Kinetic studies on the effect of heparin and fibrin on plasminogen activators

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1. Possible interactions between fibrin(ogen) and heparin in the control of plasminogen activation were studied in model systems using the thrombolytic agents tissue-type plasminogen activator (t-PA), urokinase and streptokinase-plasminogen activator complex and the substrates Glu- and Lys-plasminogen. 2. Both t-PA and urokinase activities were promoted by heparin and by pentosan polysulphate, but not by chondroitin sulphate or hyaluronic acid. The effect was on K_m . 3. In the presence of soluble fibrin (and its mimic, CNBr-digested fibrinogen) the effect of heparin on t-PA was attenuated, although not abolished. In studies using a monoclonal antibody and 6-aminohexanoic acid, it was found that heparin and fibrin did not seem to share a binding site on t-PA. 4. The activity of t-PA B-chain was unaffected by heparin, so the binding site is located on the A-chain of t-PA (and urokinase). 5. Fibrin potentiated the activity of heparin on urokinase. The activity of streptokinase-plasminogen was unaffected by heparin whether or not fibrin was present. 6. If these influences of heparin and fibrin also occur *in vivo*, then, in the presence of heparin, the relative fibrin enhancement of t-PA will be diminished and the likelihood of systemic activation by t-PA is increased.

INTRODUCTION

Plasminogen activators are serine proteinases with restricted substrate specificity that act by catalysing the hydrolysis of the Arg⁵⁶⁰–Val⁵⁶¹ bond in plasminogen. The resultant two-chain enzyme, plasmin, has a relatively broad, trypsin-like specificity and the physiological substrate, fibrin, may act as a cofactor to regulate the interaction between plasminogen and the endogenous activator, t-PA (Hoylaerts *et al.*, 1982). It is often suggested that t-PA may also exhibit ‘fibrin-selectivity’ when used at therapeutic doses, whereas the other principal thrombolytics, urokinase and streptokinase (as the activator complex streptokinase-plasminogen), are said to demonstrate no specific affinity for fibrin (Collen & Verstraete, 1983; Fox *et al.*, 1984). However, the enzymic activity of streptokinase-plasminogen activator complex is promoted by fibrin (Fears *et al.*, 1985), and the enhancement of t-PA activity may not be specific for fibrin. Thus it has been demonstrated fairly recently that heparin can bind plasminogen and t-PA (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986), with consequent promotion of plasminogen activation. The evidence for this effect of heparin is limited, but the interaction may be important, since heparin is often used as an ancillary therapy in thrombolysis.

It was the purpose of the present studies to quantify, in kinetic terms, the action of heparin on the principal plasminogen activators and to determine whether the effect was shared by other sulphated polysaccharides. The assays were extended to define the interaction with fibrin and fibrinogen and to examine the activation of both Glu- and Lys-plasminogens; Glu-plasminogen is normally the predominant circulating form of the substrate, whereas Lys-plasminogen may be the principal fibrin-bound species (Thorsen *et al.*, 1984; Holvoet *et al.*, 1985).

MATERIALS AND METHODS

Materials

t-PA (two-chain) was provided by Dr. J. H. Robinson of this Division and was purified from the recombinant Bowes melanoma cell-line TRBM6 (Browne *et al.*, 1985); the major species had an apparent M_r 63 000. The origin and purification of the other proteins used and the preparation of soluble fibrin, by digestion of clotted human fibrin using NaBr, have been described previously (Fears *et al.*, 1985). Polysaccharides were obtained from Sigma (Poole, Dorset, U.K.), and a monoclonal antibody (IgG₁) against t-PA, ESP 5, was obtained from Bioscot Ltd. (Edinburgh, Scotland, U.K.).

The method for the preparation of a CNBr digest of fibrinogen was adapted from the method described by Nieuwenhuizen *et al.* (1983); human fibrinogen (1 g) was allowed to react with CNBr (1.3 g) in 100 ml of 70 % (v/v) formic acid at 18 °C for 17 h. Unchanged CNBr and formic acid were removed by dialysis against deionized water for 24 h and the product was freeze-dried. Six degradation fragments were identified after SDS/polyacrylamide-gel electrophoresis (10 % separating gel) followed by staining for protein; these were at apparent M_r 88 000, 70 000, 38 000, 36 000 and < 20 000 (two bands). The total recovery of protein was 90 %. The fragments were dissolved in 0.05 M-NaOH and diluted to 5 mg/ml with 0.05 M-Tris/0.1 M-NaCl, pH 7.4. These fragments resemble the soluble fibrin preparations used previously (Fears *et al.*, 1985) in allowing the kinetic study of plasminogen activation under equilibrium conditions (Zamarron *et al.*, 1984), but the CNBr digest of fibrinogen is more convenient to use because, unlike soluble fibrin, it can be stored at –40 °C with retention of enhancing activity (R. Fears & R. Standing, unpublished work). Thus a single batch provides a reproducible stimulus in a succession of experiments.

