Physiology of Haemostasis: Plasmin-Antiplasmin System

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Fibrin as a Substrate

Plasmin is a potent trypsin-like serine protease that cleaves any substrate after lysyl or arginyl bonds. It activates growth factors and prohormones, actions that are outside the scope of this review, but its main substrate in vivo is fibrin. Many of the cleavage sites in fibrin have been revealed by the study of fibrinogen, which, as a soluble protein, is easier to analyse (reviewed by [1]). The ordered degradation pattern (Fig. 5.1) is detailed here as it is essential to our understanding of what is measured in assays of D-dimer and other fibrin degradation products (FDP). The first cut is to the α-chain of fibrinogen, releasing the αC fragments; the remainder of the molecule is called fragment X (~260 kDa). Fragment X is then cut in the α-, β- and γ-chains across the coiled coil that connects the central E and terminal D domains of fragment X. The cleavage occurs in two steps, first splitting the molecule asymmetrically to generate fragment Y (~160 kDa) and fragment D (~100 kDa) and then cleaving fragment Y into a second fragment D and fragment E (~60 kDa), which contains the amino terminal portion of all six polypeptide chains.

Newly formed fibrin is degraded by plasmin with the same cleavage pattern as fibrinogen, indicating that no major structural reorganization occurs during fibrin polymerization [2, 3]. In contrast, when fibrin is cross-linked by the transglutaminase factor XIIIa (Fig. 5.1, right), it is cleaved at a slower rate and different degradation products arise. D-dimer, which consists of two fragments D from adjacent fibrin monomers, cross-linked via their γ-chains remnants, is generated. This covalent dimer, bound non-covalently to fragment E, is the DD/E complex. This fragment also occurs in long arrays held together by uncleaved coiled coils [4]. Larger FDP have the capacity to reassociate with one another and with fibrin [5], so the substrate for fibrinolysis is not a single entity but a complex and dynamic one, in which both formation and degradation occur simultaneously. The clearance of FDP from the circulation is via the kidney and also liver, depending on the actual fragment [6, 7].

Fibrin as a Vital Surface for Plasmin Generation and Activity

Fibrin is at the heart of the lytic cascade and plays a vital role in "orchestrating its own destruction" [1]. This behaviour will be explained by considering the proteases and inhibitors that regulate

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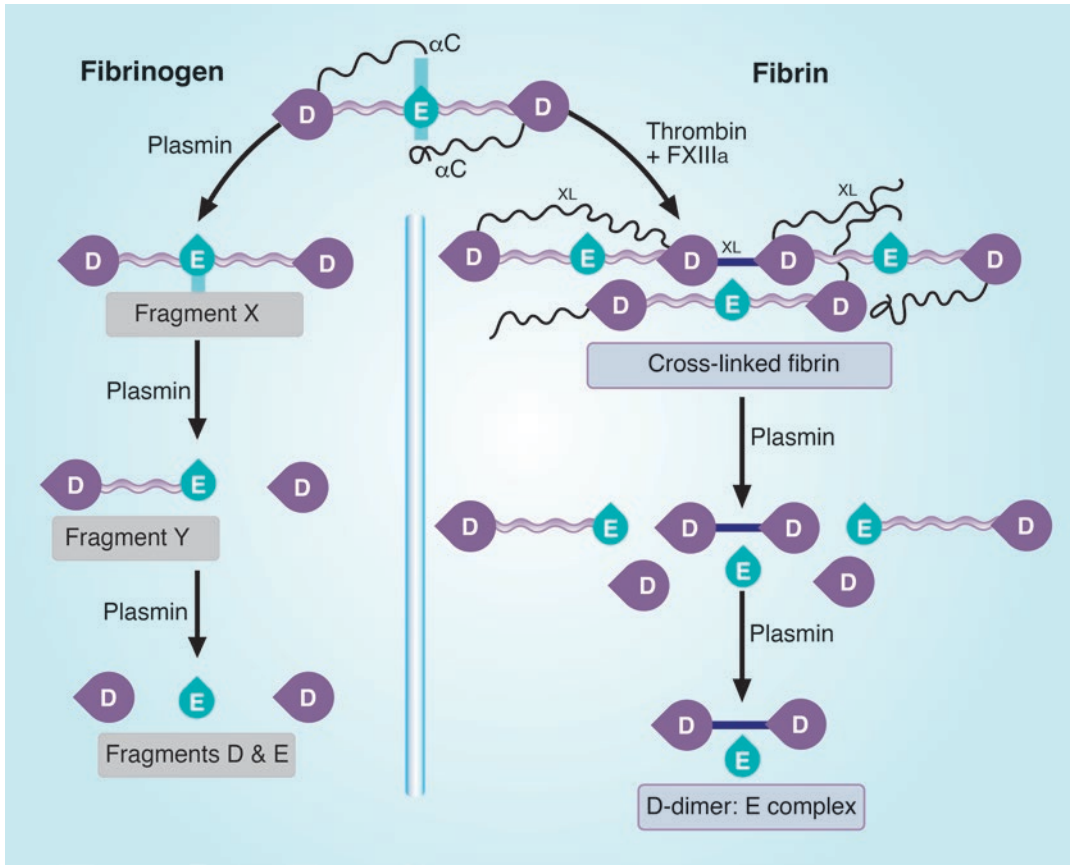

Fig. 5.1 Plasmin degradation of fibrin(ogen). Fibrinogen (**top**) is a three-domain globular protein with extending αC domains. Fibrinogen is degraded asymmetrically (**left panel**). Plasmin initially cleaves the αC domains from fibrinogen generating fragment X, which consists of all three domains connected by coiled coils, but lacks the Aα-chains and the Bβ1–42 sequence. The second cleavage occurs across the coiled coil that connects the central E and terminal D domains, generating fragment Y, which is composed of the central E domain connected by a coiled coil to the D domain. Fibrin is formed by cleavage of fibrinopeptide A and B from fibrinogen by thrombin (**right panel**). Thrombin also activates the transglutaminase factor XIII (FXIIIa) which cross-links (XL) fibrin longitudinally between the D domains and within the α-chain extensions. Cleavage of the two-stranded protofibrils by plasmin initially removes the cross-linked α-chains, followed by the coiled coils to liberate a series of fibrin degradation products (FDP), the smallest being DD/E. Larger complexes, such as DY/YD, are also released from cross-linked fibrin and are subsequently degraded to the DD/E moiety

the system, stressing throughout the governing role of fibrin.

