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Thrombin Generation, Fibrin Clot Formation and Hemostasis

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Abstract

Hemostatic clot formation entails thrombin-mediated cleavage of fibrinogen to fibrin. Previous *in vitro* studies have shown that the thrombin concentration present during clot formation dictates the ultimate fibrin structure. In most prior studies of fibrin structure, clotting was initiated by adding thrombin to a solution of fibrinogen; however, clot formation *in vivo* occurs in an environment in which the concentration of free thrombin changes over the reaction course. These changes depend on local cellular properties and available concentrations of pro- and anti-coagulants. Recent studies suggest that abnormal thrombin generation patterns produce abnormally structured clots associated with an increased risk of bleeding or thrombosis. Further studies of fibrin formation during *in situ* thrombin generation are needed to understand fibrin clot formation *in vivo*.

Keywords

thrombin; fibrinogen; fibrin; plasmin; fibrinolysis; clot structure; hemophilia; thrombosis

Fibrinogen and fibrin clot formation

Fibrinogen is a 450 Å, 340 kDa trinodular protein present at high (2 – 4 mg/mL) concentrations in plasma. Fibrinogen consists of pairs of three different disulfide-linked polypeptide chains: Aα, Bβ, and γ. These six polypeptide chains are arranged with their N-termini converged in a central "E" domain of the molecule. The C-termini of the Bβ and γ chains extend outward into distal "D" domains. The C-termini of the Aα chains are globular and situated near the central E domain of fibrinogen where they interact intra-molecularly.[1] Comprehensive reviews on the biochemistry of fibrinogen and fibrin structure have been published.[2–4]

Mechanisms of fibrin production have been elucidated in studies conducted by adding exogenous thrombin to purified fibrinogen. These studies have shown that thrombin removes N-terminal peptides from the fibrinogen Aαl and Bβ chains, causing the spontaneous formation of half-staggered, double-stranded protofibrils followed by thickening of protofibril chains.[5] Initial formation of protofibrils occurs during a "lag" phase in which no turbidity increase is detected. Subsequent lateral aggregation of fibrin protofibrils causes a turbidity increase. The magnitude of the turbidity increase relates to the structure of the formed clot; formation of thicker fibers causes a greater increase in final turbidity.[6,7] Fibrin formation can be followed

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spectrophotometrically [8,9]; while, fibrin structure can be determined using electron or confocal microscopy.[10–15]

The thrombin concentration influences fibrin clot formation, structure, and stability

Fibrin clot structure depends on a number of variables, including pH, ionic strength, and concentrations of calcium, fibrinogen, and thrombin present during gelation.[5,8,10,16–21] In particular, the thrombin concentration influences both the fiber thickness and density of the fibrin clot. Low thrombin concentrations produce very turbid, highly permeable fibrin clots that are composed of thick, loosely-woven fibrin strands (Figure 1). Higher thrombin concentrations produce relatively non-turbid, less permeable clots that are composed of a dense network of relatively thin fibrin strands (Figure 1).[11,21–23] The differential structures of these clots has been shown to impart varying degrees of stability (viscoelastic properties and resistance to fibrinolysis) on the clot.

Individual fibers have significant extensibility and elasticity [24,25]; however, the potential influence of fiber thickness on the properties of individual fibers is not yet known. In contrast, the influence of clot structure on viscoelastic properties has been extensively examined. These studies have shown the relationship between fibrin structure and clot stiffness to be highly complex, depending on fiber thickness, length, density, and degree of branching, and extent of cross-linking (reviewed in [21,26]). Generally, increased thrombin concentrations are associated with the formation of stiffer clots [27]; however, Ryan *et al*. observed that clots formed in the presence of thrombin concentrations above 0.25 U/mL (composed of thin fibers) are less stiff (smaller G′) compared to clots formed with lower thrombin concentrations (thicker fibrin fibers).[21] The differences in findings from these studies may relate to differences in the experimental conditions (fibrinogen, thrombin and calcium concentrations, ionic strength, and pH).

