Laboratory Evaluation of Hemostasis and Thrombosis

THIRD EDITION

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Preface

Since the second edition of this book was published in 1974, I have continued to see many patients with hemostatic problems and have become increasingly interested in patients with thrombotic problems. It is for this reason that the title of the book has been changed to include our experience with the laboratory investigation of the latter. I have continued to teach medical students and residents and medical technologists, and have worked with my coauthor, Reaner Shannon, to establish the Docent Hemostasis Laboratory at the University of Missouri-Kansas City School of Medicine.

My primary laboratory interest continues to be in those studies that can be performed accurately in the clinical laboratory and that are useful in the diagnosis of disease states and the care of patients. We have performed many new procedures and have done comparative studies in several areas to determine which procedures are the most accurate, convenient to perform, and economical. In this book, we have included primarily those tests with which we have personal experience and which we consider useful. It is so easy to initiate tests because they are fashionable and because kits are available that make the procedures seem less difficult. The ordering physician and the laboratory technologist must understand what a test is measuring, the principles of the method used, the accuracy of the method, and the relationship of the test to others that are being performed. The separation of the laboratory from the clinical arena has discouraged this joint responsibility.

New research in the areas of hemostasis and thrombosis has broadened and changed our understanding of hemostatic processes. In Chapter I, we have included much of this new information and have integrated it with older facts that are still accurate. I am sure the future will bring additional changes. Chapter II is greatly increased in length because of the explosion of clinical information about bleeding and clotting disorders. The remaining chapters, which relate to the techniques of testing, include remarks concerning the principles involved, the specific methods to be used, and discussions of how to use and interpret results. Many tests are based on clotting end points, but the availability of synthetic substrates and radioimmunoassay procedures has expanded and significantly changed the methodology of the hemostasis laboratory. We have made a major effort to standardize

procedures and reagents, as well as the style of describing methods. The major equipment in our laboratory has not changed significantly since 1974.

I wish to thank Betty Steinman and the Audio-Visual Department of the University of Missouri-Kansas City School of Medicine for their invaluable help in the development of new illustrations for this edition. Also, for frustrating hours spent in typing this manuscript, I wish to thank my secretary, Gladys Burns. It has been a tremendous help to be able to share the responsibility of this edition with Reaner Shannon with whom I have now worked in the laboratory evaluation of hemostasis and thrombosis for 12 years. Much of the research work in the area of hypercoagulability which we have done in the Docent Hemostasis Laboratory has been supported by the Lettie V. McIlvain Trust.

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Nothing is more frustrating to a medical technologist than attempting to follow a test procedure and finding that all the details and directions are not provided or that the procedure is difficult to comprehend. This has been my experience on several occasions when trying to develop or implement new procedures from those described in the medical literature. Therefore, in helping to write this book, I have placed particular emphasis on procedural details. Every attempt has been made to be as clear, precise, and thorough as possible in describing all procedures in order to make it easier for those who wish to use them. I hope this objective has been accomplished.

I express my gratitude and appreciation to Dr. Marjorie Sirridge for the opportunity to share the authorship of this book and for the knowledge and experience I have gained in the years I have worked with her. I consider myself fortunate, for it is a rare opportunity to be associated with such an investigator and physician.

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CHAPTER 1

Mechanisms of Hemostasis and Thrombosis

Blood is normally fluid; in the body it circulates throughout the vascular system under pressure. The prevention of spontaneous bleeding and the control of traumatic hemorrhage are referred to as hemostasis. Until recently, most research efforts related to hemostatic mechanisms were directed toward determining and studying those abnormalities that result in bleeding problems due to disturbances of hemostasis. It has become apparent, however, that far more important is the study of processes and changes that result in the formation of intravascular thrombi in intact, nontraumatized arteries, veins, and capillaries. This is referred to as thrombosis. Hemostasis is known to be dependent primarily on the following:

1. Normal resistance and contractility of blood vessels and an adequate supportive framework for them.
2. Normal platelet activity, which includes adequate numbers and function.
3. An adequate coagulation system.
4. Stability of the clot.

Vessels, platelets, and the coagulation system are all important in thrombus formation, which is the major mechanism for hemorrhage control. After vessel injury, the exact sequence of events is not always the same, and the following factors have been shown to be of variable importance.

1. Location of the injured vessels and the chance of continuing trauma (i.e. oral cavity, joints).
2. Size of vessels, flow patterns, and blood pressure within them; also the potential for contraction of the vessel wall.
3. Intrinsic abnormalities of the vessels.
4. Abnormalities or damage to surrounding tissues.
5. External pressure on vessels due to edema or hemorrhage into surrounding tissues.
6. Application of external pressure or surgical intervention.

