

The cell-based model of coagulation

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Abstract

Objective – To review the current model describing coagulation processes, including the critical contributions of cells.

Data Sources – Original research articles, scientific reviews, and textbooks.

Data Synthesis – Normal hemostasis is vital for prevention of blood loss, but controls are necessary to limit coagulation to the site of injury. The previous cascade model of blood coagulation, although refined and updated over the last few decades, is flawed as a description of how hemostasis occurs in vivo. The recently proposed model incorporates the vital role of cells in coagulation processes, and corrects deficiencies of the older cascade models.

Conclusions – The cell-based model of coagulation provides a description of coagulation that more likely reflects hemostatic processes as they occur in vivo.

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Introduction

The hemostatic system is a vital protective mechanism that is responsible for preventing blood loss by sealing sites of injury in the vascular system. However, hemostasis must be controlled so that blood does not coagulate within the vasculature and restrict normal blood flow. Understanding of hemostatic mechanisms has progressed substantially over the last century, with the majority of investigations occurring in static, cell-free in vitro systems. Recent advancements in the ability to study coagulation have dramatically expanded information regarding coagulation mechanisms. New models that include the contributions of cells in vitro and systems that involve real-time in vivo imaging of coagulation have significantly modified current understanding of how hemostasis occurs in vivo.¹

Previous Coagulation Models

History of the understanding of blood coagulation

Initially it was thought that exposure of blood to air was the initiator of coagulation after injury. It was not

until the mid-19th century that the concept was proposed that the ability to clot blood was a function of a specific component of blood. Coagulation models developed in the early part of the 20th century described the terminal portions of coagulation where fibrinogen is converted to fibrin by thrombin, but discovery of the specific upstream components (formerly described together as prothrombin activity) responsible for generation of thrombin did not occur until the 1940s and 1950s.² Newly identified coagulation factors were named for either their discoverer, or for the first patient that was described with a deficiency of that factor, and numbered in order of their discovery. Over these decades the plasma components of coagulation were gradually sorted into specific proenzymes that could be converted to enzymes, and procofactors that could be converted to cofactors but were without enzymatic activity. In the 1960s, 2 different groups proposed the cascade model that defined a series of steps where enzymes and their cofactors cleaved subsequent proteins.³

The cascade model

The cascade model consists of a sequence of steps where enzymes cleave zymogen substrates (also known as proenzymes) to generate the next enzyme in the cascade. The majority of the steps in the cascade occur on phospholipid membrane surfaces and require calcium. The cleavage of fibrinogen by thrombin is the

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Table 1: Abbreviations for participants in coagulation

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| aPC: activated protein C |
| AT: antithrombin |
| BK: bradykinin |
| FII: prothrombin |
| FIIa: thrombin |
| FV: factor V |
| FVa: activated factor V |
| FVII: factor VII |
| FVIIa: activated factor VII |
| FVIII: factor VIII |
| FVIIIa: activated factor VIII |
| FIX: factor IX |
| FIXa: activated factor IX |
| FX: factor X |
| FXa: activated factor X |
| FXI: factor XI |
| FXIa: activated factor XI |
| FXII: factor XII |
| FXIIa: activated factor XII |
| Fg: fibrinogen |
| Fb: fibrin |
| Gla: glutamic acid |
| HMWK: high molecular weight kininogen |
| HSPG: heparan sulfated proteoglycans |
| K: kallikrein |
| PAI: plasminogen activator inhibitor |
| PC: protein C |
| PK: prekallikrein |
| ProS: protein S |
| TAFI: thrombin activatable fibrinolysis inhibitor |
| TF: tissue factor |
| TFPI: tissue factor pathway inhibitor |
| TM: thrombomodulin |
| vWF: von Willebrand factor |

most notable exception to the membrane requirement. Some enzymes cleave their substrates poorly without binding to their required cofactor. The model was divided into the familiar extrinsic and intrinsic pathways. The extrinsic system was localized outside (or extrinsic from) the blood, and consisted of TF (for abbreviations see Table 1) and FVIIa. The intrinsic system was localized within the blood (or intrinsic to) and was initiated through the contact activation of FXII on negatively charged surfaces, which subsequently activated other contact components. Either pathway could activate FX to FXa, which in turn (with its cofactor FVa) could activate prothrombin to thrombin, which then cleaved fibrinogen to form fibrin. This latter portion was generally referred to as the common pathway⁴ (Figure 1).

