REVIEW ARTICLE

Binding of plasminogen activators to fibrin: characterization and pharmacological consequences

Robin FEARS

Beecham Pharmaceuticals Research Division, Great Burgh, Epsom, Surrey KT18 5XQ, U.K.

INTRODUCTION

Physiological fibrinolysis

The fibrinolytic pathway maintains vascular patency by the proteolytic degradation of fibrin, the end-product of coagulation. Formation of a fibrin mesh during haemostasis is part of the physiological response to vascular injury, whereas thrombosis is a pathological obstruction of blood flow, but the biochemical and cellular participants are believed to be similar. Physiological fibrinolysis is co-ordinated by an interaction of enzymes, zymogens and inhibitors to produce the local lysis of fibrin deposits. Plasminogen activators are serine proteases with restricted substrate specificity, catalysing the hydrolysis of the Arg-561–Val-562 bond in the zymogen, plasminogen (Fig. 1). The resultant two-chain enzyme, plasmin, which is also a serine protease, has a trypsin-like specificity so that the physiological function of fibrinolysis requires some localization of plasminogen activation within the fibrin matrix.

Fibrin can act as a cofactor, as well as substrate, increasing the catalytic efficiency of plasmin production by forming a ternary complex with plasminogen and the endogenous plasminogen activator, t-PA (tissue-type plasminogen activator) (Hoylaerts *et al.*, 1982). Fibrin also interacts with a second type of endogenous plasminogen activator, scu-PA (single chain urokinase-type plasminogen activator), possibly by nullifying the effect of a plasma inhibitor (Lijnen *et al.*, 1986) or by inducing a conformational change in plasminogen, enhancing activation (Pannell *et al.*, 1988). Hence, although scu-PA may have no specific affinity for fibrin, it may, like t-PA, at physiological concentrations have some selectivity for the activation of plasminogen bound to fibrin. These mechanisms, together with the relative protection of

fibrin-bound plasmin from the principal inhibitor, α_2 -antiplasmin, provide a molecular rationale to substantiate the original concept (Alkjaersig *et al.*, 1959) of fibrinolysis as the local activation of fibrin-bound plasminogen in an inhibitor-free environment.

Use of plasminogen activators in therapy

There has been a revival of interest in the use of thrombolytic agents to treat patients with acute myocardial infarction following acceptance of the evidence that an occlusive coronary thrombus precipitates most infarctions. Clinical trials of plasminogen activators, administered early after the onset of symptoms and in sufficiently high doses, have shown that recanalization can be achieved with the prospect of salvage of compromised myocardium and improved heart function. Recently, large placebo-controlled trials have established that thrombolytic agents can improve survival rates after myocardial infarction (AIMS Trial Study Group, 1988; ASSET Study Group, 1988; GISSI, 1987; ISIS-2 Collaborative Group, 1988).

It has been suggested that, even when used in therapeutic amounts, t-PA retains fibrin selectivity by comparison with the other agents that were used in the initial clinical trials, t-PA (two-chain urokinase-type plasminogen activator) and SK (streptokinase) (Sobel *et al.*, 1984). The bacterial protein SK has no proteolytic activity itself but forms an activator species by complex formation with endogenous plasminogen (Fig. 2). It has been asserted that SK demonstrates no specific affinity for fibrin (e.g. Collen & Verstraete, 1983; Fox *et al.*, 1984; O'Neill *et al.*, 1988) but early studies appeared to show that SK–human plasminogen complex could bind to fibrin in human plasma clots (Müllertz, 1956; Lassen, 1958; Blix, 1962). Enzyme kinetic studies have found

Fig. 1. Schematic representation of structure of Glu-plasminogen

The Figure emphasizes the five repetitive, triple-cysteine polypeptide domains (Kringles, K_1 – K_5). Plasminogen activator-cleavage site is indicated by Δ and primary plasmin-cleavage site (yielding Lys-78-plasminogen) by $\blacktriangle$.

Abbreviations used: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; scu-PA, single-chain urokinase-type plasminogen activator; t-PA, two-chain urokinase-type plasminogen activator; SK, streptokinase; APSAC, anisoylated Lys-plasminogen streptokinase activator complex.

Fig. 2. Maturation of the activator complex formed by SK

SK + human plasminogen combine stoichiometrically (1:1) to form an initial, inactive, complex that undergoes a conformational rearrangement, disclosing the active centre in SK-plasminogen*, the nascent activator species. Activation of plasminogen by the complex is promoted by fibrin(ogen) (Chibber *et al.*, 1985; Fears *et al.*, 1985b). SK-plasminogen** is a subsequent conformation that is less able to bind to clots (Fears *et al.*, 1985a) or to be potentiated by fibrin. Intramolecular cleavage of the plasminogen moiety at Arg-561-Val-562 produces SK-plasmin that, in plasma, is subject to progressive proteolysis of the SK moiety (Strandring *et al.*, 1988), producing SK*-plasmin fragments.

that binding of SK-human plasminogen by fibrin increases catalytic efficiency (Fears *et al.*, 1985b). It should be appreciated that there is a sequence of conformational changes during maturation of the SK-activator complex (Fig. 2) and an interaction with fibrin may be greatest for the nascent activator. Temporary stabilization of the nascent complex can be achieved by insertion of an acyl group into the active centre, using *p*-amidinophenyl esters as 'inverse' acylating agents (Smith *et al.*, 1981). The novel thrombolytic agent APSAC [anisoylated Lys-plasminogen streptokinase activator complex; Eminase (a trade mark of Beecham Group plc)] is, therefore, a proenzyme which is protected from degradation and inhibition while in the acyl form (Strandring *et al.*, 1988).

The purposes of this review are to characterize the fibrin affinity of plasminogen activators with regard to methods of measurement and structure-function relationships, to examine the association between fibrin affinity and other pharmacological attributes, and to consider the contribution of fibrin affinity to clinical efficacy, that is, potency and side effects.