For example, in large arterial vessels in which the blood pressure is high, usually the flow of blood cannot be slowed sufficiently to allow an occluding thrombus to form without the application of a tourniquet, the use of external pressure at the bleeding site, or some type of surgical intervention. Occasionally, enough tissue

damage surrounds the vessel, with accumulation of blood in this tissue, to produce local tamponade of an artery with eventual cessation of bleeding. With repeated trauma, however, bleeding is easily reactivated. With the use of local pressure, hemostasis is more easily accomplished in large veins that have been traumatized than in large arteries; but usually some type of surgical intervention is also required. Local tamponade by accumulated blood in tissues is more effective in producing venous occlusion. In small venules and capillaries, hemostasis may be accomplished by the simple adhesion of endothelial surfaces and the local adhesion and aggregation of platelets with the deposition of stabilizing fibrin strands. In small arteries and arterioles, however, spontaneous control of bleeding requires a complex interaction among the components of the vessel wall, the platelets, and both the intrinsic and extrinsic pathways of coagulation for the formation of occluding thrombi (Figure 1-1).

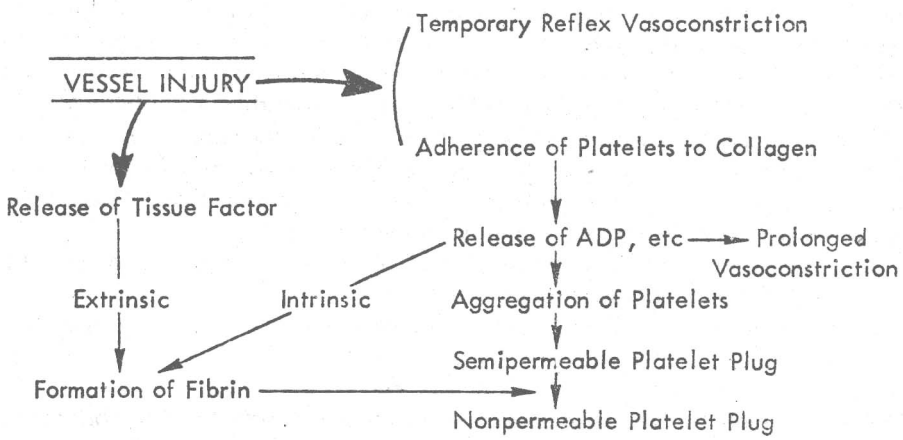

Figure 1-1. Interrelationships in hemostasis in small arteries and arterioles.

Many of the factors involved in hemostasis are also important in the process of thrombosis. In large arterial vessels, there may be gradual narrowing of the lumens by the adherence of platelets and fibrin and the formation of atherosclerotic plaques, which can eventually result in the complete occlusion of these vessels by local thrombus formation. In the intact venous system, particularly in the lower extremities, the spontaneous formation of large venous thrombi is an important clinical problem usually related to altered flow patterns, resulting in stasis. Thrombi form in intact small arteries, veins, and capillaries due to local changes in vessels and to systemic stimuli, which may result in disseminated microvascular clotting.

The process of thrombus formation, which occurs during both hemostasis and thrombosis, usually proceeds in the following way:

1. A change in the endothelium of the vessel.
2. Adherence of platelets to collagen, basement membrane, and subendothelial microfibrils. (Fibronectin is an adhesive glycoprotein in connective tissue, which is required for normal platelet interaction with collagen.)
3. Initiation of the platelet release reaction by collagen and other stimulating substances present at the site (within 3 seconds).

4. Further aggregation of platelets by ADP, thromboxane A_2, and other substances released from platelets with the formation of a reversible platelet plug.
5. Generation of thrombin, probably initially by the extrinsic pathway.
6. Further aggregation of platelets by thrombin, formation of fibrin by thrombin, and stimulation of clotting via the intrinsic pathway.
7. Formation of a stable fibrin-platelet plug (within 1 to 4 minutes).
8. Continued formation of fibrin by both extrinsic and intrinsic pathways.
9. Entrapment of RBCs and more platelets in fibrin strands with thrombus formation.
10. Eventual lysis or organization and recanalization of the thrombus.

