INVITED REVIEW

Basic mechanisms and regulation of fibrinolysis

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Summary. Fibrinolysis appears in many diverse physiological situations, and the components of the system are well established, along with mechanistic details for the individual reactions and some high-resolution structures. Key questions in understanding the regulation of fibrinolysis surround mechanisms of initiation and propagation, the localization of fibrinolysis reactions to the fibrin clot, and the influence of fibrin structure and clot composition on thrombolysis. This review covers these key areas with a focus on recent developments on fibrin structure and binding, the effects of a variety of cell types, the consequences of histones and DNA released by neutrophils, and the influence of flow. A complete understanding of the regulation of fibrinolysis will come from the building of detailed mathematical models. Suitable models are at an early stage of development, but may improve as model clots increase in complexity to incorporate the components and interactions listed above.

Keywords: fibrin; fibrinolysis; plasminogen; thrombolytic therapy.

Introduction

Fibrin is a substrate in fibrinolysis in two senses of the word, being both a surface for the binding and development of key reactions, and also a substance that an enzyme, plasmin, acts upon. These two features may also be viewed as delineating the two key steps of fibrinolysis which are the generation of plasmin followed by the digestion of fibrin. It is likely that the main players in the fibrinolysis pathways have been identified, and individual reactions have been extensively studied. The regulation of

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fibrinolysis involves different mechanisms, including protease action, serpin inactivation and conformational changes. Fibrin fiber diameter and clot architecture influence fibrinolysis, so clot stability and resistance is predetermined to a significant degree at the clot formation stage. This brief review covers selected aspects of fibrinolysis with a focus on recent developments that improve our understanding of regulation. Space does not allow for many important historic citations which are replaced by reference to more recent reviews and apologies are given to original authors.

Fibrin binding and the initiation of fibrinolysis

Tissue plasminogen activator (tPA) is probably the most widely studied plasminogen activator, as well as being extensively used clinically as a therapeutic thrombolytic (Alteplase), so much detail is available on its mechanism of action. Key in understanding the regulation of tPA activity is its colocalization with plasminogen on a fibrin surface [1] (elaborated in Fig. 1), leading to stimulation in activity of 10^2- to 10^3-fold, probably as a random-order process [2]. Early investigations identified the main fibrin binding sites, which are located primarily in the finger domain and kringle 2 of tPA [3], and one or more of the five kringle domains in plasminogen (see below). Kringle domains often (though not always) contain lysine binding sites (LBS) that bind to internal and C-terminal lysine residues. C-terminal lysine residues generated by plasmin are particularly important as a positive feedback mechanism for the stimulation of fibrinolysis.

