

Slow Clearance of Acylated, Hybrid Thrombolytic Enzymes

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Key words

Fibrinolysis – Pharmacokinetics – Thrombolytic agents

Summary

Two hybrid plasminogen activators, plasmin A-chain/t-PA B-chain and plasmin A-chain/u-PA B-chain have been synthesized and purified in sufficient yield to permit measurement of clearance in small laboratory animals. Each hybrid enzyme was reversibly acylated at the active centre to allow the pharmacokinetic profile to be followed using an activity-based method without interference from plasma inhibitors. The acylated plasmin/u-PA hybrid had a clearance half-life ($t_{1/2}$) in guinea pigs of approximately 80 min, whereas acyl u-PA had a $t_{1/2}$ of 3 min. The pharmacokinetic profile of the acylated plasmin/t-PA hybrid was measured in guinea pigs, rats and rabbits; the half-lives in all three species were 60–80 min compared to half-lives of acylated, native t-PA that were in the range 0.5–1.0 min. Thus, plasmin A-chain-containing, acylated hybrid enzymes are cleared some 30- to 100-fold more slowly than the acylated parent activators.

Introduction

A new generation of thrombolytic agents comprising the anisoylated plasminogen-streptokinase activator complex, APSAC (Eminase**) (1), tissue-type plasminogen activator, t-PA (2) and the pro-, or single chain (sc), form of urokinase, u-PA (3) are currently under evaluation for the treatment of acute myocardial infarction (4–6). Unlike Eminase (7), both t-PA and scu-PA share the clinical disadvantage that they are rapidly cleared from the bloodstream, probably necessitating their prolonged infusion in large doses (8, 9). By contrast, their substrate, plasminogen, is slowly cleared (10). We reasoned that it might be possible to construct hybrid enzymes combining in one molecule the fibrin-binding and slow clearance properties of plasminogen with the enzyme specificity of the plasminogen activators. We published a preliminary report on the synthesis of three hybrid enzymes; plasmin A-chain/t-PA B-chain, plasmin A-chain/u-PA B-chain and t-PA A-chain/u-PA B-chain (11). Nakayama et al. also provided a preliminary report on synthesis of a plasmin A-chain/u-PA B-chain hybrid (12). More recently, Robbins and Tanaka (13) and Robbins and Boreisha (14) have described in more detail the synthesis and *in vitro* properties of plasmin/urokinase and plasmin/t-PA species. The biological properties of these novel hybrid enzymes have not yet been fully described. We now report the *in vivo* clearance of active centre-acylated derivatives of plasmin/t-PA and plasmin/u-PA enzymes in laboratory animals.

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Materials and Methods

Materials

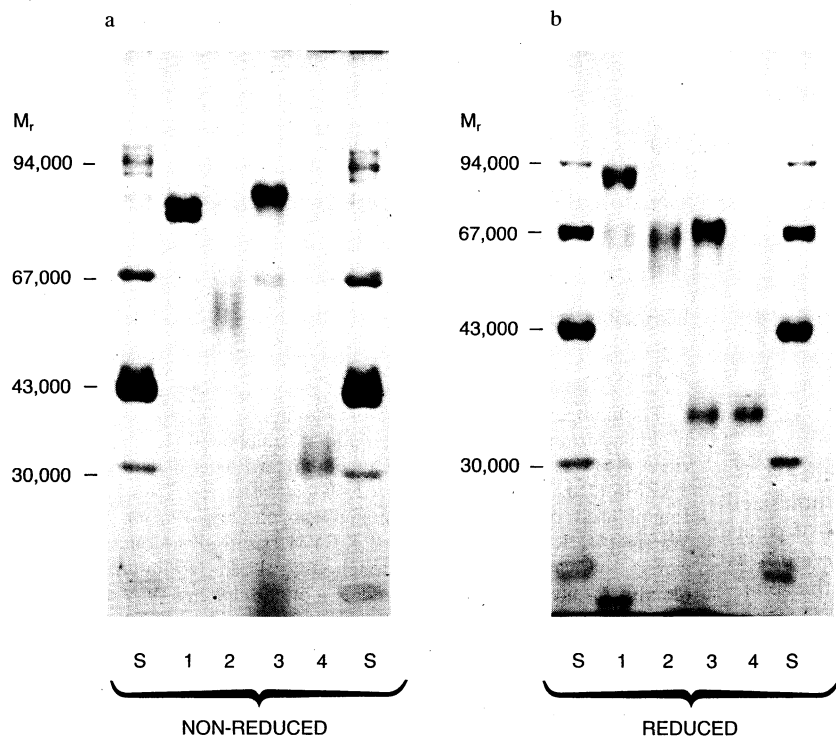
Plasminogen was obtained from Immuno, Vienna, Austria, and urokinase from Serono, Freiburg, Federal Republic of Germany. Purified rt-PA (15) was obtained from the recombinant Bowes melanoma cell line TRBM6 (16). Lysine Sepharose CL4B has been described before (15). Immobilised para-aminobenzamide was purchased from Pierce and Warriner (UK) Ltd., Chester, U.K. YM10 ultrafiltration membranes were from Amicon Ltd., Stonehouse, U.K. 4'-amidinophenyl-4-aminobenzoate (AP-AB) and 4'-amidinophenyl-*N,N*-dimethyl-4-aminobenzoate. HCl (AP-DAB) were kindly supplied by Dr. R. A. G. Smith, Beecham, U.K. Sodium Heparin BP was obtained from Evans Medical Ltd., Greenford, U.K. Sodium pentobarbitone was from May and Baker, Dagenham, U.K.; "Hypnorm" from Janssen Pharmaceuticals Ltd., Oxford, U.K. and urethane from Sigma Chemical Company Ltd., Dorset, U.K.

