/S: Bagsvaerd, Denmark) can be used for rapid correction of coagulopathy induced by Coumadin and may also have a role in the correction of ATC secondary to the association of exposed tissue factor.[47] There have been two randomized controlled trials using rFVIIa in traumatic hemorrhage, demonstrating a moderate reduction in total units transfused, but no mortality benefit.[48,49] In addition to reduction in packed red blood cells (pRBCs) and plasma requirements, these studies demonstrated a decreased incidence of multiorgan failure and acute respiratory distress syndrome, which may be attributed to the molecular control of ATC, protein C, and other unknown contributors. While thromboembolic events are thought to be a consequence of factor VII use, these events occurred with similar frequency in the trauma groups receiving rFVIIa and the control group.[49,50–52] Interestingly, a subset of patients will see no correction of their PT despite receiving rFVIIa. These patients have a significantly higher 24-hour and 30-day mortality and are considered nonresponders to this agent.[53,54]

It has also been demonstrated that rFVIIa is impaired by acidosis; "nonresponders" in some studies demonstrate a lower mean pH than those patients who achieved hemostasis with rFVIIa.[55,56] It is widely understood that the role of rFVIIa in trauma patients has decreased for two reasons: The randomized studies showed no survival benefit, and the iatrogenic resuscitation injury of excessive crystalloid and unbalanced plasma and RBC transfusion is less frequent than in previous years.

PCC was originally developed for hemorrhagic complications of hemophilia B and is rich in factors II, VII, IX, X, and there are several different formulations in use.[57] It also has a significant role in warfarin reversal because of its composition of vitamin K–dependent clotting factors.[58] There may be increased thromboembolic risk associated with its use, but this has not been well substantiated.[59,60] Fries et al. in 2006 corrected coagulopathy in the porcine model with the use of PCC. This model showed that clot formation was enhanced, blood loss decreased, and mortality improved with administration of PCC in uncontrolled hemorrhage.[61] While the indications for use after injury in humans have not been completely delineated, the porcine model and observational study through clinical application are provocative.[61–63]

ATC has a well-definable antifibrinolytic component, so it is reasonable to use antifibrinolytic agents as adjuncts in the correction of traumatic hemorrhage. Tranexamic acid (TXA) is an antifibrinolytic that targets the lysine binding pocket of plasminogen and has been shown to significantly improve survival after trauma.[64,65,66,67] Fibrinolysis is well documented in the trauma patient by the thromboelastogram, indicating a swifter than expected breakdown in clot formation.

In 2010, Shakur et al. published the CRASH-2 study, which randomized trauma patients to receive TXA versus placebo. All-cause mortality was decreased from 16% in placebo to 14.5% in the treatment group, with the risk of death from bleeding significantly reduced and no reported safety issues.[67] Approximately, 50% of the patients randomized in the study were not transfused any blood products. Unfortunately, when TXA was given more than 3 hours after injury, death rates were increased.[65,66,67] These data influenced others to carefully examine the use of TXA in the trauma population.

It is now known that a prolonged Ly30 on thromboelastography in a patient in hemorrhagic shock corresponds to a severe state of fibrinolysis and increased mortality.[64] Higher doses of TXA may lower seizure threshold, and this requires further investigation in trauma patients.[67,68] Aminocaproic acid, which has not been extensively evaluated in traumatic hemorrhage, is a similar lysine-based plasminogen binding inhibitor. This agent has been shown to decrease hemorrhage during elective surgery.[65] Its application to the acutely hemorrhaging trauma patient is an interesting avenue of research.

Apoprotinin is a nonspecific protease inhibitor originally used for coronary bypass–associated coagulopathy. It has been studied in traumatic coagulopathy, where it demonstrated reduction in total transfusion requirements, but no other outcome differences.[69,70] It therefore cannot be yet extrapolated for use in trauma.

While many of these agents potentially show promise for application to the trauma population, none other than TXA has been shown in this population to make a difference in outcome. More studies are anticipated before universal use can be recommended.

IATROGENIC COAGULOPATHY

As discussed above, traumatic coagulopathy was traditionally attributed to the "lethal triad" of hemodilution, hypothermia, and acidosis, leading to an imbalance and consumption of clotting factors.[13,71,72] Dilutional iatrogenic coagulopathy occurs in part by massive crystalloid resuscitation.[13,73] Large crystalloid volume followed by multiple units of transfused pRBCs further augments the coagulopathy because pRBC units are equally devoid of coagulation factors.[74] Traditionally, there was no defined limitation to crystalloid resuscitation, and the guidelines for massive transfusions encouraged coagulopathy-inducing ratios of fresh frozen plasma:platelet:RBC. Crystalloid volumes in the range of 20 to 30 L over 24 hours were not uncommon. Resultant dilution of all aspects of the complex coagulation system including the procoagulant, anticoagulant, and fibrinolytic systems created a complex coagulopathy, prompting investigation of targeted protocols for transfusion and resuscitation.

It has been shown that thrombin activity is decreased with traditional large volume crystalloid-based resuscitations, although it may be that thrombin dysfunction is partially counteracted by inhibitory actions of antithrombin.[75,76] In addition, reduced fibrinogen levels as well as impaired fibrinogen bioavailability have been reported as a result of trauma and subsequent resuscitation-based dilution.[77,78] Fibrinogen levels are decreased earlier and more significantly, compared with other coagulation factors, in massive transfusions and situations in which large volume of crystalloid is administered.[78] The functionality of fibrinogen is depleted at 150% of blood volume loss, whereas

critical levels of coagulation factors and platelet count do not occur until 200% blood volume loss.[77,78] It should be noted that fibrinogen level of 100 mg/dL, which is defined by most massive transfusion protocols as being the level at which function is altered, is an arbitrary definition with few data to support it. The polymerization of fibrinogen is the final common pathway for hemostasis, clot formation, and thrombin activation.[79] Functional fibrinogen deficiency can be caused by dilution with crystalloid and colloid infusion leading to abnormal fibrin polymerization.[34] Additional studies, both *in vivo* and *in vitro*, have demonstrated that increasing fibrinogen concentrations incompletely reverses dilutional impairment of clot formation.[79,80] Dilutional effects on coagulopathy may even occur at small resuscitation volumes of <500 mL as discussed in the Acute Traumatic Coagulopathy section.[81]

The second contributor to iatrogenic coagulopathy is hypothermia. Hypothermia, defined as core body temperature <35°C, remains a significant problem in trauma patients.[82,83] Acute blood loss results in decreased heat production. In addition, patients are often brought in from the field suffering from evaporative heat loss due to environmental factors. These same patients are then exposed in the trauma bay for evaluation, causing further heat loss by convection and radiation. Many patients are then taken to the operating room; open body cavities result in additional evaporative losses. Exacerbating heat loss is the administration of room temperature or colder resuscitative fluid.[84] Compounding all these factors is the loss of endogenous heat generation, secondary to shock. At 33°C, even if clotting factors are at normal levels, the clotting process is functionally equivalent to a factor IX deficiency, meaning that the functionality of these factors is only around 30%.[85,86] In addition, it has been shown that thrombin production decreases by 10% for each hypothermic degree.[87] There is a known synergism of hypothermia, dilution, and acidosis leading to impaired coagulation and poor outcomes.[88] Hypothermia is additionally an independent predictor of mortality in trauma, as shown by Gentilello and Jurkovich et al. in several studies involving hypothermia and active rewarming.[84,89] Additionally, there may be an inhibitory effect of hypothermia on platelet aggregation and the clotting cascade.[89,90]

Trauma patients suffer from significant metabolic acidosis. Acidosis in traumatic injury is multifactorial but is known to be caused by shock. Acidosis contributes to impaired thrombin production and disrupted coagulation.[71] Crystalloid exacerbates acidosis, which is why initial hemorrhage should not be treated with any volume of crystalloid replacement.[13,71,72] Clotting dysfunction becomes apparent at a pH below 7.2, which is not uncommon in hemorrhagic shock or severely injured patients, and its effects are persistent. However, correcting pH does not completely reverse the effects of the acid-induced coagulopathy.[56,82,91]

Taken together, metabolic perturbations and ATC, which cause coagulopathy after trauma, represent a significant contribution to bleeding and worsened outcome after injury. Considerable focus has been directed toward modification of resuscitation and surgical intervention of hemorrhage with the intent to mitigate the coagulopathic state.

DIFFUSE INTRAVASCULAR COAGULATION

"Consumptive coagulopathy," otherwise known as diffuse intravascular coagulation, describes impaired clot formation with simultaneous fibrinolysis. It is one probable cause of traumatic coagulopathy, although its specific role early in the trauma patient's clinical course continues to evolve.[71] The consumptive mechanism seen in traumatic coagulopathy appears similar to that seen in disseminated intravascular coagulation (DIC) on multiple laboratory tests.[92] It is possible, though not proven, that the tissue injury, which induces ATC, also predisposes patients to DIC. Following injury, tissue factor is exposed, which activates the extrinsic coagulation cascade, and increases thrombin production. "Thromboplastins" may also be released at the time of severe injury; these are defined as lipids from bone marrow, amniotic fluid, and brain phospholipids, all of which may contribute to DIC.[93]

In contrast to this multifactorial causation of DIC, ATC is usually only seen when tissue injury and systemic hypoperfusion occur simultaneously.[32] ATC is independent of thrombin production or consumption. Consumption is also not likely to be the sole or primary cause of ATC because ATC is known to occur sporadically in patients with varied degrees of both tissue damage and thrombin production. Additionally, fibrinogen levels are not generally decreased on initial presentation after injury in patients with ATC. In fact, platelet counts and fibrinogen levels have been shown to be normal after initial injury in studies of ATC.[22,94,95] It is possible that ATC may be an early player on the spectrum of DIC, but DIC, by definition, requires thrombocytopenia or hypofibrinogenemia, which may not occur in ATC.[96–98]

DIC and ATC are likely overlapping entities but are mechanistically different, and the strict criteria needed for DIC does not account for the dynamic changes seen in ATC.[99,100] DIC is a diagnosis made based on laboratory criteria including low platelets and fibrinogen, prolonged PT, and elevated fibrin/fibrinogen split products.[97] Conversely, in patients presenting with ATC, fibrinogen levels are rarely decreased, and platelet counts are generally normal. D-dimer levels are frequently elevated in DIC, which may indicate fibrinolysis, but fibrinolysis is unusual immediately after traumatic injury.[94,95]

THROMBOEMBOLIC COMPLICATIONS IN TRAUMA: THE PROCOAGULANT STORY

DVT/PE

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are two complications of trauma that are more common than historically appreciated.[101–103] Lower extremity DVT has been reported in an average of 1% of those with multisystem trauma, rising to 15% in severely injured patients; pulmonary embolus with hemodynamic compromise is seen in about 1% of patients with multitraumatic injuries.[104]

There are several potential reasons for why trauma patients are susceptible to the development of DVT and PE. There are known hypercoagulable abnormalities seen as early as the first admission labs.[105,106] Thromboelastography is currently being used at the University of Texas at Houston Health Science Center. Maximum amplitude (MA) is the point in time at which an aliquot of the patient's blood reaches its full thrombotic strength. MA higher than 65 with more than 2 non–weight-bearing extremities puts patients at an increased risk of DVT and subsequent PE and may be an indication for early inferior vena cava filter.[107] Extremity fractures, rib fractures, and pulmonary contusions also likely cause microvascular damage to the venous structures, predisposing patients with these injuries to thrombus

formation.[108–111] The immobility associated with traumatic injury is also a risk factor for DVT and PE.[112,113] Up to 62% of spinal cord injured patients and 80% of patients with femoral fractures developed DVTs in a study of 716 trauma patients.[108] The risk of DVT and PE exists throughout the patient's entire hospitalization and even in the final phases of his recovery. Severely injured patients who survive the initial hypocoagulation or coagulopathy associated with shock and transfusion are at high risk of late thromboembolic events as well.[43,114–116]

TREATMENT OF BLEEDING AFTER TRAUMA

Hemorrhagic shock was classically treated with crystalloid bolus, followed by RBCs, and then at a later time, plasma, platelets, and cryoprecipitate. This resulted in a disorganized administration of blood product. The initial coagulopathy of trauma was worsened by this approach, and it became clear that new transfusion protocols were needed. Previously recommended ratios of plasma:pRBC of 1:4 or 1:10 and sporadic platelet transfusions were replaced.[117,118] As the understanding of ATC has progressed, so have the resuscitation protocols developed from the ongoing military experience focused on "hemostatic resuscitation" or "damage control resuscitation."[119]

Based on results from the military, new algorithms and transfusion protocols have emerged over the last 9 years, changing the way many trauma centers transfuse blood products.[120] Surgical bleeding must be addressed and corrected. Strategies of permissive hypotension, limitation of crystalloid infusion, and maintenance of balanced transfusions of plasma, platelets, and RBCs in a balanced infusion scheme to avoid unbalanced resuscitation patterns are now employed.[121,122]

There are many studies demonstrating civilian and military utilization of 1:1:1 plasma:platelet:RBC ratios in massive transfusion protocols; however, it should be stressed that there is still considerable debate in the literature regarding the best ratio combination.[117,123,124] Holcomb et al. reported the largest and broadest of these civilian studies in support of a 1:1:1 ratio in 2008. They are currently completing an observational study at 10 trauma centers.[125] The original 2008 study of 466 massively transfused patients at 16 major US level I trauma centers showed decreased mortality in higher transfusion ratios. Patients with ≥1:1 plasma:pRBC had 2% and 25.5% 6-hour and in-hospital mortality, compared to 15.2% and 41.1% in those transfused with 1:4 to 1:1, and 37.3% and 54.9% in patients transfused with ratios <1:4. In addition, higher plasma:pRBC transfusion ratios were associated with lower overall units transfused (18 vs. 13 within the first 24 hours).[126] Multiple other civilian and military studies have corroborated these findings showing association with improved early and late survival.[121,122,127]

Debate exists in the literature regarding the most appropriate ratio to use for massive resuscitation. Borgman et al. demonstrated improved survival in 246 massively transfused military patients who received 1:1.4 plasma to pRBC transfusion ratio versus a more standard 1:3 ratio.[128] Additionally, some groups demonstrate no survival advantage to ratios that approximate 1:1.[129,130] Snyder et al.[131] suggested that the beneficial findings of high ratio resuscitation might be due to a survival bias of patients who receive plasma in their initial resuscitation. Kashuk et al.[129] reported a survival benefit as the ratio approached 1:2 but increased mortality at 1:1, in a small single center study. Finally, Riskin et al.[118] suggested that the benefit might derive from early aggressive therapy rather than the ratio itself. Ho and colleagues have recently reviewed most of the trauma transfusion data and concluded that survival bias threatens most of these studies. Because of the time-sensitive nature of rapid bleeding and highly variable ordering and supply of blood products from blood banks, randomized studies are required to answer the important questions raised by the retrospective transfusion studies.

