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Isolation, Identification and Pharmacokinetic Properties of Human Tissue-Type Plasminogen Activator Species: Possible Localisation of a Clearance Recognition Site

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Key words

Tissue-type plasminogen activator – Fibrinolysis – Pharmacokinetics

Summary

Purified preparations of recombinant tissue-type plasminogen activator (t-PA) from the recombinant Bowes melanoma cell line TRBM6 were shown to contain multiple species of plasminogen activator. Using a combination of chromatography on Sephadex G25, Sephadex G75 and Heparin Sepharose CL6B we have isolated two fibrinolytically active species, which, under non-reduced SDS PAGE, have apparent M_r = 38,000 and 56,000. Double immunodiffusion studies indicated that both species were closely related to both the t-PA B chain and t-PA itself. N-terminal sequencing identified the M_r = 38,000 species as ala_{160}-t-PA (essentially ΔFGKI t-PA) and the M_r = 56,000 species as ser_1-tyr_2-gln_3-gly_x-cys_{51} t-PA (ΔF t-PA), the latter probably produced by alternative splicing of the t-PA gene. The pharmacokinetic properties of *N,N* dimethyl-4-aminobenzoyl (DAB) derivatives of these activators and native t-PA were determined in the guinea pig. Whereas DAB → ΔF t-PA showed a similar, rapid plasma disappearance profile to that of DAB → t-PA, DAB → ΔFGKI t-PA was cleared significantly slower. These results suggest that a rapid clearance recognition site resides on either the growth factor or kringle 1, or both, domains of t-PA.

Introduction

Plasminogen activators play an important role in the maintenance of haemostasis. One of these activators, tissue-type plasminogen activator (t-PA), is attracting attention as a treatment for acute myocardial infarction (1–3) and it is currently being evaluated in the clinic using infusion regimes of several hours duration. There is now interest in the development of second-generation t-PA molecules capable of bolus administration (4, 5). The apparent necessity for infusion of t-PA is a consequence of its rapid hepatic clearance from the circulation (6); therefore attention has been directed towards identification of the putative clearance site on the t-PA molecule. Rijken and Emeis (7) showed a small difference in the clearance of the A and B chains suggesting that the recognition site of t-PA probably resided on the A-chain.

Native t-PA is a glycoprotein with M_r = 63,000/65,000 (8, 9), the two species differing in their extent of glycosylation (10). Other forms of t-PA have been noted including an $M_r \simeq$ 55,000 (11–13) and $M_r \simeq$ 37,000 (9, 13) but none of these have been isolated and characterised. We previously described the purification (14) of t-PA from the recombinant Bowes melanoma cell line TRBM6 (15). In some of the purified preparations we detected t-PA-like species similar to those described by other workers. It seemed possible that these species were truncated forms of native t-PA and that further analysis might provide us with some insight into the regions of the t-PA molecule responsible for its rapid clearance. This paper describes the isolation, identification and pharmacokinetic properties of these t-PA-like species.

Materials and Methods

Materials

The recombinant Bowes melanoma cell-line TRBM6 has been described before (15). Sephadex G25 (Medium grade), Sephadex G75 (Fine grade) and Heparin Sepharose CL6B were purchased from Pharmacia (Great Britain) Ltd, Milton Keynes, UK. YM10 ultrafiltration membranes were from Amicon Ltd, Stonehouse, UK.

4-amidinophenyl-*N,N*,-dimethyl-4-aminobenzoate. HCl (AP-DAB) was kindly prepared by Dr R. A. G. Smith, Beecham, UK. Lysine Sepharose CL4B has been described before (14). Sodium Heparin BP was obtained from Evans Medical Ltd, Greenford, UK. S2288 was from Flow Laboratories, Irvine, UK. PAGE Blue 83 was from BDH Ltd, Eastleigh, UK.