Because fibrin functions as both a cofactor and a substrate for the fibrinolytic enzyme plasmin, the fibrin structure influences the clot's susceptibility to fibrinolysis.[12,28–30] Compared to thick fibers, thin fibrin fibers support a slower rate of tPA-mediated plasmin generation, reducing the rate of fibrinolysis.[31] An elegant study by Collet *et al*. (2000) showed that while thin fibers are lysed more quickly than thick fibers, clots composed of thin fibers are more resistant to fibrinolysis than clots composed of thick fibrin fibers.[12] These studies indicate that clots formed in even "simple" purified systems with single thrombin concentrations exhibit complex functionality.

Normal thrombin generation *in situ*

In vivo, thrombin generation is an intricate and highly dynamic process. Coagulation involves specific interactions between cellular surfaces and plasma-derived zymogens and cofactors. The reactions are typically described in three sequential phases: initiation, amplification, and propagation. During the initiation phase, active tissue factor is exposed to plasma, permitting formation of a complex between factor VIIa and tissue factor. The factor VIIa/tissue factor complex activates factors IX and X. During the amplification phase, the factor VIIa/tissue factor-activated factor Xa promotes small-scale thrombin generation, which activates cofactors VIII, V and platelets. Phosphatidylserine exposed on activated platelets provides binding sites for procoagulant zymogens and enzymes, including factors IXa, VIIIa, X, Xa, Va, and II.[32–35] During the propagation phase, intrinsic activities supported by the activated platelet surface, produce a large-scale thrombin burst that catalyzes the conversion of fibrinogen to fibrin.[36,37] Thrombin produced during these phases also activates the transglutaminase factor XIII, which catalyzes formation of covalent bonds between γ– chains and between γ– and Aα–

chains.[38] Cross-linking increases the elasticity of individual fibers in the clot[24], as well as the overall viscoelasticity of the fibrin clot.[16] Ultimately, fibrin promotes the generation of plasmin to degrade the clot, and re-establish a blood supply for new cells proliferating at the site.

During a coagulation reaction, the concentration of free thrombin present can range from less than 1 nM to greater than 500 nM. When thrombin generation is followed using chromogenic or fluorogenic substrates, a characteristic pattern emerges.[39–42] This pattern correlates with the phases of coagulation described above. The small amounts of thrombin produced during the initiation/amplification phases are not detectable in amidolytic assays (lower limit of detection ~1 nM), resulting in a "lag" phase in the thrombin generation profile. The large burst of thrombin generation that occurs in the propagation phase on the activated platelet surface produces a "maximum rate of increase" in detectable free thrombin. When the rates of thrombin production and thrombin inhibition reach equilibrium, the thrombin generation profile exhibits a "peak." Finally, the drop in thrombin production allows the amount of free thrombin to decrease, which is seen as a return to baseline in the thrombin generation profile. The total amount of free thrombin measured during the reaction course is referred to as the "area under the curve" (AUC) or "endogenous thrombin potential" (ETP).

The turbidimetric pattern of fibrin clot formation during *in situ* thrombin generation resembles the pattern seen after exogenous thrombin is added to purified fibrinogen. A lag phase, followed by a rapid increase in turbidity indicates clot formation in both situations. However, the "lag" phase of clot formation during *in situ* thrombin generation is more complex, reflecting not only protofibril formation, but also the time required to generate thrombin on the tissue factor-bearing cell surface, activate cofactors and platelets, and commence thrombin generation on the surface of activated platelets.

The biochemical environment required for *in situ* thrombin generation can profoundly affect fibrin formation. Assembly of procoagulant complexes and generation of thrombin requires calcium, which shortens the onset of clotting and produces thicker fibrin fibers than are seen in the absence of calcium.[18] Additionally, several plasma proteins can directly or indirectly affect thrombin activity and fibrin formation. Antithrombin inhibits free thrombin, effectively lowering the apparent thrombin concentration, and resulting in prolonged times to gelation and thicker fibrin fibers.[43–47] Albumin, γglobulin, and hemoglobin shorten the onset of fibrin clot formation, a phenomenon hypothesized to be caused by their influence on macromolecular interactions in the clotting solution.[45,48] Further, these proteins cause significant differences between magnetic birefringence curves of fibrin polymerization in recalcified plasma and those of pure fibrinogen and thrombin solutions.[45] Cells can influence local fibrin structure via direct interactions between integrins and fibrin(ogen). Cellular integrins organize fibrin into tighter bundles near the cell surface than are seen more distally within the clot. Furthermore, cell-associated fibrin is more resistant to fibrinolysis than distally-located fibrin.[49–51] Cells also release intracellular stores of soluble proteins that influence clot formation and stability, including factor XIII, plasminogen activator inhibitor-1 (PAI-1), and fibrinogen.[49,52–55]