QUANTIFICATION OF FIBRIN BINDING

Plasminogen

Because of the similarities in methodology used and the role of fibrin-bound plasminogen in fibrinolysis, an understanding of the behaviour of the zymogen contributes to an understanding of the pharmacology of the activators.

The native form of the substrate, Glu₁-plasminogen (Fig. 1) binds to fibrin with less affinity than the partly-degraded natural variant, Lys₇₈-plasminogen, when binding is measured during clot formation from human plasma (Rákóczi *et al.*, 1978) or purified fibrinogen (Thorsen, 1975). Lys-plasminogen also binds more than Glu-plasminogen to fibrin suspensions, devised to minimize non-specific binding (Lucas *et al.*, 1983) and to other fibrin preparations used to minimize artefacts relating to speed of diffusion (Bok & Mangel, 1985). Most studies have measured, or assumed, an overall binding stoichiometry of 1:1 and the fibrin dissociation constant of a high-affinity, lysine-binding, site on Lys-plasminogen is in the range 0.2–10 μM (Suenson &

Thorsen, 1981; Garman & Smith, 1982; Lucas *et al.*, 1983; Bok & Mangel, 1985) by comparison with 20–40 μM for Glu-plasminogen (Suenson & Thorsen, 1981; Lucas *et al.*, 1983; Suenson *et al.*, 1984; Bok & Mangel, 1985). There are, also, lower-affinity binding sites.

The initial plasmin digestion of fibrin creates new, high-affinity binding sites for Glu-plasminogen although quantification of the dissociation constant depends on the assumptions made about stoichiometry (Bok & Mangel, 1985; Suenson *et al.*, 1984; Tran-Thang *et al.*, 1986). It has been proposed (Christensen, 1984) that C-terminal lysine residues of fibrin fulfil the ligand structural requirements of the high-affinity, lysine-binding, sites situated within the region encompassing Kringles 1–4 of Glu-plasminogen (Fig. 1). It is suggested that the action of plasmin on partially-degraded fibrin may be facilitated, therefore, by formation of C-terminal lysine at the plasmin-cleavage sites (and by conversion of Glu- to Lys-plasminogen). Initiation of lysis may, however, depend on the aminohexyl-ligand-binding site, on Kringle 5 (Christensen, 1984).

Binding of plasminogen activators during clotting of purified fibrinogen

One of the earliest quantitative comparisons of t-PA (from pregnant hog ovaries) and tcu-PA was made by incubating activator with clotting fibrinogen at 37 °C for up to 30 min (Thorsen *et al.*, 1972). When unbound plasminogen activator was measured by a functional assay (clot lysis time), t-PA was found to have bound more strongly than tcu-PA. A similar clotting method has been used by many other workers, but the long incubation time sometimes used (e.g. 1 h; Rijken *et al.*, 1982) will permit proteolysis to occur unless an exogenous inhibitor, such as aprotinin, is present or the fibrinogen is rigorously purified free from plasminogen. Thus, current assays vary according to the concentration of plasminogen present and the amount of fibrinolysis occurring. The assays also vary according to the method used to measure the unbound concentration of activator, e.g. immunoassay (Rijken *et al.*, 1982), fibrin zymography (Verheijen *et al.*, 1986; Kalyan *et al.*, 1988), radiometric assay (Larsen *et al.*, 1986) and assay of amidolytic activity (Verheijen *et al.*, 1986). Non-specific

Table 1. Fibrin affinity of plasminogen activators

Plasminogen activator	Source	Fibrin(ogen) preparation	Dissociation constant (nM)	Reference
Direct binding assay				
t-PA	Human melanoma cells	Fibrin clot	400	Rijken <i>et al.</i> (1982)
t-PA	Pig	Fibrin bound to activated PVC	1400	Angles-Cano (1986)
t-PA	CHO cells	Fibrin clot	400	Higgins and Vehar (1987)
t-PA	African green monkey kidney cells	Fibrin clot	300	Larsen <i>et al.</i> (1988)
t-PA	Human melanoma cells (t-PA bound to PVC)	FCB-2 (CNBr fragment of fibrinogen)	140	Bosma <i>et al.</i> (1988)
Enzyme kinetic assay				
t-PA	Human uterus	Fibrin film (substrate: Glu- or Lys-plasminogen)	140	Hoyleaerts <i>et al.</i> (1982)
t-PA	Human melanoma cells	CNBr-digested fibrinogen (Glu-plasminogen)	180	Zammaron <i>et al.</i> (1984)
tcu-PA (M_r 53000)	Human melanoma cells	CNBr-digested fibrinogen (Glu-plasminogen)	1300	Lijnen <i>et al.</i> (1986)
t-PA		CNBr-digested fibrinogen (Lys-plasminogen)	60	Cassels <i>et al.</i> (1987)
SK-plasmin(ogen)		CNBr-digested fibrinogen (Lys-plasminogen)	15	Cassels <i>et al.</i> (1987)

binding in the assay system can be evaluated using a protein with no affinity for fibrin, e.g. albumin (Larsen *et al.*, 1988) or tumour necrosis factor (Higgins & Vehar, 1987): it is important to define the range of fibrinogen concentrations most appropriate for measuring specific binding because use of higher concentrations can promote non-specific entrapment as a result of increasing clot rigidity.

Estimates of the dissociation constant are in reasonable agreement for various preparations of t-PA (Table 1). In general, t-PA binds to fibrin with higher affinity than does plasminogen although marked variations in fibrin structure (Angles-Cano, 1986) may impair binding. Neither scu-PA (Gheysen *et al.*, 1987; Nelles *et al.*, 1987) nor tcu-PA (Robbins & Tanaka, 1986; Nelles *et al.*, 1987) bind appreciably to fibrin.

