This chapter will discuss coagulation in the context of a hemostatic response to a break in the vasculature. Coagulation is the process that leads to fibrin formation; this process involves controlled interactions between protein coagulation factors. Hemostasis is coagulation that occurs in a physiological (as opposed to pathological) setting and results in sealing a break in the vasculature. This process has a number of components, including adhesion and activation of platelets coupled with ordered reactions of the protein coagulation factors. Hemostasis is essential to protect the integrity of the vasculature. Thrombosis is coagulation in a pathological (as opposed to physiological) setting that leads to localized intravascular clotting and potentially occlusion of a vessel. There is an overlap between the components involved in hemostasis and thrombosis, but there is also evidence to suggest that the processes of hemostasis and thrombosis have significant differences. There are also data to suggest that different vascular settings (arterial, venous, tumor microcirculation) may proceed to thrombosis by different mechanisms. Exploitation of these differences could lead to therapeutic agents that selectively target thrombosis without interfering significantly with hemostasis. Other chapters of this book will discuss some of the mechanisms behind thrombosis.

Healthy vasculature

Intact vasculature has a number of active mechanisms to maintain coagulation in a quiescent state. Healthy endothelium expresses ecto-ADPase (CD39) and produces prostacyclin (PGI$_2$) and nitric oxide (NO); all of these tend to block platelet adhesion to and activation by healthy endothelium [1]. Healthy endothelium also has active anticoagulant mechanisms, some of which will be discussed below. There is evidence that the vasculature is not identical through all parts of the body [2]. Further, it appears that there can be alterations in the vasculature in response to changes in the extracellular environment. These changes can locally alter the ability of endothelium to maintain a quiescent state.

Even though healthy vasculature maintains a quiescent state, there is evidence to support the idea that there is ongoing, low-level activation of coagulation factors [3]. This ongoing activation of coagulation factors is sometimes termed “idling” and may play a role in preparing for a rapid coagulation response to injury. Part of the evidence for idling comes from the observation that the activation peptides of factors IX and X can be detected in the plasma of healthy individuals. Because levels of the factor X activation peptide are significantly reduced in factor VII deficiency but unchanged in hemophilia, the factor VIIa complex with tissue factor is implicated as the key player in this idling process.

Tissue factor is present in a number of tissues throughout the body [4]. Immunohistochemical studies show that tissue factor is present at high levels in the brain, lung, and heart. Only low levels of tissue factor are detected in skeletal muscle, joints, spleen, and liver. In addition to being distributed in tissues, tissue factor is expressed on vascular smooth muscle cells and on the pericytes that surround blood vessels. This concentration of tissue factor around the vasculature has been referred to as a hemostatic envelope. Endothelial cells in vivo do not express tissue factor, except possibly during invasion by cancer cells. Also, there is evidence to suggest that tissue factor may be present on microparticles in the circulation. The nature and function of this circulating tissue factor is being actively researched by a number of groups. The information to date suggests that this tissue factor...
accumulates in pathological thrombi. Further, there is general agreement in these studies that circulating tissue factor levels are extremely low in healthy individuals. Limited data suggest that tissue factor does not incorporate into hemostatic plugs [5], unlike the accumulation of tissue factor seen in thrombosis; and so, the model of hemostasis described in this chapter does not include a role for circulating tissue factor in hemostasis.

Given the location of tissue factor, it seems plausible that the processes associated with idling may not be intravascular but may rather occur in the extravascular space. At least two mechanisms are known that can concentrate plasma coagulation factors around the vasculature (Plate 1.1). Coagulation proteins enter the extravascular space in proportion to their size; small proteins readily get into the extravascular space, whereas large proteins do not seem to reach the extravasculature [6]. Because tissue factor binds factor VII so tightly, it can trap factor VII that moves into the extravascular space. This means that blood vessels already have factor VII(a) bound [7]. Also, factor IX binds tightly and specifically to the extracellular matrix protein collagen IV; this results in factor IX being concentrated around blood vessels [8]. A role for this collagen IV-bound factor IX in hemostasis is suggested by the observation that mice expressing a factor IX that cannot bind collagen IV have a mild bleeding tendency.

