The Biochemical and Physical Process of Fibrinolysis and Effects of Clot Structure and Stability on the Lysis Rate

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Abstract: The effectiveness of fibrinolysis results from the combination of regulated enzymatic activity and the physical properties of the fibrin scaffold. Physiologically, clots or thrombi are dissolved from within *via* internal lysis. In contrast, with therapeutic thrombolysis, lytic agents are introduced at one surface and lysis proceeds across the thrombus. In the latter case, there are complex changes that take place at the lysis front in a narrow zone. However, at the microscopic level the mechanisms for either general type of fibrinolysis appear to be similar. Fibrinolysis proceeds by fibers being transected laterally, rather than digestion of fibers by surface erosion from the outside. A molecular model to account for these observations together with what is known from the biochemical characterization of fibrinolysis involves the movement of plasmin laterally across fibers, binding to sites created by its own proteolytic activity. Fibrin clots can have a great diversity of structural, biological, physical, and chemical properties depending on the conditions of formation, and the rate and nature of fibrinolysis is related to these properties. In general, the rate of lysis appears to be faster for clots made up of thicker fibers than for clots made up of thinner fibers, but the lysis rate is not simply a function of fiber diameter and also depends on other physical properties of the clot. Platelet aggregation and clot retraction have a dramatic effect on the structure of fibrin and hence on fibrinolysis.

Key Words: Fibrinogen, fibrinolysis, plasminogen, tissue type-plasminogen activator, thrombolysis, microscopy, fibrin structure, blood clot.

BIOCHEMICAL PROCESS OF FIBRINOLYSIS AND ITS REGULATION

Formation of fibrin is an intermediate step in hemostasis followed by fibrin dissolution after a clot is made [1, 2]. In order to prevent blocking of the lumen of blood vessels and continuous cessation of blood flow, the biochemical "ramrod" exists, called the fibrinolytic system. This is a powerful enzymatic ensemble designed to dissolve fibrin and recanalize the blood vessels. The phenomenon of spontaneous lysis of the cadaveric blood clots was first described by Morgagni in 1769 [3] and the term "fibrinolysis" was introduced by Dastre in 1893 [4].

Fibrinolysis is the process of fibrin digestion resulting in dissolution of a clot or a thrombus, the latter process referred to as thrombolysis. The central enzyme in fibrin lysis is plasmin (Pn), a serine protease formed from its inactive precursor, named plasminogen (Plg), upon the action of activators. After activation, Pn cleaves a variety of substrates such as fibrin and extracellular matrix proteins, activates other proteases and growth factors. As a result, in addition to fibrinolysis, Plg and Pn are involved in a number of other physiological and pathophysiological processes such as wound healing, inflammation, cell migration, ovulation, embryogenesis, tumor growth and metastasis, angiogenesis, and atherosclerosis [5-9].

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Fibrinolysis occurs in two major steps, which are the conversion of Plg to Pn by a plasminogen activator (PA) and the degradation of fibrin by Pn. The first step occurs predominantly on the fibrin fibers or on a cell surface and the Pn activity is further reduced or eliminated by inhibitors. Attachment of Plg and the tissue-type plasminogen activator (t-PA) to the surfaces is mediated by the C-terminal lysine residues on a surface and the specific lysine-binding sites on the Plg and t-PA molecules. Structural differences between fibrinogen and fibrin affect the initiation of fibrinolysis [10].

There is a system of interdependent biochemical reactions comprising fibrinolysis and its regulatory mechanisms (Fig. **1**). First, a very small number of Pn molecules are generated from a portion of Plg that is initially present in the clot and is partially bound to fibrin. Because Pn cleaves peptide bonds formed by the carboxyl group of lysine, the new C-terminal lysines become exposed in the fibrin fibers, which provide additional binding sites for Plg and t-PA. Therefore, most of Pn activity is generated on the fibrin fibers after formation of a ternary complex of fibrin, t-PA, and Plg (Fig. **2**). The surface-bound Pn accomplishes the formation of a positive feedback loop and significantly accelerates the reaction by cleaving more fibrin and exposing new C-terminal lysine residues, which provide additional binding sites both for t-PA and Plg. Eventually fibrin is degraded to soluble fibrin degradation products as the clot is lysed.

The activation of Plg proceeding on fibrin and on cell surfaces is a highly regulated process. There are a number of mechanisms to moderate the activity of the primary profibrinolytic components, the tissue-type (t-PA) and urokinase-

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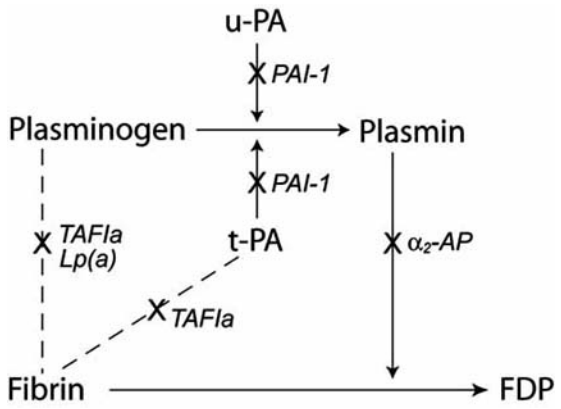

Fig. (1). Schematic representation of the major reactions of fibrinolysis and their regulation. Arrows show the biochemical conversions (horizontal arrows) under the action of the enzymes (vertical arrows). Dashed lines show the binding interactions. Crisscrosses show the inhibitory effects. t-PA – tissue-type plasminogen activator, u-PA – urokinase-type plasminogen activator, PAI-1 – plasminogen activator inhibitor-1, TAFIa – the active form of the thrombin-activatable fibrinolysis inhibitor, Lp(a) - lipoprotein(a), α_2-AP - α_2-antiplasmin, FDP - fibrin degradation products.

type (u-PA) Plg activators (Fig. **1**). Plasminogen activator inhibitor-1 (PAI-1) is the most potent inhibitor of t-PA and u-PA, whereas α_2-antiplasmin (α_2-AP) directly inhibits Pn. A thrombin-activatable fibrinolysis inhibitor (TAFI or TAFIa upon activation) cleaves C-terminal lysine residues from partially degraded fibrin and thus inhibits fibrinolysis by preventing the lysine-dependent binding of Plg with fibrin. Lipoprotein(a) [Lp(a)] has a structural homology with Plg and, therefore, may compete with Plg for binding to lysine residues and impair fibrinolysis [11]. As a result of these concerted reactions, the fibrinolytic system functions as a highly effective and vitally important mechanism in maintaining homeostasis and adaptability of an organism in various physiological and pathological states.

PLASMINOGEN (Plg) AND PLASMIN (Pn)

Plg is a 92-kDa glycoprotein present in many tissues and body fluids but mostly in blood plasma at a concentration of about 0.2 mg/ml (2 μM) [12, 13]. It is synthesized predominantly in the liver as a single polypeptide chain containing 810 amino acid residues, but during secretion a 19 amino acid peptide is cleaved, resulting in the 791 amino acid protein [12, 14]. The schematic structure of the human Plg molecule is presented in Fig. **3**. At the N-terminus the protein contains glutamate, hence named Glu-Plg. Native human Glu-Plg comprises the N-terminal ("preactivation") peptide (residues Glu1–Lys77), five homologous kringle domains (K1-K5) and the catalytic protease domain (residues Val562-Asn791) [15]. The kringle domains of approximately 80 amino acids are formed between residues Lys77 and Arg 561, each being cross-linked with three disulphide bonds [16]. The kringles are not unique to Plg, and have been identified in other proteins associated with hemostasis, such as prothrombin, t-PA, u-PA, factor XII, as well as Lp(a) and hepatocyte growth factors [17]. The kringle domains mediate Plg binding to various substrates, cofactors, and receptors, e.g., fibrin(ogen), bacterial proteins, mammalian cell surfaces, as well as small molecules like Cl^- and α,ω-diamino acids [18-21]. The binding of kringle domains to proteins occurs *via* C-terminal lysine residues with affinities in the 10^{-7}–10^{-5} M range [22, 23] and is followed by a great enhancement of the Plg activation rate due to a ligand-induced conformational change. Free lysine analogues, such as ε-aminocaproic acid, also bind to the kringle domains of Plg, causing the conformation-dependent enhancement of Plg activation [24, 25]. Interactions of human Plg with lysine-containing ligands take place with all kringles, except for kringle 3. Kringles 1 and 4 exhibit the strongest affinities for the lysine residues, physiologically relevant for binding to fibrin [26, 27], to extracellular matrix components [20], and to cells [28, 29]. The lysine-binding site of Plg kringle 1 consists of three aromatic residues, Tyr64/Trp62/Tyr72, lining the hydrophobic trough of the lysine-binding site and the double charged anionic (Asp55/Asp57) and cationic (Arg35/Arg71) centers located at opposite ends of the hydrophobic trough [30]. Kringles 2 and 5 have some affinity for soluble lysine analogues, but it is too weak to anchor Plg to surface lysines [31].

In order to study structure-function relationships, recombinant Plg derivatives can be expressed that contain only one kringle (mini-Plg) or no kringles (micro-Plg) [32, 33]. Three-

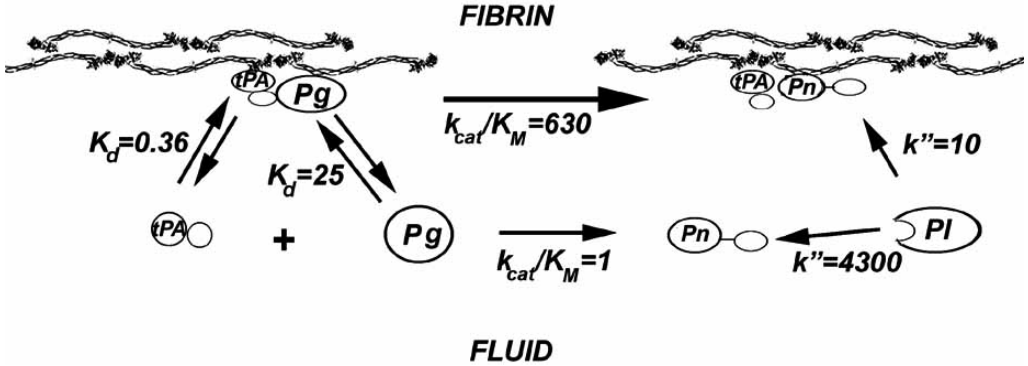

Fig. (2). Effects of fibrin on the generation and inhibition of plasmin. Abbreviations: Pg, plasminogen; Pn, plasmin; PI, α_2-plasmin inhibitor. The values for dissociation constants (K_d, μM) are from [359] for plasminogen and [74] for t-PA, catalytic efficiency (k_{cat}/K_M, $mM^{-1}.s^{-1}$) from [147], second-order reaction constant for inhibition of plasmin (k'', $mM^{-1}.s^{-1}$) from [360]. The empty circles in the structure of t-PA and plasmin indicate active protease domains. Reproduced with permission from [331].