The binding of t-PA to fibrin, like the binding of Glu-plasminogen, appears to be augmented by initial fibrinolysis (Suenson *et al.*, 1984) but the increment in binding, relative to the zymogen, requires further quantification. One-chain t-PA may be bound to fibrin to a slightly greater extent than two-chain t-PA (Rijken *et al.*, 1982; Higgins & Vehar, 1987) but both forms are bound to new sites with high-affinity (and low prevalence) after the initial digestion of fibrin (Higgins & Vehar, 1987).

Binding of plasminogen activators to plasma clots

The measurement of binding to clots forming from purified fibrinogen is relatively simple but it is not clear how relevant the findings are to the binding of proteins to pre-existing thrombi. It is known that the binding of plasminogen to purified fibrin is greater than to clotted plasma (Rákóczi *et al.*, 1978). The structure of fibrin produced on clotting plasma may differ from that produced from purified fibrinogen (Carr, 1988) and the potential influence of other plasma proteins may be difficult to simulate in purified systems. Furthermore,

plasminogen can bridge between two monomer units of polymerizing fibrin whereas it cannot do so when added to established clots (Garman & Smith, 1982) and t-PA binds to the fibrin matrix less well once a clot has formed (Brommer, 1984).

Although it is possible to examine the binding of plasminogen activators to pre-formed plasma clots in lysing conditions (Cassels *et al.*, 1987; Fears *et al.*, 1987b), valuable quantitative comparisons can be made without the complication of a lysing matrix, and some recent findings (Fears *et al.*, 1987b) are summarized in Fig. 3. It should be appreciated that, in the presence of aprotinin, SK-plasminogen will form an active centre-inhibited complex. In these conditions, the clot uptake of tcu-PA is much less than the uptake of the other plasminogen activators; the binding of the stabilized SK-activator complexes are of the same order as the binding of t-PA but significantly greater than the binding of the corresponding plasminogen species. Although fibrin affinity cannot now be characterized in terms of a dissociation constant, the model system is particularly useful for the study of certain variables (Fears *et al.*, 1987b), for example the influence of clot age and clot cell content. The acquisition of data on clot affinity in such systems also facilitates the investigation of the relationship between fibrin binding and fibrinolysis.

Derivation of dissociation constants from enzyme kinetic studies

In purified systems, fibrin decreases the K_m of t-PA for plasminogen, consistent with the formation of a ternary complex (Hoyleaerts *et al.*, 1982). The K_m of SK-plasmin(ogen) for plasminogen also decreases in the presence of fibrin, although to a lesser extent (Fears *et al.*, 1985b). By contrast, the activation of Lys-plasminogen by tcu-PA is unaffected by fibrin (Fears *et al.*, 1985b); a small increase in the rate of activation of Glu-plasminogen by

Fig. 3. Uptake into human plasma clots

Binding of ^{125}I -protein after 30 min incubation at 37°C was measured (Fears *et al.*, 1987b) for: (i) t-PA (8×10^{-9} M), (ii) APSAC (3×10^{-8} M), (iii) SK-Lys-plasminogen (3×10^{-8} M), (iv) Lys-plasminogen (3×10^{-8} M), (v) tcu-PA (6×10^{-8} M). Clots ($20 \mu\text{l}$) were incubated in $100 \mu\text{l}$ of autologous plasma in the presence of $10 \mu\text{M}$ -aprotinin and activators were compared at concentrations that have been found equilytic in a system, *in vitro*, where lysis was allowed to proceed. Results are shown $\pm$ S.E.M. ($n = 6-12$).

tcu-PA can be attributed to a conformational change in the substrate on binding rather than to formation of a cyclic ternary complex (Lijnen *et al.*, 1984).

When the initial rate of activation at infinite plasminogen concentration is related to fibrin concentration, a dissociation constant can be calculated (Table 1). Use of soluble preparations of fibrin permits kinetic investigations in equilibrium conditions that may not be possible with solid-phase fibrin; the fibrin mimetic CNBr-digested fibrinogen is more convenient to use and gives reproducible results (Zamarron *et al.*, 1984; Cassels *et al.*, 1987; Fears, 1988). The binding of SK-plasmin(ogen) to the site revealed by CNBr cleavage of fibrinogen appears to be at least as strong as the binding of t-PA (Table 1). One advantage in measuring dissociation constants from enzyme kinetic assays is that the reaction mechanism can also be defined. It was demonstrated (Hoylaerts *et al.*, 1982; Cassels *et al.*, 1987) that t-PA and SK-Lys-plasmin(ogen) catalyse plasminogen activation in a similar manner, that is by an obligate reaction sequence where fibrin binds before plasminogen.

Because of early problems with characterization, reproducibility and stability of fibrin preparations, attempts were made to use poly-lysine to study the kinetic mechanism in the expectation that a polymeric lysine analogue will present structures homologous with the region in fibrin responsible for binding plasminogen and activator (Allen, 1982). It was confirmed (R. Fears, unpublished work) that poly(D-lysine) (M_r 100000) enhanced plasminogen activation by t-PA but not by tcu-PA (M_r 54000). Maximum enhancement was obtained at $0.1-0.2 \mu\text{M}$ -poly(D-lysine), similar to the concentrations of soluble fibrin (Fears *et al.*, 1985b) and CNBr-digested

Fig. 4. Schematic representation of six-chain structure of fibrinogen

The disulphide knot, incorporating the *N*-terminal regions, yields the dimer core fragment E on plasmin digestion. Fragments D comprise the disulphide-linked remnants of $\text{A}\alpha$, $\text{B}\beta$ and γ chains of fibrinogen. The CNBr-cleavage fragment of fibrinogen, FCB-2, is composed of disulphide-bonded sequences $\text{A}\alpha$ 148-207, $\text{B}\beta$ 191-305 and 95-265. The action of thrombin on fibrinogen, liberating the *N*-terminal fibrinopeptides A and B, initiates fibrin formation; the polymer is stabilized by factor XIIIa-catalysed cross-linking to produce dimers and α polymers

fibrinogen (Fears, 1988) giving peak enhancement. Other analogies have been drawn between the effector functions of poly-lysine and fibrin (Petersen *et al.*, 1985) but poly-lysine may be an unreliable surrogate for fibrin. For example, the isolated B-chain of t-PA is not bound by fibrin and the activity is not stimulated by fibrin, yet B-chain activity is enhanced by poly(D-lysine) (Dodd *et al.*, 1986), possibly because of an ionic interaction between the polycation and a sequence in the B-chain rich in acidic residues.