Initiation

A break in the vasculature exposes extracellular matrix to blood and initiates the coagulation process (Plate 1.2). Platelets adhere at the site of injury through a number of specific interactions [9]. The plasma protein von Willebrand factor (VWF) can bind to exposed collagen and, under flow, undergoes a conformational change such that it binds tightly to the abundant platelet receptor glycoprotein Ib. This localization of platelets to the extracellular matrix promotes collagen interaction with platelet glycoprotein VI. Binding of collagen to glycoprotein VI triggers a signaling cascade that results in activation of platelet integrins. Activated integrins mediate tight binding of platelets to extracellular matrix. This process adheres platelets to the site of injury.

In addition to platelet processes, plasma concentrations of factors IX and X are brought to the preformed factor VIIa/tissue factor complexes at the site of injury. Factor VIIa/tissue factor activates both factor IX and factor X; the activated proteins play distinct roles in the ensuing reactions. Factor IXa moves into association with platelets, where it plays a role in the later stages of hemostasis. Factor Xa forms a complex with factor Va to convert a small amount of prothrombin to thrombin. The source of factor Va for this reaction is likely protein released from the alpha granules of collagen adherent platelets [10]. Platelet factor V is released in a partially active form and does not require further activation to promote thrombin generation [10]. Thrombin formed on pericytes and in the extravascular space can promote local fibrin formation but is not sufficient to provide for hemostasis throughout the wound area.

The factor VIIa/tissue factor complexes are, over time, inhibited by tissue factor pathway inhibitor (TFPI). TFPI participates in a ternary complex with factor Xa and factor VIIa bound to tissue factor.

Deficiencies of tissue factor have not been seen in humans, and a knockout of the tissue factor gene in mouse models leads to embryonic lethality. Factor VII deficiency is associated with a bleeding phenotype, and many patients with <1% factor VII activity have spontaneous, severe bleeding.

Amplification

The thrombin formed in the initiation phase acts as an amplifier by acting on platelets and proteins to facilitate platelet-driven thrombin generation (Plate 1.3). Thrombin has a tight specific interaction with platelet glycoprotein Ib [11]. When bound to glycoprotein Ib, thrombin undergoes a conformational change that alters the activity of the protein and may protect it from inhibition. This conformational change enhances the ability of thrombin to cleave either of the two platelet protease-activated receptors (PARs). PARs are members of the seven transmembrane domain G-coupled family of proteins [12]. Cleavage of a PAR creates a new amino terminal, which can fold back on itself and bind to a receptor site in the transmembrane domain. This intramolecular binding initiates a signaling cascade. In platelets, cleavage of PAR1 leads
to signaling that results in platelet activation. This process is initiated after exposure of platelets to very small amounts of thrombin.

Platelet activation leads to numerous significant changes. Platelets undergo cytoskeletal changes leading to a shape change. There are regulated changes in the platelet membrane such that expression of phosphatidylserine on the outer leaflet of the platelets is significantly enhanced [13]. Phosphatidylserine induces allosteric changes in the procoagulant complexes that significantly increase their activity. Platelets degranulate, releasing the contents of both alpha granules and dense granules. Dense granule contents, especially released-ADP, participate in a positive feedback loop either on the same platelet or on nearby platelets to further promote platelet activation. Among the alpha granule contents released when platelets are activated is partially activated factor V.

In addition to its action on platelet receptors, thrombin can also activate procoagulant cofactors. Platelet factor V or plasma factor V bound to platelets is activated by thrombin cleavage to release the B domain. VWF, in addition to participating in platelet adhesion, acts as a carrier of factor VIII. It seems reasonable that VWF bound to glycoprotein Ib might bring factor VIII into proximity of thrombin, also bound to glycoprotein Ib. Thrombin cleavage releases factor VIII from VWF as well as activating factor VIII. So the amplification phase results in activated platelets that have cofactors Va and VIIIa bound to the surface.