Utility of the cascade model

The cascade model was extremely useful in advancing the understanding of how coagulation enzymatic steps occur in plasma-based *in vitro* coagulation. The understanding of the calcium dependence of coagulation allowed for prevention of coagulation in blood collection

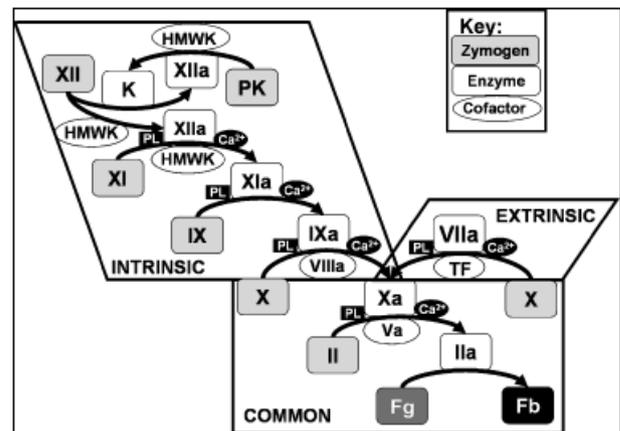


Figure 1: The cascade model of fibrin formation. This model divides the coagulation system into separate redundant pathways (extrinsic and intrinsic) either of which can result in generation of FXa. The common pathway results in generation of thrombin and subsequent cleavage of fibrinogen to fibrin. Many of the enzymes and enzymatic complexes require calcium (Ca^{2+}) and binding to active membrane surfaces (PL) for full activity. See Table 1 for abbreviations. For simplicity, feedback activation of procofactors to cofactors and the many inhibitors of the various enzymes have been omitted.

using calcium chelators. The description of the cascade model has also allowed for clinically useful interpretation of laboratory tests for plasma coagulation abnormalities. Specifically, deficiencies in the extrinsic or common pathways are identified using the prothrombin time, while deficiencies in the intrinsic or common pathways are reflected with prolongation of the activated partial thromboplastin time (aPTT). Additional less commonly used tests such as the Russell's viper venom time, the thrombin time, and assays for function of specific factors, have allowed for further isolation of the exact site of coagulation defects.

The description of the cascade model has also allowed for isolation and study of specific steps in protein-dependent coagulation processes, leading to better understanding of the properties of individual enzyme complexes. Continued experimentation based on the cascade model has led to the discovery of previously unidentified cross-interactions between different components, and to identification of and understanding of the roles of the more recently discovered specific inhibitors of coagulation.

The cascade model, as modified over the last few decades to include these new intersections between intrinsic and extrinsic pathways, functions reasonably well to explain the way coagulation occurs in plasma or purified protein-based fluid systems where the fluid is static and does not interact with vascular wall or cell surfaces.⁵

Deficiencies in the cascade model

While separating the various enzymatic processes of coagulation into this Y-shaped cascade was useful in further understanding of how coagulation processes occur in plasma-based *in vitro* coagulation, it is obvious that this model does not adequately explain the hemostatic process as it occurs *in vivo*.^{1,3,6,7} The cascade model suggests that the extrinsic and intrinsic pathways operate as independent and redundant pathways, while clinical manifestations of individual factor deficiencies clearly contradict this concept. Deficiencies in the initial components of the intrinsic pathway (FXII, HMWK, or PK) cause marked prolongation of the aPTT, but they are not associated with a tendency for bleeding in mice or humans.⁸ Furthermore, FXII is clearly not required for normal hemostasis because some mammalian species (such as whales and dolphins) do not have this protein.⁸ Deficiency of the next downstream enzyme FXI (hemophilia C) is associated with variable hemostatic deficits in humans, with some individuals experiencing bleeding. In contrast, deficiency in either of the next downstream components of the intrinsic pathway (FVIII and FIX) results in the serious bleeding tendencies seen with hemophilia A and B, despite the fact that these patients have an intact extrinsic pathway.⁸ Similarly, deficiency of the primary enzyme of the extrinsic pathway (FVII) can be associated with bleeding, despite the presence of an intact intrinsic pathway.⁹ The clinical manifestations of isolated abnormalities in either the intrinsic or extrinsic pathways argues against the idea that these enzymatic systems operate as independent generators of FXa.