IDENTIFICATION OF BINDING SITES ON FIBRIN

The greater affinity of t-PA and SK-plasminogen complexes for fibrin than fibrinogen can be attributed to a latent binding site on fibrinogen that becomes exposed on fibrin formation (polymerization) or when the structure is cleaved by CNBr. Plasminogen binding to fibrin(ogen) has been characterized in some detail (Suenson & Petersen, 1986; Bosma *et al.*, 1988) and the interaction with the zymogen is mediated by regions (Doolittle, 1973; Williams, 1981) (Fig. 4) that include a sequence in the E domain (binding Kringle 1), part of the CNBr-cleavage fragment of the D domain, FCB-2 (binding Kringles 1-4) and a sequence elsewhere in the D domain with affinity for the aminohexyl-binding site on plasminogen.

Information obtained from enzyme kinetic assays has also been useful with regard to identifying the sites on fibrin(ogen) mediating the enhanced activation of plasminogen by t-PA. By a process of selective chemical modification of amino acids in clotted fibrin, it was found that lysine residues were important in the stimulation of activity of t-PA isolated from pig heart (Radcliffe, 1983). On analysing the influence of individual fibrinogen CNBr fragments, the site mediating enhancement of t-PA (two-chain from human uterus) was found to reside in the sequence $\text{A}\alpha$ 148-197 of FCB-2 (Nieuwenhuizen *et al.*, 1983), although the maximum en-

hancement obtained with this peptide was less than with fibrin monomer or the unfractionated CNBr digest of fibrinogen. The lysine residue at position A α 157 seemed to be particularly important in the promotion of t-PA activity (two-chain from human melanoma cells) (Voskuilen *et al.*, 1987) but this residue does not conform to the ligand structural requirements for the high-affinity binding site on plasminogen, that is, it is not C-terminal. Therefore, rate-enhancement by this residue may be explicable by an interaction with the aminohexyl-binding site on plasminogen, or by the binding of t-PA or, perhaps, by the adoption of a spatial conformation whereby the appearance of a C-terminal lysine is simulated (Voskuilen *et al.*, 1987).

The locations of the binding sites on fibrin for t-PA and plasminogen have recently been compared (Bosma *et al.*, 1988) using immobilized t-PA (two-chain from human melanoma cells) or Lys-plasminogen, passively adsorbed onto poly(vinyl chloride) microtitre plates. These direct measurements of binding substantiate the conclusions based on the measurement of fibrin-induced acceleration of plasminogen activation and implicate a region of the A α chain that is situated around the outer disulphide ring terminating the coiled-coils and connecting the E-domain with the D-domains (Doolittle, 1973; Williams, 1981) (Fig. 4).

IDENTIFICATION OF FIBRIN BINDING SITES ON t-PA

Influence of carbohydrate

The evaluation of the properties of t-PA with regard to glycosylation status is important because the cell lines used to produce t-PA may differ in their post-translational modifications (Pannekoek *et al.*, 1988). The amino acid sequence of t-PA indicates four possible sites for N-linked oligosaccharide, at Asn-117, -184, -218 and -448 (human melanoma species). Asn-218 is not glycosylated because of an adjacent proline; the other residues are glycosylated in variant II of t-PA but Asn-184 does not bear oligosaccharide in variant I (Little *et al.*, 1984; Rijken *et al.*, 1985; Rasmussen *et al.*, 1988). There may also be O-linked oligosaccharide.

The enzymic removal of most N-linked oligosaccharide or synthesis of a partially aglyco form in the presence of tunicamycin did not affect fibrin binding or fibrin stimulation of activity (Little *et al.*, 1984; Rijken *et al.*, 1985). However, the influence of modifying individual oligosaccharide substituents is beginning to be examined (Rasmussen *et al.*, 1988) and there is some evidence that the presence of carbohydrate can affect the function of individual domains (Collen *et al.*, 1988; Kalyan *et al.*, 1988).

Influence of chain nature

Like other serine proteases, t-PA is synthesized and secreted in the one-chain form. By contrast with other serine protease zymogens, one-chain t-PA seems to possess enzymic activity (Rijken *et al.*, 1982), but the properties of one-chain t-PA have been difficult to study because plasmin cleavage at Arg-275 produces the two-chain form (Fig. 5). Further analysis of the attributes of one-chain t-PA has been expedited by the use of site-directed mutagenesis to produce Glu-275 (Tate *et al.*, 1987) or Gly-275 (Petersen *et al.*, 1988) mutants that are not susceptible to plasmin cleavage at this position. It

Fig. 5. Schematic representation of structure of t-PA

The figure emphasises A-chain domains: fibronectin-like finger (F), epidermal growth factor-like sequence (G), Kringles 1 and 2 (K₁, K₂). Plasmin-cleavage site is indicated by ▲.

was observed that the fibrin binding and enhancement of these analogues were much higher than of both the native two-chain form and the two-chain variant produced by the action of *Staphylococcus aureus* V8 protease on the Glu-275 mutant (Tate *et al.*, 1987). Although these findings are consistent with the possibility that fibrin binding can induce an activated conformational state in one-chain t-PA (Petersen *et al.*, 1988) and a mechanistic explanation has been advanced (Pannekoek *et al.*, 1988), it should be appreciated that the one-chain mutants may still be susceptible to cleavage by plasmin, e.g. at Lys-277, and further evaluation is necessary.