Some schemes of coagulation do not describe amplification as a separate step. But work from the Maas-trich group, which was expanded on by Dale and colleagues, shows that platelets can be activated to different levels of procoagulant activity [13,14]. This suggests that in vivo the procoagulant activity of platelets may be modulated by local conditions. It also suggests that aspects of platelet activation could be targeted to reduce thrombin generation in pathological settings. So, amplification is included in this model as a discrete step.

**Propagation**

The activated platelet with activated cofactors is primed for a burst of thrombin generation (Plate 1.4). Factor IXa formed during the initiation phase binds to activated platelets. One component of this binding is a saturable, specific, reversible site independent of factor VIIIa [15], and the other component of this binding is factor VIIIa. The factor IXa/VIIIa complex activates factor X on the platelet surface. This platelet surface-generated factor Xa can move directly into a complex with platelet surface factor Va. In the presence of prothrombin, this factor Xa is protected from inhibition by antithrombin or TFPI. Recent data suggest that these factor Xa/Va complexes are very stable for even extended times and, in the presence of a new supply of prothrombin, can immediately act to promote thrombin generation [16]. Platelet surface-generated factor Xa plays a different role than factor X activated by factor VIIa/tissue factor. Because of the rapid inhibition by TFPI of factor Xa that is not in a complex, it is likely that factor X generated by factor VIIa/tissue factor cannot reach the platelet surface. This conclusion is supported by the observation that, in hemophilia, when platelet factor Xa generation is absent or severely defective, the clot is very poor even though factor VIIa/tissue factor activity is normal and fibrin deposition can be observed at the margins of hemophilic wounds [17].

The burst of thrombin during the propagation phase leads to cleavage of fibrinopeptides from fibrinogen. Cleavage of these fibrinopeptides exposes new binding sites that fit with complementary sites on other fibrin molecules [18]. These interactions lead to fibrin molecules assembling in long, branched chains anchored at the platelet receptor glycoprotein IIb/IIIa. This process stabilizes the initial platelet plug into a consolidated fibrin plug. The nature and stability of the fibrin plug appear to depend on the rate of thrombin generation during the propagation phase [19].

In addition to its role in cleaving fibrinopeptides, thrombin generation participates in a positive feedback loop by activating factor XI on the platelet surface [20]; this factor XIa can activate factor IXa to enhance factor Xa generation. And the high levels of thrombin generated during the burst phase can cleave PAR4. Signaling downstream from PAR4 contributes to platelet shape changes that might be important in stabilization of the hemostatic plug. Finally, high levels of thrombin generated during the propagation phase bind to fibrin and, when bound, are protected from inhibition by antithrombin. This fibrin-bound thrombin

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**Basic principles underlying coagulation**
provides an important role in maintaining hemostasis. Disruption of a plug brings fibrinogen into contact with the bound thrombin, where fibrin formation can be initiated immediately without the need for thrombin generation. One aspect of the bleeding associated with hemophilia may be both the initial poor structure of the fibrin plug and the lack of bound thrombin to stabilize the plug.

Deficiencies of proteins in the propagation phase are associated with bleeding. X chromosome-linked hemophilia in males is associated with deficiencies in factors VIII and IX (hemophilia A and B, respectively). Because both genes are located on the X chromosome, the hemophilic phenotype results from a single-gene defect in males. Bleeding risk in hemophilia A and B is linked to factor level. Factor XI deficiency is also associated with bleeding risk. However, bleeding in factor XI deficiency shows a somewhat weak association with factor level [21]. The proposed model is consistent with this observation in that factor XI is not primary to the pathway leading to thrombin generation, but rather contributes through the positive feedback loop to boost thrombin generation.

**Localization**

A hemostatic plug should, by definition, seal the break in the vasculature but not continue platelet accumulation and thrombin generation to the point that the entire vessel is occluded. Thrombin released from a platelet plug into flowing blood is swept downstream. At plasma concentrations of antithrombin, the expected half-life of thrombin in blood is well under a minute. Also, factor Xa, either released into the blood or generated on healthy endothelium, is rapidly inhibited by TFPI in solution or TFPIβ, which is associated with the endothelial cell surface through a glycosylphosphatidylinositol linkage [22].