Isolation of t-PA Fragments

Purified t-PA (14) in 0.02 M Tris/0.5 M NaCl/0.5 M L-arginine/0.01% Tween 80 pH 7.4 was concentrated by ultrafiltration using YM10 membranes. The concentrate (30 ml; 21 × 10^6 SU, see below) was buffer-exchanged into 0.05 M NH_4HCO_3 using Sephadex G25 (id, 5 cm; h, 20 cm) and the t-PA activity (3.7 × 10^6 SU) that eluted in the void volume of the column was lyophilised.

Isolation of M_r = 38,000 Species

Part of the lyophilisate (1.6 × 10^6 SU) was reconstituted in 3.0 ml 0.02 M Tris/0.05 M NaCl/0.5 M L-arginine/0.01% Tween 80, pH 7.4 buffer and chromatographed on a column (id, 2.5 cm; h, 63 cm) of Sephadex G75 equilibrated in the same buffer. The velocity of flow was 2.6 cm h^{-1}. The fractions enriched in the smallest t-PA species were pooled (total volume, 45 ml), ultrafiltered to a small volume (2.0 ml), buffer-exchanged into 0.05 M NH_4HCO_3 (3.0 ml) and lyophilised.

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Table 1 N-terminal amino acid sequences of various t-PA species. Row A denotes the amino acid sequence (one-letter code) of t-PA (9). The open triangle represents the region homologous with the Type I structure in fibronectin (the fibronectin finger). Row B shows the N-terminal sequence of the fibronectin finger-lacking t-PA derived from cDNA prepared from Detroit 562 mRNA (12). Row C is the N-terminal sequence of the t-PA species of apparent M_r = 56,000 described in this paper. Where identification of the amino acid was equivocal (†) all selected residues are given e.g. R, P, S. Where identification was impossible, the position is given the letter X

	Sequence
	1 ⌈⌉ 51 60
A:	S Y Q\.../C S E P R C F N G G T C Q Q A
B:	S Y Q G C S E P R C F N G G T C Q Q A
	S† R†
C:	D Y Q G X S E P X X F N G G X X Q Q A
	S

Isolation of M_r = 56,000 Species

The fractions from the Sephadex G75 column that contained the higher M_r plasminogen activators were pooled, ultrafiltered and buffer-exchanged into 0.05 M NH_4HCO_3. This was mixed with similarly prepared material to give a total volume of 32 ml containing 2.1×10^6 SU and applied to a column (id, 16 mm; h, 100 mm) of Heparin Sepharose CL6B equilibrated in 0.05 M NH_4HCO_3. The column was developed using (a) 0.05 M NH_4HCO_3 (b) a gradient of 0.05 M to 1 M NH_4HCO_3 (of total volume 2Vt) and (c) 1 M NH_4HCO_3. Fractions (approx. 3 ml) were analysed by S2288 assay and by SDS PAGE followed by fibrin zymography (17). Fractions containing the t-PA species of M_r approx 56,000, were pooled to give 17 ml at 47,000 SU/ml and were lyophilised. Fractions that contained the t-PA species of M_r approx 65,000 (activity about 500,000 SU) were pooled, ultrafiltered, buffer-exchanged into 0.05 M NH_4HCO_3 and finally lyophilised.

Lyophilisates were usually reconstituted in 0.05 M sodium phosphate/0.1 M NaCl/0.01% Tween 80 pH 7.4 (phosphate/saline).

Synthesis of Acyl Plasminogen Activators

All t-PA species were reversibly blocked at the active centre serine of the molecule using AP-DAB. Specifically, the M_r = 38,000 species (5.5 nmoles) in phosphate/saline (2.0 ml) 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 phosphate/saline using Sephadex G25 and the product was stored at −70° C. The other t-PA 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 (16). Under these conditions the pseudo-first-order rate constant for all acyl activators was approx 5×10^{-5} sec^{-1}.