Function of individual domains in the A-chain

The B-chain of t-PA contains the active site whereas the A-chain accounts for the affinity to fibrin (Dodd *et al.*, 1986). The A-chain comprises distinct domains (Fig. 5), homologous to structures in other blood proteins. A finger domain resembles the structures in fibronectin involved in fibrin binding and a growth factor domain shows homology with human and murine epidermal growth factor; two Kringle structures are homologous with their counterparts in plasminogen (Fig. 1). t-PA can, therefore, be considered as a mosaic protein with functional modules arising as a result of exon shuffling during evolution (Gilbert, 1978).

Limited proteolysis, removing a sequence of approx. 10 kDa from the N-terminus, abolished the fibrin affinity (clotting fibrinogen) of t-PA (Banyai *et al.*, 1983) and although the precise sequence was not identified, it was assumed that the fibronectin finger-like domain was removed. By contrast, an analogue of t-PA lacking the finger domain appeared to retain fibrin affinity (fibrin-celite column) although no direct comparison was made with parent t-PA (Kagitani *et al.*, 1985). More detailed analysis of a mutant where the finger domain had been specifically deleted (Verheijen *et al.*, 1986) showed a diminished affinity, especially at low concentrations of fibrin, although binding was partly restored by the presence of plasminogen. The activity of this mutant could be stimulated by the CNBr cleavage fragment of fibrinogen, FCB-2, on extended incubation, that is in conditions where the effector is subject to proteolysis (Beckmann *et al.*, 1988), generating new high-affinity binding sites.

A function for Kringle 2 in mediating the binding of t-PA to fibrin was proposed on the basis of affinity on lysine-Sepharose columns (Ichinose *et al.*, 1986). A comparison of various deletion mutants (van Zonneveld *et al.*, 1986a) indicated that fibrin binding and enhance-

ment (on extended incubation) were determined by both the finger and Kringle 2 domains but only Kringle 2 mediated binding to lysine-Sepharose (van Zonneveld *et al.*, 1986b). Thus, misleading conclusions may be drawn from an analysis of lysine-Sepharose binding alone. An analogy has been made (van Zonneveld *et al.*, 1986b; Verheijen *et al.*, 1986) between the function of Kringle 2 of t-PA and Kringles 1-4 (lysine-binding sites) of plasminogen. Accordingly, Kringle 2 of t-PA interacts with C-terminal lysine or arginine exposed after initial plasmin digestion of fibrin but the initial binding of t-PA is mediated by the finger domain. The amount of fibrin binding or enhancement of activity measured for an analogue of t-PA will, therefore, depend on the amount of fibrin degradation (Gething *et al.*, 1988); the relationship between binding and fibrinolysis will also be influenced by the experimental conditions (Kalyan *et al.*, 1988; Larsen *et al.*, 1988).

Further information on the function of the domains was obtained from a study of natural variants of recombinant t-PA produced in human melanoma cells (structures identified by Dodd *et al.*, 1988). The fibrin enhancement of plasminogen activation was measured for a brief incubation time (Fears *et al.*, 1985b) to minimize proteolysis of the effector. In agreement with previous studies (Fears *et al.*, 1985b; Fears, 1988), plasminogen activation by parent t-PA (M_r 63000; Dodd *et al.*, 1988) was markedly enhanced, at low substrate concentrations, by soluble fibrin and by CNBr-digested fibrinogen (Fig. 6). The activity of a natural, truncated, variant of M_r 38000 (K_2 -B) was not stimulated in these conditions and a variant of M_r 56000 (GK_1K_2 -B) was only slightly stimulated. Therefore, the finger domain is confirmed as an important determinant of the initial fibrin enhancement of t-PA and, by inference, initial fibrin binding.

Deleting both the finger and growth factor domains can decrease the affinity of the remnant for fibrin by up to 100-fold compared with native t-PA (Collen *et al.*, 1988; Larsen *et al.*, 1988) but selective deletion of the growth factor domain decreased affinity to the same extent as selective deletion of the finger domain (Larsen *et al.*, 1988). The most likely explanation is that removal of the growth factor domain affects the behaviour of adjacent domains by inducing alterations in their conformation or by introducing steric hindrance. Thus, in general, because of the possibility of secondary changes, the loss of a specific function on deletion of a domain is not sufficient evidence for the normal function of that domain (Gething *et al.*, 1988).

In summary, it is not yet possible to be certain as to which structures in t-PA mediate binding to fibrin. The comparison of the work of different groups is difficult because of the differences in assay as well as differences in precise construction of mutants (Gething *et al.*, 1988) and choice of cell for protein expression. It is likely, however, that the fibronectin-like finger and Kringle 2 domains make some contribution to the interaction of t-PA with fibrin. The role of Kringle 1 remains to be resolved (Gething *et al.*, 1988).

IDENTIFICATION OF FIBRIN-BINDING SITES ON SK-PLASMINOGEN COMPLEXES

The formation of an activator complex with SK imparts additional fibrin affinity to plasminogen (Cederholm-Williams, 1981). The affinity of SK-plasmin(ogen)

Fig. 6. Effect of A-chain structure on plasminogen activation by t-PA

Lys-plasminogen was activated in the presence of 0.1 μM-CNBr-digested fibrinogen (▲), 0.05 μM-soluble fibrin (■) or in the absence of effector (●): (a), t-PA (M_r 63000); (b) t-PA variant (M_r 56000); (c) t-PA variant (M_r 38000).

for CNBr-digested fibrinogen (Table 1) and for soluble fibrin (Cassels *et al.*, 1987) is at least as high as that of t-PA. However, the complex between SK and the isolated B-chain of plasmin(ogen) demonstrates no fibrin affinity (Robbins & Boreisha, 1987).