There still exists considerable debate on the best transfusion method, but most clinicians agree on an early, balanced, aggressive plasma, platelet, and RBC-based resuscitation with limitation of crystalloid. Although there is a significant literature detailing the known risk of transfusion-related lung injury, acute respiratory distress syndrome, and cardiac volume overload associated with transfusion of plasma and platelets, the majority of this work was conducted at a time when crystalloid administration was not as limited in hemorrhagic shock as it is now.[132–137] Many centers are reporting decreases in crystalloid use during the initial phases of resuscitation, and these same patients are demonstrating corresponding decreases in organ failure and improved survival.[138,139,140]

FUTURE DIRECTIONS

Trauma-related coagulopathy and definitive hemostasis are complex and diverse subjects. Not only are the mechanisms of each not completely understood, but there are diverse and widespread molecular interplays that contribute to ongoing coagulopathy. Its consequences can be deadly or lead to extensive morbidity, and its treatment is incompletely elucidated. There is opportunity for continued research in all aspects of its mechanism, treatment, and complications. Algorithms for massive transfusions have dramatically changed over the last 5 years. The entities of ATC and of iatrogenic coagulopathy have been defined, and hemostatic resuscitation guidelines have been developed. Integrated basic and clinical research efforts are needed to deliver rapid, accurate, and predictive laboratory data, understand early hypocoagulopathy, decrease the number of hemorrhage-related deaths, prevent late venous thromboemboli, and optimize how we use blood products.

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CHAPTER

129 Blood Management in the Cardiovascular Surgical Patient

LAWRENCE TIM GOODNOUGH AND L. HENRY EDMUNDS, JR.

Bleeding is a potential complication of any surgery and a major concern of all surgeons. All surgeons are trained to control visibly bleeding vessels, but additional skills and information are necessary for successful management of complex operations, trauma victims, and patients who require mechanical circulatory assistance. Surgical hemostasis now requires knowledge of perioperative coagulation disorders, blood conservation techniques, and the risks and benefits of blood component replacement and transfusion therapy. This chapter outlines a practical approach to the principles of blood management related to all surgery, but particularly cardiac surgery.

Blood management is defined as the appropriate use of blood and blood products, with the goal of minimizing their use.[1] The conservation of blood is historically based on principles related to blood risks, blood inventory, and blood costs. Cardiothoracic surgery programs led the development of intraoperative and postoperative salvage techniques because of concerns regarding blood availalbity.[2] Subsequently, recognition of blood-transmitted hepatitis and HIV infections fueled further efforts to limit blood transfusions[3,4] (Table 129.1). Accurate accounting of blood costs has identified that alternatives to allogeneic blood (e.g., autologous blood salvage and reinfusion) may be cost equivalent.[5] Blood risks and known and unknown emerging pathogens will continue to drive blood conservation and blood transfusion practices.[6,7]

Considerable variation in transfusion practices has been identified in several studies over the last 20 years,[8,9,10] despite guidelines emphasizing more prudent transfusion practices, with suggested transfusion triggers of 6 to 8 g/dL in the perioperative setting.[11–13] A randomized trial in patients undergoing cardiac surgery demonstrated that a restrictive transfusion strategy (Hgb ≤ 8 g/dL) resulted in equivalent patient outcomes, compared to patients treated with a liberal (Hgb ≤ 10 g/dL) transfusion strategy.[14] This trial supported a previous randomized trial in critical care patients that demonstrated similar clinical outcomes in patients transfused for Hgb ≤ 7 g/dL compared to Hgb levels <10 g/dL.[15] Point-of-care testing (FIGURE 129.1) linked with a transfusion algorithm for plasma and platelet transfusions has improved patient care and reduced transfusions of blood and blood components[16] along with substantial cost savings.[17]

PREOPERATIVE ASSESSMENT

Preoperative planning is essential to reducing or avoiding perioperative allogeneic transfusion.[1,18] Preadmission testing should take place as far in advance as possible (e.g., 30 days) of elective surgery to allow time for adequate identification, evaluation, and management of anemia,[19] as illustrated in FIGURE 129.2. The estimated equivalent to number of units "saved" by various blood conservation strategies available in blood management of the surgical patient in the preoperative, intraoperative, and postoperative intervals is listed in Table 129.2.[20]

The preoperative evaluation of a patient who requires surgery varies with the type and urgency of the operation. For all patients, a thorough history is the best method to discover clinically significant bleeding disorders. A good history reveals bleeding related to previous surgical and dental procedures; epistaxis; menorrhagia; excessive bleeding with major trauma; and easy bruising or joint or muscle swelling after minor trauma. Positive answers raise the possibility of hereditary or acquired coagulation deficiencies, antibodies to specific coagulation proteins, von Willebrand disease (vWD), or platelet disorders. The patient's medication profile may reveal ingestion of inhibitory drugs, such as aspirin or aspirin-containing compounds, nonsteroidal anti-inflammatory drugs (NSAIDs), warfarin, chronic steroid use, platelet inhibitors, or injections of standard or low molecular weight heparin. The history may also reveal acquired hemorrhagic conditions associated with parenchymal liver disease, renal failure, or myeloproliferative syndromes.

The physical examination includes assessment of a possible bleeding tendency. One may find purpura, which is particularly important if not associated with a known history of trauma. Other findings include petechiae, splenomegaly, hepatomegaly, lymphadenopathy, joint deformities, lack of mobility, palpable collections of blood arising as deep hematomas, and evidence of gastrointestinal (GI) bleeding on digital rectal examination. Discovery of blood in stool is the most effective way to detect occult sources of GI bleeding from esophageal varices, duodenal ulcer, gastritis, diverticulitis, polyps, cancer, ingestion of NSAIDs, and other disorders. Comorbid diseases, such as uremia, cirrhosis, aortic stenosis, polycythemia vera, or continuous flow ventricular assist devices, may enhance bleeding from local lesions in the GI tract.

The results of the history and physical examination and the proposed operation determine the selection of preoperative laboratory tests for coagulation disorders and the need for special preoperative or intraoperative therapy.

Blood must be typed, and a sample sent to the blood bank for antibody screening (type and screen/crossmatch) for all patients prior to scheduled surgery. A complete blood count is obtained in all patients, and traditionally both a prothrombin time (PT) and activated partial thromboplastin time (aPTT) are measured. A prolonged PT detects deficiencies of the extrinsic coagulation pathway, which may be due to vitamin K deficiency or liver disease; a prolonged aPTT detects deficiencies of proteins in the intrinsic coagulation pathway. Results of these measurements

Table 129.1

Risks of major infectious and noninfectious complications of transfusion in the United States

Complications	Risk
Transfusion-transmitted Disease Agents	
Human immunodeficiency virus	1:1,800,000
Hepatitis C virus	1:1,600,000
Hepatitis B virus	1:220,000
Bacteria (platelets, apheresis)	1:75,000
Noninfectious Complications	
Acute hemolytic reaction	1:25,000
Mistransfusion[a]	1:19,000
Transfusion-related acute lung injury	1:5,000
Transfusion-associated circulatory overload[a]	1:400
Fever/allergy	1:100

[a]Can occur in association with transfusion of autologous blood as well as allogeneic blood.
Modified from Uhl L. Patient blood management: a 68 year old woman contemplating autologous blood donation before elective surgery. *JAMA* 2011;306:1902–1910.

are generally laboratory specific, and the predictive value for bleeding is low, but values exceeding the normal range should be investigated. For example, familial factor XI deficiency may not be detected by a bleeding history, but does prolong the aPTT. A history of bleeding episodes or physical signs of bleeding mandate a full bleeding workup and consultation with a hematologist to assess platelet numbers and function and the presence of inherited or acquired coagulation factor deficiences.[21]

The role of erythropoiesis-stimulating agents (ESAs) in management of preoperative anemia patients undergoing cardiovascular surgery and its safety in this setting are unresolved issues. In a European trial,[22] the authors found no differences in mortality, thrombotic events, or serious adverse events among their 76 patients, whether treated with ESA or placebo. A US study also concluded that no differences in adverse events between ESA- and placebo-treated patients were observed and that ESA therapy was well tolerated.[23] The safety of ESA in surgical settings has been under re-review since the completion of the SPINE study, in which patients scheduled for elective spine surgery had higher rates of thrombovascular events with an ESA versus placebo in subjects who did not receive prophylactic ESA therapy during spinal surgery.[24] Nevertheless, there was an uneven distribution of adverse events between the two treatment groups. Use of ESA remains a valuable tool for patients with special requirements, such as Jehovah's Witness patients for whom blood transfusion is not an option.[18] Until additional

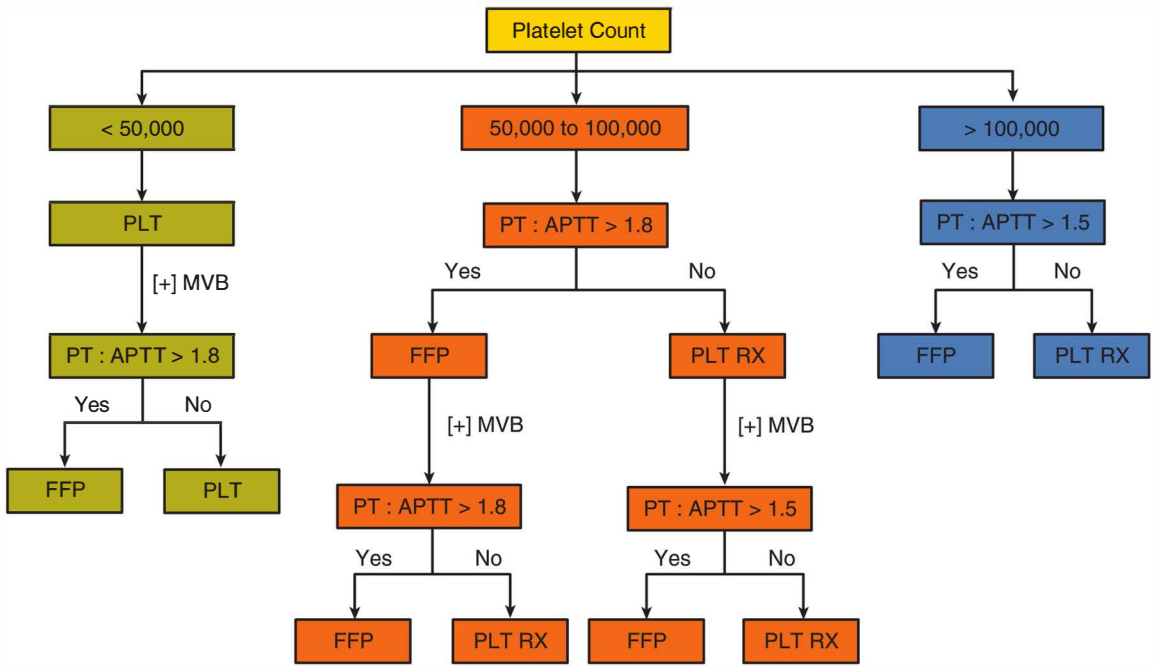

FIGURE 129.1 Transfusion algorithm based on preoperative platelet count. An initial platelet count of <50,000 (*left arm*: severe platelet deficiency) warrants platelet transfusion. If the initial platelet count is >50,000 (*middle* and *right arms*), the PT:aPTT ratio (patient vs. normal reference) would delineate whether platelet therapy or plasma was to be administered. *Platelets*, Platelet transfusion (6 random donor units); PLT RX, platelet therapy (6 random donor units or 0.3 mg/kg DDAVP at physician's discretion); FFP, 2 units of thawed plasma; [+] MVB, persistent microvascular bleeding. (Reprinted from Despotis GJ, Santoro SA, Spitznagel E, et al. Prospective evaluation and clinical utility of on-site monitoring of coagulation in patients undergoing cardiac operation. *J Thorac Cardiovasc Surg* 1994;107:271–279, with permission.)