Abbreviations used: Glu-plasminogen, human plasminogen with *N*-terminal glutamic acid (residues 1–790); Lys-plasminogen, human plasminogen with *N*-terminal lysine (residues 77–790); t-PA, tissue-type plasminogen activator.

Fig. 1. Dependence of enhancement on CNBr-digested-fibrinogen concentration

Lys-plasminogen (0.05 μM) was activated by t-PA. Results are means ± S.E.M. for four determinations.

Plasminogen activation

The procedure for the calculation of kinetic constants was the same as that described previously (Fears *et al.*, 1985), except that stock solutions of enzymes were prepared by using 0.05 M-NaH₂PO₄/0.1 M-NaCl/0.01 % (w/v) Tween 80, pH 7.4, with the consequence that calculated maximum velocities of activation were higher than before. In summary, plasmin production was monitored at 1 min intervals for 5 min at 37 °C by using the substrate S-2251 (*H*-D-Val-Leu-Lys *p*-nitro-anilide · 2HCl). Initial rates of plasminogen activation were obtained by plotting $A_{405}^{1\text{cm}}$ versus (time)². The concentration of activator in the assay was 5×10^{-10} M for t-PA, and 10^{-10} M for urokinase and streptokinase · Lys-plasminogen.

The method used to measure the kinetics of plasminogen activation can be classified as a one-stage assay where plasmin is protected from autodegradation (Jespersen *et al.*, 1986). Furthermore, initial velocities were measured for a sufficiently short period (up to 5 min) to minimize sequential changes in reactivity evoked by changes in fibrin or fibrinogen structure (Norrman *et al.*, 1985; Suenson & Petersen, 1986).

In preliminary studies, the relationship between concentration of CNBr-digested fibrinogen and the degree of enhancement of plasminogen activation by t-PA was examined. At low substrate concentrations (for example, 0.05 μM-Lys-plasminogen; Fig. 1) maximum enhancement was obtained at 0.3–0.4 μM CNBr-digested fibrinogen, but at higher concentrations of plasminogen the contents of the cuvette became turbid at concentrations of CNBr-digested fibrinogen greater than 0.2 μM. This effect may be attributable to plasminogen–fibrinogen-fragment cross-linking, and for this reason 0.1 μM CNBr-digested fibrinogen was the concentration used routinely.

RESULTS AND DISCUSSION

Response to sulphated polysaccharides

The activation of Lys-plasminogen by t-PA was promoted by heparin (sodium salt from pig intestinal

Fig. 2. Dependence of enhancement on heparin concentration

Lys-plasminogen (0.5 μM) was activated by t-PA in the presence of various concentrations of the sodium salt of heparin obtained from pig intestinal mucosa. Results are means ± S.E.M. for four determinations.

mucosa; Fig. 2), in agreement with the essentially qualitative results obtained by others (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986). The range of heparin concentrations encompassing the maximum effect is comparable with the concentrations customarily achieved *in vivo*, for example 3 units/ml, initially, after a bolus dose of 10000 units (O'Reilly, 1985). A similar concentration response (not shown) was obtained for heparin from another source (sodium salt from bovine lung) and for another polysaccharide, pentosan polysulphate (studied up to 120 μg/ml), where maximum stimulation occurred at 23 μg/ml, a concentration equivalent to heparin at 4 units/ml. There was no stimulation, however, by chondroitin sulphate (from whale/shark cartilage; mixed isomers; up to 120 μg/ml) or hyaluronic acid (from human umbilical cord; up to 120 μg/ml).