Because the interaction of SK-activator complex with fibrin(ogen) decreases as the activator ages (Fig. 2), studies on fibrin and clot binding are facilitated by stabilization of the nascent activator in a pro-enzyme form. The binding of the stabilized acyl-enzyme, APSAC,

to fibrin is at least as great as the binding of t-PA (Fears *et al.*, 1987b) and significantly greater than the binding of the zymogen, Lys-plasminogen (Fig. 3) in conditions where the lysis of fibrin, and the disclosure of new binding sites, is prevented.

Relatively little is known concerning the conformational change induced in plasminogen on binding to SK, but the high affinity of APSAC for fibrin is attenuated by 6-aminohexanoic acid (Fears *et al.*, 1987b). It is interesting to note that the activation of Glu- or Lys-plasminogen to an active site-inhibited plasmin species (Suenson & Thorsen, 1981) also results in a marked increase in fibrin binding, even though gross conformation is unchanged. The conversion of Val-442-plasminogen (K5-B) to active site-inhibited plasmin does not increase fibrin affinity.

When fibrin binding was inferred from the sustained fibrinolytic response induced by transient exposure of clotted human plasma to plasminogen activator (Fears *et al.*, 1985a), the binding of APSAC was also much greater than the binding of the truncated variant containing Val-plasminogen. Sustained fibrinolysis, *in vitro*, following brief exposure of a clot to activator has also been seen for t-PA (Baldus *et al.*, 1988) but not for tcu-PA (Fears *et al.*, 1985a; Baldus *et al.*, 1988) or scu-PA (Gurewich & Pannell, 1986).

The binding of APSAC (containing Lys-plasminogen) to fibrin was significantly greater than the binding of the Glu-plasminogen homologue, partly because of the presence of additional 6-aminohexanoic acid-sensitive sites (Fears *et al.*, 1987a). The uptake of APSAC into human plasma clots, *in vitro*, was also significantly greater than the uptake of SK-Glu-plasmin(ogen), the form of the complex that is initially produced when SK is administered to patients. The additional fibrin affinity of APSAC, in comparison with the Glu-plasminogen homologue, presumably accounts for the greater fibrinolytic activity measured *in vitro* (Fears *et al.*, 1985a).

BINDING OF PLASMINOGEN ACTIVATORS TO OTHER MACROMOLECULES

Endogenous plasminogen activator activity has been implicated in a variety of biological processes, including inflammation, metastases, ovulation, neurodevelopment, cell migration, insulin biosynthesis and tissue remodeling. Any involvement of t-PA is assumed to require an effector, because activation of fluid-phase plasminogen is kinetically unfavourable, but it is not possible to review all of the literature describing the binding of activators to other macromolecules. Some recent examples of the binding and potentiation of t-PA activity are listed in Table 2. Although caution should be exercised when extrapolating from results obtained in purified systems, a consideration of these interactions may be important in understanding the response to pharmacological amounts of activators used in thrombotic disorders as well as to understanding the functions of activators in physiological processes.

Binding of t-PA, together with plasminogen, to immobilized proteins and cell surfaces (Table 2) enhances enzyme activity by an effect on K_m and there are further analogies with the effector functions of fibrin. For example, initial plasmin digestion reveals new binding sites on thrombospondin (Silverstein & Nachman, 1987) and IgG (Feigert & Harpel, 1988) and plasmin is

Table 2. Binding and potentiation of t-PA activity by other macromolecules

t-PA-binding ligand	Comments	Reference
Histidine-rich glycoprotein, Thrombospondin	t-PA binds with K_d 10–40 nM; scu-PA does not bind. Surface-associated plasmin is protected from α_2 -antiplasmin	Silverstein <i>et al.</i> (1985)
Fibronectin	tcu-PA binds poorly by contrast to t-PA	Gilboa & Kaplan (1985), Salonen <i>et al.</i> (1985)
IgG	Plasmin pretreatment of IgG uncovers new binding sites for t-PA	Feigert & Harpel (1988)
Human endothelial cells	Affinity not mediated by domains responsible for binding of t-PA to fibrin	Hajjar <i>et al.</i> (1986, 1987)
Human platelets	Generation of plasmin degrades glycoproteins Ib and IIb/IIIa	Stricker <i>et al.</i> (1986)
Glycosaminoglycans, e.g. heparin	tcu-PA also binds but SK-plasminogen does not	Andrade-Gordon & Strickland (1986), Pâques <i>et al.</i> (1986), Fears (1988)

protected from fluid-phase inhibitors (Silverstein *et al.*, 1985). The surface binding of t-PA to platelets and the endothelium is probably partly mediated by fibrinogen and thrombospondin. t-PA and plasminogen also bind to other cells, e.g. fibroblasts (foreskin and smooth muscle cells (internal mammary artery) (Hajjar *et al.*, 1986, 1987).