Plasminogen

Plasminogen is a 92-kDa glycoprotein, abundant in plasma (Table 5.1). It is a classic zymogen, a single-chain molecule, activated by cleavage of one peptide bond to produce plasmin, in which the two chains are held together by two disulphide bonds. It is composed of several discretely folded domains. From the N-terminus, these are the activation peptide, a pan apple domain, kringles 1–5 and the protease domain (Fig. 5.2). The crystal structure of plasminogen indicates that two chloride ions in association with the pan apple and serine protease domain hold the zymogen in an inactive closed conformation [8]. The kringles, particularly kringle 1 [8], endow plasminogen

Table 5.1 Plasma balance of the principal proteins of the fibrinolytic system

Protein	Mr (kDa)	Molar concentration	Plasma $t_{1/2}$	Function
Plasminogen	92	2 μM	2.2 d	Zymogen
tPA	68	70 pM	4 min	Protease
scuPA	54	40 pM	7 min	Function
PAI-1	52	200 pM	8 min	Inhibitor
α_2-Antiplasmin	70	1 μM	3 d	Inhibitor
TAFI (pro-CpU)	60	75 nM	10 min[a]	Inhibitor
C1-inhibitor	105	1.7 μM	3 d	Inhibitor
α_2-Macroglobulin	725	3 μM	2–3 d	Inhibitor
PAI-2	46/70	<70 pM	–	Inhibitor
Factor XII	80	375 nM	2–3 d	Zymogen
Prekallikrein	88	450 nM	7–10 d	Zymogen
HMW kininogen	110	600 nM	9 h	Cofactor

[a]Activated form

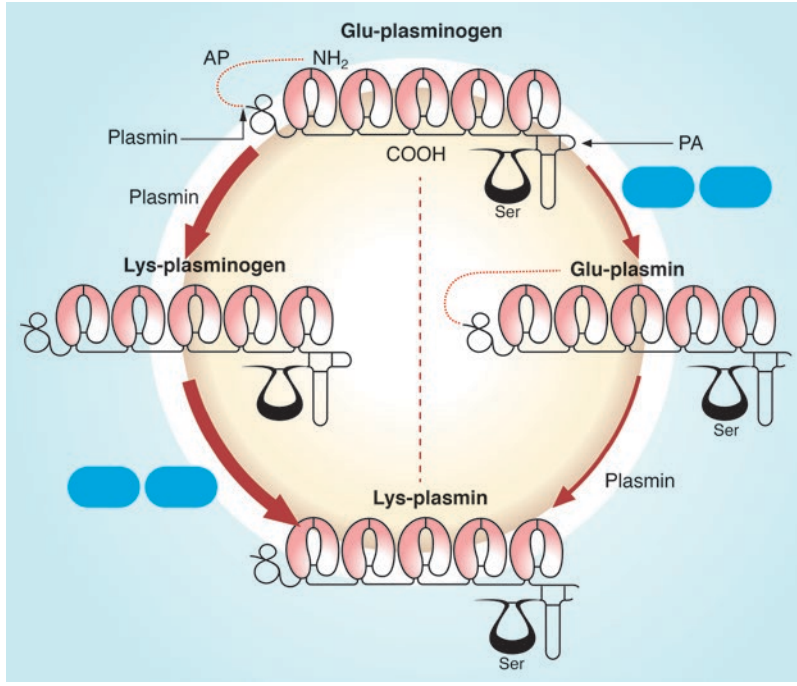

Fig. 5.2 Plasminogen activation. Plasminogen activator (PA) cleaves at Arg 561-Val 562, separating the B (light, protease or catalytic) and the A (heavy, kringle) chains. Glu-plasminogen and Glu-plasmin forms both contain the amino-terminal activation peptide from Gln1 to Lys 76 (shown in red). Plasmin can cleave this activation peptide (**left side**), generating Lys-plasminogen, an intermediate form that interacts with fibrin more efficiently and is more readily cleaved by tPA and uPA. It is this pathway of plasmin generation that occurs more readily, as indicated by the shading and heavyset arrows. Plasmin can also cleave the activation peptide from Glu-plasmin, generating Lys-plasmin (**right side**). The five kringle structures of the A-chain modulate binding of plasminogen to both fibrin and cell receptors. The catalytic centre contains the typical Ser-His-Asp residues and is the major site of interaction with its principal inhibitor, α2-antiplasmin

with the capacity to bind to cells and other proteins; the most relevant to this chapter are fibrin, α_2AP and TAFI. Such binding has profound effects on plasminogen activation. Plasminogen is primarily produced by the liver and is classified as an acute-phase protein [9]. Cells other than hepatocytes can produce plasminogen, for example, eosinophils, kidney, cornea, brain and adrenal medulla; such plasminogen is more likely to have local effects acting on substrates other than fibrin [10–13]. Human deficiency of plasminogen is uncommon, but when it occurs, it is often in association with fibrin deposition, for instance, in ligneous conjunctivitis [14].

Native plasminogen has several variants, in terms of limited proteolysis, degree of glycosylation and genetic polymorphism. For the purposes of this review, we will consider only the two main variants, Glu-plasminogen, the full-length form, and Lys-plasminogen, which has been processed to a variable extent at the N-terminus by trace plasmin. These two forms differ markedly in how efficiently they are activated (Fig. 5.2). Glu-plasminogen is a relatively closed structure [15], whereas Lys-plasminogen is more flexible and open; it binds to the plasminogen activator approximately tenfold more effectively [16–18]. Lys-plasminogen also binds to fibrin with higher affinity than Glu-plasminogen. The same is true of binding to plasminogen receptors, a group of proteins that are exposed on cell surfaces and bind to plasminogen via lysine residues [19]. Thus, through several mechanisms, Lys-plasminogen is activated more readily, especially on the fibrin or cell surface [20].

Plasminogen Activators

The principal plasminogen activators are tPA and uPA, while the contact pathway plays a role in some contexts. Activation of plasminogen is always by cleavage of Arg561-Val562 bond, yielding the two-chain active form, plasmin. It may be helpful to consider the life cycle of a plasminogen activator in terms of synthesis and release into the circulation, neutralization by inhibitors and clearance from the circulation by receptor-mediated mechanisms.

tPA is produced by endothelial and other cells as a single chain but is exceptional in that it is an active serine protease and not a true zymogen [21]. It circulates at low concentrations, mostly in complex with its primary inhibitor, PAI-1 [22, 23]. The plasma half-life is very short (Table 5.1) and shows a circadian rhythm, with lowest levels at night. Plasma tPA can be increased approximately fourfold under experimental conditions by venous occlusion or by drugs that induce acute endothelial release, such as bradykinin, histamine and β-adrenergic agents [24, 25]. Exercise also augments adrenalin-mediated tPA release, but also decreases clearance from the circulation [26]. Both tPA and tPA-PAI-1 complex are cleared by the low-density lipoprotein-related protein receptor (LRP) system [27].

tPA contains a finger domain and two kringle domains; the finger domain is the basis for its affinity to fibrin [28, 29]. This characteristic is crucial because tPA is a poor plasminogen activator in solution and requires fibrin to function as a cofactor in the reaction. Fibrinogen is not able to accelerate plasminogen activation by tPA, as the sites are encrypted in the precursor form [30]. Single-chain and two-chain tPA bind to fibrin in a comparable way [31] with plasminogen increasing the affinity of tPA for fibrin some 20-fold [32], as a result of ternary complex formation. In the absence of fibrin, the K_M values range from 9 to 100 μM plasminogen [33–35]. In most studies, this K_M value is three- to fourfold lower with two-chain tPA than with the single-chain form, a difference that essentially disappears in the presence of fibrin, when both forms of tPA yield K_M values ranging from 0.16 to 1.1 μM plasminogen [33, 35]. These concentrations are readily achieved in blood (Table 5.1). One clear reason for the experimental range in these data is that the kinetics are non-linear [34, 36, 37], with a dual-phase activation. Starting with Glu-plasminogen and tPA in the presence of fibrin, the initial K_M of 1.05 μM plasminogen was observed. Following plasmin formation and

generation of partially digested fibrin, binding of both plasminogen and tPA increased [38–42], so that the K_M was decreased to 0.07 μM plasminogen, with no change in k_{cat} [37].