Isolation of A Chain of M_r = 56,000 t-PA

A lyophilisate of the M_r = 56,000 t-PA was reconstituted in H_2O to 2 mg/ml, diluted with an equal volume of 0.1 M Tris/0.15 M NaCl/20% (v/v) glycerol/0.01% Tween 80 pH 7.4, and treated with 10 mM dithiothreitol for 1.5 h at 4° C. The mildly reduced M_r = 56,000 t-PA was chromatographed on a column (id, 15 mm; h, 51 mm) of lysine Sepharose CL4B that had been equilibrated in phosphate/saline. The M_r = 56,000 t-PA A-chain was eluted using 0.1 M Tris/6 M Guanidine. HCl/2 mM EDTA pH 8.1. The isolated A-chain was then further reduced by treatment with 10 mM dithiothreitol for 30 min at 37° C and S-carboxymethylated using iodoacetamide (20 mM) for 2 h at 37° C in the dark, after readjusting the pH to 8.0. The S-carboxymethylated A-chain was buffer-exchanged into 0.05 M NH_4HCO_3 using Sephadex G25 and lyophilized. Amino acid analysis (14) indicated that the lyophilisate contained about 10 nmoles of A-chain.

Protein Sequencing

The N-terminal amino acid sequence of the plasminogen activators was determined by automated sequencing. The M_r = 38,000 species was sequenced by Sequal Ltd., Aberdeen, UK, the M_r = 56,000 species A chain by Applied Biosystems Ltd., Warrington, UK. Both utilized Applied Biosystems gas-phase sequencing systems.

Plasminogen Activator Assays

Essentially two types of activity assay were used in these studies; both are described elsewhere (17). Briefly the first uses the chromogenic substrate S2288 and activities are expressed either in substrate units (SU) or in arbitrary units if the rapid non-standardized microtitre plate-based assay is used. Approximate specific activities for single and two chain t-PA species are 50,000 and 200,000 SU/mg protein respectively. Variant t-PA species appear to be mainly two chain. The second assay uses plasminogen-containing fibrin plates. Activities are expressed in International Units (IU) by reference to the International Standard for t-PA, Lot 83/517.

Determination of Pharmacokinetic Properties of Acyl Plasminogen Activators

Male Dunkin Hartley guinea pigs (350–450 g) were anaesthetized with urethane (25% w/v solution; 6 ml/kg i.p.). One carotid artery was cannulated for collection of blood samples. One femoral vein was cannulated for injection of heparin (50 U/kg i.v.) and agent under test. Approximately 5 min after heparinization, a pre-dose blood sample was taken and mixed with 0.1 volumes 129 mM trisodium citrate. The compound under test was then injected (1 ml/kg) over 10 s. Further blood samples were taken exactly 2, 4, 8, 16, 30 and 60 min later. Heparin treatment (50 U/kg i.v.) was repeated after the 30 min sample to maintain cannula patency. All citrated blood samples were kept on ice until the end of experiment, then centrifuged at 1700 g for 15 min at 4° C to obtain plasma. The euglobulin fraction was precipitated by adding 0.1 ml of each plasma to 1.82 ml ice-cold 0.011% (v/v) acetic acid in water. After 30 min standing in ice, all tubes were centrifuged at 1700 g for 15 min at 4° C. The supernatants were poured away, the inner walls of each tube carefully wiped dry and each precipitate redissolved in 0.4 ml phosphate-buffered saline, pH 7.4, containing 0.01% (v/v) Tween 80. Aliquots (30 µl) were then applied to fibrin plates and incubated at 37° C for approx 18 h. Fibrinolytic activity was determined by reference to a calibration curve obtained by adding known amounts of the compound under test to the pre-dose plasma of each animal. These standards were processed using the same methods, including euglobulin precipitation, and at the same time as the experimental samples. To construct the calibration curve, diameters (mm) were plotted against log concentration of compound. The plasma concentration of compound in each experimental sample was expressed as a percentage of the initial concentration expected on the assumption of 45 ml plasma/kg body weight for each guinea pig.