The binding and enhanced activity of t-PA in the extracellular matrix may be mediated by thrombospondin, and by proteoglycans (Knudsen *et al.*, 1986). It has been shown in model systems that heparin binds and potentiates the activity of t-PA and tcu-PA but does not affect SK-Lys-plasmin(ogen) (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986; Fears, 1988). The effect of heparin on t-PA is attenuated (Fears, 1988) or nullified (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986) in the presence of fibrin so that fibrin-selectivity may be diminished. The binding of t-PA from human melanoma cells to heparin is dependent on a domain in the A-chain, probably not Kringle 2 (Fears, 1988). By contrast with the results obtained in purified systems, it has been shown that a clinically-relevant concentration of heparin does not influence the binding of t-PA to human whole blood clots and does not affect the activation of plasminogen in plasma (Fry & Sobel, 1988), a finding consistent with the lack of effect of heparin on t-PA-induced fibrinogenolysis in human whole blood (R. Fears & H. Greenwood, unpublished work). It is conceivable that the effective heparin con-

Table 3. Novel thrombolytic agents with fibrin affinity

Plasminogen activator		Comments	Reference
Protease sequence	Sequence conferring fibrin affinity		
u-PA B-chain	Plasmin A-chain	Bound and activity enhanced by fibrin, unlike t-PA	Robbins & Tanaka (1986)
t-PA B-chain	Plasmin A-chain	Binding slightly less than t-PA	Robbins & Boreisha (1987)
tcu-PA (low- M_r)	Fibrin-specific monoclonal antibody 64C5 (<i>N</i> -terminus on β chain) and Fab' fragment	10^3 -fold increase in potency (fibrin monomer-Sepharose) relative to t-PA	Bode <i>et al.</i> (1987)
t-PA	Fibrin-specific monoclonal antibody 59D8 (<i>N</i> -terminus on β chain)	10-fold increase in potency (fibrin monomer-Sepharose), 2-fold increase in thrombolytic potency relative to t-PA	Runge <i>et al.</i> (1987, 1988)
u-PA 144-411	1-263 t-PA (recombinant hybrid, CHO cells)	Fibrin-binding weaker than t-PA but greater than scu-PA, tcu-PA; activity stimulated by CNBr-digested fibrinogen	Nelles <i>et al.</i> (1987), de Vries <i>et al.</i> (1988)
147-411	1-262 t-PA (recombinant hybrid, mouse Ltk ⁻ cells)		
134-411			
136-411	1-67 t-PA (recombinant hybrid hamster kidney cells)		
tcu-PA (high- M_r)	Lys-plasmin (conjugated at active centre)	Fibrin-binding similar to Lys-plasminogen; activity stimulated by CNBr-digested fibrinogen	Ferres <i>et al.</i> (1988)

centration in blood is decreased below that needed to enhance the activity of t-PA by binding to other proteins such as antithrombin III.

DESIGN OF NOVEL PLASMINOGEN ACTIVATORS WITH FIBRIN AFFINITY

As a result of the resurgence of clinical enthusiasm for thrombolytic therapy there are, currently, many attempts to construct novel activators with improved properties. Many of these attempts involve augmenting the fibrin binding of t-PA or conferring fibrin binding on u-PA (Harris, 1987; Krause, 1988; Pannekoek *et al.*, 1988): Table 3 exemplifies some of the types of approach adopted. When analysing structure-function relationships, it should be appreciated that the acquisition of a specific function on receipt of a discrete domain provides stronger evidence for the natural function of that domain than does the loss of function accompanying deletion of the domain.

Plasmin A-chain hybrids with the B-chain of t-PA or u-PA possess the fibrin affinity of plasminogen. The initial results of Robbins and colleagues (1986, 1987), demonstrating fibrin enhancement, have been substantiated (R. Fears, unpublished work) for the hybrids prepared by Robinson *et al.* (1988). The presence of the entire A-chain of plasmin in a complex with u-PA confers greater fibrin affinity than does the truncated sequence comprising Kringles 1-3 (Nakayama *et al.*, 1986). Conjugation of tcu-PA to the active centre of plasmin (Ferres *et al.*, 1988) also produces an agent with fibrin affinity comparable to that of the parent plasminogen and the acquisition of fibrin binding was accompanied by fibrin enhancement of enzyme activity and increased fibrinolytic potency *in vitro*.

As the potential fibrin-selectivities of t-PA and scu-PA are determined by different mechanisms, it was thought to be advantageous to synthesize a hybrid from the *N*-terminus of t-PA and the *C*-terminus of scu-PA (Nelles *et al.*, 1987). However, such an agent bound to fibrin less well than t-PA and clot selectivity was similar to that of scu-PA. The corresponding scu-PA hybrid bearing only the finger domain of t-PA (Gheysen *et al.*, 1987) showed no fibrin affinity whereas studies on another hybrid in which the finger and growth factor domains of t-PA were replaced by growth factor and Kringle domains of tcu-PA (Lee *et al.*, 1988) indicated the intrinsic importance of the finger domain. As discussed previously, the comparison of studies may be partly confounded by differences in expression system (Table 3) and assay methodology. Furthermore, although there is some flexibility in the functional requirements of the modular structure (Lee *et al.*, 1988), the individual domains may not act autonomously and some interaction with the B-chain may even be necessary for the normal folding of the A-chain of t-PA.

Specific monoclonal antibodies may have an affinity for fibrin up to 10^3 -fold greater than t-PA (Runge *et al.*, 1988). Although conjugation of tcu-PA and t-PA with a monoclonal antibody (Table 3) increased fibrinolytic potency, the potentiation of lysis was much less when using human blood clots than when using fibrin monomer. The explanation for this discrepancy may be that fibrin degradation products compete for binding to the activator. However, it has also been suggested (Mao *et al.*, 1988) that the use of antibodies directed to the *N*-terminus of fibrin (Table 3) induces binding to fibrin monomer but not to polymeric fibrin because the *N*-terminus may not be exposed within the clot matrix. An increase in thrombolytic potency in animal models has

been found for monoclonal antibody-conjugates (Runge *et al.*, 1987) but it is difficult to distinguish between the influences of increased fibrin binding and slower clearance half-life, a problem that has also complicated the interpretation of the response to other agents endowed with fibrin affinity (Nakayama *et al.*, 1986).