Kinetic transitions

The promotion of the basal Lys-plasminogen-activating activity of t-PA by heparin was mediated by an effect on K_m (Table 1). In order to extend the studies to quantify any interaction between heparin and fibrin, however, it was necessary to take into account the variable influence of fibrin alone on t-PA kinetics (Fears *et al.*, 1985). For both soluble fibrin (Fears *et al.*, 1985) and the CNBr digest of fibrinogen, a fibrin mimic, there was a concave-downward deviation in the double-reciprocal plots (Fig. 3). In the present studies, which employed two-chain t-PA, curvilinear plots were only obtained for Lys-plasminogen in the presence of enhancer; double-reciprocal plots were linear for the basal activation of Lys-plasminogen and for the enhanced activation of Glu-plasminogen, in agreement with other results (Rånby, 1982). The kinetic changes observed in Fig. 3 are characteristic of negative co-operativity (Levitzki & Koshland, 1969), and it has been postulated (Rånby, 1982) that Lys-plasminogen induces an allosteric modification of t-PA, so that, in the presence of fibrin, plasminogen activation proceeds via some complex containing t-PA and two molecules of plasminogen. This

Table 1. Kinetics of plasminogen activation by t-PA and urokinase

Results are means $\pm$ S.E.M., where indicated, for the designated number of separate experiments. Each experiment comprised two to four replicate assays for a range of plasminogen concentrations. Final concentrations (where appropriate) were: heparin, 4 units/ml; pentosan polysulphate, 23 μ g/ml; CNBr-digested fibrinogen, fibrinogen, 0.1 μ M. 'x' values represent the relative increase.

Activator	Plasminogen substrate	Potentiator	n	K_m (μ M)	V_{max} (nmol $\cdot$ min $^{-2}$ $\cdot$ amidolytic unit $^{-1}$)	V_{max}/K_m
t-PA	Lys	—	8	18.6 $\pm$ 1.9	1.3 $\pm$ 0.4	0.07
	Lys	Heparin	2	7.2	1.5	0.21 ($\times$ 3.0)
	Lys	CNBr-digested fibrinogen	6	0.04 $\pm$ 0.005	1.1 $\pm$ 0.4	28
	Lys	CNBr-digested fibrinogen + heparin	3	0.025 $\pm$ 0.002	1.2 $\pm$ 0.5	48 ($\times$ 1.7)
	Lys	CNBr-digested fibrinogen + pentosan polysulphate	2	0.028	1.4	50 ($\times$ 1.8)
	Glu	Fibrinogen	3	13.4 $\pm$ 1.8	0.19 $\pm$ 0.02	0.014
Urokinase	Glu	Fibrinogen + heparin	2	2.2	0.18	0.082 ($\times$ 6.0)
	Lys	—	6	5.6 $\pm$ 0.3	17.0 $\pm$ 1.9	3.0
	Lys	Heparin	4	2.5 $\pm$ 0.3	15.8 $\pm$ 1.1	6.3 ($\times$ 2.1)
	Lys	CNBr-digested fibrinogen	2	5.9	15.3	2.6
	Lys	CNBr-digested fibrinogen + heparin	4	0.74	14.9 $\pm$ 0.5	20 ($\times$ 7.7)
	Lys	CNBr-digested fibrinogen + pentosan polysulphate	2	0.52	15.4	30 ($\times$ 11)
	Glu	Fibrinogen	2	11.0	1.9	0.18
	Glu	Fibrinogen + heparin	2	7.0	6.3	0.9 ($\times$ 5.3)

explanation, in terms of homotropic interaction, is consistent with other results showing two binding sites for Lys-plasminogen per fibrin monomer (Lucas *et al.*, 1983; Bok & Mangel, 1985) and one t-PA binding site (Hoylaerts *et al.*, 1982). Thus the K_m values presented in Table 1 represent an analysis of the linear region at relatively low substrate concentrations, corresponding to the normal hyperbolic saturation of the first binding site.

Fig. 3. Lineweaver-Burk plot for t-PA

Lys-plasminogen was activated in the presence of 0.1 μ M CNBr-digested fibrinogen (●) or in its absence (■). Results are means for four to eight determinations and, for the enhanced rate, the line drawn is the best fit for initial velocities in the range of substrate concentrations 0.01–1.0 μ M.

Control of t-PA activity

Both heparin and pentosan polysulphate modestly further promoted the enhanced activity of t-PA observed in the presence of CNBr-digested fibrinogen, and the effects were, reproducibly, on K_m (Table 1). Heparin was less stimulatory in the presence, than in the absence, of the fibrin mimic, but there was some additive effect in contrast with the results of others using fibrinogen fragments and fibrin-degradation products (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986). It should be noted, however, that the concentration of fibrin mimic used routinely (Table 1) was submaximal (Fig. 1), so, if heparin competed with fibrin for the binding site on t-PA (Andrade-Gordon & Strickland, 1986), the additive effect might disappear at higher fibrin concentrations. In fact, it was found (Fig. 4) that the additive effect of heparin was maintained at the maximum stimulating concentration of CNBr-digested fibrinogen. The results, using CNBr-digested fibrinogen as a convenient fibrin mimic, have been confirmed by using fibrin monomer preparations. For example, heparin also decreased the K_m of t-PA for Lys-plasminogen in the presence of 0.05 μ M soluble fibrin obtained by NaBr digestion of a human fibrin clot (K_m 0.013 μ M versus 0.032 μ M).