uPA is synthesized by several cell types, particularly those with a fibroblast-like morphology, but also by epithelial cells [43], monocytes and macrophages [44, 45]. uPA can activate solution-phase plasminogen; it does not require fibrin as a cofactor. This behaviour, which is in marked contrast with tPA, is sometimes interpreted to suggest that uPA is unimportant in fibrinolysis and certainly it has roles in other processes, such as extracellular matrix degradation, cell migration, wound healing, inflammation, embryogenesis and invasion of tumour cells and metastasis [46, 47].

uPA has three domains: an epidermal growth factor (EGF) domain, a kringle and a protease domain. The uPA kringle has no affinity for fibrin. Its main binding, via the EGF domain located in the amino-terminal fragment, is with a specific uPA receptor, uPAR, described later in this chapter. uPA is expressed in its single-chain (sc) form, which has trace proteolytic activity; full activity requires cleavage of Lys158-Ile159 [48]. This can be achieved by several enzymes, the most relevant being plasmin [49, 50], factor XIIa and kallikrein [51]. Normal plasma contains scuPA at relatively stable concentrations of 2–4 ng per mL [52, 53] with little circadian fluctuation [54]. While endothelium is not a major source of uPA, there are reports of increased uPA following venous stasis [53], DDAVP infusion [55] and strenuous physical exercise [56], probably explained by decreased clearance from the circulation by receptor-mediated mechanisms. Under normal circumstances ,uPA activity is not detected in plasma, but both leukocyte-associated and free scuPA are elevated in leukaemia [57] and other disorders, including liver disease [58]. If generated, uPA is rapidly cleared from plasma, in a manner that depends on hepatic blood flow [59]. The LRP system binds and internalizes scuPA and uPA-PAI-1 complexes [27, 60, 61]. The asialoglycoprotein receptor, on parenchymal liver cells, also removes nonsialated uPA from the circulation [59].

Contact activation is a distinct process resulting from the interactions of four proteins, factor XII (FXII), prekallikrein (PK), factor XI (FXI) and high-molecular-weight kininogen (HK). Negatively charged surfaces such as polyphosphate [62, 63], RNA [64], misfolded proteins [65] and collagen [66] stimulate reciprocal activation of FXII to FXIIa and of PK to kallikrein (PKa) in association with its non-enzymatic cofactor, HK. The process is accelerated by zinc ions which induce a conformational change in FXII [67–71] and HK [72–74], thereby augmenting surface interactions. The downstream targets of these proteases have been debated as this pathway is associated with coagulation via cleavage of FXI to yield FXIa, inflammation by generation of bradykinin from HK and fibrinolysis.

Of note, while FXII is classified as a coagulation factor, it is structurally related to tPA, uPA and plasminogen [75, 76] and can function in plasminogen activation by different mechanisms. FXIIa directly activates plasminogen (Fig. 5.3) albeit relatively poorly compared to tPA and uPA [77–79]. However, the reaction is markedly enhanced by negatively charged surfaces such as dextran sulphate [80] and importantly by platelet-derived polyphosphate [81]. Circulating plasma concentrations of FXII are four orders of magnitude higher than tPA and uPA and, combined with the increase in plasma half-life, suggest that in certain environments or conditions, in vivo FXIIa could be a relevant plasminogen activator [82].

PKa generated by FXII-dependent [51, 83] and FXII-independent [84] pathways is a kinetically favourable activator of scuPA (Fig. 5.3) which in turn activates plasminogen. Finally, the vasoactive peptide bradykinin, described above in the inflammatory arm of the contact pathway, also indirectly impacts fibrinolysis by stimulating tPA release from endothelial cells [85, 86]. These three functionally distinct mechanisms implicate the contact pathway as a modulator of plasminogen activation, but further studies are necessary to unravel its contribution in different milieu.

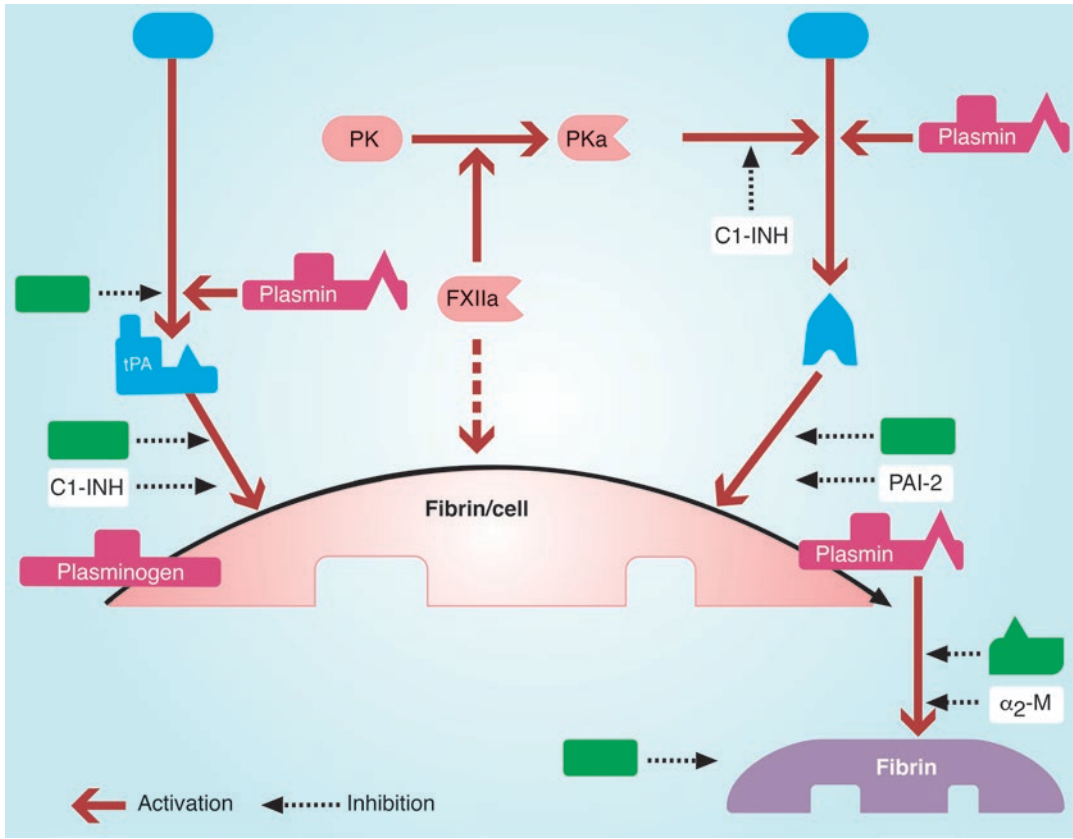

Fig. 5.3 Significant players in the fibrinolytic system. scuPA, single-chain urokinase plasminogen activator; sctPA, single-chain tissue plasminogen activator; α_2AP, α_2-antiplasmin; α_2M, α_2-macroglobulin; C1-INH, C1-inhibitor; PAI-1, plasminogen activator inhibitor 1; PAI-2, plasminogen activator inhibitor 2; TAFI, thrombin-activatable fibrinolysis inhibitor; FXIIa, activated factor XII; PK, prekallikrein; PKa, kallikrein. Activation of plasminogen to plasmin usually occurs on a surface, either fibrin or a cell membrane. Once formed, plasmin degrades fibrin as described in Fig. 5.1