Synthesis and Purification of Lys₇₇Plasmin A Chain/t-PA B Chain Hybrid

Lyophilised, salt-free lys₇₇plasminogen was dissolved at 15 mg/ml in 0.05 M Tris, 0.1 M NaCl, 25% (v/v) glycerol, pH 9.0 and activated using u-PA (final concentration 500 IU/ml) at 27° C for 1 h. Mild reduction of plasmin to its constituent A- and B-chains and their subsequent separation were performed essentially as previously described (17). The isolation of the B-chain of rt-PA has been reported elsewhere (18). The lys₇₇A-chain and the rt-PA B-chain pools were treated with glycerol to 25% (v/v), followed by addition of 0.05 M Tris base, 0.02 M L-lysine and 0.003 M EDTA. On the basis of protein analysis (19) using a bovine serum albumin calibration curve, 5 nmol of each chain were mixed in a total volume of 3 ml and subjected to mild oxidation in the presence of air by dialysis for 3 days at 5° C against 30 vol of 0.04 M Tris, 0.02 M L-lysine, 0.08 M NaCl, 0.003 M EDTA, 25% (v/v) glycerol, pH 9.0 followed by a further dialysis for 5 days against fresh buffer. The dialysed retentate was diluted with 5 vol 0.02 M phosphate, 0.15 M NaCl, 0.01% Tween 80, pH 7.4 (PBS-Tween). This solution was chromatographed on a column (i. d., 10 mm; ht, 37 mm) of lysine Sepharose that had been equilibrated in PBS-Tween. After application of the dialysed retentate the column was washed with PBS-Tween followed by 0.02 M Tris, 0.5 M NaCl, 0.01% Tween 80, pH 7.4. The hybrid was then desorbed using 0.02 M Tris, 0.5 M NaCl, 0.5 M L-arginine, 0.01% Tween 80, pH 7.4. All parts of the chromatography were carried out at 5° C at a velocity of 150 cm h⁻¹. Fractions corresponding to the arginine-containing eluate were pooled and further purified using a column (Vt, 2 ml) of solid-phase anti t-PA B-chain monoclonal antibody (ESP2, Bioscot Ltd., Edinburgh) that had been equilibrated in PBS-Tween. The column was then washed sequentially with (i) PBS-Tween and (ii) 0.1 M glycine, pH 2.0. All parts of the chromatography were carried out at 5° C at a velocity of 100 cm h⁻¹. The eluate of buffer (ii) was buffer-exchanged into 0.05 M NH₄HCO₃ and lyophilised.

Synthesis and Purification of Lys₇₇Plasmin A Chain/u-PA B Chain Hybrid

Lys₇₇plasmin A chain was prepared as described above. u-PA B chain was prepared as follows. 2 mg u-PA (20 mg/ml in deionised H₂O) was diluted with an equal volume of 0.02 M 2-mercaptoethanol, 0.1 M Tris-HCl, 0.3 M NaCl, 0.04 M EDTA pH 8.0 and incubated at 21–24° C for 17 h. The incubate was then diluted with 9 volumes 0.02 M Tris, 0.003 M 2-mercaptoethanol pH 7.4 (TME) and applied to a column (Vt, approx. 4 ml) of p-amino-benzamide Sepharose that had been equilibrated in