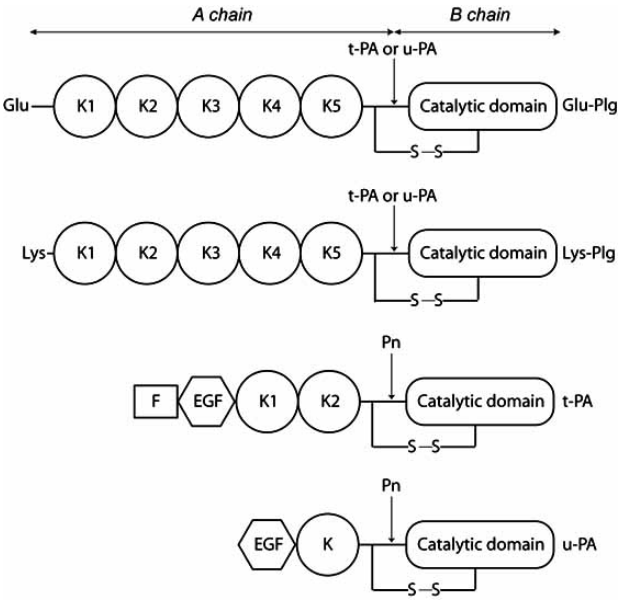

Fig. (3). Modular structure of Glu- and Lys-Plg, t-PA, and u-PA. K - kringle domains, F - finger domain, EGF - epidermal growth factor domain. Arrows point to the sites of cleavage resulting in transformation of the enzymes form single-chain to two-chain forms.

dimensional structures, obtained using X-ray crystallography and NMR, of the isolated recombinant kringles and the catalytic portion of human Plg have become available [34-41].

Plg has two major isoforms, type I and type II, which differ in the type of glysocylation [42]. Plg I contains one oligosaccharide chain attached to Thr345 and one attached to Asn289 [43, 44], whereas Plg II contains two glycans attached to Ser248 and Thr345 [45]. The binding sites for the O-glycans (Ser248 and Thr345) are located between kringles 2 and 3 and between kringles 3 and 4, respectively, while the site for the N-glycan (Asn289) is located in kringle 3 of Plg [45, 46]. Glycosylation of Plg is known to modulate its functionality. For example, the Plg type II is bound stronger to α_2-AP than type I (K_d=0.2 and K_d=0.9 μM, respectively) [47]; Plg type I and type II display different kinetics of activation by u-PA [48] and t-PA [46]. In addition, Plg II has at least six glycoforms differing in their sialic acid content, which decreases the catalytic efficiency of Plg activation by t-PA, u-PA, and streptokinase (SK) [49].

The conversion of Plg to proteolytically active Pn involves cleavage of the Arg561-Val562 bond near kringle 5, resulting in generation of the enzyme, which contains a heavy A chain of 561 amino acids and a light B chain of 230 amino acids (Fig. **3**) [50]. The A and the B chains in Pn are linked together by two disulphide bonds, Cys549-Cys666 and Cys558-Cys566. The active catalytic triad of Pn, as a serine protease with broad substrate specificity, is located in the B chain and consists of His603, Asp646, and Ser741. The substrate specificity of the activating enzymes towards Plg is determined by the Cys558-Cys566 disulphide bond, responsible for the cyclic nature of the peptide sequence surrounding the Plg activation site at Arg561-Val562 [51].

Another reaction concomitant with Plg activation is the Pn-catalyzed cleavage at either Lys62, Arg68, or Lys77 residues which yields the truncated forms, altogether referred to as Lys-Plg (Fig. **3**) [52, 53]. Native Glu-Pg is a compact molecule with intramolecular interactions between the N-terminal 1–77 peptide and kringle 5 in the C-terminal region [54, 55] and between kringles 3 and 4 [56], arranged in a spiral-type conformation [25, 54, 57]. This structure has been shown to protect the rupture bond (Arg561-Val562) from easy access of Plg activators [58]. Therefore, the proteolytic removal of the N-terminal peptide during Glu-Plg to Lys-Plg conversion results in transition from compact to an extended open conformation, which becomes more readily activated than Glu-Plg by either t-PA [59] or u-PA [60]. Because of improved fibrin binding and activation of Lys-Plg compared to Glu-Plg, the formation of Lys-Plg within a clot constitutes a positive feedback mechanism that can further stimulate the activation of Plg by t-PA as fibrinolysis proceeds. Obviously, the structure and the molecular weight of Glu-Pn or Lys-Pn depend on whether an enzyme was generated directly from Glu-Plg or Lys-Plg, respectively.

The development of gene-targeted mice either lacking or expressing altered forms of hemostatic factors has provided information on the biological role of Plg and Pn *in vivo* [61]. Such studies have demonstrated that Plg is critical for fibrinolysis and that disruption of Plg leads to widespread terminal vessel thrombosis in mice [62]. Although Plg plays a role in many other biological processes, and the consequences of its deficiency are severe, Plg is not essential for normal development, survival to adulthood or reproduction.

PLASMINOGEN ACTIVATORS

The activators of Plg are serine proteases of tissue-type (tissue-type plasminogen activator, t-PA) or urokinase-type (u-PA) and bacterial proteins that acquire proteolytic activity after the interaction with human Plg or Pn (streptokinase, SK, and staphylokinase, SAK). Plg activators are segregated into fibrin-selective and non-fibrin-selective enzymes. The fibrin-selective activators, including t-PA and SAK, preferentially activate fibrin-bound Plg. Then surface-generated Pn remains bound to fibrin and is protected from rapid inhibition by α_2-AP. The non-fibrin-selective enzymes, including SK and two-chain u-PA (tcu-PA), activate both Plg in the circulating blood and fibrin-bound Plg. The two main endogenous activators of Plg in the human body are t-PA and u-PA.

t-PA

t-PA is synthesized mainly by vascular endothelial cells and secreted into blood as a 70-kDa 527-residue single-chain glycoprotein [63, 64]. The circulating level in plasma is about 5 ng/ml (70 pM) [13]. It is a multidomain protein consisting of five distinct modules: a finger domain (Serl-Lys49), an epidermal growth factor-like domain (Ser50-Asp87), two kringle domains (Thr88-Gly176, K1, and Asn177-Cys261, K2), and a C-terminal trypsin-like catalytic domain (Ser262-Pro527) (Fig. **3**). The single-chain t-PA (sct-PA) can be converted to the two-chain form (tct-PA) by Pn-catalyzed cleavage at the Arg275-Ile276. High-resolution three-dimensional

structures of t-PA fragments have become available [35, 38, 65-71].

Unlike other trypsin-like serine proteases, the single-chain form of t-PA ("pro-enzyme") exhibits considerable catalytic activity of about 10% that of the two-chain form, which is due to the intramolecular interactions between of Lys156 and Asp194, which stabilize the enzymatically active conformation of the sct-PA [72, 73]. Both sct-PA and tct-PA act by forming a ternary complex with fibrin and Plg and catalyze the conversion of Plg to active Pn by cleaving the Arg561-Val562 bond. Upon binding, Plg and t-PA undergo conformational changes, which result in hundred-fold enhancement of Plg activation. Binding of t-PA to fibrin has been mainly localized to the finger and the kringle 2 domains [74, 75]. In fibrin there are high-affinity Lys-dependent t-PA binding sites (K_d=16-33 nM) in the compact portion of fibrin(ogen) αC region within residues Aα392-610 [76] and low-affinity Lys-independent t-PA binding sites located in the γ312-324 region (K_d~10^{-6} M) [77]. All these sites are cryptic in fibrinogen and become exposed in fibrin. Because t-PA and Plg bind to the same sets of sites, the zymogen and its activator are brought into close proximity, which results in efficient local generation of Pn. These mechanisms of surface activation of Plg are designed to minimize degradation of circulating fibrinogen and confine fibrinolysis to the places of fibrin deposition [78].

u-PA

u-PA can be synthesized and secreted by various cell types, such as endothelial and epithelial cells, vascular smooth muscle cells, monocytes and macrophages, leukocytes, fibroblasts, the level of expression depending on (patho)physiological conditions [79-82]. In human plasma, u-PA antigen concentrations range from 2 to 4 ng/ml [83, 84] and it is also found in urine [85]. u-PA is secreted as a single-chain proenzyme (pro-u-PA) containing 411 residues with an apparent molecular weight of 53 kDa [86]. The single-chain form (pro-u-PA or scu-PA) can be converted to a two-chain protein (tcu-PA) by cleavage of the Lys158-Ile159 bond of scu-PA to produce chains A and B held together by a disulphide bond (Fig. **3**) [87]. The activation of pro-u-PA is primarily catalyzed by Pn [88] but the reaction can also be promoted, at least *in vitro*, by other proteases, including plasma kallikrein, factor XIIa, trypsin, and cathepsins, although the physiological significance of these interactions remains uncertain [89, 90]. Pro-uPA is a true zymogen with a very low protease activity for Plg, which is less than 1% that of the two-chain form [91-93]. tcu-PA activates circulating and fibrin-bound Plg by cleaving the Arg561-Val562 bond with a similar rate [94], but the role of u-PA extends much further than its action as a proteolytic enzyme (see below).

u-PA consists of three functionally independent domains (Fig. **3**), an amino-terminal growth factor domain (GFD, Ser9–Asp45)*, a kringle domain (Lys46-Glu143)* and a C-terminal serine protease domain (SPD, Lys144-Lys411)* [95]. The first two domains largely correspond to the amino terminal fragment (ATF), which can be released upon cleavage of pro-uPA at Lys134–Lys135. The rest of the molecule, the C-terminal protease domain, forms most of the low-molecular weight u-PA with full specific activity [96]. The N-terminal A chain in tcu-PA includes the GFD and the kringle domain, while the B chain contains the proteolytic domain. GFD has no protease activity but binds to the high-affinity cell surface u-PA receptor (u-PAR) and mediates tissue remodeling and cell migration [97]. The kringle domain contains a sequence that interacts with the inhibitor PAI-1 [98] and mediates heparin binding [99]. The proteolytic domain includes the active site of the enzyme represented by a serine protease amino acid triad His204, Asp255 and Ser356 and maintains the ability to activate Plg irrespective of whether tcu-PA is bound or not to a cell surface [100, 101]. This proteolytic domain of tcu-PA cleaves and activates not only Plg, but also other proteins, such as growth factors and matrix metalloproteinases (MMPs) [97, 102, 103]. The high-resolution three-dimensional structure of u-PA, its fragments, and complexes have become available [104-112].

Streptokinase (SK)

SK is a single-chain 47-kDa protein containing 414 amino acids produced by various strains of β-haemolytic *Streptococci*. Unlike u-PA and t-PA, which possess proteolytic activity themselves, SK is not an enzyme. It acquires the ability to activate human Plg indirectly by forming a 1:1 stoichiometric complex with Plg or Pn, in which the zymogen catalytic site is activated non-proteolytically by an intramolecular cleavage of the Arg560–Val561 bond [113-116]. The resultant activator complex is a highly specific enzyme, which binds free Plg and converts it into Pn by proteolytic cleavage [117]. Pn binds tightly to SK [118, 119] and the SK-Pn complex further propagates proteolytic Plg activation [119]. In addition, Pn cleaves the SK molecule generating the N-terminal 59-residue fragment, which remains covalently attached to the rest of the molecule, precluding binding of the SK-Plg complex with fibrin and making SK a fibrin non-selective Plg activator [120]. Thus, the SK-Plg complex is converted to SK-Pn complex, both of which are equally efficient activators of Plg. The mechanism is regulated by intrinsic differences in affinity of SK for Glu-Plg, Lys-Plg, and Lys-Pn [116, 117, 121]. SK binds preferentially to the extended conformation of Lys-Plg compared to compact Glu-Plg through the lysine binding site [118]. The conformational activation of Plg by SK is supposed to be triggered by the insertion of Ile1 of SK into the Plg N-terminal binding cleft through sequence-specific interactions of the first 10 SK residues [122]. High-resolution three-dimensional structures of SK and its complexes with micro-Plg and Pn are available [123-125].