Results

Preparations of t-PA purified from TRBM6 cells using zinc chelate and lysine Sepharose (14) contain mainly one species of t-PA (M_r = 63,000). Other species with apparent molecular weights under non-reducing conditions of M_r = 65,000, 56,000 and 38,000 could be detected, principally by SDS PAGE followed by fibrin zymography. Attempts to buffer-exchange purified t-PA into 0.05 M NH_4HCO_3 using Sephadex G25 were only partially successful as the majority of the t-PA was retarded. However subsequent analysis of the individual fractions from the column revealed that only the M_r = 63,000 species was retarded. The reason why just this single species is retarded is not known. We took advantage of this observation to obtain enriched preparations of the minority species.

Thus, the material eluting in the void volume from the Sephadex G25 column was suitably formulated and chromatographed on Sephadex G75 (Fig. 1). The difficulty in separating the different M_r species on the basis of A_{280} or amidolytic activity

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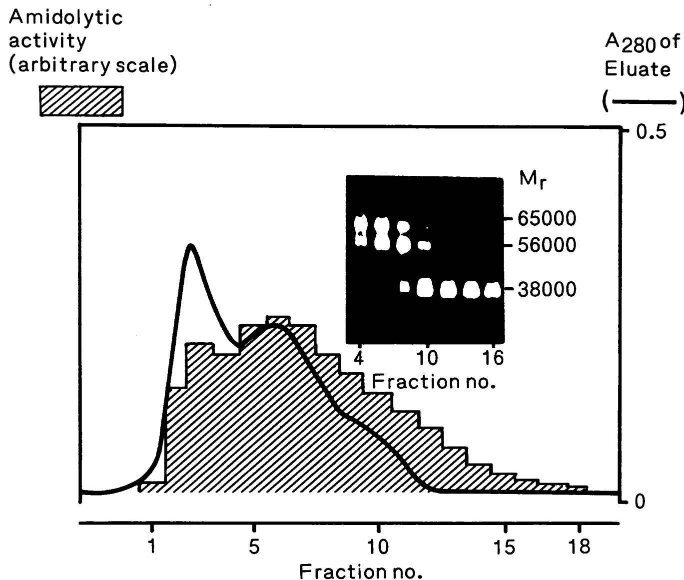

Fig. 1 Chromatography of minority t-PA species on Sephadex G75. Suitably formulated t-PA that had previously eluted in the void volume of a Sephadex G25 column was chromatographed on a column of Sephadex G75. The eluate from the column was continuously monitored at 280 nm. Fractions were analysed for S2288 activity and by SDS PAGE followed by fibrin zymography (inset). Full details are given in the Methods section

was resolved by analysing the fractions using fibrin zymography (Fig. 1 inset). This allowed the identification and subsequent preparation of first, highly purified M_r = 38,000 plasminogen activator and, second, an enriched mixture of the M_r = 56,000 and 65,000 species alone. Chromatography on Heparin Sepharose CL6B was used to separate the two higher M_r species. The M_r = 56,000 species eluted at a lower ionic strength than the M_r = 65,000 species (Fig. 2). Preparations of both species were subsequently formulated for further studies. Analysis by SDS PAGE