Effect of the thrombin generation pattern and location on fibrin clot formation and structure

Differences in cellular procoagulant activity and plasma factor levels can alter the relative influences of extrinsic and intrinsic activities during coagulation.[13,14,41,56–61] Variations in these activities produce different patterns of thrombin generation, causing variations in the concentration of thrombin present during protofibril and fiber formation. Fibrinopeptide release may occur under low, medium, or high thrombin concentrations, resulting in significantly different kinetics of fibrinopeptide release and fibrin polymerization compared

to assays in which a single thrombin concentration catalyzes the release of all fibrinopeptides. Since fibrinopeptide release dictates protofibril formation and lateral aggregation, clots produced during *in situ* thrombin generation contain considerably heterogeneous fibrin structures.[13,14,45,62,63] Thus, the composition of a given clot may be quite specific to the circumstances under which it formed. Additionally, following formation of the initial clot, fibrin-bound thrombin released during clot lysis can modulate subsequent platelet procoagulant activity and fibrin deposition.[64–66]

It is currently hypothesized that extrinsic activities (on the tissue factor-bearing cell) and intrinsic activities (on the surface of activated platelets) play specific, independent roles during different stages of thrombin generation and fibrin formation.[67–70] Low thrombin concentrations (less than 1 nM) are sufficient to trigger the onset of fibrin formation, and can be rapidly produced via extrinsic activities on surface of tissue factor-bearing cells. Thus, the onset of clot formation depends on the nature and procoagulant properties of the tissue factor-bearing cells. Since different tissue factor-bearing cells support different levels of procoagulant activity, they differ in their ability to initiate fibrin formation.[68,71–73] Interestingly, Ovanesov *et al*. (2005) showed that the rate of thrombin generation on tissue factor-bearing cells does not influence the rate of propagation of fibrin into the plasma milieu.[68] Rather, propagation of fibrin away from the site of initiation depends on the plasma composition and procoagulant activity of the surrounding milieu.[68] These findings suggest that thrombin generated on the surface of platelets during the propagation phase dictates that rate of fibrin clot formation away from the initiating cells.[67–70]

Fibrin formation during cell-mediated thrombin generation likely includes a complex spatial component as well. It is likely that this physical progression of procoagulant activity from the tissue factor-bearing cell (low levels of thrombin generation) to the activated platelet surface (rapid burst of thrombin generation) induces the formation of a thrombin gradient in space. Given the direct effects of thrombin concentration on fibrin formation, this gradient may therefore, cause the formation of a range of fiber thicknesses across a region of growing thrombus.

Pathologic effects of altered thrombin generation on fibrin clot formation, structure and stability

Many studies have shown that the fibrin structure strongly influences the mechanical and fibrinolytic stability of the clot. Current thinking suggests that these differences in stability have significant physiologic implications. Dense, highly stable clots are associated with thrombosis; while, loosely-woven, unstable clots are associated with bleeding disorders.[12,28–30,74–78] We and others have correlated abnormal patterns of thrombin generation with the formation of abnormal fibrin clots. We hypothesize that the altered thrombin generation pattern contributes to thrombotic or bleeding complications via its influence on fibrin structure and stability.

Hyperprothrombinemia

The G20210A mutation in the 3′ untranslated region of prothrombin causes increased plasma levels of prothrombin. Several studies have identified an increased risk of venous thrombosis in individuals with this mutation.[79–81] In *in vitro* assays, elevated prothrombin levels increase the maximal rate, peak and area under the curve of thrombin generation.[13,41,59,82] We have shown that elevated (pro)thrombin levels trigger the formation of densely-packed fibrin clots composed of thin fibrin fibers compared to normal clots.[13] Increased thrombin generation in these individuals also increases activation of the thrombin-activatable fibrinolysis inhibitor (TAFI) *in vitro*.[83] Activated TAFI downregulates fibrinolysis by cleaving C-terminal lysine

residues from fibrin and reducing the number of tPA and plasminogen binding sites on fibrin. It has been suggested that the combination of abnormal structure and increased TAFI activation reduces the rate of fibrinolysis and contributes to the increased risk of thrombosis in these individuals.