Further information on whether heparin and fibrin bind to the same site on t-PA was sought by examining the response to agents known to interfere with fibrin binding to the Kringle-2 domain of t-PA. A monoclonal antibody, ESP 5, preincubated with t-PA, at 20-fold molar excess (for 10 min at 4 °C), markedly impeded subsequent stimulation by CNBr-digested fibrinogen, but not by heparin (Table 2). 6-Aminohexanoic acid also inhibited the stimulation of t-PA activity by CNBr-digested fibrinogen, in agreement with published results (Ichinose *et al.*, 1986). Although 6-aminohexanoic acid

Fig. 4. Comparison of effect of heparin at maximal and submaximal concentrations of CNBr-digested fibrinogen

Lys-plasminogen was activated in the presence of 0.1 μM CNBr-digested fibrinogen with (▲) or without (●) 4 units of heparin/ml or in the presence of 0.4 μM digest with (▼) or without (■) 4 units of heparin/ml. Results are means for two to four determinations; for clarity the S.E.M. is shown only at one substrate concentration.

Table 2. Effects of a monoclonal antibody and 6-aminohexanoic acid on the potentiation of t-PA activity by heparin and the CNBr digest of fibrinogen

Results are expressed as the percentage change between mean values for at least eight replicate assays. Statistical significance is indicated by * $P < 0.05$ and ** $P < 0.001$. Control stimulated rates ($A_{405} \cdot \text{min}^{-2} \cdot \text{amidolytic unit}^{-1}$) were 0.0065 ± 0.0003 in the presence of CNBr-digested fibrinogen (0.05 μM -Lys-plasminogen substrate) and 0.0044 ± 0.0002 in the presence of heparin (1 μM -Lys-plasminogen), these rates being significantly greater ($P < 0.001$) than the rates in the absence of potentiator.

Potentiator	Change in initial velocity of plasminogen activation (%)	
	ESP 5 (20-fold molar excess)	6-Aminohexanoic acid (10 mM)
CNBr-digested fibrinogen (0.1 μM)	-79**	-98**
Heparin (4 units/ml)	-3	-38*

also impaired the potentiation of activity by heparin, the interference was relatively less, and the net rate was still significantly higher than the basal rate. Thus there is little evidence, in this experimental system, to indicate that the CNBr fragment of fibrinogen and heparin bind to the same site on t-PA.

It has not proved possible to saturate t-PA with Glu-plasminogen alone (at concentrations up to 50 μM), but the effect of heparin on the activation of Glu-plasminogen

was measured in the presence of human fibrinogen in order to provide conditions analogous to those in the bloodstream. The relative effect of heparin on the K_m in this model system (Table 1) was greater than the effect in the presence of fibrin (using Lys-plasminogen as substrate) and consistent with the augmented fibrinolytic activity obtained by Pâques *et al.* (1986).

Control of urokinase activity

Heparin also promoted plasminogen activation by urokinase (M_r 54000; two-chain, of urinary origin). The relative influences on the basal activation of Lys-plasminogen and on Glu-plasminogen in the presence of fibrinogen corresponded to the effects on t-PA, except that the promotion of Glu-plasminogen activation appeared to comprise a change in V_{max} , as well as K_m (Table 1). CNBr-digested fibrinogen did not enhance Lys-plasminogen activation by urokinase, in agreement with published results (Lijnen *et al.*, 1984), but the combination with heparin or pentosan polysulphate produced a large stimulation of activity, mediated by an effect on K_m .

Other activators

The plasminogen-activating activity of the isolated B-chain of t-PA, prepared as described previously (Dodd *et al.*, 1986) was not influenced by heparin or pentosan polysulphate or by mixtures of polysaccharide and CNBr digest of fibrinogen (results not shown). It is likely, therefore, that the heparin-binding site of both t-PA and urokinase (Andrade-Gordon & Strickland, 1986) is located on the A-chain.