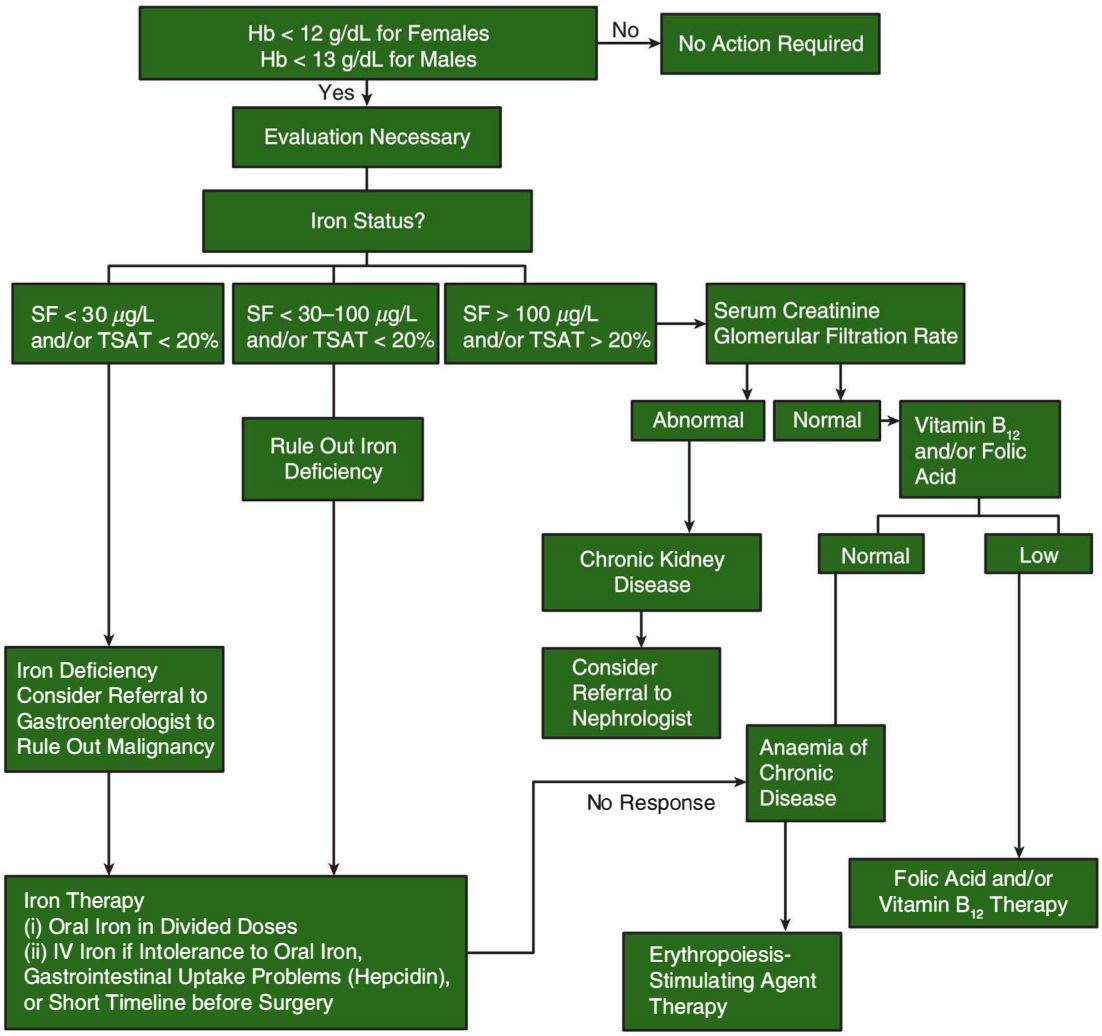

FIGURE 129.2 Algorithm for the detection, evaluation, and management of preoperative anaemia. SF, serum ferritin; TSAT, transferrin saturation. (From Goodnough LT, Maniatis A, Earnshaw P, et al. Detection, evaluation, and management of preoperative anaemia in the elective orthopaedic surgical patient: NATA guidelines. *Br J Anaesth* 2011:106:13–22.)

safety data are forthcoming, however, the off-label use of ESA in patients undergoing cardiac or vascular surgery in the United States cannot be recommended.[25]

CONDITIONS THAT INCREASE PERIOPERATIVE BLEEDING

Genetic and Acquired Clotting Abnormalities

Hereditary coagulation deficiencies are usually identified by history or screening test and are confirmed by specific laboratory evaluation. The most common inherited coagulation protein deficiencies are hemophilia A and B and vWD. Patients with hemophilia A or B may develop IgG antibodies to the deficient coagulation proteins, FVIII or FIX, from chronic replacement therapy. Deficiencies in coagulation proteins are usually treated by replacement therapy, and many of these are now available as recombinant human proteins. Inherited deficiencies in fibrinolytic inhibitors, α_2-antiplasmin or plasminogen activator inhibitor-1, may also cause substantial bleeding due to excessive fibrinolysis, and these are effectively treated by lysine analogs, epsilon aminocaproic acid, or tranexamic acid.[26] A variety of genetic defects may affect platelets and disturb platelet numbers and function.[27] Some may respond to desmopressin or require platelet transfusions, but consultation from an expert hematologist is needed, particularly since patients may develop antibodies against the diminished receptors from prior transfusions. Osler-Weber-Rendu and Ehlers-Danlos syndromes may cause troublesome bleeding problems during surgery even though circulating coagulation factors and platelets are normal. Marfan syndrome is not associated with increased risk of bleeding.[27]

Acquired bleeding disorders of coagulation proteins, fibrinolysis, and platelets occur more often than hereditary bleeding diseases. Drug-induced antibodies (quinidine, quinine, some antibiotics) against specific coagulation proteins are uncommon. Surgeons, however, may encounter patients with antibodies to factor V due to prior use of topical bovine fibrin glue for local hemostasis.[28,29] Advanced liver disease may diminish nearly all

Table 129.2

Approximate contributions of selected modalities to blood management in the surgical patient

Options	Number of Blood Units
Tolerance of anemia (reduce transfusion trigger)	1–2
Increase preoperative red blood cell mass	2
Preoperative autologous donation	1–2
Intraoperative options	
Meticulous hemostasis and operative technique	1 or more
Acute normovolemic hemodilution	1–2
Blood salvage	1 or more
Postoperative options	
Restricted phlebotomy	1
Blood salvage	1

Modified from Goodenough LT, Spence R, Shander A. Bloodless medicine: clinical care without allogenic blood transfusion. *Transfusion* 2003;43:668–676.

coagulation and regulatory proteins of the coagulation system to levels that affect hemostasis and/or promote severe fibrinolysis.[30] However, drugs prescribed to inhibit platelet function cause the most common acquired bleeding disorders that concern surgeons.

Surgeons must also be aware of a large menu of genetic procoagulant clotting disorders that increase the likelihood of thrombotic and embolic complications. Factor V_{Leiden} mutation, which impairs activation of the natural anticoagulant protein C, is prevalent (2% to 5%) in Northern European whites and their descendants and the prothrombin 20,210 mutation is prevalent (1% to 3%) in Southern Europeans.[31] Both mutations increase the risk of venous thrombosis.[31] Lupus anticoagulant and anticardiolipin antibodies may produce thrombotic complications during and after major surgery.[32]

Platelets. Thrombocytopenia or platelet dysfunction that are not drug related occur in patients with myeloproliferative diseases, uremia, acquired vWD, antiplatelet antibodies (e.g., immune thrombocytopenia), posttransfusion purpura, and cirrhosis.[33] Aspirin and some NSAIDs irreversibly inactivate platelet cyclooxygenase for the life of the platelet (~10 days). Cyclooxygenase blockade partially inhibits platelet function by preventing endoperoxide and thromboxane A2 synthesis.[34] Aspirin prolongs the bleeding time[35] and blocks platelet aggregation,[36] but may increase the bleeding time to a greater extent if other, mild hemostatic deficiencies (e.g., vWD) are present. Aspirin resistance caused by genetic or acquired mechanisms occurs in approximately 10% of patients with acute coronary syndromes.[37]

Major surgery, including cardiac surgery, in patients with aspirin-inhibited platelets may increase perioperative bleeding.[36,38,39,40] Nearly all patients presenting with acute coronary syndromes receive aspirin as part of their initial therapy, but usually the impact on surgical hemostasis is minor.[38] Aspirin-related bleeding is treated by platelet transfusions, since aspirin is cleared from blood in <90 minutes.[41]

Clopidogrel and more recently prasugrel are thienopyridines that are irreversible platelet inhibitors that prevent adenosine diphosphate binding to platelet $P2Y_{12}$ receptors. Both drugs have rapid platelet inhibition, but the kinetics of prasugrel are faster and more consistent and the drug is preferred for management of acute coronary syndromes.[42,43] Median plasma half-life is 7 to 8 hours, but platelet inhibitory effects vary and last 3 to 10 days after the drug is discontinued.[33] Both drugs are routinely used in combination with aspirin to reduce adverse cardiac ischemic events.[44] Major bleeding complications from the combination of aspirin and prasugrel are slightly, but significantly higher (2.4%) than with aspirin and clopidogrel (1.8%).[42] For this reason, the Society of Thoracic Surgeons' guidelines recommend stopping thienopyridines at least 3 days and preferably 5 days before elective coronary arterial bypass surgery.[39] However, reversal of thienopyridines is highly variable between patients. A point-of-care device, which assesses platelet responsiveness to ATP, showed reduced platelet aggregation in some patients who had stopped thienopyridines within 7 days of operation.[45]

Platelet GPIIIa/IIb ($\alpha_{IIb}\beta_3$) receptor antagonists (abciximab, eptifibatide, and tirofiban) inhibit most platelet functions and prolong bleeding times considerably longer than aspirin.[46] Abciximab (Reopro) rapidly binds to the platelet receptors and remains bound for the life of the platelet.[47] Eptifibatide and tirofiban are reversible inhibitors and relatively short acting. Inhibitory effects of abciximab last approximately 48 hours, whereas that of eptifibatide lasts <1 hour.[47] Certain prostaglandins inhibit platelets by activating platelet adenylate cyclase or inhibiting cyclic nucleotide phosphodiesterases (e.g., prostacyclin and dipyridamole).[33] All of these platelet inhibitors increase the bleeding time.[33]

Warfarin. For minor or superficial operations, it is usually not necessary to reverse warfarin, but for major operations, warfarin should be stopped for 3 to 5 days before surgery, if the likelihood of a clotting event within that interval is low.[13] The PT should be checked before operation to be sure that most, if not all, of the warfarin effect has been reversed. In patients that cannot safely tolerate interruption of anticoagulation (e.g., mechanical heart valves), intravenous heparin may be used to maintain anticoagulation until 2 to 3 hours before incision.

Oral vitamin K (2 to 4 mg) administration usually corrects the PT to normal within 24 hours.[48] In an emergency, warfarin anticoagulation can be reversed by intravenous vitamin K (10 mg IV, infused slowly over 30 minutes) and plasma infusion (15 to 30 mL/kg, or 4 to 8 units in an adult) over 4 to 6 hours, with careful attention to volume status of the patient.[49,50]) Transfusions of prothrombin complex concentrates (PCCs), some of which contain high concentrations of vitamin K–dependent coagulation factors II, VII, IX, and X, have also been utilized. PCC acts rapidly but may have a risk of thrombosis.[51,52] If replacement therapy is not effective, administration of recombinant factor VIIa (rFVIIa) (2 mg IV, or 20 to 40 μg/kg for 100 to 50 kg patients) is another alternative in patients with emergency or life-threatening bleeding[39,53]; however, this agent also has defined risks of arterial thrombotic complications, particularly in the elderly or at higher doses.[54]

Dabigatran is a recently approved, oral inhibitor of thrombin that does not require blood monitoring.[55–57] The drug is taken twice daily and is effective prophylaxis in patients with paroxysmal or chronic atrial fibrillation (AF),[58] in treatment of acute venous thromboembolism,[57] and in acute coronary

syndromes.[59] Plasma half-life is 12 to 17 hours,[56] and the drug is cleared by the kidney.

Rivaroxaban is a new, oral factor Xa inhibitor that is rapidly absorbed and has a half-life of 9 to 12 hours and can be given as a fixed dose.[59,60]. Most of the drug is eliminated in urine and the remainder in feces. Rivaroxaban has been approved for preventing stroke in patients with AF (ROCKET-AF trial),[61] as it reduced the incidence of stroke and intracranial bleeding compared with warfarin treatment (0.49 vs. 0.74%). If emergency surgery is required before either dabigatran or rivaroxaban is eliminated, rFVIIa may be attempted to control bleeding, but its value is not certain as an antidote for anticoagulants.[62]

Fibrinolytic drugs. If operation occurs within 12 hours of the last dose of streptokinase or tissue-plasminogen activator (t-PA), the risk of surgical bleeding is increased.[63] If excessive bleeding occurs in such patients, plasma fibrinogen can be replaced with cryoprecipitate, fresh frozen plasma, or fibrinogen concentrate (available in Europe). Platelet transfusion may also be needed, especially if a long-acting platelet inhibitor such as a thienopyridine or abciximab is circulating. Antifibrinolytic drugs are not generally utilized, as the thrombolytic agent will have been cleared from the circulation in most cases. Fibrinolytic drugs are cautiously recommended for treating thrombotic strokes. Potential benefits appear to outweigh the risk of intracranial hemorrhage or surgical site bleeding.[64–66]

ANTICOAGULANTS USED DURING CARDIAC AND VASCULAR SURGERY

Standard, unfractionated heparin (UFH) is universally used to maintain the fluidity of blood during mechanical circulation outside the body.[67] Heparin greatly accelerates inhibition of thrombin and to a lesser extent activated coagulation factors IXa and Xa by the binding action of antithrombin, which is an abundant plasma protein. The drug does not inhibit thrombin bound in fibrin clots.[68] UFH acts rapidly, can be given intravenously, and is reversed by protamine sulfate (PS), but disadvantages include incomplete suppression of thrombin formation; heparin resistance (low antithrombin concentrations); interaction with platelets, complement, and various plasma proteins that variously affect clearance; and stimulation of IgG antiplatelet factor 4 antibodies in some patients. "Heparin resistance" occurs when plasma antithrombin concentrations are deficient from either malnutrition in cyanotic infants or cachectic patients or from sustained heparin administration just prior to operation.[69]

Low Molecular Weight Heparin

Low molecular weight heparins (LMWHs) catalyze antithrombin, but primarily inhibit factor Xa. Theoretically, these heparins should prevent thrombin formation, but they fail to do so during extracorporeal circulation, as monitored by protein fragment, F1.2, in recirculated human blood[70] or in baboons.[71] LMWHs can be given intravenously, act rapidly, are long acting (plasma half-life 4 to 8 hours), are monitored by anti–factor Xa activity, are not completely reversed by PS, may generate antiplatelet IgG antibodies (10% as frequently as with UFH), do not reliably prevent clot formation in the circuit, and are associated with excessive postoperative bleeding.[72,73] LMWH anticoagulants are not suitable for cardiopulmonary bypass (CPB).