Fibrin binding sites

A molecule of fibrinogen is a dimer where each subunit is composed of three polypeptide chains forming distinct structural regions, crucially a central E domain, composed of N-terminal regions from each half of the dimer and two symmetric distal D-domains (for a review of fibrinogen structure, see [4] and Fig. 1A). Each fibrinogen chain contains 104 lysine residues, but intact fibrin initially has no C-terminal lysines. This initial fibrin structure demonstrates only a weak affinity for the native full length form of plasminogen (Glu-plasminogen), with K_d
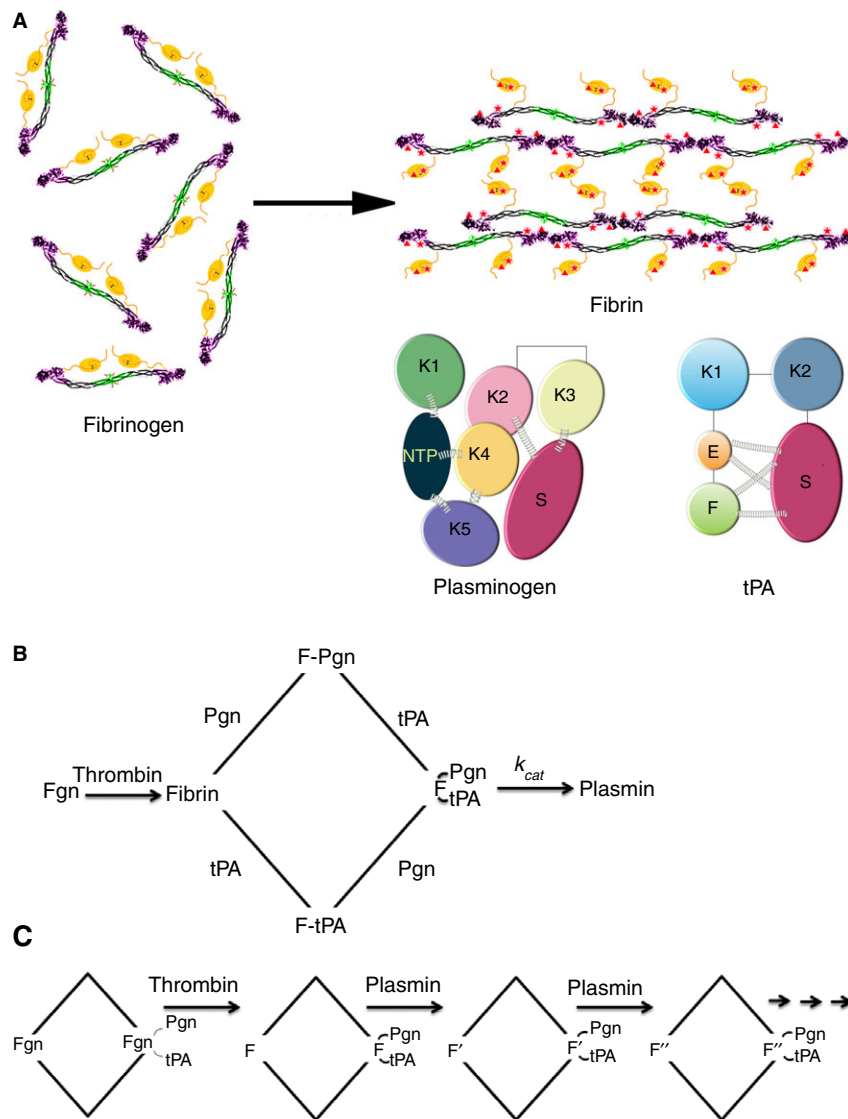
Fig. 1. Proposed scheme for initiation and propagation of fibrinolysis. Panel (A) shows the formation of fibrin highlighting the central (E-) domain (green) of fibrinogen with the N-terminal fibrinopeptides A and B (red) that are cleaved from the Aα- and Bβ-chains by thrombin when fibrinogen is converted to fibrin. The distal (D-) domains (purple), composed of the C-terminal portions of the Bβ- and γ-chains, interact with the E- and D-domains of adjacent fibrin monomers to form double-stranded protofibrils. The αC-domains (yellow) meet in the central part of the fibrinogen molecule, initially connecting non-covalently in fibrin to form an extensive 3D network. At a later stage, FXIIIa forms isopeptide bonds between γ- and α-chains in adjacent monomers. The location of potential initial binding sites for plasminogen (stars) and tPA (triangles) is indicated. Also shown are models of Glu-plasminogen and tPA indicating intramolecular bonding of domains, adapted from [15,16] and [61], respectively. Panel (B) shows reaction sequences occurring during fibrinolysis as a template mechanism where a ternary complex of fibrin-tPA-plasminogen (F-tPA-Pgn) forms to stimulate the generation of plasmin. The series of reactions in C shows the change from fibrinogen (with weak, kringle-dependent binding sites [62,63]) to fibrin and subsequent series of fibrin degradation products (F, F′, F″, etc.). Early events in fibrin formation include exposure of cryptic binding sites, indicated in panel (A). Plasmin generates C-terminal lysines providing a positive feedback mechanism through enhanced plasminogen binding. Fibrin degradation leads to aggregate formation to focus the binding of tPA (via finger domain) and plasminogen (at C-terminal lysines) around amyloid-like cross-β structures to complete fibrinolysis through a series of fibrin degradation products, culminating in DDE the smallest FDP to bind tPA and plasminogen.

values in the 10^{-5} M range, higher than the plasma concentration of plasminogen (around 2 μM) [1,5]. However, published K_d values for the binding of plasminogen and tPA molecules to fibrin are highly variable (e.g. reviewed [6]).

Models for the initiation and propagation of fibrinolysis have been developed, built upon binding and kinetic studies using fibrinogen and fibrin fragments, synthetic peptides, X-ray structure data, monoclonal antibodies and natural variants of fibrinogen [4,7], and are summarized in Fig. 1. Work with fibrinogen peptides drew attention to the sequences Aα148-160 (as a kringle-dependent binding site, most likely for plasminogen) and γ312-324 (as a binding site for tPA via the finger domain).