Figs. 1a, b SDS PAGE of purified plasmin/rt-PA hybrid. The hybrid was purified using lysine Sepharose and an anti-t-PA B-chain monoclonal antibody. All samples were analysed with or without prior reduction using 2-mercaptoethanol by SDS PAGE using 10% polyacrylamide gels and protein bands stained with Coomassie Brilliant Blue R250 followed by silver staining. The silver-stained gel is shown. Lanes marked S are protein molecular weight markers (low molecular weight kit, Pharmacia). Lanes 1, 2, 3 and 4 are respectively lys_{77} plasminogen, lys_{77} plasmin A-chain, plasmin/rt-PA hybrid and rt-PA B-chain. The right hand panel (b) shows the same samples analysed under reducing conditions

TME. The column was developed with (a) TME (b) 0.02 M Tris, 0.5 M NaCl, 25% (v/v) glycerol, 0.003 M 2-mercaptoethanol pH 7.4 and (c) as (b) but additionally containing 0.5 M L-arginine. The peak arginine-containing fractions (vol. 4.9 ml) were augmented with 0.05 M Tris, 0.02 M L-lysine, 0.003 M EDTA by addition of 0.245 ml of a 20-fold-concentrated stock solution. The pH was noted as 8.5. Protein content was 82 $\mu\text{g}/\text{ml}$. This solution was regarded as the u-PA B-chain pool. The

synthesis and purification of the plasmin A chain/u-PA B chain hybrid were the same as described above for the t-PA B chain hybrid except that the lysine Sepharose-purified u-PA hybrid was further purified on pABA Sepharose. Conditions of the chromatography were similar to those used for the isolation of the u-PA B-chain itself.

Synthesis of Acyl Hybrid Plasminogen Activators

Both hybrid plasminogen activators and reference two-chain t-PA (tct-PA) and urokinase (tcu-PA) were reversibly blocked at the active centre serine of the molecule using either AP-AB (plasmin/u-PA and tcu-PA) or AP-DAB (plasmin/t-PA and tct-PA). Specifically, the plasmin/t-PA hybrid (200 $\mu\text{g}/\text{ml}$ in PBS-Tween) was treated with a 3-fold molar excess of AP-DAB for 30 min at 25° C. Free acylating agent was removed by buffer-exchange into PBS-Tween containing 0.02 M ϵ -ACA using Sephadex G25 and the product was stored at -70° C. Other plasminogen activator species were acylated in a similar way.

Determination of Deacylation Rate Constants of Acyl Plasminogen Activators

Deacylations were carried out in phosphate/saline at 37° C but otherwise as described previously (20). Under these conditions the pseudo-first-order rate constant for tct-PA and the plasmin/t-PA hybrid was approx. $5 \times 10^{-5} \text{ sec}^{-1}$, and for u-PA and the plasmin/u-PA hybrid, approx. $4 \times 10^{-5} \text{ sec}^{-1}$.

Determination of Pharmacokinetics of Plasminogen Activator Species in Guinea Pigs

Male Dunkin Hartley guinea pigs (350–470 g) were anaesthetised with urethane (1.5 g/kg i. p.). Heparin (50 U/kg) was injected via a cannulated jugular vein, followed by the plasminogen activator (1 ml/kg over 15 sec). At the times shown, blood (0.6 ml) was drawn from a cannulated carotid artery, mixed with 0.1 vol. 0.13 M trisodium citrate and centrifuged at 1700 g for 15 min at 4° C to obtain plasma. The euglobulin fraction was precipitated by adding 50 μl of each plasma sample to 0.9 ml ice-cold 0.01% (v/v) acetic acid in water and leaving for 30 min on ice. After centrifugation at 1700 g for 15 min at 4° C, the precipitates were redissolved in 0.2 ml (Fig. 3) or 0.5 ml (Fig. 4) PBS-Tween. Aliquots (30 μl) were assayed on human fibrin plates (18) by reference to calibration curves for the appropriate acylated activator serially diluted in