Staphylokinase (SAK)

SAK is a 15.5-kDa protein consisting of 136 residues produced by *Staphylococcus aureus* [126]. SAK itself does not possess enzymatic activity, but it forms a 1:1 stoichiometric complex with trace amounts of Pn on fibrin clot that activates other Plg molecules [127]. SAK binds to the B chain of Pn, which contains the catalytic active site, and this binding induces a conformational change of the active site of Pn to that of a Plg activator. In plasma, in the absence of

* Domain borders and their connecting peptides are not strictly defined.

fibrin, the trace amounts of free Pn and the Plg activator activity in the SAK-Pn complex are rapidly inhibited by α_2-AP [128]. However, in the presence of fibrin, generation of the active SAK-Pn complex is facilitated because the initial trace amounts of fibrin-bound Pn are protected from α_2-AP and inhibition of the complex by α_2-AP at the clot surface is delayed more than 100-fold. Moreover, the Plg activation by SAK-Pn complex is enhanced by fibrin 2- to 3-fold and 30-, 38- and 8.5-fold by proteolytic fragments and complexes of fibrin, the (DD)E complex, DD and E fragments, respectively [129, 130]. These mechanisms make SAK a fibrin-selective Plg activator. The crystal structure of the human micro-Pn-SAK complex has been elucidated using X-ray diffraction [131]. SAK has a central α-helix (residues Lys57-Thr71), a five-strand β-sheet, and the connecting loops. The major residues in human micro-Pn, which are responsible for the affinity for SAK are Arg719 and Arg767 [132]. The minor amino acids in Pn, which are involved in binding with SAK, are Asn169, Tyr171, Asn173, Glu180, Leu217 and Lys224. These amino acids of micro-Pn are in close contact with the most critical patch consisting of Tyr24, Met26, Asn28, Glu46, Tyr44 and Ile128 in SAK. Both Arg94 and Gly174 in micro-Pn are located in close proximity to Glu75 and the Glu88:Ile128 pair of SAK, respectively [133].

MODULATORS OF FIBRINOLYSIS

The fibrinolytic system is regulated by a number of proteins, either directly inhibiting proteolytic activity or modulating the substrate-binding interactions that affect specificity and activation/inactivation rates of the fibrinolytic enzymes.

Thrombin-Activatable Fibrinolysis Inhibitor (TAFI)

Human TAFI is synthesized in the liver and secreted as a single-chain pro-peptide consisting of 401 amino acids with apparent molecular weight of 60 kDa [134]. The plasma concentration has been reported as 75 nM [135], 275 nM [136] or 220 nM [137]. The protein belongs to the metallocarboxypeptidase family and is also known as plasma procarboxypeptidases B, R, and U [134, 138, 139]. TAFI is heavily glycosylated at Asn22, Asn51, Asn63, and Asn86, which is important to maintain its function [140]. The protein is an effective regulator of fibrinolysis which acts *via* several mechanisms. First, it removes surface-exposed C-terminal lysine residues from degrading fibrin [141, 142] and perhaps from cells [143-145], thus eliminating Plg-binding sites. Second, it is known that Pn bound to C-terminal lysine residues is partially protected from inhibition by α_2-AP, therefore, by eliminating these binding sites, the activated TAFI shortens the half-life of Pn [146]. Third, the activated TAFI slows down the conversion of Glu-Plg to Lys-Plg by Pn, which is a much better substrate for t-PA [147]. Finally, at relatively high concentrations the activated TAFI directly inhibits Pn [148].

The proteolytic activity is exclusively assigned to the activated form of the enzyme (TAFIa), although the TAFI zymogen has been recently also shown to possess the carboxypeptidase activity [149-151]. The zymogen is activated through proteolytic cleavage, resulting in the activation peptide (Phe1-Arg92) and the catalytic portion TAFIa (Ala93-Val401) [134, 152]. The enzymatic activity of TAFIa is remarkably unstable, with a half-life of the order of minutes at 37°C, hours at 22°C, and several days at 0°C [135, 153-155]. This inactivation is likely caused by a change in the intrinsic structure of TAFIa, rather than by a proteolytic event [154, 155]. The structural change appears to expose regions with affinity for α_2-macroglobulin and pregnancy zone protein and may function *in vivo* as a mechanism to mediate the clearance of TAFIa from the circulation [156]. TAFI and TAFIa can be cross-linked to fibrin by factor XIIIa to localize and perhaps stabilize the activity [157].

The proenzyme, TAFI, can be activated by trypsin-like proteases, such as thrombin and Pn, by cleavage at Arg92 [134, 152]. Thrombin is a relatively weak activator of TAFI, but in the presence of thrombomodulin the activation by thrombin is enhanced more than 1000-fold [158, 159]. The (thrombin-thrombomodulin)-mediated activation of TAFI is calcium dependent [141, 160]. The catalytic efficiency of Pn is about 8-fold higher than the activation by thrombin alone and increases even further in the presence of heparin. However, the catalytic efficiency is still 10-fold lower compared to that of the thrombin-thrombomodulin complex. In contrast to the TAFI activation induced by thrombin-thrombomodulin, the Pn-mediated activation is independent of the presence of calcium ions [161]. The half-maximal effect of the inhibitory effect of TAFIa on fibrinolysis *in vitro* occurs at only 1 nM [158], but the ability of TAFIa to prevent fibrinolysis from proceeding to the propagation phase has been shown to depend on whether the TAFIa activity remains above or below a threshold level [162].

Plasminogen Activator Inhibitors 1 and 2 (PAI-1 and PAI-2)

PAI-1, the major inhibitor of u-PA and t-PA, belongs to the serpin (serine protease inhibitor) superfamily of proteins [163]. PAI-1 is expressed as a single-chain 379-residue 50-kDa glycoprotein in endothelial cells, hepatocytes, and many other cell types surrounding the vasculature [164-166]. Its normal plasma concentration is low (0.4 nM), but in platelet-rich thrombi the local concentration of PAI-1 can be relatively high [167] due to its continuous synthesis in platelets [168]. It can be also accumulated at the sites of vascular injury due to tight interaction with vitronectin, an extracellular matrix protein [169, 170]. These local mechanisms are thought to protect the developing thrombus from premature lysis. As the clotting and initial fibrinolysis proceed, more t-PA and Plg bind to fibrin within the thrombus, which makes t-PA hardly accessible and in excess over PAI-1 and results in massive generation of Pn and fibrin dissolution [163, 171].

Like other serpins, PAI-1 forms a stable 1:1 acyl-enzyme complex with t-PA or u-PA [172] with association rate constant of greater than $2x10^7$ $M^{-1}s^{-1}$ [173]. PAI-1 is also able to directly inhibit Pn [174]. The tertiary structure of active PAI-1 includes an exposed reactive center loop (RCL) that acts as a bait substrate for the target protease. When attacked by a protease, the PAI-1 Arg-Met reactive center bond is cleaved and the N-terminal portion of the RCL undergoes a conformational change transferring the protease to the opposite pole of the serpin [175-177]. This shift impairs the protease active site, which slows further cleavage of the acyl-enzyme

bond and prevents release of the protease from the serpin [172, 178-180]. Once cleaved, PAI-1 is no longer active as an inhibitor and is eliminated from circulation as the serpin-protease complex followed by intracellular degradation [181-185].

PAI-1 exists in active, latent, and protease-complexed conformations [186-188]. Active PAI-1 is highly unstable and spontaneously looses its inhibitory activity [189]. In plasma PAI-1 is bound to vitronectin [190, 191], which delays its transition to the latent conformation and abates fibrinolysis by sustaining activity and increasing half-life of PAI-1 [192]. Vitronectin-bound PAI-1 maintains the inhibitory activity toward Plg activators [193, 194] but, upon formation of a protease-PAI-1 complex, its affinity for vitronectin is dramatically reduced [186, 195]. Binding of PAI-1 to vitronectin converts the latter to an activated form that readily interacts with integrins, which makes PAI-1 an important modulator of cell adhesion and motility [196-198].

PAI-2 is another inhibitor of the serpin family; *in vitro* it inhibits u-PA and tct-PA [199-202], although 10 to 50 times slower than PAI-1 [203]. It is mainly produced by monocytes [204] and trophoblast cells in placenta [205, 206]. The major part of PAI-2 exists as a 43-kDa abundant intracellular non-glycosylated protein [203], lacking a classical N-terminal signal sequence, but it can be secreted as a 60-70-kDa glycoprotein upon stimulation by thrombin [207-209]. PAI-2 contains a unique glutamine-rich sequence that has been shown to make PAI-2 a substrate for tissue transglutaminases and factor XIIIa, which crosslink PAI-2 to fibrin and fibrinogen [210]. The binding was shown to be mediated by Gln83, Gln84, and Gln86 in PAI-2 [211] and all cross-linking of PAI-2 is to the lysine residues (148, 176, 183, 239, and 457) in the fibrinogen Aα chain [212]. Increasing evidence now supports a major physiological role of PAI-2 as a modulator of intracellular proteolytic events associated with a number of cellular functions, not related to fibrinolysis [9].

α_2-Antiplasmin (α_2-AP)

α_2-AP, also widely called α_2-plasmin inhibitor, a member of the serpin family, is the primary physiological inhibitor of Pn. α_2-AP is expressed in the liver as a 452-residue single-chain 63-kDa glycoprotein and circulates at a concentration of about 1 μM (70 μg/ml) with a long half-life of ~3 days [213, 214]. It acts by three mechanisms. First, it forms a very stable inactive α_2-AP-Pn complex with an association constant of 10^7 $M^{-1}s^{-1}$ [215]. Second, it can be covalently cross-linked with fibrin, making it more resistant to lysis [216-218]. Third, it can competitively prevent Plg interactions with fibrin due to the presence of exposed lysine residues.

The reason for the high affinity of α_2-AP for Plg and especially for Pn (K_d = ~2 x 10^{-10} M for α_2-AP-Pn) is the unique C-terminal peptide consisting of 51 amino acid residues, which tightly binds to Plg and Pn *via* the Lys-binding sites in their kringle domains [219]. The Plg- and Pn-binding region of α_2-AP has six lysine residues, one of which is the C-terminal residue. The Lys436 was shown to be the main residue involved in binding to Plg or Pn [220], while other works conclude that the C-terminal Lys452 is the most important in this interaction [221, 222]. The crosslinking of α_2-AP with fibrin is catalyzed by factor XIIIa by formation of an isopeptide bond between the N-terminal Gln2 in α_2-AP and Lys303 in the α chain of fibrin [223].