Bivalirudin (Hirulog) is a synthetic, 20–amino acid, reversible, direct thrombin inhibitor,[74] which acts rapidly, has a half-life in plasma of 25 to 36 minutes,[75,76] is cleared by proteolysis and the kidneys,[77] but has no antidote.[76] Dosage is preferably monitored by ecarin clotting time,[78,79] but it has also been successfully monitored with the activated clotting time (ACT)[80,81] and aPTT. Bivalirudin is essentially nonantigenic and has no major side effects except bleeding. The drug has been evaluated in patients with acute coronary artery syndromes,[82] during off-pump coronary arterial surgery,[83] in cardiac surgery with CPB,[84,85] and during extracorporeal membrane oxygenation.[86] The pharmokinetics of the drug are attractive for use during CPB, but very careful and rigorously monitored protocols must be followed to prevent clotting within the perfusion circuit. Drug dosage remains under investigation: initial dosing schedules combine 1.5 mg/kg iv and 50 mg in the pump prime and a continuous infusion at 2.5 to 3.5 mg/kg/h.[84,87]

Lepirudin (recombinant hirudin) directly binds and inactivates thrombin, including thrombin within fibrin clots. The drug has a plasma half-life of approximately 80 minutes and is primarily excreted by the kidney.[88] The duration of anticoagulant activity of lepirudin is inversely proportional to creatinine clearance and is prolonged up to 3 to 5 hours by anesthesia.[88] Plasma concentrations are monitored by ecarin clotting time or by aPTT.[89] There is no antidote, the major side effect is bleeding, and allergic reactions are minimal. Lepirudin is approved by the US Food and Drug Administration for use during CPB in patients with heparin-induced thrombocytopenia (HIT) or heparin-induced thrombocytopenia with thrombosis (HITT) and is the most widely used alternative to standard heparin.[88,90]

Argatroban is a synthetic, low molecular weight, reversible, direct thrombin inhibitor that avidly binds thrombin even in fibrin clots.[91] Given intravenously, the drug acts rapidly, has a half-life in plasma of approximately 45 minutes,[92] and is metabolized by the liver. Anticoagulant activity is monitored by aPTT, but when high doses are used, ACTs are recommended.[91,92] There is no antidote, bleeding is the major side effect, and no toxic or allergic reactions are known. Argatroban has been used for CPB in patients for whom heparin was contraindicated,[93] but difficulties with excessive bleeding or clotting within the circuit are reported.[94,95]

EXCEPTIONAL SURGICAL BLEEDING PROBLEMS

Heparin-Induced Thrombocytopenia

An unexpected low platelet count in patients who received heparin 5 to 15 days earlier raises the possibility of HIT and the possibility of complicating thrombosis (HITT).[96] HIT is mediated by IgG antibodies to a complex of heparin and platelet factor 4 (PF4).[87,97] The disease affects approximately 2% of all cardiac surgical patients.[98–100] HIT is strongly suggested when the platelet count precipitously decreases by 30% to 50% or by 100,000/μL after exposure to heparin.[87] A serotonin release test or enzyme immunoassay to detect the presence of anti-heparin/PF4 IgG antibodies[101,102] is mandatory before more heparin is given. Thrombocytopenia and thrombosis due to massive platelet consumption may produce platelet counts below 30,000/μL, but platelet transfusions are not recommended.[103]

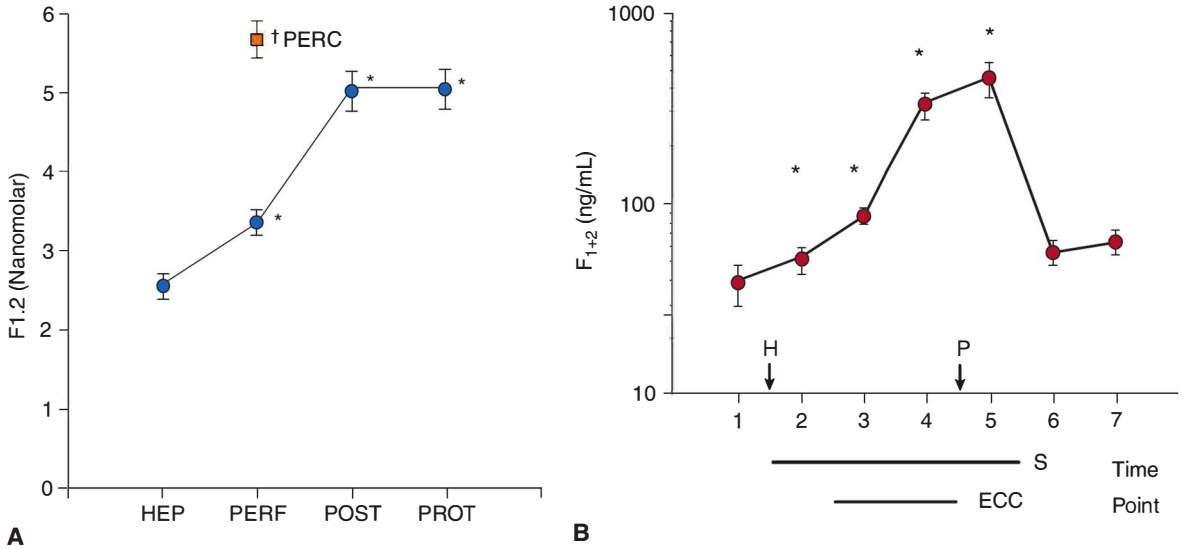

FIGURE 129.3 **A:** F1.2 concentrations taken from the perfusion circuit during cardiac surgery. Thirty to forty-five minutes after beginning CPB, simultaneous samples were drawn from the pericardial well surrounding the heart (PERC) and the circuit (PERF). HEP, heparin injection; post, immediately after CPB stopped; PROT, at the time of protamine administration. (Reproduced from Chung JH, Gikakis N, Drake TA, et al. Pericardial blood activates the extrinsic coagulation pathway during clinical cardiopulmonary bypass. *Circulation* 1996;93:2014, with permission.) **B:** Serial measurements of prothrombin fragment, F1.2, during CPB. S, duration of operation; ECC, duration of CPB; H, heparin injection; P, protamine administration. (Reproduced from Boisclair MD, Lane DA, Philippou H, et al. Thrombin production, inactivation and expression during open heart surgery measured by assays for activation fragments including a new ELISA for prothrombin fragment F1+2. *Thromb Haemost* 1993;70:253, with permission.)

Disseminated intravascular coagulation (DIC) is a consumptive coagulopathy that may complicate severe trauma, particularly if brain is injured, or complicate postoperative septicemia. The underlying disease (e.g., trauma or infection) initiates thrombin generation primarily by tissue factor exposed in injured tissues[105,106] and on monocytes and endothelial cells.[107–110,111,112] DIC may coexist with a fibrinolytic state.[105,113] Effective therapy requires control of the initiating cause of the coagulopathy. Except for life-saving transfusions, replacement of blood constituents is usually futile until the etiology of DIC is brought under control.[114] Preparation for surgery varies, but consumed procoagulants should be replaced, and if intravascular thrombosis is present, heparin or a direct thrombin inhibitor to control thrombin generation may be required. In a multi-institutional, prospective, randomized trial, tranexamic acid significantly reduced both bleeding and death in trauma victims, who had a defined, significant risk of major hemorrhage.[115] Emerging therapy includes the use of rFVIIa in patients with massive trauma and coagulopathy.[116–118]

CPB and cardiac surgery expose the blood to synthetic surfaces of the perfusion circuit and to the open wound, and coagulation of blood must be prevented by use of systemic patient anticoagulation. High doses of UFH are universally used, but do not completely prevent generation of thrombin.[119,120] Endothelial cells maintain the integrity of the vascular system and fluidity of blood by active metabolic processes, which are absent when circulated blood passes through the heart–lung machine or contacts the surgical wound.[67,121] The net result is continuous generation of thrombin; fibrinolysis;[122] and activation of platelets, complement, neutrophils, and monocytes.[67] Platelets adhere to surface-adsorbed fibrinogen and other adhesive proteins, become activated, release granule contents, and are partially destroyed by shear forces. At the end of CPB and open heart surgery, platelet counts are typically reduced by 40% to 60%, circulating platelets are dysfunctional, and template bleeding times are approximately twice normal.[98,99] During CPB and cardiac surgery, thrombin is generated in both the perfusion circuit and the surgical wound, but most of the thrombin is produced in the wound[111,123] (FIGURE 129.3A). Protein fragment F1.2, which is produced when prothrombinase cleaves prothrombin to α-thrombin, rises continuously during perfusion and peaks at the time PS is given after CPB[119,124] (FIGURE 129.3B).

Thrombin stimulates endothelial cells to produce t-PA, which cleaves plasminogen into the active enzyme, plasmin. D-Dimer, a marker of plasmin activity, and fibrin/fibrinogen degradation products appear in high concentrations in the surgical wound (FIGURE 129.4A),[125] as compared to the circulating perfusate (FIGURE 129.4B). Circulating D-dimer levels peak when PS is given,[126] as does F1.2, but fibrinolysis continues for hours after CPB ends. Thus cardiac surgery with CPB produces a consumptive coagulopathy that primarily occurs in the surgical wound, but also peaks in the perfusate when heparin is reversed by PS.

INTRAOPERATIVE MANAGEMENT OF BLEEDING

Wound Management

Meticulous surgical hemostasis is the cornerstone of any blood conservation program. During open heart surgery, careful

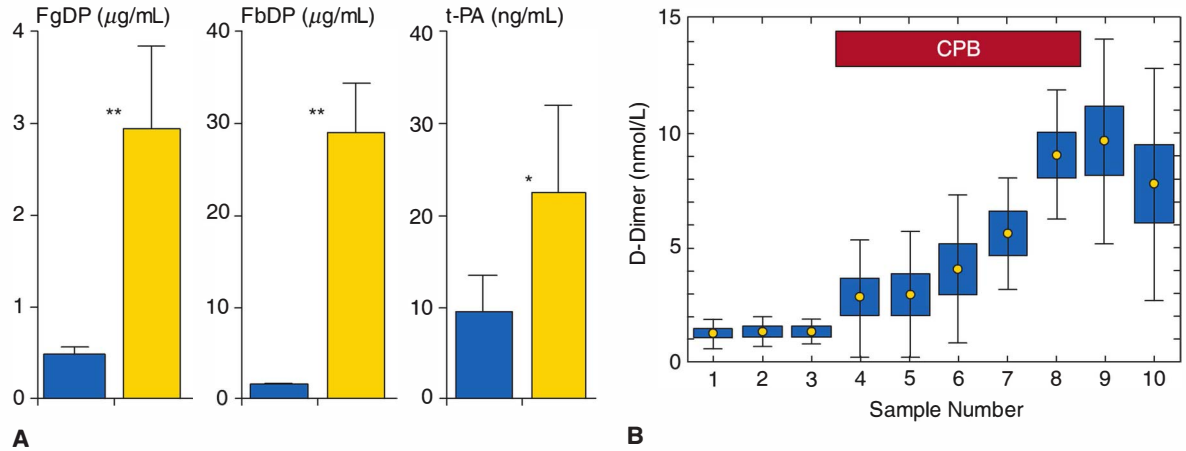

FIGURE 129.4 A: Simultaneous measurements of fibrinogen degradation products (FgDP), fibrin degradation products (FbDP), and tissue-type plasminogen activating factor (t-PA) drawn from the perfusion circuit (*blue bars*) and from the wound (*yellow bars*). (Reproduced from Tabuchi N, Haan J, Boonstra PW, et al. Activation of fibrinolysis in the pericardial cavity during cardiopulmonary bypass. *J Thorac Cardiovasc Surg* 1993;106:828–833, with permission.) **B:** Ten serial measurements of D-dimer during cardiac surgery with CPB (*blue bar*). Protamine was given at sample 9; sample 10 was taken 2 hours later. (Reproduced from Chandler WL, Valen T. Plasmin generation and d-dimer formation during cardiopulmonary bypass. *Blood Coagul Fibrinolysis* 2004;15:583–591, with permission.)

hemostasis before heparin and CPB is important to minimize bleeding during the pump run, but general oozing from cut tissues is expected. The electrocautery or ultrasonic scapel and hemostatic clips speed the process of dissection and facilitate hemostasis.[127]

Reinfusion of Shed Wound Blood

Three methods are used to recover heparinized blood that accumulates in the surgical wound during surgery with CPB. Aspirated blood is either filtered in a reservoir and returned directly to the perfusate or is processed by centrifugation or ultrafiltration. Centrifugation recovers largely red cells (hematocrit ~65 to 70), whereas ultrafiltration saves proteins and red cells.[39] Comparison reinfusion studies of coronary revasculation operations[128] fail to show significant differences in postoperative blood loss, hematocrit, or platelet count between the three methods.[129] Direct comparison of centrifugation versus ultrafiltration also fails to show differences in postoperative blood losses or transfusion requirements.[39] No data are available regarding the superiority of the three methods of blood salvage in more complicated operations.[128]

Intraoperative Tests

For most noncardiac surgery procedures, intraoperative tests of the coagulation system are not necessary unless unexpected, substantial, or nonsurgical bleeding occurs. In cardiac patients managed with CPB, tests for platelet number, hemoglobin concentration, and coagulation tests (aPTT and PT) are obtained and replacement therapy prescribed as indicated. Platelet counts <100,000/μL prompt platelet transfusion if bleeding is a problem at the time. PS reversal of heparin anticoagulation is monitored by ACT measured in the operating room. New point-of-care devices are also available to monitor fibrinolysis, which may occur in some noncardiac patients, who have large surgical wounds or who develop a consumptive coagulopathy from massive trauma or sepsis.