Inhibitors of Plasmin Generation and Activity

The proteases of the system are controlled by inhibitors, most of which act directly on the proteases and form inactive complexes with them. PAI-1 and α_2AP are members of the serpin family, which inhibit plasminogen activators and plasmin, respectively, via a reactive centre loop that mimics the protease substrate (reviewed by [87]). A second mode of action, exemplified by TAFIa, is modulation of the generation of fibrinolytic activity.

PAI-1 is the principal inhibitor of tPA and uPA and inhibits both with second-order rate constants greater than 10^7 M^{-1} S^{-1}, close to the diffusion limit [88]. It does not inhibit scuPA, which is largely inactive, but it does associate with scuPA non-covalently [89]. It is an unusual serpin in that it spontaneously loses activity by insertion of its reactive centre loop into the core of the molecule [90]. This inactive form was originally termed "latent", which unfortunately gives an impression that the latent material is physiologically activated. Reactivation is indeed possible, but only by chemical denaturation and

refolding [91]. It was characterized originally as a product of endothelial cells but it is synthesized by most cells in culture, including megakaryocytes [92], endothelial cells [93], hepatocytes [94] and adipocytes [95–97]. PAI-1 is synthesized in its active form and circulates in plasma in complex with vitronectin, which stabilizes the active form substantially lengthening its plasma half-life [98].

PAI-1 plasma concentrations are approximately 20 ng per mL [99–101] but reported values range, even in normal individuals, from barely detectable to 40 ng per mL. The variations may be circadian; PAI-1 plasma concentration peaks in the morning [102–104], and in addition, PAI-1 is an acute-phase protein [105]. Understanding its behaviour in response to stress is complicated by the fact that it is synthesized by a wider range of cells than the classic acute-phase proteins and that it is responsive to many stimuli. Some variations in PAI-1 measurements may be methodological. It is necessary to exclude platelets and their release products in analysis of plasma PAI-1, since platelets are the major pool (more than 95%) of circulating PAI-1 antigen [106]. PAI-1 in plasma is in excess over tPA (Table 5.1); therefore, most of the tPA is in complex with PAI-1. Immunological assays of either protein generally measure both free and complexed forms, requiring care in interpretation. Gram-negative septicaemic patients have dramatically elevated plasma PAI-1 concentrations, as much as 50-fold over normal, and are associated with high mortality [107]. High circulating PAI-1 is associated with a range of disease, including cardiovascular disease [108, 109] and cancer [110]. The causal significance remains unclear, and it seems that high PAI-1 does not independently predict disease when factors like obesity, diabetes and elevated triglycerides are taken into account [111]. There is a guanine insertion/deletion polymorphism at position 675 in the PAI-1 promoter [112], which is associated with differences in circulating PAI-l [113], but the predictive power of this polymorphism appears to be low [111, 114]. Deficiency of PAI-1 in humans is rare but it causes a lifelong bleeding disorder, characteristically after a delay, consistent with normal clotting but premature lysis of haemostatic plugs at sites of vascular trauma [115–118]. Fibrinolytic inhibitors such as tranexamic acid decrease plasminogen activation and therefore are effective in normalizing haemostatic function in such patients [117, 118].

α_2AP is the principal inhibitor of plasmin, the term fast-acting being used to stress the rapid inhibition, with a second-order rate constant of 4×10^7 per M per second [119]. Its plasma concentration is 1 μM, about half the molar concentration of plasminogen; it has to be remembered that plasma plasminogen is seldom, if ever, entirely converted to plasmin, so the inhibitor is usually in excess. It is synthesized in the liver and consequently decreased in patients with advanced impairment of hepatic function. The $t_{1/2}$ of the native inhibitor is approximately 3 days, whereas the covalent plasmin/α_2AP (PAP) complex is cleared with a $t_{1/2}$ of approximately 0.5 days [120].

α_2AP circulates in several forms, depending on limited proteolysis at N- and C-termini. The processing of the inhibitor has little impact on the inhibitory capacity of α_2AP which depends on the reactive centre loop. Newly produced α_2AP (Met form) has 12 residues at the N-terminus that can be cleaved to yield N-terminal Asn [121] by an antiplasmin cleaving enzyme (APCE) [122]. Both forms are equally represented in plasma [123]. The N-terminal cleavage is important because it reveals Gln2, in the processed, Asn form, the Gln2 being cross-linked to Lys 303 of the fibrin(ogen) Aα-chain by FXIIIa [124, 125]. In contrast, in the Met form, Gln2 is blocked [126]. Fibrin to which α_2AP is cross-linked resists lysis by plasmin, and this observation was central to the discovery of the first human deficiency of α_2AP [124]. Consistent with this, antibodies that react specifically with cross-linked α_2AP stimulate lysis of fibrin [127].

Comparison of α_2AP with other members of the serpin family reveals that it has a C-terminal extension of some 50 residues [121]. This full-length form and a shortened form are both detectable in normal human plasma [128]. The full-length form binds plasminogen but the processed form, which is still a potent inhibitor of plasmin, cannot bind plasminogen [129].

The enzyme responsible for this C-terminal cleavage has not yet been characterized. The ratio of two forms, plasminogen binding to non-binding, is approximately 2:1 in plasma. This was still true even in advanced liver cirrhosis [58], despite the impaired synthesis of α_2AP in these patients.

Binding of α_2AP to plasminogen competes with the plasminogen-fibrin interaction, as it occurs via the same lysine binding site (Fig. 5.4). Plasmin formed on fibrin is therefore relatively protected from the action of α_2AP [130], a key finding in the control of fibrinolysis [130]. The experimental basis for this concept used lysine analogues, in the presence of which α_2AP was about 100 times less effective in inhibiting plasmin [119]. The exact Lys residues responsible for binding the C-terminal region of α_2AP to plasminogen are not conclusively defined. Studies have shown a major effect of Lys452, but that other internal Lys residues "tether" the kringles [131, 132]. A different study, in which Lys residues were systematically mutated, suggested that Lys436 had the greatest effect [133].