The Contribution of Cells to Coagulation

The incorporation of the role of cells in coagulation allows for an integrated understanding of the mechanisms by which coagulation may occur in the dynamic vascular system. This model also allows for inclusion of the role of specific platelet binding sites for coagulation proteins. The cell-based model further incorporates the concept that some coagulation proteases, while able to participate in the enzymatic cascade of coagulation, may function primarily in roles outside of hemostasis, such as inflammation, vessel wall function, and cell proliferation.

Cellular localization of coagulation reactions

In order to control hemorrhage in response to injury, hemostatic processes are required to create an obstruction at the site of injury. Lack of regulation of hemostasis has the potential to initiate coagulation (and consequently impede blood flow) at sites where no injury is present. Appropriate hemostasis consequently

requires that coagulation control and regulation be localized specifically at a site of injury. The control responsible for localization is accomplished primarily via the contribution of membrane surfaces to coagulation processes.

Interactions of coagulation proteins with membranes

The speed at which many of the enzymatic reactions in coagulation proceed is profoundly affected by the presence of an appropriate membrane surface. These enzymatic reactions are enhanced by membrane binding of the participating proteins in part because localization to a membrane surface helps properly align the participating proteins. TF is the only coagulation protein that is permanently attached to the membrane surface.⁹

Other coagulation proteins (eg, FVII, FIX, FX, prothrombin, protein C, protein S, protein Z) contain glutamic acid (Gla) residues that allow for binding of the protein to a membrane surface via interaction between calcium and negatively charged phospholipids. Calcium binding of the Gla regions of these proteins requires that their Gla residues be carboxylated in a post-translation modification via the vitamin K cycle in the liver. Without complete carboxylation, these Gla proteins do not develop the required ability to properly bind calcium. As calcium binding is necessary for interaction of the Gla residues with the membrane surface, Gla proteins that have not been adequately carboxylated are unable to properly bind the activated membrane surface. The importance of carboxylation in membrane binding is dramatically illustrated by the profound adverse impact that vitamin K antagonists, such as warfarin, have on the ability of Gla proteins to function in hemostasis.

Some cofactors (FV and FVIII) also have regions that interact with phospholipids, allowing for formation of the fully functional enzymatic complex on the membrane surface. This mechanism of membrane binding by cofactors is less well described than that for Gla proteins.

Membrane surfaces

All cells are surrounded by lipid membrane bilayers that contain a large number of constitutively expressed membrane surface proteins. The composition and distribution of membrane phospholipid molecules is tightly controlled. In the inactive resting membrane state, neutral phospholipids (primarily phosphatidylcholine, sphingomyelin, and sugar-linked sphingolipids) are located on the external leaflet of the membrane, and phosphatidylserine (PS) and phosphatidylethanolamine (PE) are localized to the inner surface of the membrane. This membrane asymmetry is essential and tightly controlled under normal

conditions. When cells are activated or injured, they shuffle the PS and PE to the outer membrane leaflet. This membrane phospholipid shuffling is controlled by a variety of enzymes. Flippase actively transports PS from the external to the internal leaflet while floppase transports PC in the opposite direction. These ATP-dependent enzymes maintain asymmetry in the resting state. When a cell is activated or injured (such as occurs when platelets are exposed to platelet activators, or when other cell types are stimulated to undergo apoptosis) the enzyme scramblase actively shuffles the phospholipids between the 2 surfaces in response to increased concentrations of calcium in the cytosol. This results in the appearance of PS and PE on the external membrane surface.¹⁰⁻¹²

Contribution of the procoagulant membrane

The expression of PS and PE on the cell surface has a profound impact on the procoagulant properties of the membrane surface. Although a full understanding of how coagulation reactions occur on activated membrane surfaces has not yet been achieved, it is known that the presence of PS on the membrane markedly increases the speed of some coagulation reactions (>thousands of times faster). Less PS is required for maximum speed when PE is present.¹³ It is currently thought that Gla proteins preferentially bind to PS clusters on the membrane surface, and that PE aids in grouping PS into these clusters. The expression of PS (particularly with PE) on the external leaflet turns the cell membrane into a procoagulant surface. Because coagulation reactions occur very slowly on membranes that do not contain PS, resting cells are essentially incapable of supporting the coagulation cascade. Under normal physiologic conditions cells do not express a procoagulant membrane. Consequently, generation of coagulation enzymes is extremely slow, and insufficient to generate enough fibrin to form a clot. Production of thrombin is consequently limited to surfaces of cells in an injured area that have been triggered to express a procoagulant membrane. As a result, the ability of cells to control the nature of their membrane surface constitutes a powerful method of regulating coagulation reactions.¹²