Suppression of Bleeding in the Surgical Wound

During cardiac surgery with CPB, the wound is the major source of thrombin generation and fibrinolysis, which continues after CPB is stopped and PS is administered.[128] Studies demonstrate that topical treatment of the surgical wound after PS administration with an antifibrinolytic drug reduces postoperative blood loss.[125,130,131] Epsilon amino caproic acid (EACA) and tranexamic acid are lysine analogs that block lysine-binding sites on plasminogen and thereby interfere with plasminogen binding to fibrin.[104] Tranexamic acid has been associated with increased postoperative seizures,[132] but both are effective in appropriate doses. EACA is not associated with toxicity even in high doses.[128] Aprotinin is a serine protease inhibitor that binds plasmin with high affinity and kallikrein with approximately 100-fold less affinity,[129] but is no longer available for systemic use in the United States. Topical treatment of the surgical wound with an antifibrinolytic reduces blood loss.[125,130,131]

Systemic lysine analogs are also used during cardiac surgery with CPB to suppress circulating plasmin and are usually given as loading doses before incision, in the priming volume of the heart–lung machine and as a continuous infusion during and up to 3 hours after stopping CPB.[134,135] This practice of "frontloading" lysine analogs during CPB is designed to suppress fibrinolysis during CPB. However, D-dimer concentrations peak when protamine is given at the end of CPB and continue to be elevated for many hours after operation.[134] Since blood clotting is desired after protamine is given, logic suggests that a high concentration of a lysine analog is needed in the wound and also in the systemic circulation during the first several hours after CPB ends.[128]

A number of topical compounds[39] are used in open wounds to reduce nonsurgical bleeding and bleeding at sites of vessel or cardiac anastomoses. "Compression hemostatic agents" are designed to provide a scaffold upon which clotting is encouraged and include bovine or recombinant human thrombin, fibrinogen, serum, or albumin in various vehicles or are designed to activate platelets.[39] Other newer topical anastomotic sealants derived from plants or chitin have also been developed, but efficacy is not yet established.[39] These "tissue glues" are directly applied to oozing surfaces.

None of these topical agents directly address the mechanism of nonsurgical bleeding in the cardiac surgical wound, which is primarily due to reduced platelet numbers and function and fibrinolysis.[67,128] Nevertheless, these products are often effective for microvascular bleeding in cardiac operations,[136] debridement of skin burns and oozing vascular anastomoses, and as a sealant for dural suture lines and parenchymal lung leaks. Use of bone wax to reduce marrow bleeding from sternotomy edges is contraindicated because of adverse effects and embolization.[137,138]

Off-Label Use of Recombinant Factor VIIa

rFVIIa is an expensive, recombinant protein that is nearly identical with plasma factor VIIa. A bolus dose (90 μg/kg) immediately increases plasma factor VIIa from 1% to about 15%.[139] rFVIIa is approved at 90 μg/kg dosage for treatment of bleeding in patients with hereditary or acquired hemophilia who have inhibitors, and it has been used increasingly off-label in a variety of patients with trauma or surgical bleeding.[140]

Gill et al.[141] recently published a RCT of rFVIIa in adult patients undergoing cardiovascular surgery with CPB. In this 30-center trial, patients with ongoing bleeding following reversal of heparinization after CPB were randomized to receive placebo ($n = 68$) or rFVIIa at doses of 40 μg/kg ($n = 35$) or 80 μg/kg ($n = 69$). Treatment with the higher rFVIIa dose significantly decreased the need for reoperation ($P = 0.04$), and patients in both groups received significantly fewer allogeneic RBC transfusions ($P = 0.01$). Mortality was not significantly different between groups, but there was a trend toward a higher rate of serious adverse events, particularly stroke, in the patients receiving rFVIIa. The authors concluded that rFVIIa should not be used for cardiovascular surgery until larger controlled clinical trials have been performed.

The risk of thrombotic adverse events (TAEs) observed in clinical trials evaluating off-label uses of rFVIIa has raised concerns. Available data suggest that the risk of TAE is increased when rFVIIa is used in off-label settings, with particular concerns being raised about arterial thrombosis in the elderly and at higher doses.[54]

Delayed Wound Closure

Occasionally, after cardiac surgery, the chest cannot be closed safely because of compression of the heart, persistent microvascular bleeding, or hemodynamic instability.[142–144] In such circumstances, the wound is packed with sponges and left open. The open wound is covered with a rubber dam or silicone membrane, which is sutured or stapled to the skin edges.[142–144] Delayed closure is frequently necessary in patients who require mechanical circulatory assistance. In one series, 46 of 86 patients had delayed wound closure after implantation of a centrifugal flow left ventricular assist device.[145] In newborns and infants, the incidence of delayed closure is approximately 4% to 9%[143]; in adults the incidence is slightly <2%.[142] Closure of surviving patients is achieved at a mean of 3 to 4 days,[142–144] but may be delayed as long as 2 weeks.[146] Wound infections are surprisingly uncommon; a few patients have superficial infections[143,144]; deep infection and poor sternal healing are rare.[144,146] A survey of 37 congenital heart surgical programs found no difference in wound infections between patients with immediate or delayed wound closures.[147]

POSTOPERATIVE MANAGEMENT

Monitoring and Initial Tests

Bleeding after most operations is most often related to delayed bleeding from a vascular suture line; from a clipped, ligated, or cauterized vessel; or from continuous generalized ooze into a hidden body cavity. Management requires prompt restoration of the circulating blood volume, control of the bleeding source, and evacuation of space occupying hematomas. Two to four percent of patients who have open heart surgery are returned to the operating room for control of postoperative bleeding.[148–151] Reoperations, complex procedures, and many aortic operations are associated with increased postoperative bleeding and the need for replacement transfusions.[128] Approximately one-third of patients who require ongoing mechanical circulatory assistance require reexploration within a few days after operation.[145,152] After cardiac surgery, the pericardium is not closed and chest tubes are routinely left in the mediastinum and opened pleural cavities and are placed on gentle (10 to 20 cm H_2O) suction to monitor the rate of bleeding and to prevent cardiac tamponade. Bleeding patients with an elevated PT or fibrinogen concentrations below 100 mg/dL receive fresh frozen plasma. An elevated aPTT is treated with an additional dose of 25 to 50 mg of PS. Platelet counts below 100,000/μL are treated with platelet transfusions in patients who are actively bleeding. If ongoing fibrinolysis is suspected, D-dimer is measured and/or EACA or tranexamic acid is given empirically.

Indications for Wound Exploration

Bleeding at the rate of 10 mL/kg in the 1st hour after operation is an indication for prompt wound exploration. If postoperative bleeding during wound closure is anticipated, a platelet count, PT, and aPTT are sent from the operating room. Any deficiencies are corrected as soon as possible. After cardiac operations, persistent bleeding that equals or exceeds 5 mL/kg/h for each of the next 3 hours or bleeding that totals 20 mL/kg in the first 4 hours after operation are indications for mediastinal exploration. Bleeding over 5 mL/kg/h for the 1st postoperative hour should prompt repeating the platelet count, PT, and aPTT. Postoperative platelet counts below 100,000/μL in a bleeding patient are treated with one or two packs of pooled platelets. Elevated PTs (INR > 1.3) are usually treated with fresh frozen plasma. Elevated aPTT (>40 seconds) is an indication for additional PS, after which the aPTT test is repeated.

Truly massive bleeding or a sudden increase in the rate of bleeding with or without hemodynamic instability is justification for opening the wound in the intensive care unit to secure a major surgical bleeding point.[153] Stabilization often is achieved

in the intensive care, and sometimes the wound may be reclosed *in situ*.[154] Bleeding is the most common early postoperative complication following implantation of circulatory assist devices, occurs in a high percentage of patients, and is frequently substantial.[145,155,156] In a series of 86 patients, 17 (20%) returned to the operating room for control of bleeding.[145] During the 1st week after implantation, chest tube drainage averaged 5,880 ± 4,480 mL for the entire cohort and required an average of 15.6 ± 12.6 units of red cells, fresh frozen plasma, and platelet packs in addition to 11.6 ± 7.5 units during operation.[145] Many of these patients have a consumptive coagulopathy[155–157]; topical treatment of the surgical wound and systemic administration of EACA or tranexamic acid guided by serial measurements of systemic D-dimer offers a mechanistic based solution to the problem and likely is safer than empirical administration of PCCs or rFVIIa.[128,158–160]

PATIENT BLOOD MANAGEMENT

Blood management has been defined as "the appropriate use of blood and blood components, with a goal of minimizing their use[1]." This goal has been motivated historically by (a) known blood risks, (b) unknown blood risks, (c) preservation of the national blood inventory, (d) and constraints from escalating costs.[161] Known risks of blood include transmissible infectious disease, transfusion reactions, and potential effects of immunomodulation (e.g., postoperative infection or tumor progression). Unknown risks include emerging pathogens transmissible by blood (e.g., new variant Creutzfeldt-Jakob disease and West Nile virus).[162–164] Additionally, several studies have linked allogeneic blood transfusions with occurrence of unfavorable outcomes including increased risk of mortality and various morbidities.[165–167] For example, studies have indicated that the risk of postoperative infections such as sepsis is as much as two to four times higher in transfused patients compared with untransfused cohorts.[168,169] Taken together, these lines of evidence support the implementation of blood management as a means to improve the clinical outcomes of the patients. Blood management was cited recently as one of the ten key advances in transfusion medicine over the last 50 years.[170]

Patient blood management encompasses an evidence-based medical and surgical approach that is multidisciplinary (transfusion medicine specialists, surgeons, anesthesiologists, and critical care specialists) and multiprofessional (physicians, nurses, pump technologists, and pharmacists).[1,171] Preventative strategies are emphasized to identify, evaluate, and manage anemia[19,172,173] (e.g., pharmacologic therapy[174] and reduced iatrogenic blood losses from diagnostic testing)[175]; to optimize hemostasis (e.g., pharmacologic therapy[176] and point-of-care testing)[177]; and to establish decision thresholds (e.g., guidelines) for the appropriate administration of blood therapy.[165,178,179]

Anemia Management

Guidelines for detection, evaluation, and management of anemia in elective surgery patients have been published (FIGURE 129.2).[19] Predictors of blood transfusion for patients have long been identified, with the most important being the preoperative circulating red blood cell mass,[180] as estimated by the patient's hemoglobin concentration. In addition to being a risk factor for blood transfusions, preoperative anemia is also an independent predictor of morbidity and mortality in patients undergoing noncardiac surgery.[181–184] Evaluation of anemia should begin with an initial assessment of iron-restricted erythropoiesis, which can occur in either the presence or absence of inflammation. Iron-restricted erythropoiesis can cause anemia due to an absolute deficiency of storage iron, an iron-sequestration syndrome due to inflammation, or a functional iron deficiency due to erythropoietin-stimulated erythropoiesis.[185,186] The evaluation of unexpected anemia must additionally consider unexpected diagnoses including chronic kidney disease or occult malignancy. In order to facilitate this, screening and detection of anemia should occur as far as possible (up to 30 days) in advance of an electively scheduled surgery.[19] This requires close collaboration among the patients' primary care physicians, surgeons, anesthesiologists, and the medical directors of the institution's preadmission testing program.

Red Blood Cell Transfusion

When feasible, the patient's own (autologous) blood can serve to reduce or eliminate the need for allogeneic blood. In preoperative autologous donation, the patients donate their own blood over a period of a few weeks preceding the elective procedure.[187] In acute normovolemic hemodilution, the donation takes place in the operating room before the surgery and reinfusion occurs before the patient leaves the operating room. In cell salvage, the reinfusion occurs during or after the surgery as patients' blood is collected and reinfused.

Preoperative autologous donation has some limitations.[174] As with allogeneic blood units, autologous blood is susceptible to acquired storage lesions, such as depletion of 2,3-diphosphoglycerate and impaired ability for erythrocyte to unload oxygen to tissues. Many predonated blood units may be wasted rather than transfused. Additionally, preoperative autologous donation induces anemia, with an attendant increased subsequent likelihood of blood transfusion with the associated risks, including errors in blood administration. Thus, preoperative autologous donation is generally not cost-effective but may be of value in selected patient populations, such as in patients previously alloimmunized with erythrocyte antibodies.[174]

Acute normovolemic hemodilution causes blood losses during surgery to be diluted, thus reducing actual total blood losses. Acute normovolemic hemodilution offers several advantages compared with preoperative autologous donation, including avoidance of blood storage lesions, no risk of blood labeling or patient identification errors, and adaptability for patients undergoing nonelective procedures.[188] However, evidence on efficacy of acute normovolemic hemodilution is mixed.[189–191] Reasons for low acceptance include lack of standardized protocols, variations in the target hemoglobin, types of fluids used, heterogeneity in surgical blood losses by procedure, and patient selection criteria. Acute normovolemic hemodilution is most effective in procedures with large blood loss.[192]

Autologous blood cell salvage and reinfusion has been demonstrated to be safe and effective in reducing allogeneic blood transfusions in a variety of patient populations.[193,194] The procedure requires the collected shed blood to be washed resulting in loss of platelets and plasma, which could potentially cause dilutional coagulopathy or thrombocytopenia with large blood volumes processed. However, reinfusion of the yielded autologous blood is still preferred rather than allowing the shed blood to be completely wasted, or using allogeneic blood which is also devoid

of platelets and plasma and carries the same if not more risks at large volumes. The American Association of Blood Banks (AABB) has Standards for Perioperative Autologous Blood Collection and Administration[195] to provide guidance on quality management for facilities who seek accreditation for these activities.

Indications for Blood Transfusion

Guidelines for blood transfusion attest to the inadequacy of discrete hemoglobin levels as "triggers" for transfusion, and in addition to recommending transfusion of one blood unit each treatment event, they also acknowledge the necessity of considering other more physiologic criteria.[196] It is generally agreed that transfusion is not of benefit when hemoglobin levels are >10 g/dL and are beneficial when hemoglobin levels are <6 g/dL.[12,13] The variability in transfusion outcomes in patients undergoing cardiothoracic surgery continues to persist even after adjusting for patient- and institution-related factors.[197,198] Moreover, prospective randomized trials in patients undergoing cardiac[14] and noncardiac[15,199] surgery have each demonstrated that such patients can tolerate perioperative anemia without transfusion to hemoglobin levels between 7 and 8 g/dL and have equivalent clinical outcomes comparable with transfusions to hemoglobin levels >10 g/dL.