Recombinant αC domain, Aα221-610, was also found to include lysine dependent but distinct binding sites for both tPA and plasminogen. Binding sites were localized to the C-terminal portion from residue 392 and demonstrated a high affinity for tPA and plasminogen (K_d 16–33 nM), but this region in fibrin is cleaved early by plasmin. However, this picture is complicated by other findings that show a high dependence of tPA binding to C-terminal lysines in fibrin degradation product, DDE domain complexes. There are 8 C-terminal lysines, and removal of four could drastically reduce the stimulation of plasminogen activation by tPA [8]. A precise, simple picture of the unmasking of specific binding sites during fibrin formation is further challenged by longstanding observations that tPA and plasminogen bind to many denatured, aggregated or modified proteins [9]. tPA binding and plasminogen activation via this mechanism has been termed the cross-β structure pathway [10]. According to this mechanism, stacked β-sheets (cross-β structures with amyloid properties) bind to tPA finger domain residues with a particular alternating charge sequence, Arg7, Glu9, Arg23, Glu32, Arg30, while plasminogen binding and activation relies on C-terminal lysines. These observations bring into focus binding to fibrin fibrils or aggregates. For example, addition of tPA to preformed clots resulted in concentration of tPA to a narrow lytic zone and the development of fibrin 'agglomerates' that tightly bind plasminogen and tPA [11,12]. More recently, confocal microscopy studies have shown green fluorescent protein fusions of tPA (tPA-GFP) bind fibrin aggregates [13], primarily through the finger domain. Fingerless tPA-GFP (ΔF tPA-GFP) interacts more weakly with aggregates, results that agree with kinetic studies where it was estimated that finger interactions account for 80% of the binding of tPA to fibrin [14]. It was also observed that aggregates form preferentially in fibrin composed of thick fibers, not fine fibers, and stain with thioflavin T, a well-known marker for cross-β structures (see Fig. 2). Together, these results suggest a mechanistic link between binding to fibrin and other protein aggregates, and highlight differences in fibrinolysis between thin and thick fibrin fibers (see below). Thus, some of the later fibrin structures shown in Fig. 1C (F″ etc.), are aggregates that concentrate tPA and plasminogen, at least in fibrin composed of thick fibers (common in plasma clots [11]). The variety of modes of interaction with fibrin and different fibrin structures involved may explain the difficulties in providing consistent estimates of K_d values for tPA and plasminogen binding [6].

Plasminogen binding

Significant progress has been made recently from the structural analysis of full length plasminogen (Glu-plasminogen, Glu1-Asn791) [15,16]. These structures shed light on long-established observations, such as the role of Cl⁻ ions in maintaining the closed conformation; the affinity of different kringles for lysine analogues; and the role of different kringles in triggering conformational changes. In particular, the structures help explain the resistance of Glu-plasminogen to activation and how fibrin binding promotes activation to plasmin. Thus, a key feature of control of plasminogen activation is termed 'conformational regulation' due to the relatively inert closed spiral structure of Glu-plasminogen in which the activation cleavage site at Arg561 is inaccessible to plasminogen activators (as is a pro-activation site at Lys77). Glu-plasminogen is a protein of seven domains,
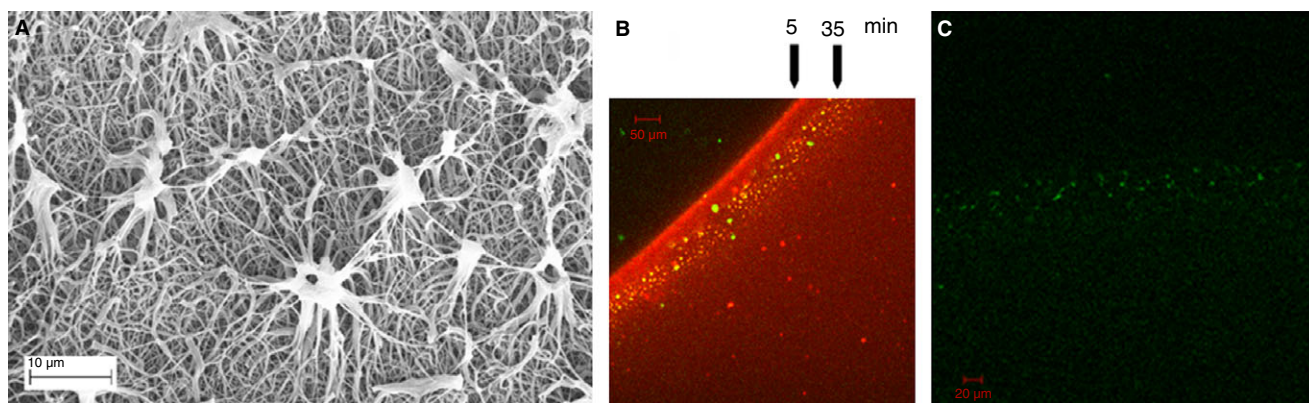
Fig. 2. Fibrin aggregate formation and characterization. Panel (A) shows fibrin aggregates from a scanning electron microscopy image following 10 min of fibrinolysis after tPA was added to the surface of a preformed, coarse fiber clot made with 5 nM thrombin. Panel (B) shows a similar clot made with orange labeled fibrinogen treated with tPA fused to jellyfish green fluorescent protein (tPA-GFP). Fibrin aggregates are red spots and when merged with the green tPA-GFP image appear yellow, illustrating the strong association of fibrin aggregates and tPA. The arrows indicate positions of the lysis front in two overlaid images showing the diffuse surface accumulation of tPA at 5 min and the appearance of aggregates after 35 min at the new lysis front. Panel C shows the staining of fibrin aggregates by thioflavin T (ThT) after 45 min of fibrinolysis with native (unlabeled) tPA. ThT fluorescence indicates the presence of amyloid-like cross-β structures, which are able to bind the finger domain of tPA (images adapted from [13]).