Healthy endothelial cells, in addition to the mechanisms described above for blocking platelet activation, have active mechanisms to downregulate thrombin generation [23]. Thrombin on the platelet surface participates in a positive feedback loop that promotes additional thrombin generation. By contrast, thrombin on healthy endothelium participates in a negative feedback loop that blocks additional thrombin generation (Plate 1.5).

Thrombin that reaches an endothelial cell binds to thrombomodulin. This binding causes a conformational change in thrombin such that it can no longer cleave fibrinogen. Thrombin bound to thrombomodulin is rapidly inhibited by protein C inhibitor [24]. This thrombin/inhibitor complex rapidly dissociates so that thrombomodulin can again bind thrombin, and thrombin bound to thrombomodulin can rapidly activate protein C. The endothelial cell protein C receptor enhances protein C activation by thrombin/thrombomodulin. Activated protein C, in coordination with protein S, inactivates factors Va and VIIIa. The net result is that thrombin generation is confined by healthy endothelium to a site of injury. Deficiencies of protein C or S, or defects that prevent cleavage and inactivation of factor V (factor V Leiden), allow for the spread of thrombi into the vasculature and are associated with venous thrombosis.

**Coagulation assays**

The two most common assays in the clinical coagulation laboratory are the Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT). In the PT assay, a large excess of thromboplastin (tissue factor) is added to plasma. There is rapid activation of factor X, leading to thrombin generation and clot formation. The assay is sensitive to deficiencies of factors VII, X, V, and prothrombin, but not factors XI, IX, or VIII. Thus, the PT evaluates the factors involved in the initiation phase (Plate 1.2).

Because the PT does not assess factors VIII or IX (the factors that are deficient in hemophilia A and B, respectively), the APTT assay was developed to diagnose hemophilia and monitor therapy. The original APTT used a dilution of thromboplastin, but kaolin was substituted in 1961 [25], resulting in a simple, reproducible, reliable assay (that no longer has a thromboplastin component). The current APTT takes advantage of the ability of factor XII and high molecular weight kininogen, even though they are not involved in physiological hemostasis, to be activated by a negatively charged surface. With this initiator, the clotting reaction proceeds through, and is sensitive to deficiencies of, factors XI, IX, VIII, X, V, and prothrombin. Thus, the APTT assays the factors involved in the platelet surface propagation phase (Plate 1.4).
Summary

This model of hemostasis views the process as having three overlapping phases: initiation, amplification, and propagation. The hemostatic plug is localized to the area of injury by healthy endothelium, which has active processes to downregulate thrombin generation. It is important to focus on the cellular location of the steps rather than the proteins involved. The protein factors overlap between the steps, but, for example, thrombin bound to platelet surface glycoprotein Ib plays a different role than thrombin bound to endothelial cell thrombomodulin. So, each of the cellular steps must contribute for the overall process to result in a coordinated hemostatic plug. A defect in initiation means that the coagulation reactions will not be started. Tissue factor deficiency is lethal in animals models, and factor VII deficiency is associated with bleeding. Platelet adhesion or activation defects, such as Scott Syndrome, are associated with bleeding. Hemophilia is a defect of factor X activation on the platelet surface during the propagation phase. Factor X activation by factor VIIa/tissue factor during initiation cannot substitute for the platelet surface reactions. Factor Xa is confined to the tissue factor bearing surface, where it is formed because, when released from the surface, it is rapidly inhibited by TFPI and antithrombin. So, for normal hemostasis, a factor X-activating complex must be formed on activated platelets. The localization process confines platelet deposition and fibrin formation to keep the clot from expanding over healthy endothelium. This is consistent with the observation that defects in antithrombin, TFPI, and proteins C and S are associated with thrombosis. The tie between this model and the standard coagulation assays is that the PT and APTT assess the initiation and propagation phases, respectively.

References

CHAPTER 1