A Cochrane meta-analysis of prospective randomized trials[200] comparing "high" versus "low" hemoglobin thresholds on more than 3,700 patients found that "low" hemoglobin thresholds were well tolerated; RBC transfusions were reduced (~37%) significantly in patients randomized to the "low" hemoglobin cohorts; infections were reduced by 34% in patients in the "low" hemoglobin cohorts; and a hemoglobin of 7 g/dL was sufficient for the majority of patients. More recently, a randomized controlled trial of 2,016 elderly patients with history or risk factors of cardiovascular disease who underwent hip surgery demonstrated that mortality rates, inability to walk independently, and in-hospital morbidity rates were similar in liberal- versus restrictive-transfused patients, despite significant fewer transfusions in the restrictive group.[199]

Patient blood management strategies for patients undergoing cardiac surgery have been shown to be safe and effective in reducing transfusion while at the same time delivering high-quality outcomes. One institution[201] reported that only 11% of patients undergoing cardiac operations received blood transfusions, in which the program ranked first in their state for lowest risk-adjusted mortality. Current initiatives in research for blood transfusions are reflected in the growing literature on adverse effects of blood storage and their possible implications for oxygen delivery by blood transfusion.[202]

Plasma Transfusion

In an evidence-based review, the Transfusion Practices Committee of the AABB recommended plasma therapy for only a few clinical indications, based on the available evidence in the literature (which was assessed to be of "weak quality"): trauma patients with substantial hemorrhage, patients undergoing complex cardiovascular surgery, and patients with intracranial hemorrhage requiring emergency reversal of warfarin-associated coagulopathy.[203] Patients with mild prolongations of the INR (<1.7) are not at risk of bleeding and do not need plasma therapy for minor procedures,[204] so that for the majority of clinical settings there is ample evidence that plasma transfusions are inappropriate. However, logistical/technical barriers that prevent effective and timely plasma therapy (possibly resulting in plasma therapies that are "too little, too late") have probably contributed to the paucity of evidence demonstrating any benefit for plasma therapy.[176,205]

The paucity of evidence for benefit of plasma transfusion therapy has been accompanied by growing evidence that risks of plasma have been underrecognized; in a prospective study, 6% of transfused patients developed transfusion-associated cardiac overload,[206] which is much higher than previously reported rates in retrospective studies.[207,208] Transfusion-related acute lung injury[209] is a significant cause of morbidity/mortality from blood transfusions, whose incidence has declined subsequently with use of plasma from male donors or female donors who have no history of pregnancy.[210]

PLATELET TRANSFUSION

Guidelines for platelet therapy have been pushlished.[12,13,211] For therapeutic platelet transfusions, algorithms for platelet transfusions based on point-of-care testing (FIGURE 129.1) have demonstrated promise in patients who have platelet-derived bleeding such as in cardiothoracic surgery[177,212] and in trauma.[213] As for the evidence-based literature for plasma therapy, additional studies in platelet transfusion are also needed.[214]

Long-Term Consequences of Transfusion

Careful retrospective studies have revealed that transfusions during and after cardiac surgery are associated with reduced life expectancy in proportion to the number of allogenic red cell units transfused, and possibly also in association with storage duration of RBCs.[215,216] A multicenter trial randomizing patients undergoing cardiothoracic surgery to transfusions with "younger" versus "older" RBC is currently underway to address this issue.[217] Additional studies indicate that reoperation for bleeding and red cell transfusion independently increase morbidity after cardiac surgery.[148,218–221]

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CHAPTER

130 Thrombohemorrhagic Events in the Elderly: Special Considerations

ISABELLE MAHE AND LUDOVIC DROUET

Our society is gradually aging, and in view of the increased incidence of thrombotic conditions associated with the elderly, knowledge of anticoagulation drugs and how to use them in older patients has become essential. The treatment of elderly patients is complex, since older patients are at increased risk for both thrombosis and bleeding.

AGE: A RISK FACTOR FOR THROMBOSIS

It has been widely demonstrated that the incidence of thromboembolic diseases increases sharply after the age of 65[1–4]: venous thromboembolism (VTE), including deep venous thrombosis (DVT) and pulmonary embolism (PE), but also atrial fibrillation (AF) and associated risk of cardioembolic stroke. Elderly patients are therefore the main target population for anticoagulation therapy. In the general population, VTE disease has an incidence of 1 to 2 per 1,000 individuals per year, but this incidence is almost 1% per annum in patients aged over 70[1,2] (FIGURE 130.1). Overall 70% of patients with VTE are over 60 years of age.[3] Age is also an independent risk factor for death in patients with PE.[4] The mortality due to PE during hospital stay is 21% in patients older than 65 years and may be as low as 2% in patients younger than 40 years.

DVT is a common age-related condition in medical inpatients: in a prospective study, the prevalence of asymptomatic DVT within 48 hours after admission was 17.8% in patients over 80 years and 0% in patients under 55 years.[5] While the reasons for this high incidence of VTE have not yet been fully elucidated,[6] it has been suggested that the causes are multiple, a combination of (a) malignancy (attributable risk of malignancy is close to 35% in this subset, compared to 20% in the general population with VTE), (b) acquired prothrombotic conditions that are much more common in the elderly (population attributable risk for comorbidities of 25%), and (c) the ageing vascular system with the prothrombotic/antithrombotic systems tilting toward the former in the elderly. The benefit-to-risk ratio of antithrombotic therapy may be difficult to assess in the elderly since they are at the highest risk of severe VTE but also of bleeding events while on anticoagulants. That was observed in the RIETE registry comparing patients older and those under 80 years treated for VTE events: 50% of all patients with fatal PE and 36% of those with fatal bleeding were >80 years old.[7] In those patients, the 3.7% incidence of fatal PE outweighed the 0.8% of fatal bleeding, supporting the favorable benefit-to-risk ratio of anticoagulants in elderly patients with VTE. Haspel et al.[8] tried to model the expected effect of vitamin K antagonist (VKA) treatment in patients older than 80 as compared with patients 60 years of age, taking into account age-appropriate thromboembolic recurrence rates after PE, major bleeding risks of warfarin use, and the contribution of falls to major bleeding episodes in anticoagulated elderly patients. Extended anticoagulant therapy for idiopathic PE is still favorable in elderly patients who tolerate the initial 6 to 12 months of therapy without bleeding complications, irrespective of fall risk.

AF affects 1% to 2% of the population. The prevalence rises dramatically with age, reaching 5% to 15% at the age of 80[9,10] (FIGURE 130.2). The lifetime risk of developing AF is 25% in patients who have reached the age of 40.[11] In addition, aging is an independent major risk factor for ischemic embolic stroke; patients aged 75 years or older have a significant thromboembolic and therefore stroke risk, even if they have no other embolic risk factors.[12,13,14] Patients over 75 years of age have an individual yearly risk of thromboembolism of 4%.[14] In the recent recommendations of the European Society of Cardiology, age was considered a major risk factor for embolic events, as was a history of cardioembolic events, accounting for 2 points in the calculation of the CHA_2DS_2-VASc score. In addition, age over 75 is associated with a worse prognosis for stroke and mortality than hypertension or diabetes.

With increasing age, the efficacy of VKA for the prevention of arterial embolic events in AF patients does not decrease whilst antiplatelet therapies lose their efficacy, becoming negligible by the age of 77.[15,16] In view of the higher incidence of cardioembolism in elderly patients, VKA treatment is at the present time, almost the only effective prophylactic agent in this population.[17,18] As the population ages, the number of elderly patients who require warfarin therapy is rising steadily, owing to an increase in the prevalence of AF and VTE, the main indications for anticoagulant therapy.

AGE: A RISK FACTOR FOR BLEEDING

Anticoagulants and Risk of Bleeding in Elderly Patients

A large number of studies performed with various anticoagulants (e.g., unfractionated heparin [UFH] and low molecular weight heparins [LMWHs], or VKA) have concluded that the risk of bleeding is higher in elderly patients.[19,20] Intracerebral bleeding is the most serious and feared complication of VKA treatment. Aging is a major independent risk factor for intracerebral hemorrhage, especially in patients aged over 85: the relative risk for intracranial bleeding in patients over 75 is 3.7 as compared to patients aged <75. The relative risk for major bleeding is 4.5 in patients over 80 versus those younger than 80.[21–25]

Aspirin is often intuitively preferred to warfarin in elderly patients because of the risk and the fear of bleeding. There are several arguments against this strategy: first, VKA is the only

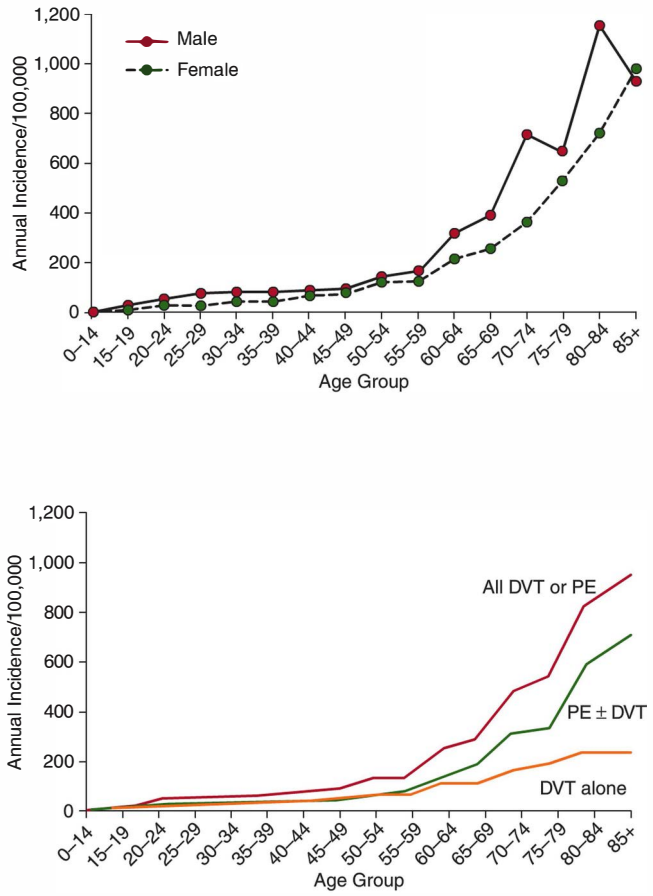

FIGURE 130.1 The increasing probability of developing VTE with age. (From Heit JA, Silverstein MD, Mohr DN, et al. The epidemiology of venous thromboembolism in the community. *Thromb Haemost* 2001;86:452–463.)

effective drug in elderly patients, and second, the risk of major bleeding with aspirin in elderly patients approaches the risk associated with warfarin, as shown in the Birmingham Atrial Fibrillation Treatment of the Aged study (2.0% per year in both groups).[25] One of the main advantages of therapeutic doses of LMWH over UFH is the lower risk of major bleeding events (1.2% vs. 2%; odds ratio [OR] 0.57; 95% confidence interval [CI] 0.39 to 0.83).[26] Elderly patients, however, are frequently renally impaired. A French Drug Agency survey showed that independent risk factors for hemorrhage in patients treated with LMWH are: age over 75, renal impairment, and prolonged duration of treatment.[27] Conversely, in the Innohep in Renal Insufficiency Study (IRIS)[28] comparing UFH and the LMWH, tinzaparin at therapeutic doses followed by VKA treatment in patients older than 70 years presenting with DVT (mean age 82.6 years), found no difference in the risk of clinically relevant bleeding at 3 months (11.9% in both arms, $P = 0.97$). In this trial, 25% of the population had severe renal impairment.[28]

The risk of major bleeding with new anticoagulants is also higher in elderly patients. Dabigatran is the first drug demonstrating a significant reduction of intracranial—including intracerebral—bleedings in the whole population with AF and also in elderly patients. However, this reduction in the risk of intracranial bleeding is counterbalanced by an increase in major gastrointestinal bleedings, the mechanism for which remains unknown.[29,30]

Risk Factors for Bleeding in Elderly Patients

The general risk factors for bleeding in association with anticoagulant therapy are described in Chapter 107. This chapter deals with the aspects specifically related to the elderly. The risk of bleeding depends on both patient- and treatment-related factors (Table 130.1). The incidence of major bleeding events also depends on the duration of anticoagulant treatment; it is

FIGURE 130.2 The increasing probability of developing AF with age. (From Go AS, Hylek EM, Phillips KA, et al. Prevalence of diagnosed AF in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) study. *JAMA* 2001;285:2370–2375.)

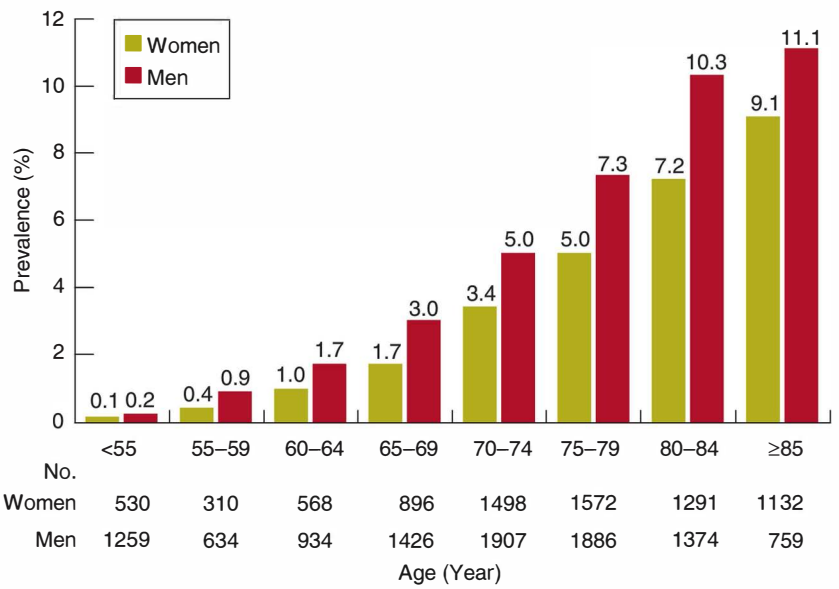

Table 130.1

Risk factors for bleeding in elderly patients receiving VKAs

Patient-Related Risk Factors	Treatment-Related Risk Factors
Age > 75 y	Intensity of anticoagulation (× 1,4 per INR-point)
Recent major bleeding	High INR fluctuation
Stroke	Duration of treatment
Uncontrolled high blood pressure	Beginning of treatment (+++ 3 first months)
Renal impairment	Concomitant treatments (antiplatelet agents)
Hepatic impairment	
Cancer	
Anemia	
Diabetes	

INR, international normalized ratio.

highest at the beginning of treatment, drops to 3% during the 1st month of anticoagulation, to 0.8% per month during the 1st year, and 0.3% per month thereafter.[24] In a recent inception cohort study, Hylek et al.[31] reported a reliable high risk (7.2%) of major bleeding during the 1st year following VKA initiation in patients ≥65 years of age with AF, with 4.7% for those <80 years, and 13.1% for those ≥80 years.