As we have come to better understand the relationship of the hemostatic mechanism to thrombotic diseases, it has become important to examine how this process affects the blood vessel walls. During the thrombotic process, collagenase and elastase enzymes become available and alter the surrounding connecting tissue. Activated platelets release a factor that is mitogenic for smooth muscle in the walls of arteries and arterioles, and this may initiate the formation of atherosclerotic plaques. Chemotaxis of leukocytes occurs. When platelets go through the release reaction, vasoconstriction caused by the release of thromboxane A_2 (TxA_2) is initiated. This effect of thromboxane A_2 may be opposed by the release of prostacyclin (prostaglandin I_2, PGI_2) from endothelial cells, since PGI_2 is not only a potent inhibitor of platelet aggregation but also causes vasodilatation. With thrombus formation, plasminogen activator is released from the vessel wall and converts plasminogen in the thrombus to plasmin. If enough plasmin is formed, the clot will be lyzed; if not, it must be organized and recanalized. Most thrombi in the microcirculation are lyzed rapidly because of their small size; but in larger vessels, organization and recanalization are more likely to occur.

BLOOD VESSELS

When blood vessels are normal, blood cells are retained within them except when actual injury occurs; however, defects in structure, permeability, contractility, and resistance may interfere with adequate hemostasis and result in hemorrhagic problems. The recognition of such defects rests primarily on careful clinical observation rather than on laboratory studies. Clinical evaluation is made not only on observation of a bleeding area, but also on inspection of the surrounding tissues and the remainder of the patient's body for evidence of generalized disease that might affect vascular function.

Problems of thrombosis related to blood vessel structure are recognized primarily by invasive techniques such as venography and arteriography which allow visualization of the size, contour, and degree of abnormalities in the deep veins of the lower extremities, the pulmonary circulation, the aorta, the coronary, cerebral and renal vessels, and others. Noninvasive techniques such as impedance phlethysmography, Doppler studies, and radionuclide scanning have also been useful in the study of arterial and venous thromboembolic processes. With the advent of successful reconstructive surgery for some vascular abnormalities and the widespread use of anti-

coagulant and fibrinolytic drugs, it has become important to determine the presence and extent of such abnormalities to plan appropriate therapy.

PLATELETS

Normal hemostasis requires that platelets be present in adequate numbers and that they be capable of fulfilling several important functions. Abnormalities in number and function can result in both hemorrhagic and thrombotic problems. Platelets normally arise from the cytoplasm of megakaryocytes in the bone marrow and are delivered into the bloodstream. The number of available platelets is dependent on the productive capacity of the megakaryocytes in the marrow and their survival in the circulation, which is normally 8 to 12 days. The marrow reserve is not excessive and can be rapidly depleted

Platelets are structurally complex, and they undergo changes in shape when they come in contact with various stimuli. Such changes render them adhesive to exposed collagen at sites of vascular injury, a process that requires the presence of a plasma protein called Willebrand factor. This factor is part of the factor VIII molecule. Normal stimulated platelets then aggregate and release several substances (among them, adenosine 5'diphosphate; ADP) which are active in inducing further aggregation. These rapid early events are reversible, but with the continuing release of ADP and other substances, and the associated formation of fibrin, more aggregation takes place and the platelet aggregates become stabilized. During these processes, phospholipid compounds (referred to as platelet factor 3) are made available on the surface of platelets. These substances become integral components of several steps in the intrinsic pathway of coagulation. Platelets also release factor V which is important in the formation of thrombin.

The release reaction is energy-requiring and is due to the formation of the endoperoxides PGG_2 and PGH_2 from arachidonic acid, which is normally present in platelet membranes. From these endoperoxides, thromboxane A_2 (Tx_2) is formed, and its release with other substances from platelets mediates continued platelet aggregation and induces the contraction of the smooth muscle of the walls surrounding arterioles. Thus, by the adherence and aggregation of platelets at sites of vascular injury and the induction of local vasconstriction, there is a slowing of blood flow which allows the initiation of clot formation. Platelets also contribute to clot retraction through the attachment of their pseudopods to fibrin strands and a reaction between adenosine triphosphate (ATP) and thrombosthenin, the contractile protein contained in their cytoplasm.

COAGULATION SYSTEM

The coagulation system is by far the most complex part of the hemostatic mechanism and involves the interaction of 10 or more different factors from plasma an tissue, as well as the regulation of these reactions by natural inhibitors. Our present concepts of the workings of the system are the result of some simple observations and the sophisticated research studies of many investigators. Some basic facts that are important in understanding coagulation include:

1. In a glass tube, blood clots in about 10 minutes.
2. In a plastic or siliconized tube, blood may take as long as 30 minutes to clot.