Lys-plasminogen activation by streptokinase-Lys-plasminogen activator complex was not affected by heparin in the presence of CNBr-digested fibrinogen: a typical result yielded K_m 0.15 μM in the presence of heparin, 0.13 μM in its absence; V_{max} 16.7 and 17.2 $\text{nmol} \cdot \text{min}^{-2} \cdot \text{fibrinolytic unit}^{-1}$ respectively. CNBr-digested fibrinogen alone enhanced the activation of Lys-plasminogen by the activator complex (basal K_m 0.40 μM ; V_{max} 18.3 $\text{nmol} \cdot \text{min}^{-2} \cdot \text{fibrinolytic unit}^{-1}$), in agreement with previous results obtained by using soluble fibrin (Fears *et al.*, 1985), but heparin alone had no significant action (K_m 0.38 μM ; V_{max} 18.5 $\text{nmol} \cdot \text{min}^{-2} \cdot \text{fibrinolytic unit}^{-1}$). The activation of Glu-plasminogen in the presence of fibrinogen was also not affected by heparin.

Physiological relevance

It has been postulated that heparin and other sulphated polysaccharides can influence fibrinolytic activity by inducing plasminogen-activator release from the endothelium and by promoting activation by the intrinsic pathway (Vinazzer *et al.*, 1982; Marsh *et al.*, 1985). The present quantitative results taken together with the initial observations (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986) indicate an additional mechanism mediated by formation of a complex between heparin, plasminogen activator and plasminogen and expressed through an effect on K_m . The demonstration that this action of heparin may be shared by certain other sulphated polysaccharides provides support for the suggestion that t-PA has a role in pathways other than fibrinolysis. For example, plasminogen activation may be involved in the degradation of matrix constituents such as basement-membrane glycoproteins and proteo-

glycans (Bar-Ner *et al.*, 1986) although other heparin-binding proteins, such as antithrombin III and histidine-rich glycoprotein, might act to attenuate the effect.

Therapeutic relevance

The interaction of heparin with t-PA and urokinase may also be significant with regard to the administration of the enzymes to patients with thrombosis, when heparin is commonly used as an adjunct therapy. It was claimed from kinetic data (Collen & Lijnen, 1984; Tiefenbrunn *et al.*, 1986) that t-PA was selective for the lysis of fibrin, whereas streptokinase and urokinase were not. However, when evaluating the clinical significance of fibrin enhancement, other factors have to be taken into account in the interpretation of the kinetics, in particular, variations in the form of substrate (all activators activate Lys-plasminogen faster than Glu-plasminogen) and the contribution by heparin. In the presence of fibrinogen and heparin, that is, in conditions that may correspond to those appertaining in the circulation, the K_m of t-PA for Glu-plasminogen is approx. $2 \mu\text{M}$ (Table 1). This value should be compared with the estimate of $65 \mu\text{M}$ that is commonly quoted as the K_m of t-PA in the absence of fibrin (Collen & Lijnen, 1984; Lijnen & Collen, 1986) and, in view of the disparity, it is not surprising that the systemic effect of t-PA observed in patients (Topol *et al.*, 1985; Williams *et al.*, 1986) is greater than had been predicted (Sobel *et al.*, 1984). The possibility of an exacerbation of systemic activation by heparin only applies to t-PA, because urokinase causes extensive plasminogen activation, even in the absence of heparin, and streptokinase-plasminogen activity is unaffected. Furthermore, because the effect on t-PA will vary according to the range of heparin concentrations achieved (Fig. 2) and because the nature of the effect, namely on K_m , depends on the concentration of substrate present, heparinization may contribute to the variability in clinical response obtained for t-PA.

The present results do not confirm the findings (Andrade-Gordon & Strickland, 1986; Pâques *et al.*, 1986) that fibrin nullifies the potentiation of activity of t-PA by heparin, but there is an attenuation that may undermine the 'fibrin-selectivity'. By using the results to calculate fibrin selectivity by comparing second-order rate constants (that is, for the activation of Lys-plasminogen in the presence of heparin and CNBr-digested fibrinogen relative to the activation of Glu-plasminogen in the presence of heparin and fibrinogen), the degree of enhancement is 600-fold for t-PA by comparison with 22-fold for urokinase and 170-fold for streptokinase-plasminogen. On this basis, whereas t-PA is clearly subject to considerable fibrin enhancement, the activity of streptokinase-plasminogen is also markedly promoted, and differences in therapeutic activity may be influenced more by the size of the dose and the clearance rate, particularly if streptokinase-plasminogen is provided as the nascent activator in an undegraded pro-enzyme form such as anisoylated Lys-plasminogen-streptokinase activator complex (BRL 26921; Eminase) (Fears *et al.*, 1985).

I thank Dr. H. Ferres and Dr. J. H. Robinson for helpful discussions and Dr. R. Standring for characterization of the CNBr digest of fibrinogen.

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