Lipoprotein (a) [Lp(a)]

Lp(a) is a plasma lipoprotein similar in structure to low-density lipoprotein (LDL), in that it is composed of a core of apolar lipid enclosed in a predominantly phospholipid monolayer with associated glycoproteins. LDL contains a ~500 kDa glycoprotein, apolipoprotein B-100 (apoB-100), while Lp(a) also contains apolipoprotein(a) [apo(a)] linked to apoB-100 covalently *via* a disulfide bond as well as through noncovalent interactions [224]. The apo(a) glycoprotein has a strong structural homology with Plg. It exists in several isoforms, containing from 12 to 51 Plg kringle 4-like units (KIV) as well as a Plg kringle 5-like unit (KV) and an inactive protease region homologous to that in Plg [225]. Instead of the scissile Arg561-Val562 bond in Plg, the apo(a) protease-like region contains a Ser-Ile substitution that may impair its recognition by activating proteolytic enzymes [226]. Some of the KIV units appear to contain either strong or weak lysine-binding sites similar to those of Plg [227].

Due to structural homology of the kringle-like structures, Lp(a) may interfere with Plg binding to lysine residues in fibrin and in cell receptors and, thereby, impair fibrinolysis and pericellular proteolysis. It was shown that free recombinant apo(a) efficiently inhibits the binding of Plg to fibrin [228] as well as to the surface receptors of endothelial cells [229], platelets [230, 231] and monocytoid cells [232]. It has been shown that apo(a)/Lp(a) inhibition of Plg binding and activation occurs as a result of competition between apo(a) and Plg for the lysine residues [11, 233, 234]. Another study demonstrated that apo(a) may inhibit Plg activation not only by displacing Plg from binding sites on fibrin but also by direct association with the catalytic complex of Plg, tPA, and fibrin [235]. Regardless of the mechanism, high concentrations of Lp(a) in plasma represent a potential source of anti-fibrinolytic activity. However, the apo(a) particles are heterogeneous in their fibrin-binding activity so that only small size isoforms display high-affinity binding to fibrin and the ability to prevent Plg activation [236].

α_2-Macroglobulin (α_2-MG)

α_2-MG with a molecular weight of ~725 kDa is a tetramer, comprised of four identical subunits [237]. It is found in circulation at a concentration of about 3 μM (2.5 mg/ml) and can bind to different proteases and their complexes with inhibitors [238]. Under certain conditions Pn and Plg activators may react with α_2-MG followed by relatively slow inhibition of their activity [239]. Cell- and fibrin-bound Pn is protected from α_2-MG [240]. α_2-MG basically works as a scavenger protease inhibitor because α_2-MG-containing protein complexes are internalized by means of low-density lipoprotein-related receptor and degraded intracellularly [241].

Other Modulators of Fibrinolysis

Some other inhibitors, such as PAI-3 (protein C inhibitor), neuroserpin, histidine-rich glycoprotein, protease nexin have been shown to have minor, local, and/or uncertain ef-

fect on the activity of the components of human fibrinolytic system [5, 7, 8, 163, 242].

CELL SURFACE "FIBRINOLYTIC" RECEPTORS

The Plg activation system appears to be widely involved in many physiological and pathological processes, other than fibrinolysis, *via* Pn generation on a cell surface. Therefore, there are many cellular binding sites or receptor molecules for Plg and its activators.

Plg Receptors

Binding of Plg to cells is mediated by a heterogeneous population of Plg receptors. Interaction of Plg with the cell-surface binding sites accelerates conversion of Plg to Pn [243, 244], enhances the catalytic activity of Pn itself [245], and protects bound Pn from inactivation by inhibitors [28, 246]. Plg receptors are present on platelets, monocytes, macrophages, neutrophils, endothelial cells, and some other cell types, not related to the vasculature. The number of Plg receptors is of the order of 10^7/cell or greater [247]. Cellular Plg-binding sites are quite diverse but, despite their heterogeneity, have a similar and relatively low affinity for Plg with K_d about 10^{-6} M. Many Plg receptors contain C-terminal lysines, which interact with the kringle-associated lysine binding sites of Plg. Therefore, lysine analogues, ε-aminocaproic acid and tranexamic acid, block cell-Plg interactions and treatment of cells with TAFI-like carboxypeptidases reduces Plg binding and activation [248, 249].

Several types of Plg binding sites on cells have been identified. These include α-enolase [248, 249], integrins αIIbβ3 [250] and $\alpha_M\beta_2$ [251], actin [252], gangliosides [253], dipeptidyl-peptidase IV (DPP IV/CD26) [254, 255], Na^+/H^+ transporter called NHE-3 [254], and histone H2B [256, 257]. Annexin II, a calcium-dependent phospholipid-binding protein, has been considered the most abundant profibrinolytic receptor that binds directly to both Plg and t-PA, functioning as a specific cofactor for Pn generation and localizing proteolytic activity to the cell surface [7, 258-260]. Alternatively, it has been proposed that S100A10 is a key Plg activation regulatory protein and that annexin A2 serves just to anchor S100A10 to the cell surface [261, 262].

u-PA Receptor (u-PAR)

The u-PAR is essential for Plg activation on the cell surface, mediated by its specific ligand, u-PA. The u-PAR (CD87) is a 55-kDa membrane receptor protein anchored by a glycosyl-phosphatidyl-inositol (GPI) linkage [263] expressed on peripheral blood cells, endothelial cells, fibroblasts, and several tumor cell lines [264]. u-PAR binds either scu-PA or tcu-PA with high affinity (about 10^{-9} M) and maintains their enzymatic potential as well as susceptibility of the active form to PAI-1 [265]. The binding results in a strong enhancement of cell surface Plg activation, the effect being dependent on the simultaneous binding of pro-uPA to u-PAR and of Plg to its cell surface receptors [266, 267]. It was recently shown that the u-PA/u-PAR-related mechanism of Pn generation works on endothelial microparticles, which may represent targeted vectors supporting Pn dissemination [268]. Besides u-PA, u-PAR also binds the adhesive glycoprotein vitronectin at a site distinct from the u-PA-binding domain [269]. Upon binding to the receptor, u-PA increases the affinity of u-PAR for vitronectin and subsequently promotes cell adhesion [270]. Thus, u-PAR appears to be the point of intersection between the components of the fibrinolytic system and cell adhesion molecules, including integrins [271, 272].

In addition to binding to u-PAR, u-PA can cleave intact three-domain u-PAR(I–III) molecules, releasing the ligand-binding domain I and leaving the cleaved form of u-PAR, uPAR(II–III), on the cell membrane. This cleavage inactivates the binding potential of u-PAR toward u-PA and vitronectin. Either intact or partially degraded u-PAR is torn off from the cell surface by cleavage at or near the GPI anchor, and the soluble form, su-PAR, has been found in biological fluids [273]. Several high-resolution structures of a ligand-bound soluble form of the human u-PAR(I–III) have been reported [274-276].

PAI-1 can couple to u-PAR-bound uPA, thereby inhibiting degradation of the extracellular matrix proteins initiated by cell-associated u-PA [277]. The inhibitory effect of PAI-1 can be abrogated either by u-PA-induced cleavage and inactivation [278] or by formation of a quaternary complex of (u-PAR)-(u-PA)-(PAI-1) with α_2-MG, which is then internalized and digested, while u-PAR is recycled to the cell surface [181, 279].

t-PA Receptors

Binding of t-PA along with Plg to cells can lead to accelerated Pn generation [264, 280, 281]. Although cell-bound high-affinity receptors for t-PA have not been identified, t-PA binds to many cellular proteins, such as mannose-6-phosphate/insulin-like growth factor II receptor, annexin II, and amphoterin [258, 282, 283]. Some t-PA interactions with cell receptors represent competition for the same binding sites with Plg [284] because both contain the lysine-binding sites. However, a number of different sites are involved in t-PA-cell interactions, especially within finger and kringle domains [285]. The dissociation constants for cell-t-PA interactions vary over a wide range from 10^{-11} to 10^{-7} M, although it appears that the highest affinity interactions are likely due to cell surface-associated PAI-1 [286]. Given the low plasma level of t-PA (70 pM), the physiological significance of putative low-affinity t-PA receptors is doubtful. For example, the t-PA (and Plg) receptor annexin A2, which binds t-PA with a K_d of 48 nM, *in vitro* causes stimulation of enzyme activity of only less than 10-fold [258]. Monocytes and monocytoid cells bind t-PA with a low affinity but high capacity and are able to stimulate t-PA enzyme activity up to around 20-fold [284, 287]. Comparing the aggregate of data on the interactions of Plg activators with cells and fibrin, it can be postulated that although u-PA and t-PA are quite similar in structure and have common substrates and inhibitors, their physiological roles are distinct. The role of t-PA has been mainly in fibrinolysis and that of u-PA in pericellular proteolysis, cell migration, adhesion, invasion, and other cell-related processes and functions [6].

THERAPEUTIC THROMBOLYSIS AND THROMBOLYTIC AGENTS

Therapeutic thrombolysis or thrombolytic therapy is dissolution of thrombi by the delivery of exogenous Plg activa-

tors into the circulation. Thrombolysis is standard treatment for acute myocardial infarction and stroke [288, 289]. More information about fibrin binding and the regulation of Plg activators during therapeutic thrombolysis is discussed in another article in this issue [290].

Streptokinase (SK), which was the first enzyme to be used as a thrombolytic agent, is still often the drug of choice for thrombolytic therapy due to its relatively low cost. Its flaw is its bacterial origin, which may result in immunological complications and ineffectiveness for repeated use. SK is also fibrin non-selective and produces generalized proteolysis with an associated bleeding tendency [291].

Anistreplase or acylated Plg-SK activator complex (APSAC) is a derivative of SK, which has been clinically approved [292]. The deacylation of the Plg active site *in vivo* is believed to provide longer plasma half-life to the molecule [293].

Recombinant staphylokinase (SAK) was shown to be more fibrin-specific than rt-PA in patients with evolving myocardial infarction. However, since SAK has a bacterial origin, infusion of SAK into humans induced an immune reaction against SAK followed by its neutralization [294]. Despite such antigenicity problems, some other microbal fibrinolytic enzymes keep attracting attention as potential thrombolytic agents [295].

The active form of u-PA (tcu-PA) was originally purified from human urine, hence the name urokinase, and used clinically [296]. Nowadays it is produced recombinantly as a thrombolytic agent. The recombinant pro-urokinase or scu-PA (saruplase**)** has been also used for thrombolytic therapy [297].

Recombinant t-PA, rt-PA, known also as alteplase **(**Activase®), is almost identical to the wild type t-PA [298]. During standard thrombolytic therapy, plasma concentrations of about 4000 ng/ml are achieved, an almost 1000-fold increase over physiological values [299, 300]. A new strategy for rt-PA delivery into the circulation implies coupling of the enzyme to red blood cells resulting in prolonged circulation, minimal extravasation, and preferential lysis of nascent versus preexisting clots in animal models [301].

Reteplase (Retavase®, Rapilysin®) is a single-chain non-glycosylated deletion mutant of rt-PA consisting only of the kringle 2 and the proteinase domain; it contains amino acids 1–3 and 176–527 (deletion of Val 4-Glu 175); the scissile Arg 275-Ile276 bond, sensitive to Pn-induced cleavage, is maintained. Reteplase has a similar plasminogenolytic activity as wild-type rt-PA, but its binding to fibrin is 5-fold lower. Reteplase and rt-PA are inhibited by PAI-1 to a similar degree [302, 303].