Role of microparticles

Microparticles (MPs) are intact vesicles derived from cells which are surrounded by membranes. They vary somewhat in size (2-20% of the size of a RBC) and arise when activated or apoptotic cells shed bits of membrane. Cytokines (such as tumor necrosis factor and interleukin-6), thrombin, shear stress, and hypoxia can stimulate MP formation. Under normal conditions MPs are primarily derived from endothelial cells, platelets,

and monocytes, but in certain disease states, MPs may arise from granulocytes and erythrocytes. The quantity of circulating MPs is increased in certain illnesses such as diabetes mellitus, sepsis, and cardiovascular disease and may contribute to pathologic coagulation in a variety of disorders.^{11,14,15}

MPs contain cell surface proteins similar to those found on their parent cell (eg, ultra large vWF monomers on endothelial cell-derived MPs, P-selectin on platelet-derived MPs, TF on monocyte-derived MPs) that can participate in coagulation reactions, especially when the MP expresses a procoagulant membrane. The contribution of MPs to normal hemostasis is currently under intensive investigation.

Other anticoagulant properties of endothelial cells

In addition to the resting membrane that does not support coagulation reactions, nonactivated resting endothelial cells express a number of other anticoagulant properties via proteins expressed on their surface. These include heparan sulfated proteoglycans (HSPGs), thrombomodulin (TM), and tissue factor pathway inhibitor (TFPI).¹⁶⁻¹⁸

Endothelial cells produce HSPGs, a small amount of which is expressed on the luminal surface in contact with the flowing blood. The HSPGs are a binding site for antithrombin (AT), which then is fully capable of inactivating thrombin produced in the vicinity of the HSPG. The inactivation of thrombin by HSPG-AT is similar to that by heparin-AT which we exploit clinically when administering soluble (nonmembrane bound) forms of heparin.

Resting endothelial cells also express TM on their surface. Thrombin, once bound to TM, converts from a procoagulant to an anticoagulant protein because the thrombin-TM complex rapidly activates protein C (aPC). aPC (with its cofactor protein S [ProS]) then irreversibly cleaves FVa and FVIIIa, preventing their further participation in generation of additional new thrombin molecules. aPC-ProS also inactivates an important inhibitor of fibrinolysis (plasminogen activator inhibitor 1) which ultimately upregulates lysis of any fibrin that is formed. It is important to note that expression of TM is 100-fold higher in capillary endothelium as compared with endothelium in the major vessels. Therefore, any thrombin circulating in large vessels will be quickly extracted when the blood passes through a capillary.¹⁶

TFPI on the endothelial cell surface prevents additional thrombin generation by acting as an upstream inhibitor of FXa and FVIIa. It irreversibly binds to FXa, then forms a quaternary complex between TFPI, FXa, FVIIa, and TF, preventing further participation of these

protein molecules in the generation of additional thrombin.⁹

The Cell-Based Model of Fibrin Formation

Our new understanding of hemostasis incorporates the role of cells. Evaluation of this model suggests that coagulation actually occurs in vivo in distinct overlapping phases. It requires the participation of 2 different cells types: a cell-bearing TF, and platelets.

Initiation

All evidence to date indicates that the sole relevant initiator of coagulation in vivo is TF. Cells expressing TF are generally localized outside the vasculature, which prevents initiation of coagulation under normal flow circumstances with an intact endothelium. Some circulating cells (eg, monocytes or tumor cells) and MPs may express TF on their membrane surface, but this TF under normal conditions is thought to be inactive or encrypted. The exact role of this blood-borne TF is controversial. Some investigators believe that circulating TF is encrypted in that it contains an additional bond that must be cleaved for activity. Others believe that circulating TF is not fully active because the membrane surface on which it resides is not a PS-containing procoagulant membrane.^{9,19}