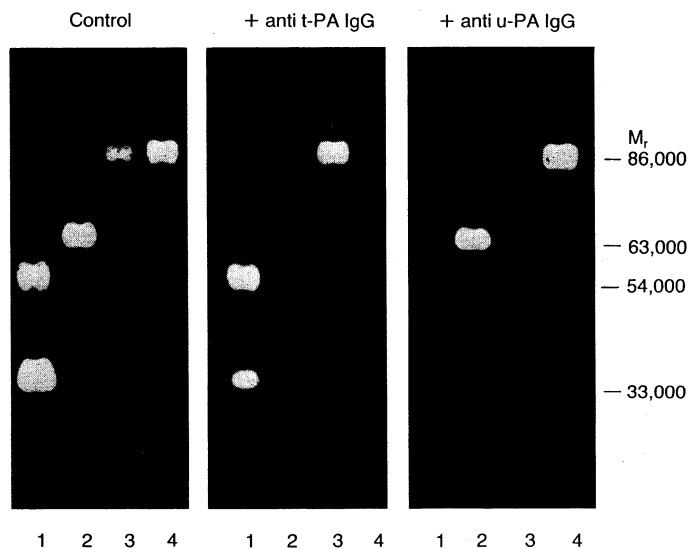


Fig. 2 Immunological identification of hybrid plasminogen activators demonstrated by fibrin zymography. Hybrid activators that had been partially purified using lysine Sepharose were analysed under non-reducing conditions by SDS PAGE followed by fibrin zymography. Specific anti-t-PA IgG or anti-u-PA IgG was incorporated into the zymograms at a final concentration of 0.2 mg/ml. The stained zymograms are shown. Lane 1, u-PA (0.2 IU); lane 2, rt-PA (1.6 IU); lane 3, plasmin/u-PA hybrid (1.6 IU t-PA); lane 4, plasmin/rt-PA hybrid (1.6 IU t-PA). N.B. Concentrations of both hybrids are expressed in t-PA IU because fibrin plate concentration-response curves for both were parallel to t-PA

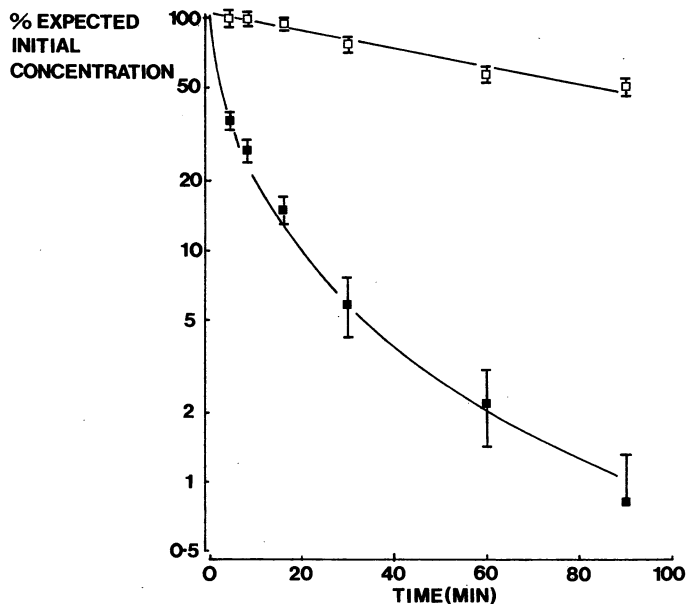


Fig. 3 Clearance of u-PA (■) and plasmin/u-PA hybrid (□) from the bloodstream of guinea pigs. The two activators were first subjected to reversible active centre acylation using AP-AB. Aliquots of plasma euglobulin fractions were assayed on human fibrin plates. Doses of activators were 0.5 nmol/kg. Each point represents the mean \pm s. e. of values from four or five animals

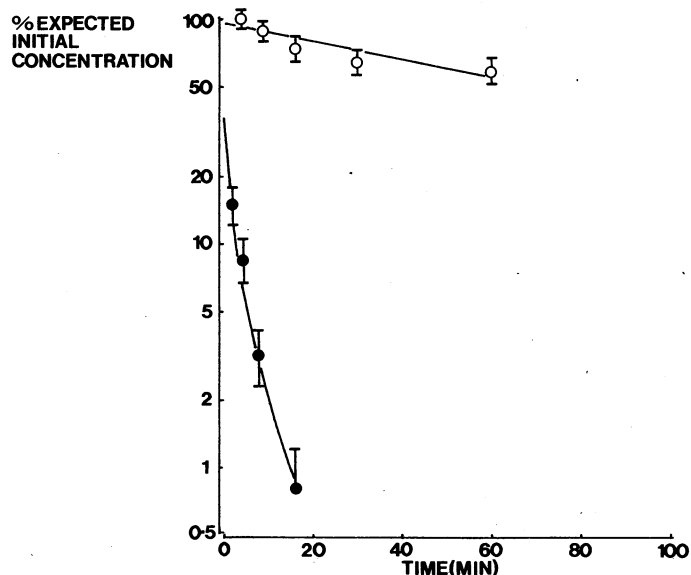


Fig. 4 Clearance of t-PA (●) and plasmin/t-PA hybrid (○) from the bloodstream of guinea pigs. Conditions same as Fig. 3 except active centre acylation was with AP-DAB to give DAB \rightarrow enzymes and dose was 0.7 nmol/kg

the pre-dose plasma of each animal. These calibration samples were processed through the euglobulin precipitation procedure using the same methods and at the same time as the clearance samples. The plasma concentrations determined were expressed as a percentage of the initial concentration expected if the total activator dose administered were distributed in 45 ml plasma/kg body weight.