N-terminal peptide (NTP), kringles 1–5 and serine protease domain, and interdomain bonds particularly between Lys50, Arg 69 and Arg70 of the NTP with kringles 4 and 5 that help maintain the closed structure (see Fig. 1A). If the NTP is cleaved at Lys77 by plasmin (generating Lys-plasminogen, Lys78-Asn791), the structure unfolds to become more linear and Arg561 is accessible to plasminogen activators. Lys77 is hidden in Glu-plasminogen but may become available following a sequence of conformational changes. It is proposed that kringle 1 initially binds fibrin and triggers undocking of kringles 4 and 5 from the NTP to expose Lys77 and Arg561. However, other interesting aspects of the activation pathway may involve differences between plasminogen glycoforms and kringle 3, which has no LBS. Glycoform I of plasminogen has two carbohydrate moieties, one at Asn289 (on kringle 3) which may destabilize the Glu-plasminogen closed conformation and enable kringle 3 to be mobile. Furthermore, two different conformations were found in the crystal structure of glycoform II, and one was partially open such that kringle 5 was available for lysine binding [15]. This is particularly interesting considering kringle 5 has a preference for internal lysines, which could be relevant to the initiation of fibrinolysis, before the generation of significant plasmin that could produce C-terminal lysines in fibrin.

Other activators

tPA is not the only plasminogen activator and it is important to appreciate the variety of mechanisms that exist to generate plasmin. Single chain urokinase plasminogen activator (scuPA) is a zymogen precursor of active 2 chain urokinase (uPA) and although scuPA/uPA is mostly linked to cell-associated fibrinolysis (in association with a specific receptor uPAR or CD87), studies with knockout mice suggest a role in intravascular fibrinolysis [17]. scuPA activity is fibrin-specific to some extent, possibly by a number of mechanisms, although uPA has no direct affinity for fibrin [18]. uPA activates plasminogen in solution, so does not rely on a colocalization mechanism like tPA, and uPA is more sensitive to the open conformation of plasminogen [14]. Thus, while 'antifibrinolytics' such as tranexamic acid or aminohexanoic acid block plasminogen binding to fibrin and inhibit tPA activity, they bind to kringle LBS to open up the inert conformation of Glu-plasminogen and enhance activation by uPA, but not tPA [14]. This conformational rather than colocalization mechanism is confirmed by studies on 'fibrinolytic cross talk' which describe scuPA or uPA bound to cells or microvesicles activating plasminogen (in an appropriate open conformation) bound to a different surface [19].