Hemophilia and recombinant factor VIIa

The bleeding disorders hemophilia A or B result from deficiency in factors VIII or IX activities, respectively. Hemophilic plasma exhibits relatively normal extrinsic (initiating) activities on tissue factor-bearing cells, but reduced intrinsic (propagating) activities on platelets. Skin biopsies of wounds in hemophilic patients show a thin peripheral layer of fibrin deposited around a central region containing relatively few fibrin fibers.[84] These findings are consistent with normal, low level thrombin and fibrin generation on and near tissue factor-bearing cells at the periphery of the wound, but deficient thrombin propagation and fibrin formation into the central region of the wound. *In vitro* assays modeling hemophilic conditions demonstrate delayed onset of measurable thrombin generation, as well as a reduced maximum rate and peak of thrombin generation.[14,41,42,85] These reduced rates and levels of thrombin generation decrease the rate of fibrinopeptide release and delay fibrin formation[85–87] Additionally, *in vitro* assays indicate that hemophilic clots are composed of abnormally thick fibrin fibers and have increased porosity compared to normal clots.[14,88,89] The fiber composition of hemophilic clots decreases their mechanical stability and makes them highly susceptible to fibrinolysis.[14,90,91] In vitro studies suggest that reduced thrombin levels limit TAFI activation, leaving clots more vulnerable to fibrinolysis.[90,92,93] The physiologic importance of TAFI in clot stability is unclear, however, since TAFI-deficient animals exhibit minimal to no bleeding.[94–96]

Recombinant factor VIIa (rFVIIa, NovoSeven) is approved for the treatment of severe bleeds in hemophilic patients with inhibitory antibodies, and has demonstrated efficacy under these conditions and in patients with acquired hemophilia.[97] By binding to the surface of activated platelets, rFVIIa reestablishes platelet tenase activity and restores thrombin generation on the platelet surface.[98] RFVIIa increases the maximum thrombin generation rate and shortens the time to reach the peak thrombin level in hemophilic conditions.[14] *In vitro* assays of hemophilic plasma and whole blood have shown that rFVIIa activity shortens the onset of clot formation and enables the formation of clots with thinner, more normal fibrin fibers, significantly improving the clot structure, porosity, and stability.[14,87,89,91] By increasing the rate of fibrin formation, rFVIIa improves fibrin clot formation during a fibrinolytic challenge.[14] RFVIIa may further protect the clot by increasing TAFI activation.[93] Recently, several “superactive” analogs of rFVIIa have been described and tested *in vitro* and in animal models. These analogs demonstrate similar procoagulant and anti-fibrinolytic properties as rFVIIa, but at significantly lower concentrations.[91,99,100]

Conclusions

In sum, current studies suggest that the thrombin generation pattern determines biochemical features of the resulting clot, including its rate of formation, structure, and mechanical and fibrinolytic stability. Understanding the relationships between parameters of *in situ* thrombin generation and clot formation is essential for determining requirements of hemostatic clot formation and developing novel hemostatic and anti-thrombotic agents.