Thrombin-activatable fibrinolysis inhibitor (TAFIa; also known as carboxypeptidase B, U, R [134] and *CPB2* gene product [135]) removes C-terminal lysyl residues from fibrin, which, as previously stressed, are important in the binding of plasminogen [136]. TAFI is produced as a zymogen (or procarboxypeptidase) and is activated by the thrombin/thrombomodulin complex [137] or by plasmin in the presence of glycosaminoglycans [138]. Its activation by thrombin makes it an important molecular link between fibrinolysis and coagulation [139]. TAFI is produced in the liver but there is considerable variation in normal circulating concentrations [136, 140] and only a fraction need be activated for full physiological impact [137]. Its activity is controlled by its instability, with an effective plasma half-life of only about 10 min [141]. The function of TAFIa was shown in clot lysis assays; potato tuber carboxy-

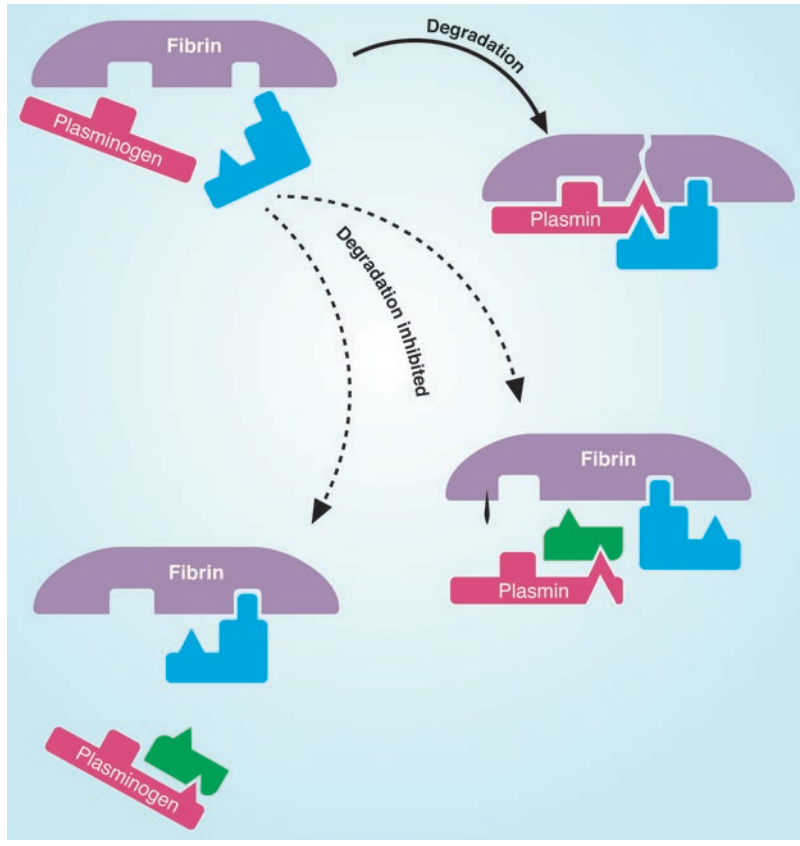

Fig. 5.4 Localization of plasmin(ogen) and tPA on fibrin; interference by α2-antiplasmin. Fibrin binds plasmin(ogen) and tPA directly and acts as a cofactor in plasminogen activation, thereby augmenting its own destruction. α_2-Antiplasmin (α_2AP) can bind to plasmin(ogen) in solution, obstructing binding to fibrin. α_2AP is also cross-linked to fibrin, via the action of factor XIIIa, which prevents plasmin from binding to fibrin and neutralizes the plasmin activity. These events hamper plasminogen activation on fibrin and/or inhibit plasmin activity, thereby limiting fibrin degradation

peptidase inhibitor relieves the inhibition [139, 142]. This approach and more sensitive and specific assays for TAFIa have shown that the carboxypeptidase must be maintained at a threshold level to be effective in modulating fibrinolysis; this level fluctuates in relation to plasmin concentration [143]. Several polymorphisms in the TAFI gene have been reported, resulting in four isoforms [144, 145]. These isoforms explain the normal wide range in concentration, but do not correlate strongly with disease [145, 146]. Thr325Ile polymorphism has been shown to be an independent risk factor for ST acute myocardial infarction in a Mexican population [147]. Elevated TAFI appears to be a mild risk factor for venous thrombosis [148], and it also increases in inflammation, correlating with other acute-phase markers [149]. Contrary to this, patients recently suffering a myocardial infarction have been shown to have lower levels of TAFI [150].

Increased fibrinolytic activity in haemophilia patients is explained by defective TAFI activation. Most thrombin is formed after clot formation, mainly by back activation of FXI by thrombin, with deficiencies in FXI resulting in a mild to moderate tissue-specific bleeding disorder (haemophilia C). In the absence of FXI, clots lyse more readily [151], which is associated with the loss in feedback activation of FXI by thrombin [152]. The enhanced generation of thrombin augments TAFI activation stabilizing clots against premature lysis [153, 154]. In line with this, defective TAFI activation in congenital haemophilia A is associated with uPA-mediated joint bleeding [155]. Addition of TAFI, thrombomodulin or factor VIII to haemophilia A plasma restores normal fibrinolysis [156]. Consistent with this, incorporation of anti-factor XI antibodies or inhibition of TAFIa in a rabbit model resulted in an almost twofold increase in endogenous thrombolytic activity [157].

We described earlier the potential contribution of the contact pathway in facilitating plasminogen activation. The role of FXIa in sustaining thrombin generation and therefore TAFI activation implicates the contact pathway in antifibrinolytic as well as profibrinolytic mechanisms. Indeed, abnormal clot structure and sensitivity to fibrinolysis have been described to help predict the risk of bleeding tendency in severe and partial FXI deficiency [158, 159].

Other Inhibitors

In most situations, α_2AP, PAI-1 and TAFI are the major gatekeepers in the regulation of plasmin generation and activity, but there are other inhibitors that may function in specific circumstances, which will now be introduced briefly.

PAI-2 is an inhibitor of uPA purified from human placenta and the cell line U-937 [160, 161]. The role of PAI-2 as a PA inhibitor has been questioned [162], as mice deficient in PAI-2 do not present any major haemostatic abnormalities [163]. The intracellular location of this serpin and the fact that it is a much poorer inhibitor of uPA and tPA [161] have led researchers to believe that its functions may lie outside the haemostatic cascade. In the circulation, monocytes are the main reservoir of PAI-2 [164] and may increase fibrin stability on migration into thrombi, particularly as PAI-2 is cross-linked to fibrin [165]. Interestingly, deficiency of PAI-2 is found to interfere with venous thrombus resolution in mice [166], most likely due to the instigated inflammatory response. PAI-2 is not normally detected in normal plasma, except in pregnancy, where it rises steadily to reach approximately 250 ng/mL by the third trimester [167]. In placental dysfunction[413,414] and intrauterine growth retardation [168–170], the rise in plasma PAI-2 is much smaller, highlighting its importance in normal foetal development. PAI-2 also occurs in plasma of patients with acute myeloblastic leukaemia (M_4 and M_5, [171]) and in patients with sepsis [172]. Local PAI-2 activity appears to be relevant to a number of cancers, and studies on the function of this serpin in these settings may provide further clues to its true biological role [173, 174].