Once an injury occurs and the flowing blood is exposed to a TF-bearing cell, FVIIa rapidly binds to the exposed TF. Note that FVIIa is the only coagulation protein that routinely circulates in the blood in its active enzyme form, with approximately 1% of total FVII circulating as FVIIa.^{9,20} All other coagulation proteins circulate solely as zymogens. The TF-FVIIa complex then activates additional FVII to FVIIa, allowing for even more TF-FVIIa complex activity, which then activates small amounts of FIX and FX. Although it occurs slowly, FV can be activated directly by FXa. The FXa generated by TF-FVIIa binds to the few generated molecules of its cofactor FVa to form the prothrombinase complex, which subsequently cleaves prothrombin and generates a small amount of thrombin. Any FXa that dissociates from the membrane surface of the TF-bearing cell is rapidly inactivated by either TFPI or AT. The FXa generated is consequently effectively restricted to the surface of the TF-bearing cell on which it was generated. However, the FIXa generated can dissociate and move to the surface of nearby platelets or other cells. FIXa is not inhibited by TFPI, and much more slowly inhibited by AT than is FXa^{3,6} (Figure 2a).

It is important to note that because TF is always expressed in the perivascular space, any FVIIa that leaves the vasculature through minor breaks in the endothelial barrier will bind to TF and potentially initiate coagulation.

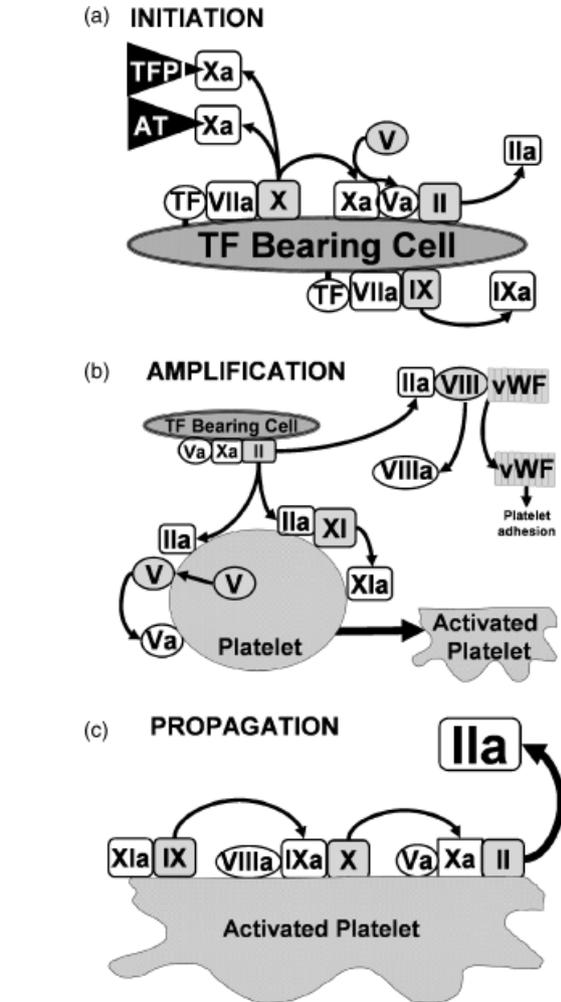


Figure 2: The cell-based model of fibrin formation. The cell-based model incorporates the contribution of various cell surfaces to fibrin formation. In this model thrombin generation occurs in overlapping phases. (a) Initiation Phase. This phase occurs on the TF-bearing cell. It is initiated when injury exposes the TF-bearing cell to the flowing blood. It results in the generation of a small amount of FIXa and thrombin that diffuse away from the surface of the TF-bearing cell to the platelet. (b) Amplification Phase. In the second phase, the small amount of thrombin generated on the TF-bearing cell activates platelets, releases vWF and leads to generation of activated forms of FV, FVIII, and FXI. (c) Propagation Phase. In the third phase the various procoagulant enzymes generated in earlier phases assemble on the procoagulant membrane surface of the activated platelet to form intrinsic tenase, resulting in FXa generation on the platelet surface. Prothrombinase complex forms and results in a burst of thrombin generation directly on the platelet. See Table 1 for abbreviations.