Duteplase is a double-chain rt-PA analogue and it has been studied in myocardial infarction [304].

Tenecteplase (TNK-rt-PA) is a mutated variant of rt-PA with Asn117→Gln, Thr103→Asn, and Lys296, His297, Arg298, Arg299 each replaced by Ala. The deletion of a glycosylation site at position 117 results in a longer half-life and the mutations of positions from 296 to 299 increase 200-fold the resistance to PAI-1. The fibrin-binding properties of TNK-rt-PA are maintained and even improved [305-307].

Lanoteplase (n-PA) is a deletion and single Asn117→Gln point mutation variant of rt-PA, lacking the finger region and the epidermal growth factor domain with an increased plasma half-life compared to alteplase [308].

Monteplase is a mutant of tPA that has only one amino acid substitution Cys84→Ser in the epidermal growth factor domain. This substitution leads to an unexpected rearrangement of disulfide bonds, loss of the fucosylation site at Thr61 and altered carbohydrate at Asn117 resulting in a prolonged biological half-life [309].

Pamiteplase is a t-PA analogue with a genetically engineered deletion in the kringle 1 domain and a point mutation at Arg275→Glu in the kringle 2 domain to produce a longer half-life and improve fibrin binding compared to wild-type t-PA [310].

Desmoteplase (DSPA) is a Plg activator derived from the saliva of the vampire bat *Desmodus rotundus* [311], which is now produced in the recombinant form [312]. Structurally, it lacks the second kringle site and the Pn-sensitive cleavage site of t-PA [313, 314]. Desmoteplase is extremely fibrin-dependent and fibrin-specific and is almost not stimulated by fibrinogen, unlike wild-type t-PA and other t-PA-derived activators [315].

Plasmin as a direct thrombolytic agent was investigated in pilot studies in humans in the 1950´s [316] and now is again considered as a drug with a potential to dissolve thrombi rapidly and completely [317]. Microplasmin, a truncated form of Pn that allows for recombinant production, has been tested in animal models, demonstrating a positive effect and less hemorrhagic complications than with rt-PA [318-320].

A promising aspect of thromboprophylaxis and therapeutic thrombolysis is the use of substances that stimulate release of endogenous t-PA, such as a derivative of vasopressin (DDAVP, desmopressin) [321], some acylated dipeptides [322], and a pyrimidine derivative [323].

MODEL SYSTEMS TO STUDY FIBRINOLYSIS

It is important to remember that there are a variety of systems that have been used to study fibrinolysis experimentally, and that each has strengths and weaknesses and is suited to the investigation of different aspects of lysis. With each of these systems, there are often several biochemical or structural methods that can be used to quantify rates of lysis.

Internal Lysis

Normally in the vasculature a clot is formed to stem the leakage of blood and then it is lysed so that it can be removed as part of the process of wound healing. To mimic this process, either t-PA mixed with plasma or t-PA and Plg mixed with fibrinogen have been clotted, so that the clot is formed and then dissolved as the t-PA activates Plg to Pn on fibrin. This system has been called internal or intrinsic lysis. The process of lysis has been followed by measurement of the decrease of turbidity or by confocal or other light mi-

croscopy or biochemically by the appearance of lysis products.

External Lysis

For therapeutic thrombolysis, a bolus of t-PA is introduced into the vasculature, so that it circulates and binds to thrombi, activating Plg to Pn on the fibrin surface. *In vitro*, t-PA can be introduced at the edge of a pre-formed clot in a chamber, commonly made of a light microscope slide and cover slip, so that lysis can be observed either by eye or by light microcopy.

Permeation

In the external lysis system just described, the t-PA enters the clot only by diffusion. However, in thrombolysis the t-PA is delivered by flowing blood, so there usually is permeation or perfusion into the thrombus. Thus, a variation of external lysis is to allow the t-PA to permeate into the clot. Commonly, the clot can be made in a tube with both ends open, so that fluid can permeate through the clot. The results can be quantified by the measurement of digestion products released or by observation of changes in clot structure as a function of time.

Animal Models

Most of the major coagulation and fibrinolytic proteins have been experimentally knocked out or modified in transgenic mouse models. Other animals have been used for experimental models of thrombosis or testing of therapeutic regimes.

MATHEMATICAL MODELING OF FIBRINOLYSIS

Computer models have been formulated to simulate several aspects of fibrinolysis. These models are useful to develop and test hypotheses about mechanisms of lysis. Most of the models include transport and binding of reagents, in addition to diffusion, and consider non-equilibrium conditions (Fig. **4**). It is important to note that the pore sizes, both within and between fibers, are large enough that free diffusion of the proteins involved is not hindered. Finally, these models can be useful to predict the results of various therapeutic agents and regimes of administration of thrombolytic agents to patients or of devices for delivery.

Models can be used to predict lysis rates of thrombi for intravenous thrombolytic regimens based on convective and diffusive penetration of the reacting and adsorbing species into one surface of the thrombus [324, 325]. One important conclusion is that pressure-driven permeation is the major mechanism responsible for dissolution of the thrombus. Without permeation, lysis with only diffusion is very slow. This model can be used to predict the rates of movement of lysis fronts across thrombi with different structures, such as those made of thin or thick fibers. Models specific for lysis rates for intravenous thrombolytic regimes based on convection and diffusion of reactants that bind to fibrin have been developed [326].

A model for the kinetics of the surface degradation of fibrin clots by different enzymes based on experimental data revealed aspects of interactions between the proteases and

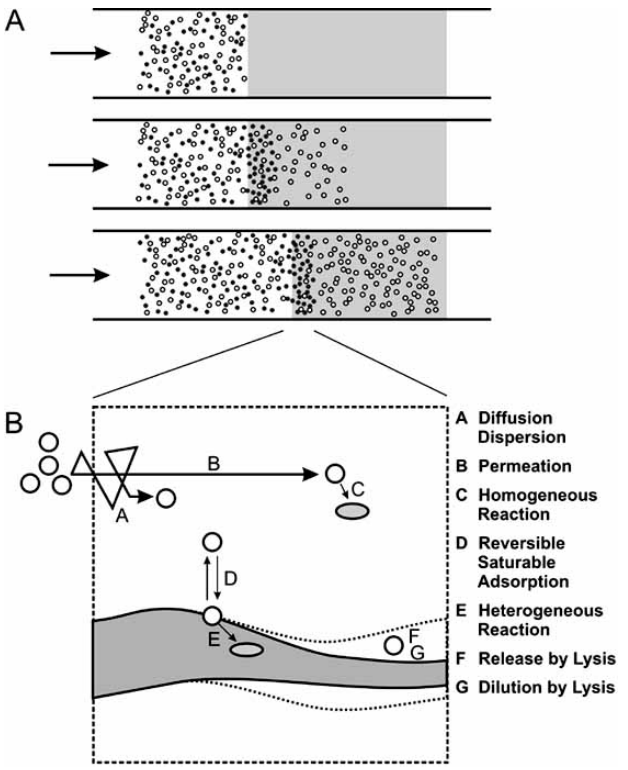

Fig. (4). Summary of transport and reaction physics during fibrinolysis. (*A*) Solutes that do not bind fibrin permeate with the solvent front, whereas fibrin-binding species accumulate at the lysis front as fluid permeates into the fibrin where they undergo continual solubilization and rebinding. (*B*) At each position of the clot, various physical and biochemical processes affect the concentration of species in the fluid and fibrin phases as well as the volume of each phase. The clot is shaded and the lysed clot is the adjacent area enclosed by the dotted lines. Reproduced with permission from [327].

their polymer substrate [327]. The kinetic parameters show a strong dependence on the nature of the protease as well as the structure of the fibrin clot. Trypsin was used for comparison, since its primary specificity is similar to that of Pn but it has a very low affinity for fibrin. This model demonstrated that thinner and more highly branched fibrin fibers are more efficiently dissolved than the thicker and less highly branched fibers. These results have not been fully appreciated by everyone in this field but are important to keep in mind with respect to the section of this review below on the effects of clot structure on lysis rates. The effects of crosslinking by Factor XIIIa are to increase the k_{cat} for Pn but to decrease the association rate constant for enzyme-substrate complex formation, which accounts for the increased proteolytic resistance of crosslinked clots. The effects of Factor XIII are described in another article in this issue [328].

Since most of the reactions in fibrinolysis occur at the interfaces between fluid and solid phases, more complex models utilizing different compartments have been formulated [242, 329]. These models include the platelet, endothelial cell and polymorphonuclear cell compartments, in addition to fluid and fibrin polymer phases.

MICROSCOPIC MECHANISMS - LATERAL TRANSECTION OF FIBERS

Plg binds more strongly to partially degraded fibrin, which is an important positive feedback mechanism in fibrinolysis, in addition to the positive feedback already mentioned as a result of the conversion of Glu-Pg to Lys-Pg. The spatial distribution of Plg has been observed and quantified by fluorescence microscopy in a model system (Fig. **5**) [330]. Clots were made by addition of thrombin to recalcified plasma in a chamber and t-PA or u-PA was applied at the edge of the resulting clot. Lysis occurred as the activating agent diffused into the clot and activated Plg to Pn on the surface of the fibrin. In these experiments, there was a large superficial accumulation of Plg at the surface being digested. This Plg-rich shell moved continuously with the surface of the digesting clot [330]. The necessity for a continuous supply of Plg from the plasma argues for the use of fibrin-specific and Plg-sparing fibrinolytic activators for thrombolytic therapy.

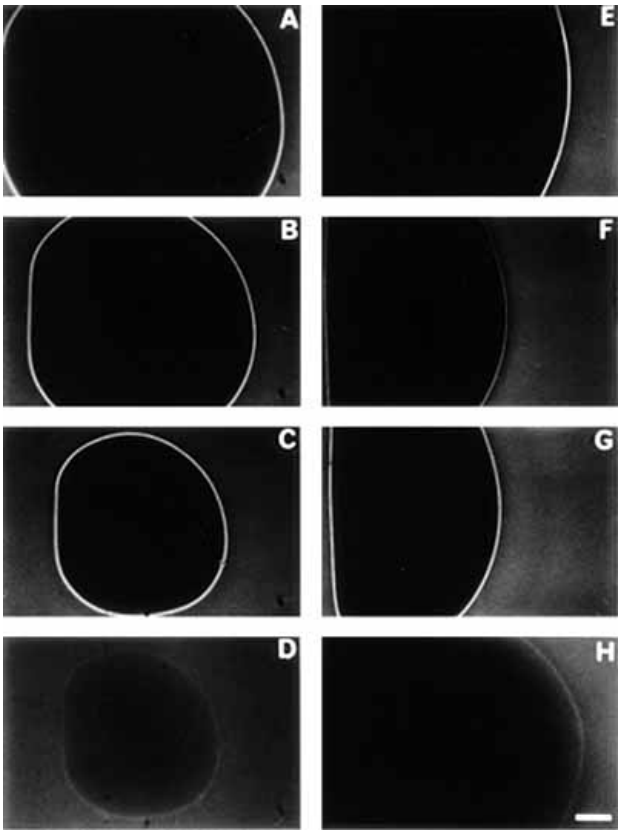

Fig. (5). Fluorescence light micrographs showing superficial accumulation of fluorescently labeled Plg during lysis of an uncrosslinked plasma clot. A through D, Images of a plasma clot after 30, 50, 70, and 100 minutes of perfusion at 37°C with plasma containing t-PA. At 20, 40, 60, and 80 minutes, new portions of plasma were used for the perfusion with freshly added t-PA. At 72 minutes, TAFI was added to the perfusing plasma. E through G, Images of a plasma clot after 4, 30, and 36 minutes of perfusion with plasma containing tcu-PA. At 32 minutes, a new portion of plasma was used for the perfusion with freshly added tcu-PA. H, A plasma clot after 60 minutes of perfusion with plasma without exogenous Plg activator. Bar=250 μm. Reproduced with permission from [332].