Bacteria have adopted a number of strategies to promote invasion that involve hijacking the host plasminogen system. Streptokinase (SK) is a first-generation thrombolytic and the therapeutic molecule, which is isolated from *Streptococcus equisimilus*, a Lancefield Group C strain, is the most widely studied. However, other SK variants have distinct mechanisms that rely on interactions with bacterial cell surface proteins such as PAM and M1, which bind plasminogen and fibrinogen, respectively [20]. Other bacterial proteins, including another binding protein, staphylokinase from *Staphylococcus* and the pla enzyme from *Yersinia pestis*, have been extensively studied, and much information regarding mechanism of action, structural–function relationships, is available [15,16,21].

Inhibition of fibrinolysis

Mechanisms are needed to reduce unwanted systemic plasmin generation to avoid excessive degradation of plasma proteins. Inert conformations of plasminogen and fibrinogen represent an important barrier to plasminogen activation, bearing in mind single chain tPA is an active enzyme, not a zymogen [22]. The two most critical serpin inhibitors in fibrinolysis are plasminogen activator inhibitor 1 (PAI-1) and alpha-2 plasmin inhibitor (α_2PI, or α_2-antiplasmin). Both these inhibitors circulate at concentrations in the same range as their potential enzyme targets and both are very potent with second-order rate constants for inhibited complex formation around 10^7 M^{-1} s^{-1}, close to the diffusion controlled limit. However, plasmin bound to fibrin, or lysine analogues, is inhibited much more slowly by α_2PI, allowing time for plasmin to act where needed, and most protection appears with the formation of C-terminal lysines [23]. PAI-1 inhibits both uPA and tPA and the rate constants quoted are also high, but these values refer to free solution and are also modulated by fibrin and fibrinogen [22]. Other serpins may form complexes with plasmin, tPA or uPA, including PAI-2 and PAI-3 and protease nexin; and α_2-macroglobulin also forms a further back up.

Within a clot, fibrinolysis may be inhibited by a metalloproteinase, thrombin-activatable fibrinolysis inhibitor (TAFIa) or carboxypeptidase U (CPU) [24,25]. This protein circulates in an inactive zymogen form (TAFI or pro-CPU) and is activated by thrombin/thrombomodulin or plasmin during on-going fibrinolysis. Once activated, the enzyme cleaves C-terminal lysines in fibrin, critical for the binding of plasminogen as discussed above, and mechanistic studies have identified a threshold behavior and the involvement of other plasma inhibitors, including α_2PI. Several approaches suggest TAFIa acts predominantly by reducing binding of plasminogen or plasmin, rather than through lysine binding of tPA via kringle 2 [14,23,26]. The crystal structure of TAFI has been solved and provides a rationale for the known thermal instability of the protein at 37 °C, which forms an important regulatory mechanism [27,28]. Both PAI-1 and TAFI, being associated with adverse cardiovascular events or cancer (in the case of PAI-1), have been targets for drug development [25].

Plasmin digestion of fibrin

The degradation of fibrin by plasmin is not well understood, probably accounted for in part by the difficulties of studying enzymology at a solid–liquid interface. As mentioned above, the αC-domains in fibrin are an early target for plasmin (cleavage of at least 10 bonds next to lysine or arginine residues results in a highly heterogeneous set of early degradation products [29]) followed by removal of a peptide from the N-terminal of the β-chain (cleaved primarily at Arg42 [30]) and cleavage of the coiled coil connector of the E- and D-domains [31] (at αLys81-βLys122-γLys59 or αArg104-βLys133-γLys63 [32]). Several lines of evidence suggest that efficient solubilization of the fibrin meshwork requires only 25% of the total E–D connections need to be broken and 50% of the fibrin monomers can remain intact [33]. However, all three polypeptide chains of the triple helical structure within a fibrin monomer must be cleaved through the same cross section in both adjacent monomers within a protofibril and in all adjacent protofibrils within a fiber. The cited low fraction of E–D connections broken at dissolution suggests that cleavage of fibrin fibers is achieved by clustering of plasmin molecules at points on a fiber rather than uniformly along the fiber, and evidence for preferential transversal cleavage of the fibers is provided by atomic force microscopic images of lysing fibrin [34]. The clustering of the enzyme optimizes the pattern of cleavage, but it has some negative impact on the kinetics of enzyme action. The macroscopic consequence of plasmin clustering was found to be a gradual decay of its lytic efficiency, which was quantitatively expressed in fractal kinetic terms as a time-dependent increase of the Michaelis constant (K_m^F) of plasmin [34]. A further observation was that low concentrations of the lysine analogue aminohexanoic acid can promote plasmin digestion of fibrin by reducing clustering, in agreement with earlier studies [35].