Factors Related to Treatment

VKA Treatment

The risk of major bleeding increases exponentially with international normalized ratio (INR) over 4 compared to an acceptably low incidence at INR between 2 and 3; it should be noted that the risk of intracranial bleeding is not significantly lower at treatment intensities <2.0.[22] Excessive dosing of VKA is common in geriatric patients: two observational studies reported an incidence of 25% among patients (aged over 80) with at least one INR result higher than five while it was <15% in patients <60 years, $P = 0.003$.[32,33]

INR Variability

Extreme INR values (both very low and very high) are more common in the elderly than in young patients, resulting in frequent measurements and dose adjustments.[32] The elderly are more prone to instability due to an increasing number of comorbidities as well as of drugs that interact with warfarin. There is a 21% fall over a 15-year period in the dose of VKA needed in the elderly to reach the same INR target.[34] This increase in sensitivity with age appears to be independent of other demographic factors.[35] Consequently, elderly patients are more sensitive to even very moderate dose adjustments. Low-dosage-VKA formulations (warfarin) are therefore recommended.[36]

Patient-related Factors

Comorbidities

Elderly patients have a higher risk of bleeding owing to an accumulation of comorbidities and polypharmacy. Acute as well as chronic comorbidities and acute concomitant diseases tend to have an impact on the sensitivity to VKA. Diseases that affect the sensitivity to VKA and that are common in elderly patients are listed in Table 130.1.[37,38] Hepatic function is also altered in the elderly and any fluctuation affects the synthesis of coagulation factors, VKA metabolism, and the anticoagulant effect. Another major point, often missed in elderly patients, is the nutritional status.[38–40] Dietary changes have long been recognized as a factor for instability of anticoagulation. In the elderly, dietary vitamin K intake is most often low, which contributes to their increased sensitivity to VKA.[39] In a 6-month randomized placebo-controlled study, a daily vitamin K supplementation in patients with unstable anticoagulation resulted in a significant improvement in anticoagulation control versus the previous 6-month period and to a greater extent than in the placebo group.[40]

Another crucial modulator is hypothyroidism, which is observed in approximately 15% of elderly individuals.[41] Fluctuations in thyroid function result in instability of anticoagulation. Increasing thyroid activity (hyperactivity of the thyroid gland or from exogenous thyroid hormones) is associated with a hypercoagulable state.[42,43] Hyperthyroidism may also increase the sensitivity to VKA, perhaps by boosting catabolism of vitamin K–dependent factors.[39] Patients with suspected thyroid dysfunction should be carefully monitored when receiving VKA and must be made aware that any change in their thyroid function may have a knock-on effect on their anticoagulant response.

Compliance with Treatment

In elderly patients, compliance with treatment is often difficult to obtain as a result of progressive cognitive impairment. Waterman demonstrated that age over 80 is predictive for out-of-range INR, resulting from insufficient compliance.[44] In practical terms, these patients should be given their pill by a caregiver.

Leukoaraiosis and Microbleeds

A recently recognized risk factor for intracerebral bleeding in elderly patients is leukoaraiosis,[45] which is defined as a diffuse, confluent white matter abnormality with low density on computed tomography (CT), and hyperintensity on T2-weighted or FLAIR magnetic resonance imaging. No consensus on the precise mechanisms linking the pathology of the vessel walls with tissue injury has been reached; ischemia, blood–brain barrier dysfunction, and endothelial dysfunction have all been suggested as causative mechanisms. The Stroke Prevention In Reversible Ischemia Trial, in which patients with cerebral ischemia were randomized to warfarin (INR 3 to 4.5) or aspirin,[46] was stopped before its scheduled end because of an excess risk of intracerebral bleeding. Leukoaraiosis was an independent risk factor for bleeding in this study (OR 9.2). Furthermore, in patients with a history of ischemic stroke, leukoaraiosis is a major and independent risk factor for subsequent warfarin-associated intracranial hemorrhage with an OR of 12.9 (95% CI, 2.8 to 59.8), as observed in a case–control study.[47] More recently, leukoaraiosis was reported to be associated with poor prognosis

after spontaneous hemorrhage.[48] In a review of the literature, O'Sullivan[45] found support for a link between gait disturbance, falls, and leukoaraiosis, which is associated with an increase in the risk of fractures and hospitalization.

In a recent systematic review on cerebral microbleeds and the risk of cerebral hemorrhage, users of antithrombotic drugs were compared to nonusers.[49] Patients with microbleeds at baseline and who were treated with antithrombotic agents had an increased risk of subsequent intracerebral hemorrhage (OR 12.1, 95% CI 3.4 to 42.5), suggesting that microbleeds may play a role in the risk of warfarin-related intracerebral bleeding.[49]

Drug Interactions

Elderly patients frequently take multiple concomitant medications. Polypharmacy is an independent risk factor for bleeding: Kagansky et al.[50] reported an OR of 6.14 (95% CI, 1.2 to 42.4) for major bleeding in elderly patients receiving more than seven medicines versus those receiving seven or less. Nonsteroid anti-inflammatory drugs (NSAIDs) and especially aspirin increase the risk of bleeding when associated with anticoagulant drugs. Many mechanisms are involved, including long duration of action, platelet function inhibition, gastric erosion and ulcerations causing gastrointestinal bleeds.[19]

Susceptibility to Falls

A susceptibility to falls is often the direct reason for not prescribing VKA to elderly patients. Falls are very common in this population, causing morbidity and mortality.[51] Dizziness and disequilibria occur frequently and are usually the cause of falls in the elderly.[52] The associated risk of bleeding is most often overstated.[53] The real risk of intracranial bleeding induced by falling is so low that a patient would have to fall 300 times per year before the risk associated with VKA outweighs the benefit of stroke prevention. This type of fall must be distinguished from syncope, which causes sudden falls without any prodromal symptoms, usually related to cardiac conduction disorders.

Renal Impairment

Renal function decreases gradually with age. A substantial proportion of elderly patients (>75 years) have moderate or severe renal impairment.[54] With age-related decline in renal function presents the issue of accumulation of renally eliminated antithrombotic drugs. Nevertheless, renal impairment on its own is a demonstrated risk factor for bleeding during anticoagulant treatment, affecting anyone of UFH, LMWH, and VKA[19,20,55,56] and even the new oral anticoagulants. It must, however, be kept in mind that this condition not only increases the risk of bleeding but also the risk for thromboembolic events and death.[57–59,60]

The optimal method of assessment of renal function in the elderly is a matter of debate. In routine setting, it is only possible to explore renal function and glomerular filtration rate (GFR). Serum creatinine is not a good indicator since total creatinine decreases with age as the muscle mass decreases. Two different methods are used to calculate GFR as a function of patient characteristics (age, sex, and serum creatinine): the Cockcroft-Gault (CG) formula and the modification of diet in renal disease (MDRD) equation. MDRD offers advantages over CG (no need for weight measurements) but the MDRD equation has never been validated in patients older than 70.[61] In addition, MDRD may underestimate creatinine clearance in elderly patients,[62,63] and clinical trials therefore use the CG equation.

Table 130.2

HAS-BLED bleeding risk score

Letter	Clinical Characteristic	Points Awarded
H	Hypertension	1
A	Abnormal renal and liver function (1 point each)	1–2
S	Stroke	1
B	Bleeding	1
L	Labile INRs	1
E	Elderly age (e.g., age > 65 y)	1
D	Drugs or alcohol (1 point each)	1 or 2

INR, international normalized ratio.
From European Society of Cardiology, www.escardio.org.

Bleeding Risk Scores

Many bleeding risk scores have been established that stratify risk by age at 60 or 65 years, but none has been fully validated in the elderly since mean age of AF patients is 75 years.[64,65] The European Society of Cardiology recently proposed the HAS-BLED bleeding risk score (Table 130.2).[14] This was developed subsequent to the Euro Hart Survey, which included 3,978 European patients with AF[66] (see also Chapter 107).

ANTICOAGULANTS IN ELDERLY PATIENTS: EFFECTIVE BUT POORLY DOCUMENTED

VKA Underuse

Despite evidence that warfarin is highly effective in preventing thromboembolism in patients with AF, it remains underused in elderly patients.[67–69] In the FALSTAF observational study performed in France from August to December 2002 in 5,893 patients with AF (FIGURE 130.3), there was a sharp drop in prescription of VKA with age from 83.9% in patients <60 years to 63.5% in patients >80 years.[67]

As a pharmacologic class, VKAs are associated with one of the highest rates of adverse events in the elderly.[70] This contributes to the fear of bleeding events and to the underuse of VKA in this population and denies elderly patients access to effective stroke prophylaxis.[71] In a survey of 142 doctors practicing internal medicine, the individual assessment of the risk of cerebral hemorrhage was 10-fold the risk observed in clinical trials for the corresponding patient.[71] In addition, the thromboembolic risk is not systematically assessed, which influences the choice of antithrombotic drug. In most studies on patients "suitable" for anticoagulant treatment or "unsuitable" for that and therefore given antiplatelet agents, for example, the ACTIVE W and ACTIVE A trials,[72,73] the $CHADS_2$ score for thromboembolic risk is the same in both groups depending on the decision of the clinician favoring anticoagulant or antiplatelet therapy. Even in the most recent AVERROES trial (apixaban vs. placebo), the $CHADS_2$ score of patients who were considered to be

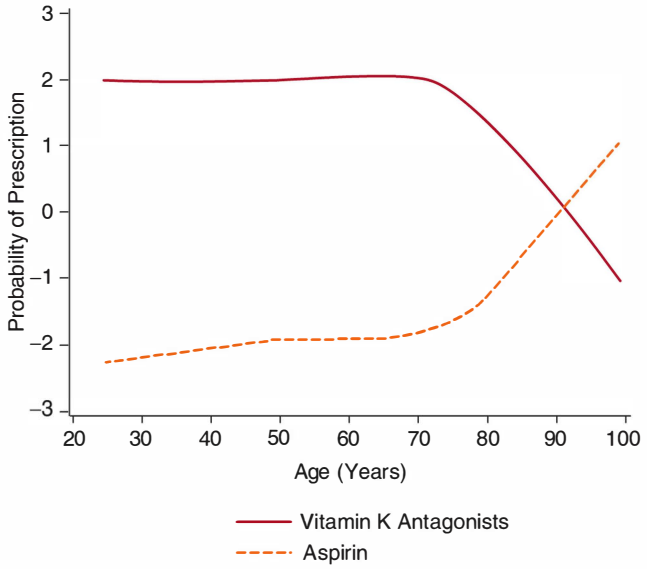

FIGURE 130.3 FALSTAF study: probability of VKA and aspirin use in patients with AF.

"unsuitable candidates" for anticoagulant therapy was similar to that of parallel studies with anticoagulant agents.[74]

Overall, there are several reasons for this under use of anticoagulant agents: first, there is the fear of bleeding based on an overestimation of the risk of bleeding. Second, thromboembolic risk is not systematically assessed in elderly patients. Third, there is some skepticism as to whether the results from clinical trials (high efficacy and acceptable risk of bleeding in elderly patients) can be extrapolated to a routine clinical setting.[71] Fourth, no single but combined geriatric characteristics contribute to the underuse of VKA in older patients with AF.[75] In addition, there is the doctor's belief that aspirin is a safe alternative to warfarin for stroke prevention in elderly frail patients.[76] The recent AVERROES study has clearly demonstrated that the hemorrhagic risk can be similar in aspirin-treated and anticoagulated elderly patients.[73] Current European guidelines plainly state that VKA is the only effective treatment for stroke prevention in the majority of AF patients, with the benefit outweighing the risk of bleeding.[14] Furthermore, aspirin is a far from safe alternative in elderly frail patients.

Clinical Trials and Real Life—a Gap in Knowledge Translation

To date, patients with high risk of bleeding have often been excluded from participation in specific clinical trials assessing anticoagulant drugs.[28,37,38,77] Hansen observed in a registry study that the risk of fatal and nonfatal bleeds was higher (3.9% per patient-year) than the usual rate reported in clinical trials.[78] Many explanations can be put forward: first, patients are included in clinical trials after a rigorous selection process minimizing the risk for bleeding in the evaluation of new anticoagulants; second, many of those patients were previously on warfarin (selection by attrition).

Observational studies like the RIETE Registry provide more realistic data on patients with VTE in terms of risk and management.[7] Of the 13,000 patients, 22% were 80 years or older. In this subset compared to the younger patients, both the incidence of major bleeding (3.4% vs. 2.1%, OR 1.7, 95% CI 1.3 to 2.1) and the risk of fatal PE (3.7% vs. 1.1%, OR 3.6, 95% CI 2.7 to 4.7) were higher. Nonetheless, the benefit-to-risk favored anticoagulant treatment in these patients. Vasco more specifically studied nonagenarians in the RIETE Registry, in which 2.8% of 21,873 patients fulfilled this criterion.[79] The most frequent thromboembolic risk factor was recent immobilization (for 4 days or more) (39%). However, only 22% of these patients had previously received thromboprophylaxis, which is an indicator of its underuse in the elderly.