CONCLUSIONS REGARDING THE FUNCTION OF FIBRIN BINDING IN THE THROMBOLYTIC EFFICACY OF PLASMINOGEN ACTIVATORS

The clinical interest in fibrin affinity as an important pharmacological attribute was stimulated by the observation that binding of t-PA plus substrate on the fibrin template increased catalytic efficiency. Higher enzyme activity in the presence of fibrin might be expected to lead to relative fibrin selectivity, that is effective thrombolysis by t-PA without marked activation of systemic plasminogen and induction of an haemostatic defect. There are, however, other relevant factors to consider with regard to the use of plasminogen activators in the therapy of thrombotic disorders.

Structure-activity relationships

Fibrin affinity is not confined to t-PA and analogues bearing certain domains of t-PA A-chain. For example, as described previously, an equivalent degree of fibrin affinity is imparted to Lys-plasminogen in the non-covalent complex with SK, particularly when the complex is stabilized by acylation to form APSAC. Furthermore, high fibrin affinity and fibrin enhancement of activity is observed for plasmin A-chain hybrids with the B-chain of t-PA or u-PA and for conjugates of plasmin with t-PA.

In general, there may be a complex relationship between fibrin binding and fibrinolysis because of sequential changes in activator, plasminogen and fibrin struc-

tures. When evaluating novel agents it is difficult to ascribe, unequivocally, changes in thrombolytic activity to changes in fibrin affinity because of concomitant changes, for example, in inhibitor binding and tissue uptake.

Mechanism of fibrin selectivity

Apart from fibrin binding, there may be other methods to achieve fibrin selectivity, exemplified by scu-PA (Lijnen *et al.*, 1986; Pannell *et al.*, 1988).

High fibrin affinity will only be associated with selective fibrinolysis if the basal enzyme activity in the absence of fibrin is low. The high basal activity of SK-plasminogen (Fears *et al.*, 1985b) induces extensive systemic activation even though the fibrin affinity is comparable to that of t-PA.

Fibrin selectivity is dose-dependent

Even for an agent such as t-PA, whose basal activity is low, the activation of fluid-phase plasminogen is dose-dependent (McNeill *et al.*, 1988). It had initially been predicted (Sobel *et al.*, 1984) that pharmacological doses of t-PA would induce little systemic activation. Although currently used doses produce less effect than SK, t-PA and APSAC, the effect can be large, for example an average 60% depletion of plasma plasminogen 3 h after the start of dosing (Rao *et al.*, 1988).

Fibrin selectivity is not equivalent to thrombus selectivity

Although biochemical selectivity is significantly greater for t-PA than for SK, t-PA and APSAC at current clinical doses, t-PA does not produce less bleeding in patients with acute myocardial infarction (Rao *et al.*, 1988; Sherry, 1987, 1988; Marder & Sherry, 1988). This is because the bleeding complications of thrombolytic therapy can be attributed to the lysis of haemostatic plugs (Fig. 7) rather than to the extent of the clotting defect

Fig. 7. Some possible biological effects of t-PA

The incidence and severity of bleeding during therapy of thrombotic states is dose-dependent and influenced by the extent of invasive procedures and by adjunct anticoagulant and antiplatelet therapy.

Fig. 8. Some possible consequences of fibrinogen depletion

These actions will also be influenced by nature of the adjunct therapy and by bioavailability of plasminogen activator and extent of recanalization.

and t-PA does not distinguish between fibrin in a thrombus and that in an haemostatic plug. Although a clotting defect might be expected to exacerbate bleeding, the adjunct use of agents such as heparin and aspirin probably negates the partial maintenance of clotting factor protein concentrations in the presence of t-PA. Furthermore, there is evidence, some of which has been discussed previously in this review, that t-PA can bind elsewhere, affecting platelet and endothelial cell function (Fig. 7) and exacerbating bleeding (Marder & Sherry, 1988).

Possible benefits of fibrinogenolysis

Any possible benefits accruing from the relative maintenance of fibrinogen levels by t-PA should be balanced against the possible merits of depleting plasma fibrinogen (Fig. 8). It is premature to consider a decrease in fibrinogen levels as indisputably advantageous, but SK has been shown to affect blood rheology when t-PA does not (Jan *et al.*, 1985) and the relative preservation of clotting activity on administering t-PA may necessitate complex dosing regimens and early heparinization to attenuate re-thrombosis.

Benefits of fibrin binding

Although it is a misconception to equate fibrin binding with fibrinogen sparing or less bleeding, there are potential clinical advantages for a plasminogen activator with fibrin affinity. One possible benefit is sustained action, that is continuing lysis once the agent has bound to the thrombus. Some of the experimental evidence for t-PA and APSAC, obtained in model systems, has already been mentioned but there is also preliminary clinical

evidence for APSAC (Brochier, 1987) to show that thrombus dissolution and increase in lumen size may continue for some hours after dosing.

In addition to prolonging activity, the net potency may also be augmented by selective binding to the thrombus. The increase in potency derives both from the increase in local concentration of activator and from the increase in enzymic efficiency achieved by the template interaction of activator and substrate ('rendezvous' mechanism). However, potency is also determined by bioavailability; that is, the longer the residence time in the circulation, the greater the opportunity for a plasminogen activator to accumulate in the lysing thrombus. Thus, the potency benefit may only be fully realized for an agent such as APSAC that is slowly cleared from the circulation (Been *et al.*, 1986). Furthermore, although fibrin affinity will also promote accumulation of agent during clot extension and re-occlusion, leading to rapid lysis of new thrombus, the advantage can only be obtained while the agent is present in the circulation.

In summary, much is now known about structure-function relationships in the fibrin binding of plasminogen activators and about the relationship between binding to fibrin and binding to other macromolecules. Fibrin binding is a desirable attribute not merely because of the possibility of fibrinogen sparing but because of the potential contribution to potency. The role of fibrin binding in clinical efficacy can, however, only be considered in conjunction with the other properties of plasminogen activators, particularly their bioavailability.

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