Examination by confocal microscopy of the fibrin network during lysis of labeled preparations of fibrinogen, Plg and Plg activators has demonstrated the changes that occur (Fig. **6**) [331, 332]. There were two phases, first prelysis during which Plg accumulated but the fibrin remained immobile, and second final lysis during which fibers moved with a tendency to shrink and disappear. The lysis zone was 5-8 μm wide with t-PA or wider with u-PA, but the prelysis zone extended much more deeply into the clot. Plg accumulated in this zone up to 30-fold in comparison with its plasma levels. t-PA also concentrated in this zone, while the u-PA bound only slightly to the digesting fibrin.

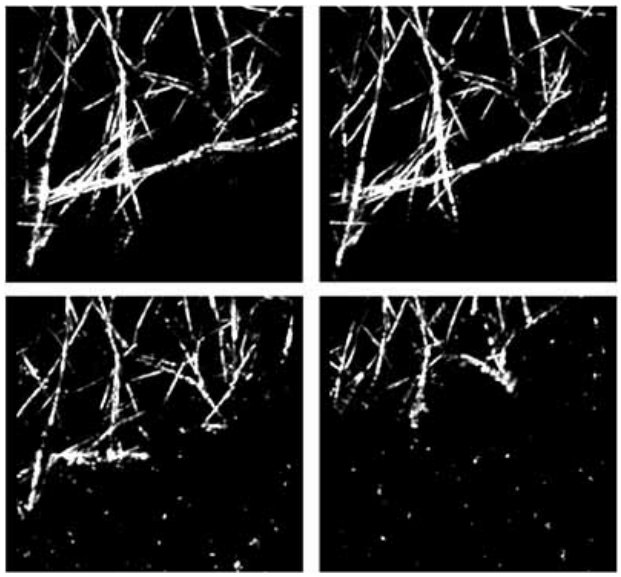

Fig. (6). Dynamic visualization of external fibrinolysis at the fiber level by confocal microscopy. Native hydrated plasma fibrin was labeled with colloidal gold particles and digested by t-PA applied at the edge of the clot. Confocal micrographs were made every two minutes. Progressive lateral transection of fibers leads to disaggregation and movement of individual fibers in the lysis zone. Aggregation and splaying of some fibers is observed. Reproduced with permission from [334].

Morphological changes in this lysis zone were visualized in more detail by electron microscopy, using either Pn or Plg together with t-PA to digest clots made of purified fibrinogen and thrombin [333]. The changes observed were correlated with biochemical characterization of the lysis process. Scanning electron microscopy of the clot surface being digested revealed many free fiber ends and gaps in the continuity of the fibers (Fig. **7**). With more extensive digestion, many free fiber segments associated laterally, with the creation of thicker fiber bundles. As a result, the pore size of the digesting clots increased over time. The supernatants of the digesting clots were negatively contrasted and visualized by transmission electron microscopy, showing large complex fragments [333]. There were large, complicated structures containing portions of multiple fibers, and individual fiber segments, as well as the smaller fragment complexes previously described from the results of biochemical methods (Fig. **8**). Some fiber pieces had sharply defined ends, indicat-

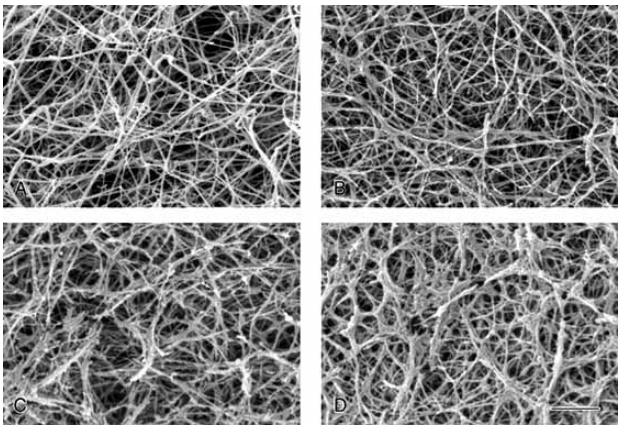

Fig. (7). Changes in the structure of fibrin clot surfaces with digestion visualized by scanning electron microscopy. Plasmin was applied to the clot surface, and samples were prepared for microscopy at various times. Clots digested by adding t-PA to clots made in the presence of Plg had the same appearance. (A) Control clot with no digestion. (B) Early stage of digestion. (C) Intermediate stage of digestion. (D) Late stage of digestion. Magnification bar = 2 μm. Reproduced with permission from [339].

ing that they had been cleaved perpendicular to the fiber long axis. Other pieces had splayed ends or a meshwork of protofibrils disassembling as their length got below that necessary for lateral aggregation of the protofibrils. Longer digestion times produced more small fragments, whose composition could be inferred from the observed structures. These results demonstrate that fibrinolysis generates larger pieces than previously identified by biochemical methods. More importantly, it appears that fibrinolysis proceeds by cutting across fibers, rather than progressive cleavage uniformly around the outside of each fiber.

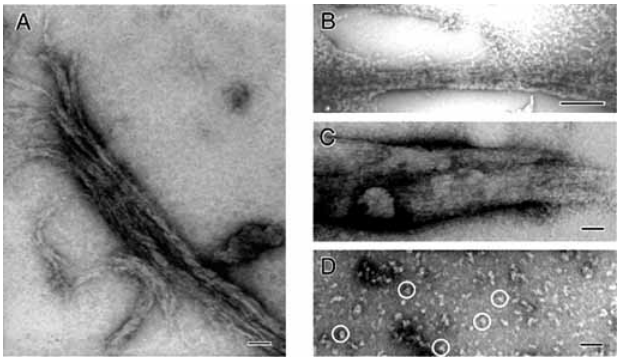

Fig. (8). Transmission electron microscopy of negatively contrasted digestant removed from fibrin clots. (A) Fiber bundles with splayed ends. (B) Fiber surrounded by a lacy meshwork of protofibrils. (C) Fiber showing sharply cut end. (D) Small pieces present at later times of digestion. Some examples of DDE complexes are circled. Magnification bar for A,C, and D = 0.1 μm. Magnification bar for B = 0.5 μm. Reproduced with permission from [339].

The foregoing experiments were all done with an external lysis model, in which the Plg activator was introduced at one surface of the clot. Normally *in vivo* clotting and lysis are initiated simultaneously and lysis occurs without any lysis front. Experiments have also been carried out to visualize the process of lysis in an internal lysis system, in which clots were made by the addition of thrombin to fibrinogen in the presence of Plg and t-PA [334]. In this case, colloidal gold was used to label the fibrinogen so that the lysis process could be analyzed quantitatively without the problems of photobleaching that often plague fluorescence microscopy (Fig. **9**). A decrease in fiber absorbance and increase of fiber diameter were observed. Lysing fibers were digested transversely and thin fibers were digested more rapidly than thick fibers. Frayed lysing fibers bound to adjacent fibers, resulting in a transient increase in average fiber diameter and a simultaneous increase in pore size [334]. At this stage of digestion, there is often a simultaneous transient increase in turbidity, but no corresponding increase in clot rigidity [335]. At the maximum decrease in fiber absorbance, the network collapsed with the release of large fragments.

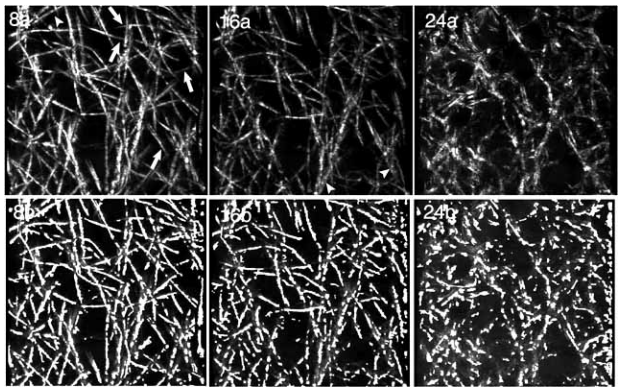

Fig. (9). Dynamic visualization of internal fibrinolysis at the fiber level by confocal microscopy. Top panels: series of micrographs showing the dynamic dissolution of purified fibrin visualized at 8, 16, and 24 minutes after the initiation of clotting and internal lysis. Bottom panels: high contrast representation of the images above, showing the detecton of disconnected fiber segments. Reproduced with permission from [336].

MOLECULAR MODEL - MOVEMENT OF Pn LATERALLY ACROSS FIBERS

Why are clots made up of thick fibers digested more rapidly than clots made of thinner fibers? The most obvious conclusion from studies of the physical process of lysis is that lateral transection of fibers means that fibers made of thicker fibers will be digested more rapidly because there will be fewer fibers to transect at the limited local sites. This is likely to be part of the mechanism. Furthermore, the progressive fiber aggregation of fibers may provide an additional mechanism for the local enhancement of lysis because cleavage proceeds laterally. Also, the rate of movement of the t-PA binding front is greater for coarse clots than for fine clots [336]. Thus, the Plg is activated earlier in these coarse clots, enhancing lysis.

Pn appears to bind in the vicinity of the end-to-end junction of two fibrin molecules. Because it is a flexible protein, it is able to reach cleavage sites on adjacent protofibrils. Since Pn cleaves at lysine residues, this degradation serves to create additional C-terminal lysines, which are new sites for

additional Pn binding. The observation that fibers are severed laterally suggests that the Pn molecules move across the fibers laterally, cleaving additional sites and leading to the transection of fibers (Fig. **10**) [337].