3. If powdered glass or other activating substances are added to plastic or siliconized tubes, blood will clot in about 2 minutes.
4. Blood to which tissue extract has been added clots in a few seconds.
5. The clotting time of recalcified plasma is much shorter than that of whole blood and shows a variability that is at least partially dependent on the speed and length of centrifugation. Platelet numbers in plasma are also known to be affected by the manner of centrifugation.
6. The recalcification time of plasma can be reduced by adding a platelet substitute, an activator substance, or tissue extract.

From these and similar observations, the following conclusions can be drawn:

1. Normal blood contains all factors needed to form a clot.
2. Some factor or factors in blood must be activated by a contact, such as with a glass tube, and this appears to be a time-consuming process. Activation is less likely to occur when blood is exposed to plastic or siliconized surfaces.
3. The process of contact activation could not require calcium, since at least partial activation occurs in blood or plasma to which a calcium-binding anticoagulant has been added.
4. Tissue factor contains a substance or substances that bypass the contact-activation process.
5. Since platelets influence the speed of the clotting process to some extent, they must also be involved in the intrinsic coagulation system.

International Nomenclature of Coagulation Factors

An international nomenclature has been established for the coagulation factors. This should be mastered before trying to understand the probable manner in which these factors interact. Most factors are regularly referred to by number, with the exception of fibrinogen (I), prothrombin (II), thromboplastin (III), and calcium (IV), and several other more recently described factors (prekallikrein, high molecular weight kininogen, Passavoy). Table 1-1 designates the factors by number and/or name and includes some of the known facts that are important in studying and understanding their functions.

The coagulation factors may be conveniently divided into three groups: the fibrinogen family, the prothrombin family, and the contact factors (Table 1-2). The fibrinogen family consists of fibrinogen itself and the three cofactor proteins VIII, V, and XIII. All are of large molecular size (MW > 250,000) and, with the exception of factor VIII, are known to be synthesized in the liver. The prothrombin family is made up of the four vitamin K-dependent clotting factors, II, VII, IX, and X. All are made in the liver, are small molecules (MW 55,000 to 70,000), and can be converted into serine protease forms. Factors XII, XI, and prekallikrein are somewhat larger than the prothrombin family proteins (MW 80,000 to 200,000), and these serine protease zymogens become activated on contact. High molecular weight kininogen (HMWK) has a molecular weight of 110,000 and acts as a cofactor for the contact-activation reactions.

Intrinsic and Extrinsic Pathways

The generation of thrombin (factor II_a), which converts fibrinogen to fibrin, represents the central event in the coagulation of blood. This occurs as a result of a

TABLE 1-1. International Nomenclature of Coagulation Factors

Factor Number	Names	Known Facts
I	Fibrinogen	MW 340,000 Synthesized in the liver T½—80–90 hours Heat labile and storage stable Concentration—2500–3500 μg/ml
II	Prothrombin	MW 70,000 (Protease) Synthesized in the liver T½—60–70 hours Heat stable and storage stable Vitamin K-dependent Concentration—100–150 μg/ml
III	Tissue Thromboplastin Tissue factor	High molecular weight lipoprotein Obtained from saline extraction of most body tissues Normally absent from plasma
IV	Calcium	
V	Proaccelerin Labile factor Accelerator globulin	MW 270,000 Synthesized in the liver T½—15–25 hours Heat labile and storage labile Concentration—5-15 μg/ml
VI	Accelerin—this factor is no longer considered in the scheme of hemostasis	
VII	Proconvertin Stable factor Serum prothrombin conversion accelerator (SPCA) Autoprothrombin I	MW 60,000 (Protease) Synthesized in the liver T½—5 hours Heat labile and storage stable Vitamin K-dependent Concentration—0.5 μg/ml
VIII	VIII:C Procoagulant Activity VIIIC:Ag—Immunologic VIII:R Factor VIII Rel.Protein VIIIR:Ag—Immunologic Willebrand factor VIIIR:RCF Ristocetin cofactor Antihemophilic factor (AHF) Antihemophilic factor A Thromboplastinogen Platelet cofactor I	MW VIII:C 285,000 VIII:R 85,000↔3,400,000 Synthesis VIII:C unknown VIII:R endothelial cells T½—8–16 hours Heat stable and storage labile Concentration—15 μg/ml
IX	Plasma thromboplastin component (PTC) Christmas factor Antihemophilic factor B Autoprothrombin II	MW 55,000 (Protease) Synthesized in the liver T½—12–24 hours Heat labile and storage stable Vitamin K-dependent Concentration—3 μg/ml