α_2-Macroglobulin (α_2M) is a non-serpin inhibitor of wide specificity. This breadth of targets and its relatively high plasma concentration (2.5 g per L, 3 μM) make it an effective stand-in inhibitor that contains the activity of many proteases, including plasmin, tPA and uPA [57]. α_2M

is a tetramer made up of a pair of dimers containing two reactive sites. When proteases are inhibited by α_2M, they generally retain activity towards small peptide substrates, but are unable to cleave larger targets.

C1-inhibitor is a highly glycosylated serpin that directly modulates the activity of C1r and C1s proteases of complement C1. It also inhibits the contact proteases, FXIIa, FXIa and PKa, as well as tPA, plasmin and uPA. It circulates in plasma at a relatively high concentration (1.7 μM). When tPA is in excess over PAI-1, complex formation with C1-inhibitor is observed [23, 58, 175]. Its diverse targets suggest that it would function in regulating contact phase–dependent fibrinolysis and the conversion of scuPA to uPA (Fig. 5.3). Indeed, peripheral blood mononuclear cells from patients with hereditary angioedema (HAE), arising from a deficiency in C1-inhibitor, express elevated levels of uPAR [176]. HAE is also associated with aberrant fibrinolytic activity as a result of dysregulated plasmin generation and inhibition. Indeed, during activation of fibrinolysis, approximately 15% of plasmin inhibition is reportedly accounted for by C1-inhibitor [177]. The increase in bradykinin generation in HAE patients will also augment tPA release from the endothelium.

Regulation of Plasmin Generation and Activity

So far, we have highlighted three important concepts: zymogen activation, protease inhibition and, crucially, the role of fibrin in promoting activation of plasminogen and protecting plasmin from inhibition. Further discussion requires consideration of particular situations, so we will now examine the balance of the various proteases and inhibitors in plasma, on platelets, cell surfaces and thrombi.

Plasma Balance

Plasminogen, the central player of the fibrinolytic system, circulates at approximately 5 orders of magnitude higher than tPA and scuPA (Table 5.1). Plasminogen is turned over relatively slowly, with a half-life of 2.2 days for Glu-plasminogen and 0.8 days for Lys-plasminogen, while tPA and scuPA have plasma half-lives of only minutes. From this we can infer that the rates of synthesis, release and clearance are low for plasminogen and much higher for the PA, illustrating the more dynamic part of the system. Similar considerations apply to the main inhibitors. PAI-1 is present in plasma at only 400 nM, while α_2AP circulates at 1 μM, and again the plasma half-lives are in marked contrast.

Fibrinolytic activity is not normally detectable in plasma because plasminogen is a true zymogen, and therefore inactive, while the one active PA in plasma, tPA, is normally controlled by an excess of PAI-1. Even if the concentration of PAI-1 were insufficient for full neutralization, then α_2AP, C1-inhibitor and α_2M would act as backup inhibitors. The other potential activator, scuPA, is not sufficiently active to initiate the process of plasminogen activation, as prior activation by plasmin or kallikrein is necessary. Any trace of plasmin generated in plasma would be quickly neutralized by α_2AP, again endorsed as necessary by other inhibitors, especially α_2M. So the quiescence of the system, in plasma, is maintained by tight control of protease activity, both at the level of existence of plasminogen as a zymogen and at the level of control by inhibitors, primarily PAI-1 and α_2AP.

Cellular and Platelet Contributions

While the central role of fibrin in controlling activation of plasminogen and protection of plasmin has been appreciated for several years [130], we are now aware that many of the same general characteristics apply to cell-based or platelet-based fibrinolysis. That is, more efficient activation of plasminogen occurs on the surface of cells, while cell-bound plasmin is protected from inhibition by α_2AP [178]. Plasminogen binding to circulating cells, including monocytes, neutrophils and platelets, was first reported in 1985 [179]. Binding to platelets is now known to occur

in distinct locations, dependent on the specific subpopulation [180]. The proteins responsible for binding vary from cell to cell but include $PlgR_{KT}$, α-enolase, S100A10 (functioning with annexin A2), actin, cytokeratin 8 and integrins $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$ (reviewed by [181]). The binding of plasminogen to these receptors tends to be low affinity but high capacity, with some cell-surface proteins only found on cells undergoing apoptosis. Not all these proteins express the C-terminal Lys that is expected of a plasminogen-binding protein. $PlgR_{KT}$ is a true membrane protein and is synthesized with a C-terminal Lys residue. tPA binding to cells occurs via annexin II and also directly to $PlgR_{KT}$ [181]. Other reports on tPA receptors, which have been characterized in less detail [182, 183], may be of the same or similar molecules.

uPA and scuPA bind to a well-characterized receptor, uPAR (CD87), with high affinity (K_D 10^{-9} to 10^{-11} M) depending on the cell type [184]. uPAR is not a transmembrane protein but is attached to membranes via a glycosylphosphatidylinositol (GPI) anchor. Binding of uPA to uPAR elicits signalling [185], via other intracellular proteins. Other proteins also bind uPAR, including vitronectin and integrins in complex with caveolin [186]. The uPA/uPAR complex on some cells is associated with Endo 180, also known as uPAR-associated protein (uPARAP) [187], and has a role in collagen IV internalization [188]. uPA bound to uPAR is still inactivated by PAI-1 but not as fast as in solution [189]. The uPA/PAI-1 complex is then internalized, while uPAR is recycled to the surface, a process that also involves LRP [61, 190]. uPAR has clear roles in migration and metastasis. In terms of fibrin degradation, we must distinguish between activation by scuPA and uPA. In the case of uPA, which can freely activate plasminogen in solution, binding to uPAR seems not to affect plasminogen activation, but the activity of scuPA is increased by two orders of magnitude when it is bound to uPAR on the surface of monocytes [191, 192]. An elegant experiment in which a uPA variant was directly anchored to the cell surface showed a stimulation of plasminogen activation similar to that achieved by binding to uPAR [193]. This is consistent with the principal function of uPAR being one of localization of uPA to the cell surface rather than enhancement of catalytic activity. The same co-localization and reciprocal activation of scuPA and plasminogen occurs on platelets [50], which do not express uPAR, indicating there are additional receptors yet to be discovered. Other studies show that cellular binding of plasminogen and (sc)uPA does not have to be on the same cells or surface to facilitate fibrinolysis [194].