Clot architecture

Fibrin structure

There is a link between fibrin structure and risk of cardiovascular events [7]. Many studies (e.g. [36]) evidence that thin fibers (formed at high thrombin concentrations for example [37]) are more difficult to dissolve on a macroscopic scale than thick fibers, despite the faster digestion of individual thin fibers. This apparent contradiction may be explained by efficient plasmin action on tightly packed monomers within a single thick cross section, avoiding slower steps where plasmin must diffuse through the pores of the network. Many factors other than thrombin concentration can regulate clot structure [7,38], including some specific cellular interactions which will be dealt with below.

Platelet effects on clot structure   In vivo, fibrin is formed at sites of blood vessel injury where platelets are also activated and bind fibrin. Thus, the strong adhesive forces between platelets and fibrin, in combination with platelet contraction, place the fibers under tension that modulates the clot structure, stiffens fibrin and increases its density in the platelet-rich areas ('clot retraction') [39]. Similar mechanical stress is exerted on fibrin on the surface of non-occluding intravascular thrombi exposed to mechanical shear generated by circulating blood, which profoundly alters the fibrin architecture: fibers become longitudinally aligned with a smaller diameter and pore size compared to the randomly running fibers in the interior of the same thrombi [40]. Stretching of *in vitro* fibrin clots can be used to model the structural consequences of platelet- and shear-related mechanical forces. Electron microscopic and small angle X-ray scattering observations evidence that mechanical stress causes unfolding of the coiled coil region in the fibrin monomers and exposure of hydrophobic residues [41]. Consequently, fluid is expelled from the vacant space among the protofibrils within the fibers resulting in a decrease in fiber diameter and an increase in the protein density of the fibers. Although unfolding generally renders proteins more susceptible to enzymatic degradation, the mechanically stressed fibrin is more resistant to plasmin digestion [40].

Proteolytic resistance conferred to fibrin by chemical modifications can also be explained by similar changes in clot morphology. Factor XIIIa (FXIIIa) is a transglutaminase of plasma or platelet origin, known to introduce γ-glutamyl-lysine cross-links between γC- and αC-domains of adjacent fibrin monomers (reviewed in [42]). This covalent cross-linking results in a significant reduction (by about 20%) of the fiber diameter without any change in the number of protofibrils in a fiber, implying that FXIIIa tightens the lateral attachment of protofibrils and decreases the volume of the vacant fluid space within the fibers [37], accompanied by a two-fold reduction of the pore size as assessed from clot permeability measurements [43]. Fibrinogen variants have been used to dissect the roles of α- and γ-crosslinks, and α-crosslinking of fibrin fibers was found to affect appearance, biophysical properties and delay fibrinolysis [44]. Platelets are also a source of polyphosphate, which when released from activated platelets results in localized changes in fibrin structure. Polyphosphate of sufficient length has been found to result in thicker fibrin strands that were more resistant to fibrinolysis [45].

In a plasma environment, additional factors appear to take a leading role as a determinant of the lytic resistance conferred by FXIIIa. α$_2$-PI can serve as a substrate of FXIIIa, and its covalent attachment to fibrin appears to be indispensable for the lytic stabilization of plasma clots [46]. Depletion of α$_2$-PI eliminates the antifibrinolytic

effect of FXIIIa, and lysis rates are inversely correlated with the amount of α_2-PI cross-linked to fibrin in plasma clots. The antifibrinolytic effect of platelet FXIIIa is also dependent on the availability of α_2-PI [47]. Further insights into conflicting results concerning the mechanism of clot stabilization by FXIII—directly altering fibrin structure and the requirement of α_2-PI—may require close evaluation of experimental methods, including the role of plasma proteins, platelets and flow.