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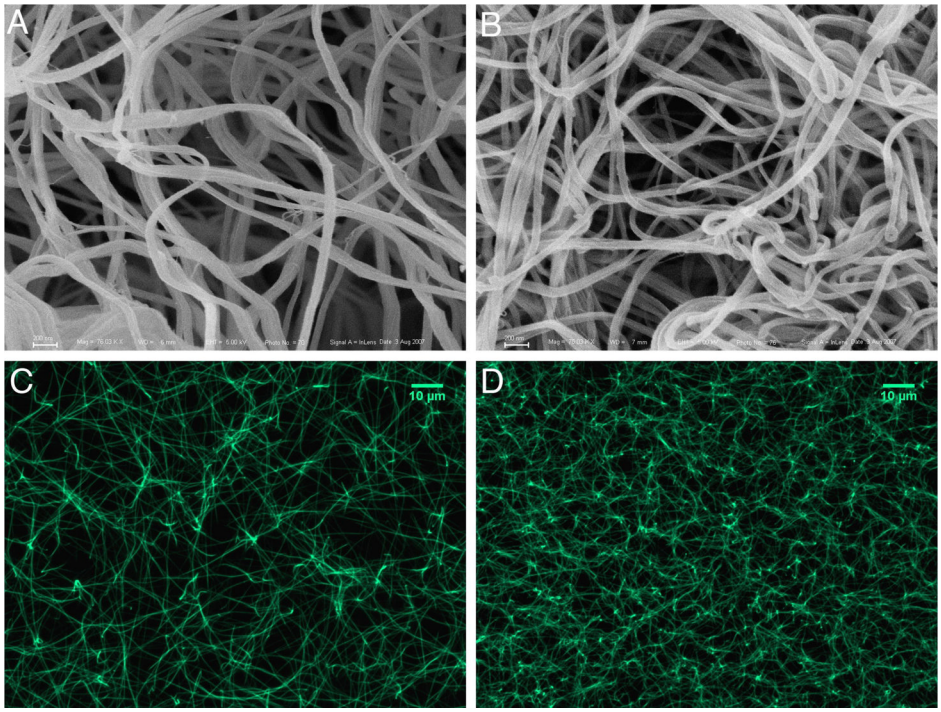

Figure 1.

The thrombin concentration present at the time of gelation dictates the fibrin clot structure. Scanning electron (A an B) and laser confocal (C and D) micrographs of fibrin clots formed by adding thrombin [2.5 nM (A and C), or 10 nM (B and D)] to purified fibrinogen (1 mg/mL) in 20 mM HEPES (pH 7.4), 150 mM NaCl. Scanning electron micrographs are at 76,030 × (micron bar is 200 nm). Laser confocal micrographs are at 63 X (micron bar is 10 μm).

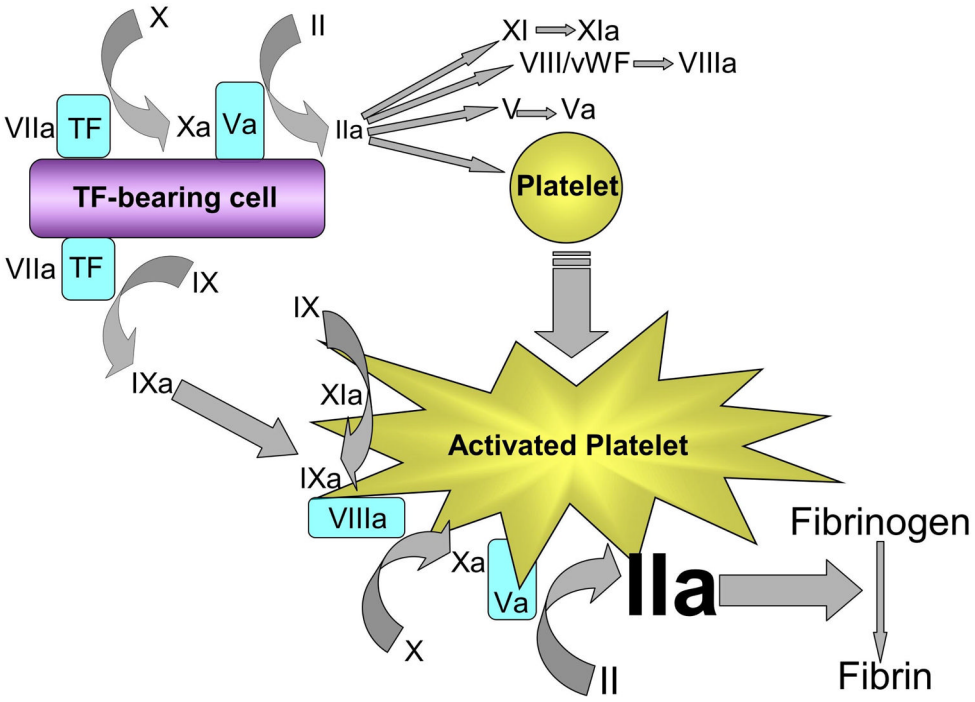

Figure 2.
Conceptual model of cellular tissue factor (TF)-initiated *in situ* thrombin generation. Please see text for details.