Platelets make several contributions to clot stability and lysis. On the profibrinolytic side, activated platelets exhibit endogenous plasmin activity [180] and surface-bound plasmin, formed from local plasminogen, is afforded protection from inhibition by α_2AP. On the antifibrinolytic side, there is the physical barrier to lysis that results from clot retraction, added to which platelets have a pool of FXIII [195] that stabilizes fibrin. Further, platelets are a source of the three main inhibitors of fibrinolysis, PAI-1, α_2AP and TAFI (Fig. 5.5). These platelet-derived pools result from synthesis and packaging of the inhibitors at the megakaryocyte stage. Indeed, it has been reported that while platelets are devoid of a nucleus, they are capable of synthesizing large quantities of PAI-1 [196]. Recent work has illustrated that despite our traditional view that PAI-1 is released from platelets, a considerable amount of active PAI-1 is retained on the activated platelet membrane [197]. In terms of activity, platelet PAI-1 is less active than plasma PAI-1, but platelets still account for some 50% of the total circulating active PAI-1. The platelet pools of α_2AP and TAFI are not as substantial, accounting for less than 1% of the total blood pool [198, 199] and may have functional significance in particular niches. The interaction of platelets and fibrin is regulated by the integrin αIIbβ3 and is key to the process of clot retraction. A recent elegant study has illustrated that the processes of clot retraction and fibrinolysis are mechanistically coupled indicating their intrinsic interaction in vivo to modulate thrombus size [200].

Studies on human thrombi reveal that the inhibitors of fibrinolysis, especially PAI-1, accumulate in great excess over proteases [201, 202],

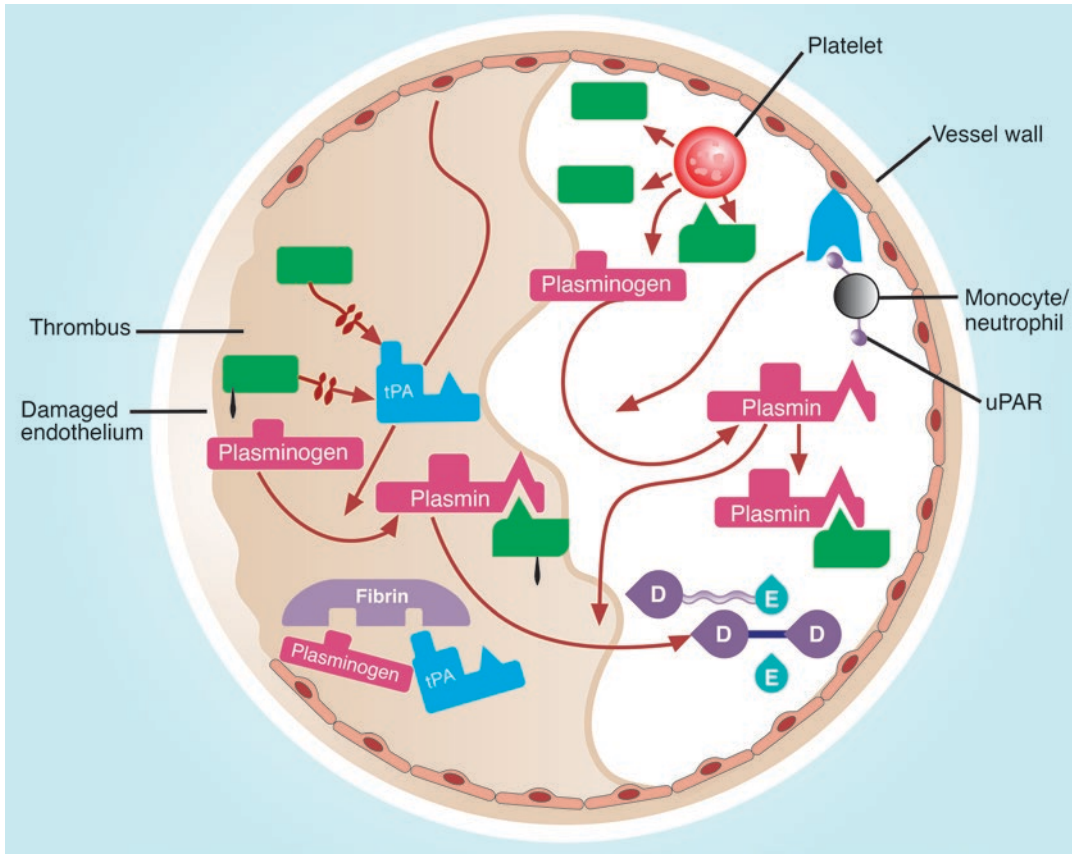

Fig. 5.5 The balance of fibrinolysis in the injured vessel wall. Schematic representation of the different modes of plasmin formation, inhibition and clearance within a damaged vessel wall, with a partially occluding thrombus. Solution, cell-surface and fibrin phases of plasmin formation are represented. Plasminogen circulates at a relatively high concentration (2 μM) and is readily incorporated into a forming thrombus by virtue of its fibrin binding capacity. tPA is largely derived from the endothelium and only circulates at low concentrations with high turnover. tPA-mediated plasminogen activation is slow in solution, but is enhanced severalfold when bound to its cofactor, fibrin. uPA is found in the circulation and is from monocytes and neutrophils. uPA does not exhibit fibrin specificity and readily activates plasminogen in solution while bound to its cell surface receptor, uPAR. Association of uPA with uPAR provides a focal point for plasmin generation. Plasmin degrades fibrin into fibrin degradation products, represented here as D dimer and DD/E complexes. The system is regulated by several inhibitors; only the principal ones are shown here for clarity. Complexes of active enzyme and inhibitor are rapidly cleared from the circulation via a low-density lipoprotein receptor. PAI-1 circulates at low concentrations, but a large pool is released from platelets upon activation. PAI-1 can inhibit tPA and uPA in solution and in the presence of fibrin or cell surfaces, but this latter process is generally less efficient. α_2-antiplasmin (α_2AP) is abundant in plasma and a minor pool is also released from activated platelets. α_2AP inhibits plasmin generation in solution, but plasmin formed on cell or fibrin surfaces is relatively protected. α_2AP is cross-linked directly onto fibrin localizing it at the site of plasmin generation. TAFI is found in plasma and platelets and can be cross-linked to fibrin. TAFI is activated by the thrombin/thrombomodulin complex or plasmin to generate TAFIa which downregulates plasminogen activation on fibrin, by removing the C-terminal lysine residues that are important for the binding of plasminogen to fibrin. This intricate sequence of events and interactions modulate fibrin accumulation in the body in a precise and coordinated manner. The many different feedback loops and surfaces involved localize reactions thereby preventing excessive plasmin generation in the circulation

providing an explanation as to why established thrombi are often resistant to lysis. Observations on human thrombi also show they retain substantial amounts of coagulant and fibrinolytic activity [203, 204]. Of course, such diverse material is taxing to work on in a quantitative way and there are obviously differences in venous and arterial thrombi and between mural and luminal thrombi.