Pharmacokinetics in Rabbits and Rats

Methods were essentially the same as for guinea pigs with the following exceptions. Rabbits were pretreated with Hypnorm (0.15 ml/kg i. m.) and anaesthetised with sodium pentobarbitone (15 mg/kg i. v.) followed by further supplements of sodium pentobarbitone as necessary to maintain anaesthesia. Rats were anaesthetised with sodium pentobarbitone (60 mg/kg i. p.).

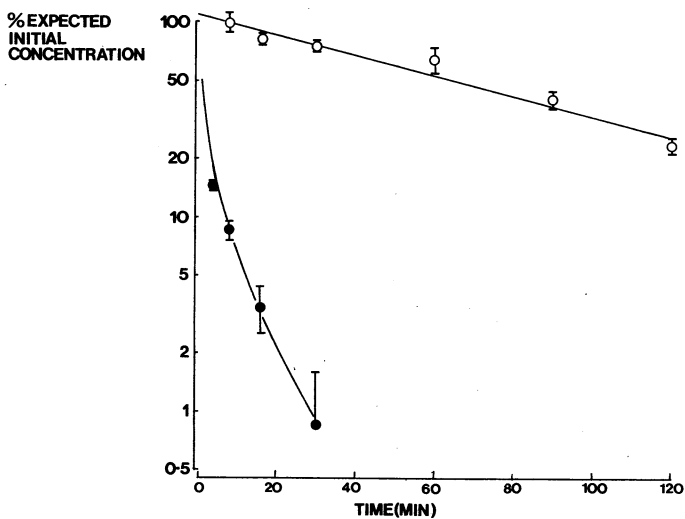


Fig. 5 Clearance of DAB \rightarrow t-PA (●) and DAB \rightarrow plasmin/t-PA hybrid (○) from the bloodstream of rabbits (0.7 nmol/kg)

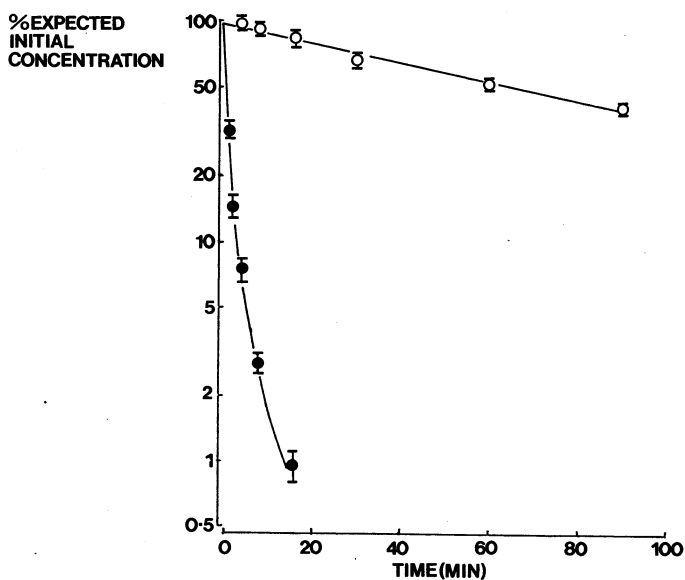


Fig. 6 Clearance of DAB \rightarrow t-PA (●) and DAB \rightarrow plasmin/t-PA hybrid (○) from the bloodstream of rats (0.7 nmol/kg)

Results

Plasmin A-chain/t-PA B-chain and plasmin A-chain/u-PA B-chain hybrid enzymes were synthesised by oxidative dialysis of isolated protein chains as described in Materials and Methods. SDS-PAGE analysis of the plasmin/t-PA hybrid showed a major protein species at $M_r = 86,000$ (Fig. 1 a, lane 3). Under reducing conditions, new stained bands corresponding to the plasmin A-chain and the t-PA B-chain were detected (Fig. 1b, lane 3), a result consistent with the linkage of the A- and B-chains by a disulphide bridge. In addition, a doublet was occasionally seen under non-reducing conditions; the two species differed by M_r approx. 3,000, had different affinities for lysine Sepharose (not shown) and presumably arose from the different carbohydrate

forms of the plasmin A-chain (21). In a typical experiment, the yield of plasmin/t-PA hybrid was approximately 30% of the initial B-chain amidolytic activity.