Red blood cells Although the size of pores between fibrin fibers (typically 160–380 nm in diameter) allows free diffusion of macromolecules, if fibrin is formed in blood, then the entrapped cells cause a significant reduction in molecular diffusion coefficients [48]. This pore-filling effect alone could further impair plasmin diffusion, a rate defining feature discussed above, but the most abundant blood cells, the erythrocytes, are more than simply inert bystanders in the clot. They are involved in active interactions with fibrin through an integrin receptor [49], and their retention in the clot requires FXIIIa [50]. Thus, fibrin can transmit the contractile force of neighboring activated platelets to red blood cells causing a change of their shape from biconcave to polyhedral [51], resulting in almost gap-free compaction of red blood cells in the vacant space between fibrin fibers and forming a structure with a stronger diffusion barrier and higher lytic resistance [52]. Red blood cells entrapped in thrombi express phosphatidylserine on their surface [53], which supports the assembly of prothrombinase complex and accelerates thrombin generation [54], and at higher thrombin concentrations, a fibrin structure with higher lytic resistance is formed. Increasing the fractional volume of fibrin occupied by red blood cells from 0 to a physiologically relevant hematocrit value of 0.4 causes a reduction of the median fiber diameter from 150 to 96 nm [52]. As discussed above in relation to mechanical and enzymological factors, such alterations of fibrin structure result in lytic resistance. The antifibrinolytic effects of red blood cells could be reversed by an integrin antagonist, eptifibatide, suggesting a therapeutic approach to thrombosis through the pharmacological blockade of the red blood cell–fibrin interactions [52].

Neutrophils Neutrophils have been identified as having a role in fibrinolysis. DNA and histones are components of 'neutrophil extracellular traps' (NETs) released by activated neutrophils at sites of infection and in intravascular thrombi (reviewed in [55]), and the effects of NET components on fibrinolysis have been summarized [56]. Briefly, histones alone or in complex with DNA result in thicker fibrin fibers and more robust clots (as shown in rheology studies), whereas DNA alone causes the opposite effects. Combinations of DNA and histones were found to have a major effect on clot lysis by being able

to hold lysing fibrin together to delay fibrinolysis. Isothermal titration calorimetry studies showed that large FDP strongly bound to histones and high molecular weight DNA, which could stabilize lysing clots, results that provide a rationale for the use of DNase as an adjunct to thrombolytic therapy [56]. Overall, the conclusions from these studies are that DNA and histones may strengthen clots and delay their complete dissolution.

Models of fibrinolysis

A number of groups have attempted to model blood coagulation (or study 'blood systems biology' [57]). Multiscale models of coagulation that include multiple reactions, flow, concentration gradients and platelets have been developed with some success, but models of fibrinolysis have lagged behind, for number of reasons. An issue with some fibrinolysis models was the approximation that fibrin was distributed homogenously rather than in fibers, which does not permit any investigation of the effects of fibrin architecture. Bannish *et al.* [58] approached this problem by developing a one-dimensional reaction-diffusion model that included a concentration of fibrin that was fixed but heterogeneously distributed into fiber patterns. As the lysis front travelled through the clot, the model was able to replicate some features seen in *in vitro* clot lysis experiments, including concentration of tPA and plasminogen at the lysis front (reported to be up to 30-fold increased for plasminogen in aggregates in the 3 μm lysis front [11]). However, the authors concluded the model was not entirely satisfactory and suggested that low concentrations of circulating tPA, amounting to three molecules per μm^3, argued in favor of a stochastic approach. Their 3D stochastic multiscale model [59] followed a number of tPA molecules binding to fibrin and activating bound plasminogen leading to transverse cutting of fibrin fibers, rather than uniform degradation that limited the earlier model. Detailed biochemical considerations, including binding and unbinding terms and uncovering of cryptic sites (C-terminal lysines), were included to develop the first stage microscale stochastic model, providing results for a subsequent macroscale stage to simulate transport of reactants and large-scale fibrin clot lysis. Very recently, we have validated the 3D stochastic model over ranges of tPA and plasminogen concentrations in clot lysis experiments using standardized clots fully characterized by scanning electron microscopy, with good results [60]. Future work will allow hypotheses to be tested and generated to better understand the regulation of fibrinolysis by the activators and inhibitors discussed above, and explore the significance of fibrin architecture, flow and cellular interactions.

This review has touched on the basic principles of fibrinolysis and highlighted current knowledge on important regulatory features. Improved understanding of regulation

may lead to advances in thrombolytic therapy, which is still widely practiced, for stroke and myocardial infarction. Infectious diseases are also major sources of human mortality and morbidity and involve interactions with host fibrinolysis pathways that provide targets for therapeutic interventions. Considering the range of physiological processes that involve fibrinolysis reactions, greater knowledge of the regulation of this system has significant potential for improving human health.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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