The gaps in the endothelial barrier under normal conditions are very small. Most of the upstream coagulation proteins are relatively small (eg, FVII: 50,000 Da) whereas some of the downstream proteins are much

larger (eg, FV, 330,000 Da; fibrinogen, 340,000 Da). This means that platelets and large proteins are sequestered from the extravascular space. Coagulation progresses beyond the generation of the small amount of thrombin that occurs with initiation only when the injury allows platelets and larger proteins to leave the vascular space and adhere to the TF-bearing cells in the extravascular area.^{3,6}

Amplification

Once a small amount of thrombin has been generated on the surface of a TF-bearing cell (the initiation phase), that thrombin diffuses away from the TF-bearing cell and is available for activation of platelets that have leaked from the vasculature at the site of injury. Binding of thrombin to platelet surface receptors causes extreme changes in the surface of the platelet, resulting in shape change, shuffling of membrane phospholipids to create a procoagulant membrane surface, and release of granule contents that provide additional fuel for the fire. Platelet granules contain a large number of proteins and other substances that include raw materials for clotting reactions and agonists to induce further platelet activation. Calcium may induce clustering of PS (increasing the procoagulant nature of the membrane), and promotes binding of coagulation proteins to the activated membrane surface. In addition to activating platelets, the thrombin generated in the initiation phase cleaves FXI to FXIa and activates FV to FVa on the platelet surface. Thrombin also cleaves von Willebrand factor off of FVIII (they circulate bound together), releasing it to mediate platelet adhesion and aggregation. The released FVIII is subsequently activated by thrombin to FVIIIa³ (Figure 2b).

Propagation

Once a few platelets are activated in the amplification phase, the release of the granule contents results in recruitment of additional platelets to the site of injury. The propagation phase occurs on the surface of these platelets. Expression of ligands on their surface results in cell-cell interactions that lead to aggregation of platelets. FIXa that was generated by TF-FVIIa in the initiation phase can bind to FVIIIa (generated in the amplification phase) on the platelet surface. Additional FIXa is generated due to cleavage of FIX by FXIa that was generated during amplification on the platelet surface. Once the intrinsic tenase complex forms (FIXa-FVIIIa) on the activated platelet surface, it rapidly begins to generate FXa on the platelet. FXa was also generated during the initiation phase on the TF-bearing cell surface. As this FXa is rapidly inhibited if it moves away from the TF-bearing cell surface, it can not easily reach the platelet surface. The majority of FXa must

therefore be generated directly on the platelet surface through cleavage by the intrinsic tenase complex. The FXa generated on platelets then rapidly binds to FVa (generated by thrombin in the amplification phase) and cleaves prothrombin to thrombin (figure 2c). This prothrombinase activity results in a burst of thrombin generation leading to cleavage of fibrinopeptide A from fibrinogen. When enough thrombin is generated with enough speed to result in a critical mass of fibrin, these soluble fibrin molecules will spontaneously polymerize into fibrin strands, resulting in an insoluble fibrin matrix.³

Additional aspects of hemostasis

Generation of enough thrombin to result in a clot is not, however, the end of coagulation. There are many steps vital to normal hemostasis that occur temporally after generation of the fibrin gel. Approximately 95% of the thrombin generation occurs after the time of fibrin gel formation as detected in routine plasma-based clotting assays such as the prothrombin time and aPTT. These assays are consequently unable to detect abnormalities associated with hemostasis that are due to deficiencies in hemostatic function that occur following initial fibrin polymerization.⁵

Thrombin generation subsequent to fibrin polymerization plays many important roles in the structure of the clot that is formed. In addition to the cleavage site for fibrinopeptide A, fibrinogen contains a site where thrombin cleaves off fibrinopeptide B. While removal of fibrinopeptide B is not strictly required for fibrin polymerization, its removal results in changes in the structure of the fibrin that is formed.²¹

Thrombin additionally activates FXIII to FXIIIa (Figure 3). This enzyme modifies the polymerized fibrin to form cross-links between fibrin strands. Cross-link formation drastically impacts the strength and elasticity of the fibrin clot that is formed. The importance of cross-linking in clot structure is demonstrated by the bleeding phenotype observed in mice and humans with FXIII deficiency.²²

Some of the thrombin generated will bind to TM on the endothelial cell surface. TM bound thrombin can activate thrombin activatable fibrinolysis inhibitor. This recently discovered enzyme acts to modify the fibrin molecules by removing their terminal lysine residues. As lysine residues are the necessary binding site for several fibrinolytic proteins, fibrin that has been acted on by activated thrombin activatable fibrinolysis inhibitor becomes markedly more resistant to fibrinolysis²³ (Figure 3).

TM bound thrombin has an additional role in termination of coagulation due to its activation of protein C. aPC forms a complex with its cofactor ProS, which

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