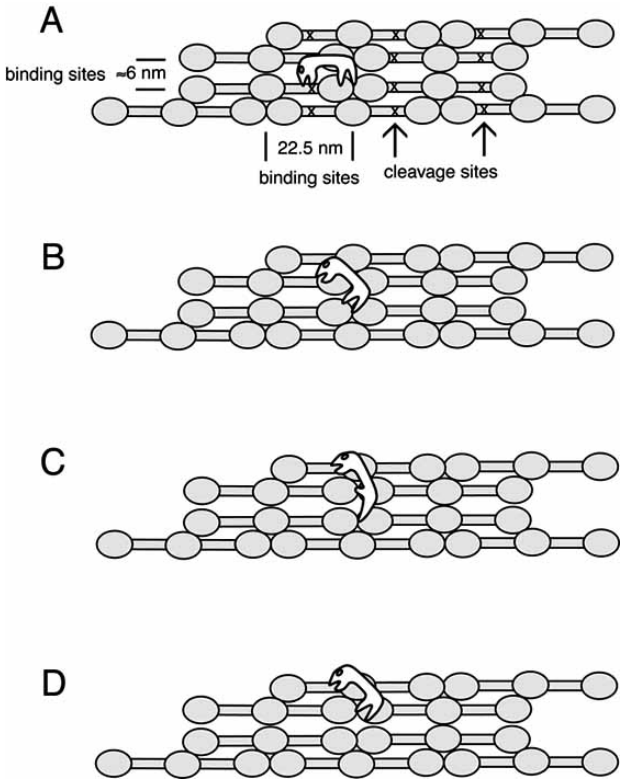

Fig. (10). Schematic representation of plasmin digesting fibrin and "crawling" across a fiber. The shaded trinodular structures are individual fibrin molecules that are interacting in a half-staggered manner to make two-stranded protofibrils. Portions of two protofibrils are shown aggregating laterally. The u-shaped plasmin molecules are represented as creatures with a proteolytic domain (head) for chewing and two fibrin binding sites (limbs). Conformational changes in plasmin allow for binding to successive sites as the limbs move across the fiber. (A) Plasmin is shown binding at the end-to-end junction of two fibrin molecules, but no matter where the binding sites are, the molecular packing and symmetry of fibrin generate equivalent binding sites at a spacing of 22.5 nm along the fiber but much less between protofibrils across the fiber. Arrows point to the major cleavage sites (marked by "X") in the middle of the coiled-coiled region that divide the D and E fragments. (B) Plasmin bound to fibrin *via* a single kringle. This is the starting point for plasmin lateral movement. (C) Conformational change allows binding of plasmin to a second site on fibrin. (D) Conformational change returns the molecule to its original state, so that it is ready for cleaving other sites or more "crawling." The additional cleavages occurring during the crawling process are not shown in this schematic representation. Reproduced with permission from [339].

Although this model has not been proven, it is consistent with structural considerations of fibrin. Equivalent Pn binding sites along the length of a fiber would be located 22.5 nm apart because of the symmetry of the fibrin. On the other hand, those on adjacent protofibrils are likely to be less than 5-10 nm apart. Thus, movement of Pn across the fibers could result from a second kringle binding to the next fibrin binding site before release of the first kringle. In this model, Pn molecules could form a bridge across two or more adjacent protofibrils. Experimental evidence for bridging of Pn comes from the precipitation of fibrin degradation products by Plg and formation of complexes. Also, the addition of Plg to polymerizing fibrin leads to clots made of thicker fibers [25, 335, 338]. Finally, Plg bound to fibrin covalently with a photoaffinity croslinker, shows examples of individual Plg molecules bound simultaneously to the ends of 3 or 4 fibrin molecules [25].

This model, which has been called "crawling" across fibrin like an inch worm [337], is consistent with the presence of multiple lysine-binding kringles in Pn and conformational changes in Pn similar to those observed already. In summary, this crawling mechanism is compatible with the Pn binding sites being closer together laterally than longitudinally, and multiple binding sites, as well as conformational changes of Pn.

CORRELATIONS OF PHYSICAL PROPERTIES OF CLOTS TO THE RATE AND NATURE OF FIBRINOLYSIS

Understanding of the relationships between clot structure and the rate of lysis must be based upon knowledge of the physical process of digestion. There have been many studies of the relationships between fibrin structure and the rate of fibrinolysis, leading to some generalizations. However, there are many exceptions, probably because of the complexity of the system, so any universal principles are still elusive.

The most common type of study of correlations between fibrin structure and the rate of fibrinolysis has involved characterization of fiber diameter, either calculated from turbidity curves or measured directly by electron microscopy. Fiber size can be easily modulated by varying the thrombin concentration, with low thrombin yielding clots made up of thicker fibers than those made with higher thrombin concentrations. Salt concentration, pH and calcium ions are common ways to obtain clots with different structural and physical properties, as is the presence of a whole host of different proteins or small molecules. Internal lysis can then be initiated by adding Plg and t-PA to fibrinogen at the same time as thrombin, so that polymerization occurs followed by lysis. In one influential study of this kind, the process was monitored by measurement of turbidity as a function of time and the rate of fibrinolysis was defined as the slope of the decrease in turbidity or mass/length ratio [339]. In these studies there seemed to be a direct correlation between the size of the fibrin fibers, as assessed by the maximum turbidity and the rate of fibrinolysis (Fig. **11**, top). Another study using plasma clots was carried out in a similar way and reached similar conclusions [340]. Both of these studies calculated the rate of fibrinolysis from the absolute slopes of the decrease in turbidity as function of time, but there are potential problems with this method. These slopes are influenced by the maximum turbidity values attained. To obtain a rate that is normalized for different maximum turbidities and hence reflects the absolute rate of dissolution of the clot, it is nec-

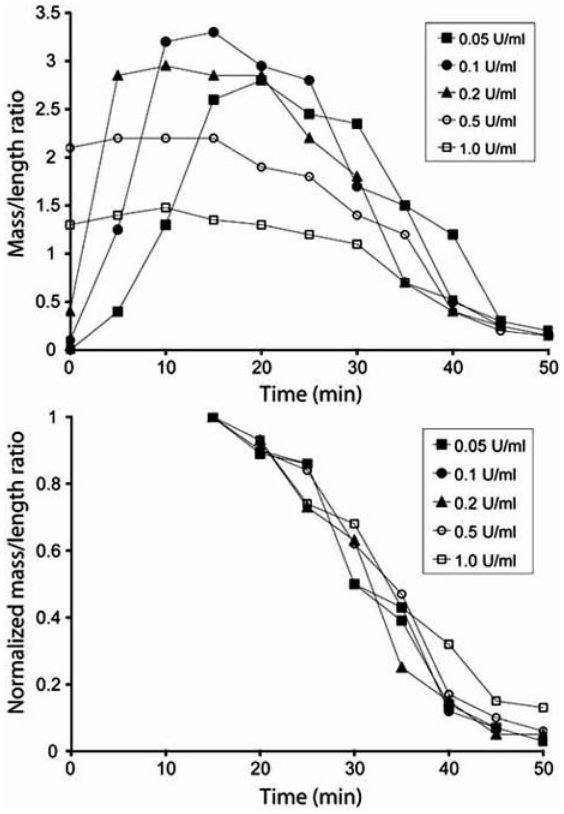

Fig. (11). Turbidity curves showing the effects of clot structure on the rates of internal fibrinolysis. Clots were made with different concentrations of thrombin, as indicated by the numbers in the insets, which are units/ml of thrombin. The top graph shows the results of the experiments. At the bottom, these curves are replotted after normalization with respect to the maximum turbidity, to correct the slopes representing the rates of lysis for the starting turbidities. Reproduced with permission from [343].

essary to divide the slopes of the turbidity decrease by the maximum turbidity. When the lysis rates are normalized for the curves in these two papers, the rates of lysis for some of the clots examined are identical or very similar [341] (Fig. **11**, Bottom). In other cases, however, the conclusion that clots made up of thicker fibers are digested more rapidly than those made up of thinner fibers is still true. In a study of three recombinant variant fibrinogens, the normalized rates of lysis were similar for all clots [342]. Thus, it is important to remember that any such generalizations must be embraced with caution.

To observe the dynamic physical and structural changes during fibrinolysis, cross-linked plasma fibrin was labeled with colloidal gold particles and lysis was followed by confocal microscopy [332]. Morphological changes were characterized at both the fibrin network and fiber levels. Global changes at the network level are directly related to bulk measurements, whereas following individual fibers as a function of time allows measurement of the rates of digestion as a function of fibrin diameter and other properties. The observation from these studies of a progressive disaggregation of fibrin fibers emphasizes that fibrinolysis proceeds by lateral transection rather than by surface erosion of the fiber, from the outside in (Fig. **6**). Plasma fibrin clots with a coarse fibrin structure made of thicker fibers were digested more rapidly than those with a dense network of thin fibers. Unexpectedly, however, individual thin fibers were dissolved at a faster rate than thick ones [332]. During the course of fibrinolysis, the t-PA binding front was broader in coarse fibrin clots and moved more rapidly than that in fine plasma fibrin clots, accounting in part for the difference in lysis rates.

There have been many studies of the effects of various dysfibrinogenemias on clot structure and the susceptibility to lysis. Generally, mutations that result in clots that are made up of thin fibers with many branch points and small pores, and are stiffer, are more slowly lysed, but there are many exceptions. Similarly, the common polymorphisms of fibrinogen and Factor XIII have some effects on clot structure and its fibrinolytic properties [343]. A splice variant of fibrinogen, γ', present in humans, has an altered C-terminal sequence in its γ chain and is more resistant to lysis than normal fibrin, possibly because the γ' fibrin specifically binds Factor XIII and thrombin. The effects of Factor XIII on lysis are described in another chapter in this issue [328].

Post-translational modifications of fibrinogen can have effects on clot structure and physical properties and hence influence fibrinolysis. These modifications include glycosylation, non-enzymatic glycation, proteolysis, phosphorylation, proline hydroxylation, tyrosine sulfation or nitration, asparagines or glutamine deamidation, glutamine cyclization, lysine acetylation, and methionine, histidine or tryptophane oxidation. The effects of some of these alternations of fibrinogen structure are discussed in another chapter of this issue [344].

The αC regions of fibrinogen have significant effects on the susceptibility of clots to fibrinolysis [345, 346]. The functions of the αC regions of fibrinogen in clotting and fibrinolysis have been determined using recombinant fibrinogen truncated at Aα 251. α251 fibrin clots were made up of thinner fibers and the densities of fibers and branch points were greater than those of control clots, suggesting that the αC regions enhance lateral aggregation. More importantly, α251 fibrin clots were much less stiff than controls and showed more inelasticity, indicating that interactions between the αC regions in normal clots play a major role in determining the clot's mechanical properties. Factor XIIIa-induced γ chain cross-linking had a significant effect on clot stiffness, although α cross-linking had a larger effect. Fibrinolysis rates at the both macroscopic and microscopic levels were greater for the α251 clots. Therefore, the αC regions play a crucial role in determining the structure and biophysical properties of clots, which in turn has an important effect on their susceptibility to fibrinolysis.

Permeation or flow of lytic agents into clots can have striking effects on the rate of lysis. Fibrin-specific lysis is greatly enhanced by strong perfusion mimicking arterial flow [347]. On the other hand, non-fibrin-specific lysis was slower and only slightly accelerated or even retarded by flow, most likely because of depletion of Plg. The efficiency of Pn, miniPn and neutrophil leukocyte elastase was also characterized under flow conditions [348]. Fibrin surface

degradation was accelerated with increasing shear rate for Pn and mini-Pn embedded into the clot but not for neutrophil leukocyte elastase. Also, with the incorporated Pn or mini-Pn the clot was abruptly disassembled at an early stage of solubilization, while the process was more gradual for the non-binding enzyme.

Since there is a delicate balance between clotting and lysis, any compounds that modulate fibrin clot structure to make it more susceptible to lysis are potentially useful as prophylactic treatments for thrombosis to shift this balance. For example, a monoclonal antibody that reacts with the C-terminus of the γ chain of fibrinogen inhibits factor XIIIa-mediated cross-linking, platelet adhesion to fibrinogen, and platelet-mediated clot retraction, facilitates thrombolysis. To understand the mechanisms by which this antibody acts, the effect of this antibody on fibrin clot structure was studied using electron microscopy and measurements of clot physical properties [349]. Clots formed in the presence of this antibody had a very unusual structure and mechanical properties, consistent with the enhanced fibrinolysis.