Plasmin/t-PA and plasmin/u-PA hybrid enzymes that had been purified using lysine Sepharose were analysed further by antibody-quenching fibrin zymography. Both hybrid enzymes showed similar electrophoretic mobility. However, only the t-PA-containing hybrid was quenched by specific anti-t-PA antibodies whereas activity of the u-PA hybrid was inhibited by anti-u-PA antibodies (Fig. 2).

In a calibrated human fibrin plate assay, both hybrid activators showed concentration/lysis lines parallel to t-PA (and not to u-PA). The specific activities of the plasmin/u-PA and plasmin/t-PA hybrids were approximately 430,000 and 160,000 IU/mg protein, respectively, compared to 500,000 IU/mg protein for t-PA.

The pharmacokinetic properties of acylated forms of the two plasmin A-chain-containing hybrid enzymes were examined in guinea pigs (Figs. 3 and 4). Both hybrids were cleared by largely monophasic processes with half-lives of 80 min each. This value was in sharp contrast to results obtained for the acylated parent activators. Both acylated parent activators displayed biphasic clearance patterns and were analysed using a graph curve peeling method (22). The majority (75%) of the injected dose of acylated t-PA was cleared with a half-life of 0.8 min; an equal proportion of acylated u-PA was cleared with a half-life of 3 min. The pharmacokinetics of the acylated t-PA hybrid were further studied in rabbits and rats; half-lives for plasma disappearance were 60 and 70 min respectively, whereas the parent, acylated t-PA was cleared very rapidly with a $t_{1/2}$ of 0.5–1 min for the majority of the dose.

Discussion

Plasmin A-chain-containing hybrid plasminogen activators are a novel class of fibrinolytic enzymes that have been described previously (11–14). These enzymes show interesting properties. For example, unlike the parent u-PA, the plasmin/u-PA hybrid enzyme exhibits significant fibrin binding (13). More unexpected are the plasminogen activating properties of the plasmin/t-PA hybrid (14). It is known that the isolated t-PA B-chain is a very poor plasminogen activator in the presence or absence of fibrin (18, 23, 24) whereas the whole t-PA molecule exhibits weak plasminogen activator activity only in the absence of fibrin (25). In the presence of fibrin, t-PA becomes a much more efficient enzyme and this process is postulated to occur by formation of a specific cyclic ternary complex between t-PA, fibrin and plasminogen (25). The substitution of the t-PA A-chain (with its specific fibrin-binding sites) by the plasmin A-chain (with different fibrin-binding sites) to form the plasmin/t-PA hybrid might have been expected to yield a fairly poor plasminogen activator; the hybrid enzyme would presumably be unable to form the specific, ternary complex that gives rise to fibrin enhancement of t-PA activity. However, the hybrid enzyme, in contrast with the isolated, t-PA B-chain, showed relatively high specific fibrinolytic activity (our results and 14). This can be explained by the fact that, in kinetic analyses, the plasmin/t-PA hybrid enzyme (like t-PA itself) showed a high degree of fibrin enhancement (R. Fears, personal communication).

We wished to determine whether the hybrid enzymes had interesting *in vivo*, as well as *in vitro*, properties. Data from this laboratory (11, 26–28) and from others (29–32) suggested that the very rapid clearance of t-PA might be mediated by a recognition site within the fibrin-binding A-chain of t-PA. Plasminogen, which contains a fibrin-binding A-chain of quite distinct structure, is very slowly cleared (10). It was not known whether this slow

clearance resulted from lack of a liver recognition site in the A-chain or whether some other feature of the molecule was responsible. Therefore, we measured, in small laboratory animals, the clearance of both hybrid enzymes and found it to be much slower than that of the parent activators. It is known that t-PA and u-PA are both rapidly cleared by a non-saturable liver-dependent pathway rather than by the inhibition of plasma proteins (33). However, t-PA and u-PA derivatives that evade this liver clearance mechanism could then be subject to plasma inhibition. In order to avoid such inhibition obscuring the pharmacokinetic behaviour of the hybrid activators, each was first reversibly inactivated by acylation of the active site (1). The acylating agent selected had a deacylation half-life (in PBS-Tween at 37° C) of about 4 h, which was sufficiently long to minimize inhibition *in vivo*, yet short enough to permit regeneration of activity *in vitro* during the subsequent functional assay of plasma samples. The rapid clearance of the acylated (DAB) form of t-PA in guinea pigs was very similar to that previously reported for the unacylated form of t-PA (34).