PRESCRIBING ANTICOAGULANT TREATMENTS IN ELDERLY PATIENTS

Low Molecular Weight Heparin

Each specific LMWH[80,81] has its unique spectrum of anti-IIa and anti-Xa activity. This variability is due to the differences in glycosaminoglycan chains (length, composition radicals, etc.), which influences both the anti-Xa/anti-IIa ratios and the differences in the mode of elimination. These chains change place with chains in the endothelial glycocalix. The higher molecular weight fractions are exchanged with the endothelial glycanic cell coat and are cleared by the reticuloendothelial system whereas the lower molecular weight chains are mostly cleared renally.[80–82] This explains kinetic differences between LMWHs such as tinzaparin (mean MW 6.5 kDa) and enoxaparin (mean MW 4.5 kDa), but also explains that LMWHs cannot have true bioequivalent counterparts. Plasma–anti-Xa activity is the usual method for assessing LMWH activity; it is sampled when at its peak concentration (~4 hours postinjection—based on studies in younger adults) but only explores the circulation of glycanic chains containing the pentasaccharide sequence that is merely one of the characteristics and modes of action of LMWHs. Although anti-Xa levels do not correlate with bleeding risk in clinical trials, very high anti-Xa–plasma levels may indicate an increased risk of hemorrhage.[59,83] Furthermore, dose-finding studies with LMWHs at therapeutic doses have demonstrated that a further increase of the dose is associated with higher anti-Xa levels and an increased risk of major bleeding.[81] In studies demonstrating the efficacy of a thromboprophylactic drug to reduce the risk of VTE, subgroup analyses consistently reached the conclusion that the elderly patients constituted the subgroup, which gained the most benefit of prevention with enoxaparin, dalteparin, or fondaparinux without a significant increase of the risk of bleeding.[84–87] In order to determine whether prophylactic doses of LMWH accumulated in elderly patients, we measured anti-Xa at peak level (Cmax) in patients who were hospitalized in internal medicine wards. We observed that more than 50% of patients had an anti-Xa activity of more than 0.5 IU/mL, only 48 hours after the initial dose.[88] Subsequently, we recruited patients >75 years of age, and found that those at the highest risk of rising anti-Xa levels were patients with severe renal impairment and low body weight.[89] Finally, we prospectively compared the pharmacokinetic and pharmacodynamic properties of two different LMWHs in patients aged 75 years and more, by measuring anti-Xa levels at baseline and after 8 days of prophylactic doses. The primary endpoint was the accumulation factor (ratio of anti-Xa Cmax on day 8 to anti-Xa Cmax on day 1), which

increased in patients receiving enoxaparin (1.22; $P < 0.001$) while it tended to remain stable in patients receiving tinzaparin (1.05; $P = 0.29$).[90] This confirms that LMWHs are not identical and vary in how they are metabolized in elderly patients. Nevertheless, the risk of bleeding in patients with severe renal impairment who are given thromboprophylactic doses is low,[91] the clinical relevance of the different anti-Xa activities observed is unknown, and neither dose adjustment nor monitoring is required for thromboprophylactic dosing in elderly patients.[92]

Although laboratory monitoring of LMWH is not routinely required, experts and guidelines have recommended this for selected patients receiving LMWH at treatment doses.[81,93] Anti-Xa measurement is suggested as a means of detecting bioaccumulation after administration of multiple doses and to avoid overdosing in the patients at highest risk, that is the elderly, particularly those with low body weight. If such monitoring is warranted, the chromogenic anti-Xa activity assay is the only test recommended.[94] Anti-Xa activity has never been shown to correlate with efficacy. Use of anti-Xa activity monitoring has not been studied in large trials. Since LMWHs are mainly excreted renally, repeated administration of LMWHs to patients with a reduced creatinine clearance may result in accumulation of the drug with an increased risk of bleeding. A meta-analysis of 12 studies and 4,971 patients demonstrated that patients with creatinine clearance <30 mL/min receiving curative doses of LMWH had elevated anti-Xa levels and an increased risk of major bleeding compared to those with higher clearance (5.0% vs. 2.4%, OR for major bleeding 2.25, 95% CI 1.19 to 4.27).[56]

Differences exist among LMWHs used at therapeutic doses in elderly patients. Mismetti et al.[95] showed a significant correlation between anti-Xa and creatinine clearance in elderly patients receiving nadroparin (180 IU/kg/day) for 9 days. Three studies, two prospective observational and one randomized, in elderly patients receiving tinzaparin at therapeutic doses suggested that this LMWH is less prone to cause accumulation in the event of renal impairment. In the first of these, Siguret et al.[96] did not detect any change in mean anti-Xa levels over the period from day 2 to day 10 in 30 subjects (mean age 87 years) receiving tinzaparin once a day (175 IU/kg) for acute VTE. Pautas followed 200 patients with a mean age of 85 years receiving tinzaparin once daily for up to 30 days. In both studies, there was no correlation between anti-Xa levels and creatinine clearance or age.[33,36] The mean creatinine clearance (CG calculation) in these studies was 41 and 51 mL/min, respectively.[33] The prospective randomized study compared the safety of UFH versus tinzaparin both followed by VKA in elderly patients with VTE and renal impairment (IRIS study).[28] The study was prematurely stopped because of a difference in mortality favoring the UFH group (11.5% vs. 6.3%, $P = 0.034$) at the predefined interim analysis after 541 patients had been enrolled. The difference in mortality could not be explained by deaths due to bleeding or VTE. There was importantly no significant difference in the risk of major bleeding at day 10 between the treatment groups, reflecting the risk related to the heparin component of the treatment. The translation of these differences between LMWHs into clinical practice is difficult to assess since severe renal impairment is an exclusion criteria for clinical trials and the elderly patients are also often excluded.

There are four possible strategies/options for the treatment of elderly patients with severe renal impairment: (a) use of UFH, as recommended by American College of Chest Physicians but the risk of bleeding is not clearly lower than with LMWHs[81]; (b) half dose LMWH, which is an empirical reduction in the dose to minimize the risk of bleeding. Few studies performed evaluating this option have shown that it is effective and relatively safe (grade 2C).[81] This suggestion has a low grade of evidence. A dose reduction by 50% in patients with severe renal impairment may underdose enoxaparin and lead to loss of efficacy; (c) anti-Xa monitoring with adjusted dose of LMWH to target the peak anti-Xa levels 4 hours after SC injection at 0.6 to 1.0 IU/mL for twice-daily administration and 1.0 to 2.0 IU/mL for once-daily administration;[81] and (d) possibly selection of a LMWH that is less likely to accumulate in case of renal impairment. Although UFH is the recommended anticoagulant treatment for patients with severe renal failure, there is a rationale for the use of an adjusted treatment with LMWH.

These alternative strategies are open to criticism: there are no data in the literature supporting a dose reduction in elderly patients treated for VTE, with all existing data derived from studies on acute coronary syndrome (ACS) setting. The studies performed with enoxaparin in ACS[97] used the dose adjustments to maintain the peak anti-Xa activity between 0.5 and 1.0 IU/mL. The resulting lower dose was not associated with an increased rate of major bleeding compared to UFH but the rate of MI and death may be higher, particularly in case of anti-Xa activity <0.5 IU/mL.[98,99] No prospective clinical trial supports adjusting the LMWH dose on the basis of anti-Xa activity. In pivotal clinical trials, the dose is adjusted to body weight, without dependence on monitoring of anti-Xa activity. Current reference on anti-Xa activity for treatment with LMWH has been extrapolated from anti-Xa activities observed in patients enrolled in clinical trials using body weight–adjusted doses.

As a general rule, it is recommended that each LMWH should be administered at the dose and for the indication used during studies. In routine practice, the safety of LMWH can be improved by measuring body weight and calculating the creatinine clearance to assess the risk of bleeding and to avoid drugs that increase this risk.

Fondaparinux

Fondaparinux has specific anti-Xa activity without any inhibition of factor IIa. The clearance is exclusively renal. There is so far no study on monitoring of anti-Xa activity in patients receiving fondaparinux. To date, the absolute contraindication is severe renal impairment. There is a rationale for reducing the dose in renally impaired patients.[100] The PROPICE study was a prospective observational study designed to establish that the thromboembolic and bleeding risk in patients with creatinine clearance is 20 to 50 mL/min and they required thromboprophylaxis in routine clinical practice. The recommended dosage in those patients is 1.5 mg compared to the standard dose of 2.5 mg.[101] The study population consisted of elderly patients (mean age 81.4 ± 6.6, 86% were older than 75 years) with renal impairment (mean creatinine clearance 39 ± 8 mL/min, 16% <30, 33% between 30 and 40, 49% between 40 and 50 mL/min).

In this high-risk population, the 1.5 mg dosage reduced the risk of bleeding without increasing the risk of thromboembolism (distal DVT 0.5% at day 10 and 0.7% at 1 month). The risk of major bleeding was 4.5% at day 10 and 5.2% after 1 month.

Vitamin K Antagonist

Warfarin is the most widely prescribed oral anticoagulant for the primary and secondary prevention of venous or arterial thromboembolism. Elderly patients require lower anticoagulant doses to achieve the therapeutic range, although the mechanism of this heightened sensitivity to warfarin observed with aging is not well understood.[102,103] In addition, these patients respond more slowly to warfarin dose adjustments.[36,102,104] In a study performed in a geriatric setting (106 patients, mean age 85 years), the mean time required to reach the steady state rose to 9 ± 4.5 days.[36] This delay must be taken into account both when starting VKA treatment and also to avoid premature dose changes while the effect of the previous dose is not completely established. This contributes not only to poor INR control in elderly patients but also to an increased risk of bleeding.

Two studies have evaluated warfarin initiation algorithms adapted for elderly inpatients. In a randomized control trial Gedge et al.[105] compared the established "Fennerty regimen" with a new lower-dose regimen in 120 patients, aged 65 to 75 years. The first dose (if INR < 1.4) was 10 mg, followed by 1 mg (INR 1.8 to 2.0) or 5 mg (INR < 1.8) with further daily adjustments. Another strategy was applied by Siguret et al.[36] in 106 even older inpatients (mean age 85), initiating with 4 mg warfarin daily for 3 days without monitoring and then adjusting according to the INR (Table 130.3). Both algorithms provided results that were advantageous regarding surrogate outcomes such as more time in the therapeutic range and fewer high INR levels but none of them was powered to demonstrate a reduced risk for thromboembolic and bleeding events. Just as the time required to reach a steady state is longer in elderly patients starting VKA treatment (vs. younger patients), when vitamin K is used to reverse VKA, the delay to achieve the maximum effect is longer than the 6 hours expected in younger patients.[106]

Education and Anticoagulation Clinics for Elderly Patients

According to the WHO, anticoagulation training allows patients to achieve and keep capacities and competencies, which will help them live better with their disease. It has been demonstrated in an observational study that insufficient patient education is an independent, strong risk factor for major bleeding events in elderly patients.[50] The specific risks, potentially adjustable with education and supportive tools, of this population are numerous, including multiple intakes of the dose, missed doses, poor nutritional status, and automedication, for example, with salicylates or NSAIDs. In addition, both patient education on VKAs and anticoagulation training have been effective in improving anticoagulation control.[107] However, education should be adapted to this population to take into account limits

Table 130.3

Algorithm for initiation of warfarin in elderly patients (>70 years)

Day	INR Value	Warfarin Dosage
0	Do not measure	4 mg[a]
1	Do not measure	4 mg
2	Do not measure	4 mg
3	<1.30	5 mg
	1.30–1.49	4 mg
	1.50–1.69	3 mg
	1.70–1.89	2 mg
	190–2.49	1 mg
	≥2.50	Measure INR daily and omit doses until INR <2.50, then give 1 mg
6	<1.60	Increase by 1 mg/day
	1.61–2.50	Maintain dosage
	2.51–3.50	
	If warfarin dosage ≥2 mg	Reduce by 1 mg/day
	If warfarin dosage = 1 mg	Maintain dosage
	>3.50	Management of supratherapeutic INR[b]

INRs were determinated in the morning, and warfarin doses were given in the evening. The target INR was 2.50.
[a]This algorithm does not apply to patients who have a pretreatment INR > 1.30.
[b]Supratherapeutic INRs are managed according to expert recommendations.
INR, international normalized ratio.
From Siguret V, Gouin I, Debray M, et al. Initiation of warfarin therapy in elderly medical inpatients: a safe and accurate regimen. *Am J Med* 2005;118:137–142.

of learning (physical and cognitive limits, etc.), low comprehension, and low motivation. The "caregiver" plays a major role, and should be involved in the management of VKA treatment and take part in the patient education.

Specialized anticoagulation clinics have been shown to be effective in reducing the risk of thromboembolic and bleeding events in outpatients.[108] A recent study demonstrated that an anticoagulation clinic is also beneficial for elderly inpatients.[109]

Perspectives on New Oral Anticoagulants

New oral anticoagulants have clear advantages over classic anticoagulants: oral route of administration, no need for monitoring, and a wider therapeutic window. Until now they have not been assessed in patients with severe renal impairment, which was an exclusion criterion in studies with dabigatran and rivaroxaban. The approval by the Food and Drug Administration of dabigatran at a dose of 75 mg twice a day for stroke prophylaxis in AF for elderly patients with severe renal impairment (creatinine clearance 15 to 30 mL/min) raises concerns about the risk of overdosing in this population. Furthermore, this is a lower dosage than the lowest dose tested in the phase III trial.[29]

In conclusion, practitioners are faced with a dilemma: elderly patients are considered to be at high risk of bleeding events. Simultaneously they are at the highest risk of thromboembolic events. Most of the time, the benefit-to-risk ratio is in favor of anticoagulant treatment. Fear of bleeding should not be a limitation to the initiation of anticoagulant treatment and give rise to a situation where patients who could benefit from intervention, are withheld from it. This and the development of new anticoagulant drugs, which would increase the benefits of treatment without increasing the risk of bleeding in the elderly, are the challenges for the future.

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