There have been a series of studies of the introduction of fibrinolytic agents into thrombi by their incorporation onto the surface of red blood cells. Normally, the half life of t-PA in the circulation is short but is greatly prolonged when it is no longer in solution. If a fibrinolytic agent such as t-PA is coupled to the surface of red cells, nascent clots are dissolved from within, in a Trojan-horse strategy, while having minimal effects on existing hemostatic clots or extracellular tissue (Fig. **12**) [301]. Thus, such approaches may allow the prophylactic administration of fibrinolytic agents. Apparently, the t-PA in the membrane can activate Plg to Pn and thus subsequently digest the fibrin in the thrombus. In a mouse and rat models, the fibrinolytic activity of the RBC-t-PA persisted at least 10-fold longer than free t-PA. In a model of venous thrombosis induced by injection of fibrin microemboli, RBC-t-PA preferentially lysed pulmonary clots lodged after injection, probably because the cells are less likely to penetrate the formed clots. Furthermore, RBC-t-PA restored circulation after occlusive carotid thrombosis, while free t-PA did not, suggesting that this method may be effective in both large and small vessels. Similar and other experiments have been carried out for other thrombolytic agents [350]. The hemodynamic stress also affects the effectiveness of the lytic agents used [351]. These approaches offer the possibility of therapeutic thrombolysis without the risks of bleeding associated with other methods.

Blood cells have many effects on fibrinolyis [352]. Platelets suppress clot lysis in both large and small arteries. Platelets release PAI-1, which inhibits lysis in the vicinity of the clot. In addition, the clot structure is modulated by the platelets, since thrombin is generated on or nearby the platelet surface [353]. Thus, there are higher concentrations of thrombin around the platelets. As a result, many fibers originate from the platelet aggregates, so that fibers are oriented radially, and the average fiber diameter is smaller (Fig. **13**) [354]. Furthermore, platelets enhance the clot stability through an increase of crosslinking by Factor XIIIa [355]. Platelets also inhibit the lysis of pulmonary microemboli [356].

Platelet-rich areas of clots are more resistant to lysis and bind less t-PA [354]. When t-PA is introduced at one edge of a platelet-rich plasma clot, the lysis front velocity is lower in the areas rich in platelets and lysis is heterogeneous, proceeding through meandering channels and sometimes leaving platelet-rich areas unlysed (Fig. **14**). The inclusion of inhibitors of platelet aggregation, such as abciximab or eptifibitide, before formation of the clot, equalized the t-PA binding and the lysis front velocity between areas rich or poor in platelets and increased the overall rate of lysis. These results demonstrate that the lysis resistance of platelet rich clots is mainly due to the heterogeneity in the clot structure between platelet-rich and platelet-poor areas of the clot.

Platelet retraction by the contractile proteins in platelets

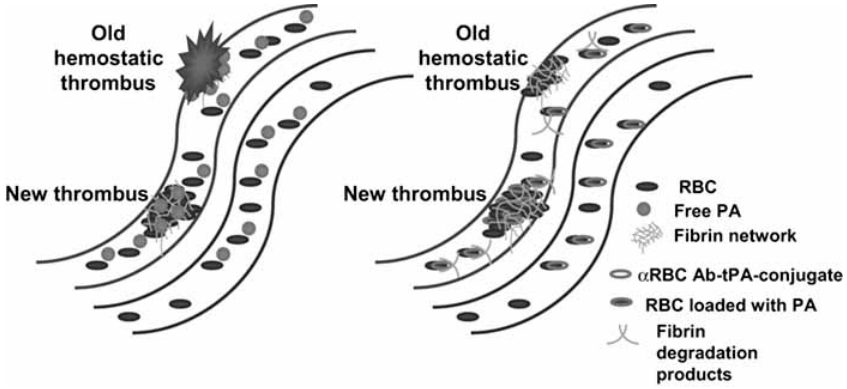

Fig. (12). A 'Trojan Horse' strategy for the prevention of vascular thrombosis. (**Left**) Administration of free t-PA to a blood vessel with an 'old hemostatic thrombus' sealing a site of vascular defect and a new obstructing thrombus. Disadvantages of injecting the free t-PA include rapid clearance from blood, side effects in tissues (brain toxicity, bleeding) and unwanted dissolution of the hemostatic clots. (**Right**) Administration of t-PA-coupled erythrocytes to a blood vessel is followed by prolonged circulation, preferential incorporation to the nascent clots, and reduced penetration to mature clots and normal tissues. The older hemostatic plug is not dissolved, as the previously incorporated erythrocytes do not contain t-PA because the thrombus was formed before the administration of t-PA-coupled erythrocytes. The vessels on the right side in each case show vessels without any thrombi, showing that with the administration of t-PA coupled to erythrocytes, there are fewer side effects because the t-PA is sequestered. Provided by S. Zaitsev (Department of Pharmacology, University of Pennsylvania School of Medicine).

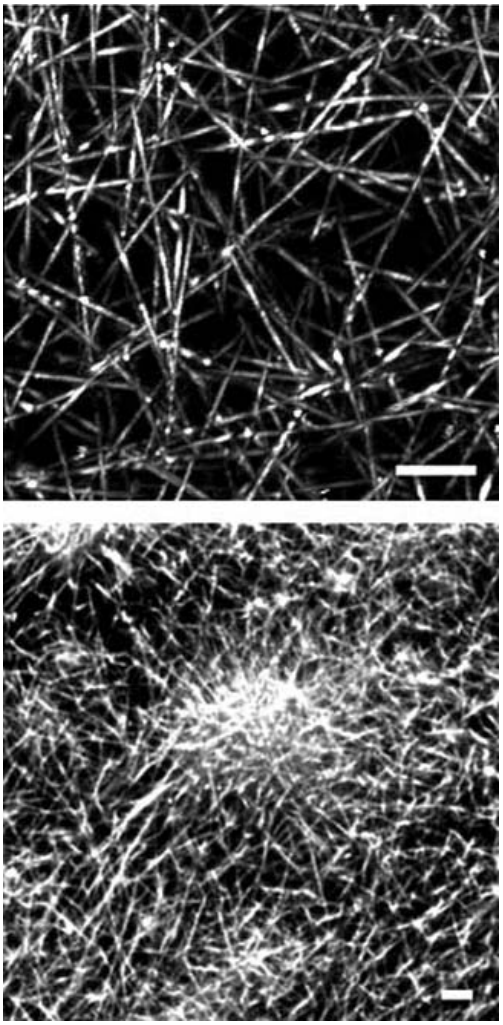

Fig. (13). Confocal micrographs of fibrin clots labeled with colloidal gold showing the difference between platelet-poor and platelet-rich plasma. These are three-dimensional reconstructions of several optical sections, so it is difficult to distinguish branch points from fibers passing each other at different depths in the clot. Note that the fibers making up the clot are nearly all straight with very little or no curvature. A. Clot made by addition of thrombin to platelet-poor plasma. Magnification bar = 5 μm. B. Clot made from addition of thrombin to platelet-rich plasma. Fibers are very dense around the platelet aggregates, with fibers radiating out from the platelets. Magnification bar = 10 μm. Reproduced with permission from [343].

has a striking effect on the susceptibility of clots to lysis [357]. Retraction greatly reduces the fluid phase of the clot, decreasing the amount of Plg available and also the rate of permeation into the clot. In arterial thrombi, the volume of whole blood can be compacted more than 500-fold [358]. Furthermore, the concentration of plasminogen and α_2-AP are a correspondingly small fraction of their original concentration.

CONCLUSIONS

The basic biochemistry of fibrinolysis and its regulation, including the action of Pg, various Pg activators and modulators, have been well defined, but many details and the complex interactions between the reactions are largely unknown. Several naturally occurring and engineered agents have been tested for therapeutic thrombolysis, with considerable success. However, it is clear that there is still much room for improvement, by the development of better drugs, and a whole new field of prophylactic thrombolysis is emerging. Mathematical models have been useful to aid our understanding of basic processes and develop therapeutic regimes. Some physical mechanisms of fibrinolysis have been discovered but these processes are less well understood than the biochemical processes. Many studies have led to some general correlations between clot structure and properties and the rate of lysis. In general, it seems that clots made of thicker fibers are digested more rapidly than those made of thinner fibers. On the other hand, there are a great many exceptions, and no general principles have emerged. It is likely that the microscopic and molecular details of fibrin are also relevant for fibrinolysis. In conclusion, some of the mechanisms that are relevant to defining the structural basis for the variable lytic susceptibility of fibrin are known but much remains to be discovered.

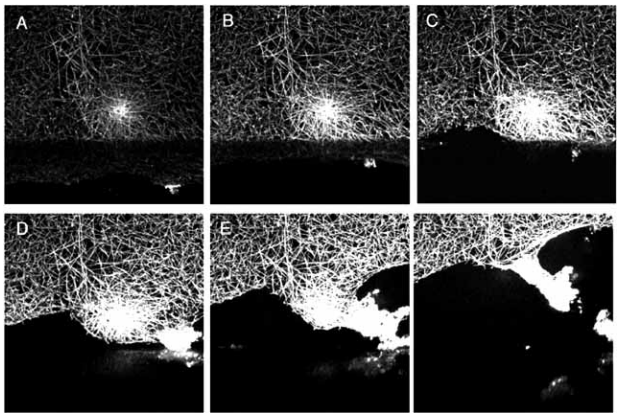

Fig. (14). Dynamic lysis of platelet-rich plasma clot visualized by confocal microscopy. Images were taken every 10 minutes. The lysis front progresses as a straight line until it reaches an area rich in aggregated platelets together with a dense meshwork of fibrin, which is digested more slowly but undergoes a complex process of agglomeration and contraction with release of big chunks of fibrin before disappearing. Reproduced with permission from [356].

ACKNOWLEDGMENTS

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ABBREVIATIONS

α_2-MG	=	α_2-macroglobulin
α_2-AP	=	α_2-antiplasmin
EGF	=	Epidermal growth factor domain
F	=	Finger domain
K	=	Kringle domains
Lp(a)	=	Lipoprotein(a)

MMPs	=	Matrix metalloproteinases
PA	=	Plasminogen activator
PAI-1 or 2	=	Plasminogen activator inhibitor 1 or 2
Plg	=	Plasminogen
Pn	=	Plasmin
SAK	=	Staphylokinase
sct-PA	=	Single-chain tisse-type plasminogen activator
scu-PA	=	Single-chain urokinase-type plasminogen activator (pro-urokinase)
SK	=	Streptokinase
TAFI	=	Thrombin-activatable fibrinolysis inhibitor (TAFIa upon activation)
tct-PA	=	Two-chain tisse-type plasminogen activator
tcu-PA	=	Two-chain urokinase-type plasminogen activator (urokinase)
t-PA	=	Tisse-type plasminogen activator
u-PA	=	Urokinase-type plasminogen activator
u-PAR	=	Urokinase-type plasminogen activator receptor

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