Although, as mentioned above, the very rapid clearance of t-PA may be mediated by a site on the A-chain, there is also evidence for a further clearance site on the B-chain. Thus, we (unpublished) and others (29) have shown that the isolated t-PA B-chain is still cleared at a rate that is significantly more rapid than that demonstrated here for the plasmin/t-PA hybrid. Similarly, lower molecular weight species of t-PA with smaller A-chains (26, 27) and t-PA A-chain mutants (28, 32) are also cleared more rapidly than the plasmin/t-PA hybrid. Furthermore, Lau et al. (35) have shown that a t-PA B-chain mutation results in a variant molecule with extended plasma half-life. There is also evidence that removal of nearly all the A-chain of scu-PA has no effect on the rapid clearance of this molecule (36), although other data (30) suggests that a site on the A-chain of t-PA may be responsible for rapid clearance. We, therefore, conclude that the very slow clearance of the plasmin A-chain hybrids can be attributed to two effects. First (especially for t-PA), the removal of a clearance recognition site in the A-chain, and, second, a further (probably B-chain-mediated) effect resulting possibly from steric hindrance of a clearance receptor by the bulky plasmin A-chain.

There is tremendous current interest in the development of recombinant, second-generation t-PA molecules capable of bolus administration (37, 38). The hybrid enzymes described here are more slowly cleared than other "second-generation" molecules and these types of enzymes can also be made by recombinant techniques; various acylated derivatives are undergoing further evaluation to assess their potential for the treatment of acute myocardial infarction.

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References

- 1 Smith R A G, Dupe R J, English P D, Green J. Fibrinolysis with acyl enzymes: a new approach to thrombolytic therapy. *Nature* 1981; 290: 505–8.
- 2 Rijken D C, Collen D. Purification and characterisation of the plasminogen activator secreted by human melanoma cells in culture. *J Biol Chem* 1981; 256: 7035–41.
- 3 Gurewich V, Pannell R, Louie S, Kelley P, Suddith R L, Greenlee R. Effective and fibrin-specific clot lysis by a zymogen precursor form of urokinase (Pro-urokinase). *J Clin Invest* 1984; 73: 1731–9.