Our studies in Chandler model thrombi showed that these thrombi lyse spontaneously, with fibrinolytic activity that could be ascribed primarily to uPA but to a lesser extent to tPA, elastase and cathepsin G [205]. This spontaneous generation of fibrinolytic activity [204] was dependent on polymorphonuclear cells, primarily neutrophils, generating local uPA activity on uPAR [205]. Plasma α_1-antitrypsin was crucial in protecting the activity from neutrophil elastase [206]. The integrin $\alpha_M\beta_2$ is important in the generation of such local activity [207]. Discovery of a role for local uPA in thrombus lysis ran counter to the usual proposition that tPA's role is fibrin degradation and uPA mediates other cellular events. There is, however, compelling support for it from a number of other studies, including failure of thrombi from uPA gene knockout mice to resolve [208]. In that model, the uPA activity was associated primarily with monocytes, which migrate into thrombi [209] and express fibrinolytic activity [44]. Indeed, monocyte-bound uPA has been shown to reduce thrombus size in a model of venous thrombosis [210].

Questions That Remain

What Initiates Fibrinolysis?

The available evidence suggests that the Glu-plasminogen to Lys-plasminogen conversion is the initiating event. It has the required features of leading to large-scale amplification as the plasminogen binding sites on fibrin are revealed by partial lysis, and formed plasmin is protected from α_2AP. Part of the same question is which PA is responsible for the first molecules of plasmin that allow Glu-plasminogen to be converted to Lys-plasminogen? In the context of fibrin, with no cells or platelets, it may be tPA, a few molecules of which may be free of PAI-1, that provides initiation, especially since its single-chain form is active and not as readily inactivated by PAI-1. This has been suggested by Thorsen (1992) in his well-established biphasic lysis [211], where a small amount of plasminogen on fibrin fibres is activated and then degrades fibrin to generate C-terminal lysine residues that bind additional plasminogen and perhaps tPA, leading to the second faster phase of tPA-mediated fibrinolysis [212]. The molecular interactions and specific binding sites involved have been extensively reviewed [30]. Experiments using tPA variants show that the finger domain of tPA plays a more dominant role in the interaction with fibrin than the kringle 2 interaction with C-terminal lysine residues [213]. This suggests that it is the binding of plasminogen to partially degraded fibrin, and thus subsequently the opening of the closed to open confirmation, that is the crucial step in the rapid second phase of fibrinolysis. This central role of plasminogen may suggest that the PA responsible for activation is less crucial than previously assumed. Our experiments with TAFI demonstrated a similar delay in lysis regardless of the PA used [214] and we interpreted this as plasminogen primarily controlling fibrin-bound plasmin generation.

If a cell membrane is present, then it may be scuPA, bound to cellular uPAR or on platelets, that yields the initial protease activity. This is suggested on the basis of several experimental systems, including data showing that the ordered addition of scuPA and then tPA [215] is potentially more effective than either agent alone. Our own work on Chandler model thrombi underlines the importance of the scuPA/uPA system in spontaneous lysis [205] but affirms the involvement of other proteases, especially tPA [204]. When in association with cellular uPAR, scuPA binds PAI-1 and other serpins reversibly [89]. This has been interpreted in terms of receptor-bound scuPA initiating proteolytic activity, with conversion to uPA achieving inhibition thereby regulating the activity [216].

How Best to Measure Fibrinolysis?

Fibrinolysis, like other cascade systems, coagulation and complement, can be studied by various means. Individual components can be quantified, either as antigen or activity, and under defined situations can provide clear answers. However, the complexities of the system mean that a change

in one factor can influence measurements of another and therefore it is important to interpret results with caution. As an example, tPA activity is challenging to measure in plasma, as it is at the limit of detection of most assays. Elevated PAI-1 may depress the activity that is measured. Frequently, a manipulation of plasma is necessary to reveal tPA activity, including acidification of plasma or preparation of a euglobulin fraction, where tPA, plasminogen and fibrinogen are retained. Most inhibitors are removed but about 50% of PAI-1 is retained [100], and these facts must be borne in mind for valid interpretation. As mentioned previously, circulating tPA is variable, whether at the level of synthesis or release; therefore, it is vital to consider the time course as each sample represents a snapshot. Rapid hepatic clearance of tPA and of tPA-PAI-1 complex from the circulation rapidly restores the system to normality, allowing key events to be overlooked.

It is often essential to measure more than one analyte for a fuller appreciation of the system. Ideally, the aim is to know how much enzyme is free and/or active and how much has been converted to a complex, such as tPA-PAI-1. A combination of ELISA and activity assays may provide a clear picture, but only if the specificity of the ELISA is known in some detail. Ideally, measurement of PA would be complemented by examining a consequence of the elevation, for instance, the fibrin degradation products produced, which of course reflects the presence of the fibrin substrate, or generation of the plasmin-α_2AP complex. The essential feature of ELISA for a complex is the use of antibody to one of the proteins, e.g. α_2AP, as a capture system and an antibody to the second moiety, e.g. plasmin, in the detection system. The capture antibody in this example will bind free α_2AP and α_2AP in complex, giving rise to potential competition and misrepresentation of the results. This element limits the use of these assays to situations where the free protein is decreased, for instance, in liver disease, where α_2AP is lower than normal. Other approaches to measuring overall fibrinolytic activity in plasma include measurement of a zone of lysis on a fibrin plate, clot lysis assays and zymography. Recently, a method which combines magnetic immunocapture of leukocyte-derived microvesicles and chromogenic measurement of plasmin generation has been described [217]. These can all be useful but there are limitations associated with most individual assays. For instance, in plasma clot lysis assays, the effects of FXIIIa cannot be reproducibly observed [218]. In addition, the overwhelming effects of α_2AP make it difficult to see inhibition by PAI-1. Failure to be alert to such considerations gives rise, in the literature, to many inappropriate interpretations about the relative importance of particular proteases or inhibitors. In all assays, the balance of enzyme to inhibitor ought to be as close to physiological as possible. When tPA is added, it should be at a low concentration, always remembering that it is a catalyst, not a reagent that is consumed. The literature abounds with examples where PA are added at high concentrations, simply to speed up the assay. This distorts a system that is designed to be delicately poised and generates artefacts of the experimental system rather than a true reflection of what goes on in vivo.

Detailed analysis of the fibrinolytic system is only practical for small numbers of samples, but, for large clinical cohorts, the aim is to obtain an insight from a limited number of assays. Not surprisingly this has promoted the use of overall measures of activity, such as global assays of fibrinolysis, which have inherent advantages and some limitations. Thromboelastography is rapid, widely available and easily applicable to large sample sizes. However, most studies add tPA as a stimulus. In this situation, added enzyme should be kept to a minimum, to avoid generating results that are far from physiological. Another global assay quantified fibrin degradation products after collection of blood samples onto thrombin [219]. Comparison of samples with and without aprotinin gives a measure of global fibrinolytic capacity, an approach that has proved useful clinically [220]. It should be noted that thrombin greatly enhances endogenous fibrinolytic activity, probably by inactivation of PAI-1 among other mechanisms. This consideration serves as a useful aide-mémoire that fibrinolysis is not an independent system. As Ratnoff reminds us, “The coagu-

lation, fibrinolysis, complement and kinin pathways are studied separately by scientists for their convenience. In life, they form a seamless web" [221]. Undoubtedly, we choose our approaches and molecules of interest to us, and may well ignore other players, by virtue of the experimental system used. These choices may be convenient, but we must bear in mind the selection bias introduced into the system.

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