- 4 Monk J P, Heel R C. Anisoylated plasminogen streptokinase activator complex (APSAC). A review of its mechanism of action, clinical pharmacology and therapeutic use in acute myocardial infarction. *Drugs* 1987; 34: 25-49.
- 5 Verstraete M, Bernard R, Bory M, Brower R W, Collen D, De Bono D P, Erbel R, Huhmann W, Lennane R J, Lubsen J, Mathey D, Meyer J, Michels H R, Rutsch W, Scharl M, Schmidt W, Vebis R, Von Essen R. Randomized trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. *Lancet* 1985; 8433: 842-7.
- 6 Van de Werf F, Vanhaeck J, De Geest H, Verstraete M, Collen D. Coronary thrombolysis with recombinant single-chain urokinase-type plasminogen activator in patients with acute myocardial infarction. *Circulation* 1986; 74: 1066-70.
- 7 Been M, De Bono D P, Muir A L, Boulton F E, Fears R, Standring R, Ferres H. Clinical effects and kinetic properties of intravenous APSAC - anisoylated plasminogen-streptokinase activator complex (BRL 26921) in acute myocardial infarction. *Int J Cardiol* 1986; 11: 53-61.
- 8 Sherry S. Recombinant tissue plasminogen activator (rt-PA): is it the thrombolytic agent of choice for an evolving acute myocardial infarction? *Am J Cardiol* 1987; 59: 984-9.
- 9 Van de Werf F, Nobuhara M, Collen D. Coronary thrombolysis with human single-chain, urokinase-type plasminogen activator (pro-urokinase) in patients with acute myocardial infarction. *Ann Intern Med* 1986; 104: 345-8.
- 10 Collen D, Tygat G, Claeys H, Verstraete M, Wallen P. Metabolism of plasminogen in healthy subjects: effect of tranexamic acid. *J Clin Invest* 1972; 51: 1310-8.
- 11 Robinson J H, Dodd I. Fibrinolytically active hybrid protein. European Patent Application 1985; EPA 0155387.
- 12 Nakayama Y, Miyazaki W, Shinohara M. Urokinase-plasmin complex adsorbable by fibrin and process for preparing same. European Patent Application 1985; EPA 0151308.
- 13 Robbins K C, Tanaka Y. Covalent molecular weight ~92000 hybrid plasminogen activator derived from human plasmin amino-terminal and urokinase carboxy-terminal domains. *Biochem* 1986; 25: 3603-11.
- 14 Robbins K C, Boreisha I G. A covalent molecular weight ~92000 hybrid plasminogen activator derived from human plasmin fibrin-binding and tissue plasminogen activator catalytic domains. *Biochem* 1987; 26: 4661-7.
- 15 Dodd I, Jalalpour S, Southwick W, Newsome P, Browne M J, Robinson J H. Large scale, rapid purification of recombinant tissue-type plasminogen activator. *FEBS Lett* 1986; 209: 13-7.
- 16 Browne M J, Dodd I, Carey J E, Chapman C G, Robinson J H. Increased yield of human tissue-type plasminogen activator obtained by means of recombinant DNA technology. *Thromb Haemostas* 1985; 54: 422-4.
- 17 Summaria L, Boreisha I, Wohl R C, Robbins K C. Recombinant human lys-plasmin and the lys-plasmin-streptokinase complex. *J Biol Chem* 1979; 254: 6811-4.
- 18 Dodd I, Fears R, Robinson J H. Isolation and preliminary characterisation of active B-chain of recombinant tissue-type plasminogen activator. *Thromb Haemostas* 1986; 55: 94-7.
- 19 Bradford M M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.
- 20 Smith R A G. An active-site titrant for human tissue-type plasminogen activator. *Biochem J* 1986; 239: 477-9.
- 21 Castellino F J. Recent advances in the chemistry of the fibrinolytic system. *Chem Rev* 1981; 81: 431-46.
- 22 Verstraete M, Bounameaux H, De Cock F, Van de Werf F, Collen D. Pharmacokinetics and systemic fibrinolytic effects of recombinant human tissue-type plasminogen activator (rt-PA) in humans. *J Pharm Exp Ther* 1985; 235: 506-12.
- 23 Rijken D C, Groenvelde E. Isolation and functional characterisation of the heavy and light chains of human tissue-type plasminogen activator. *J Biol Chem* 1986; 261: 3098-102.
- 24 MacDonald M E, Van Zonneveld A-J, Pannekoek H. Functional analysis of the human tissue-type plasminogen activator protein: the light chain. *Gene* 1986; 42: 59-67.
- 25 Hoylaerts M, Rijken D C, Lijnen H R, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. *J Biol Chem* 1982; 257: 2912-9.
- 26 Dodd I. Degraded species of tissue-type plasminogen activator. European Patent Application, 1986, EPA 0196920.
- 27 Dodd I, Nunn B, Robinson J H. Isolation, identification and pharmacokinetic properties of human tissue-type plasminogen activator species: possible localisation of a clearance recognition site. *Thromb Haemostas* 1988; 59: 523-8.
- 28 Browne M J, Carey J E, Chapman C G, Tyrrell A W R, Entwisle E, Lawrence G M P, Reavy B, Dodd I, Esmail A, Robinson J H. A tissue-type plasminogen activator mutant with prolonged clearance *in vivo*. *J Biol Chem* 1988; 263: 1599-1602.
- 29 Rijken D C, Emeis J J. Clearance of the heavy and light polypeptide chains of human tissue-type plasminogen activator in rats. *Biochem J* 1986; 238: 643-6.
- 30 Kalyan N K, Lee S G, Hum W-T, Hartzell R, Levner M, Hung P P. *In vitro* studies on the binding of tissue-type plasminogen activator (t-PA) and urokinase (u-PA) to liver membranes. *Thromb Haemostas* 1987; 58: 217 (Abstr).
- 31 Cambier P, Van de Werf F, Larsen G R, Collen D. Coronary thrombolysis in dogs with a nonglycosylated variant of human tissue-type plasminogen activator lacking the finger and growth factor domains. *Thromb Haemostas* 1987; 58: 271 (Abstr).
- 32 Larsen R, Metzger M, Blue Y, Henson K. Pharmacokinetics of genetically modified t-PA in rat. *Thromb Haemostas* 1987; 58: 491 (Abstr).
- 33 Korninger C, Stassen J M, Collen D. Turnover of human extrinsic (tissue-type) plasminogen activator in rabbits. *Thromb Haemostas* 1981; 46: 658-61.
- 34 Nunn B, Esmail A, Fears R, Ferres H, Standring R. Pharmacokinetic properties of anisoylated plasminogen streptokinase activator complex and other thrombolytic agents in animals and humans. *Drugs* 1987; 33 (Suppl 3): 88-92.
- 35 Lau D, Kuzma G, Wei C-M, Livingston D J, Hsiung N. A modified human tissue plasminogen activator with extended half-life *in vivo*. *Bio/technology* 1987; 5: 953-8.
- 36 Stump D C, Kieckens L, De Cock F, Collen D. Pharmacokinetics of single chain forms of urokinase-type plasminogen activator. *J Pharm Exp Ther* 1987; 242: 245-50.
- 37 Ezzell C. US patents in the balance. *Nature* 1987; 328: 189.
- 38 Klausner A. Second-generation t-PA race heats up. *Bio/technology* 1987; 5: 869-70.

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