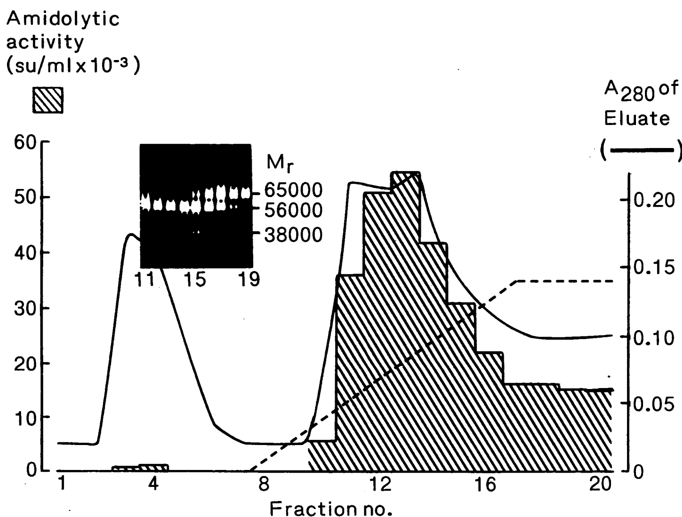

Fig. 2 Chromatography of apparent M_r = 56,000 and 65,000 t-PA species on Heparin Sepharose CL6B. An enriched preparation of the higher molecular weight t-PA species from Fig. 1 was chromatographed on Heparin Sepharose CL6B. The column was developed with a linear gradient from 0.05 to 1 M NH_4HCO_3 (---) and the individual fractions were analysed by S2288 assay and by SDS PAGE followed by fibrin zymography (inset). Fractions containing the two purified t-PA species were pooled separately. Full details are given in the Methods section

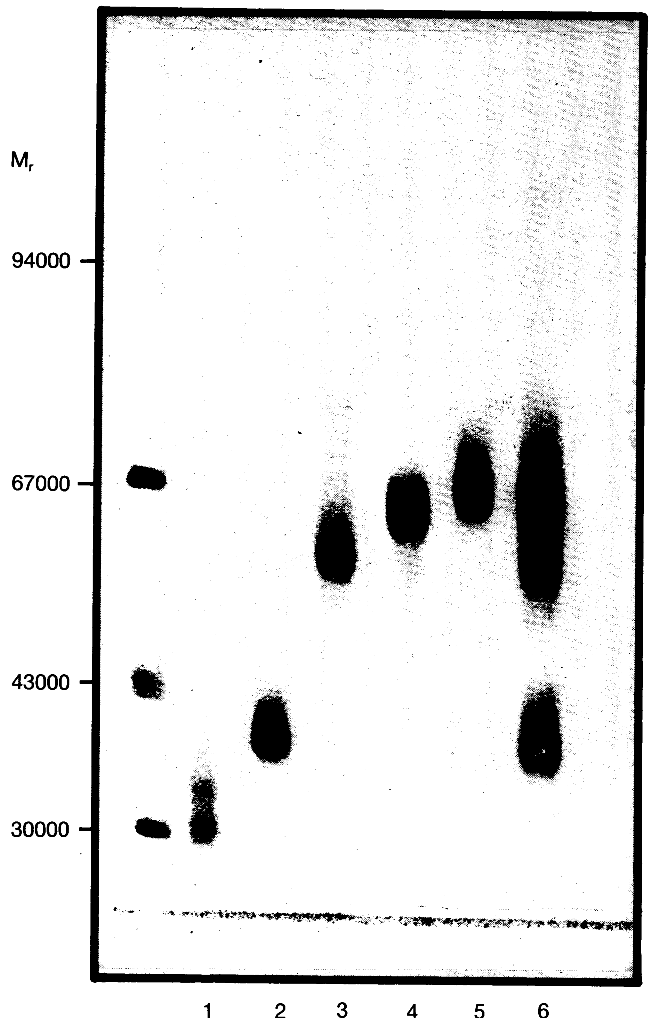

Fig. 3 SDS PAGE of purified t-PA species. Concentrated preparations of purified t-PA species were examined by SDS PAGE under non-reducing conditions followed by staining for protein using PAGE Blue 83. The individual lanes are 1. B chain, 2. M_r = 38,000, 3. M_r = 56,000, 4. M_r = 63,000, 5. M_r = 65,000 and 6. a mixture of lanes 2 to 5

under non-reducing conditions followed by staining for protein (Fig. 3) or fibrin zymography (Fig. 4) indicated that all three plasminogen activators were pure enough for further characterisation.

All three species were shown to comprise mainly two chain material by SDS PAGE under reducing conditions (results not shown). The apparent M_r = 65,000 species contained two major polypeptides both of $M_r \simeq$ 36,000. The apparent M_r = 56,000 species contained three major polypeptides, one at M_r = 36,000 – the presumed B-chain (17) – and a doublet at M_r = 31,000 and 33,000. The apparent M_r = 38,000 species contained two major polypeptides, one at M_r = 36,000 and another at M_r = 15,000.

The interaction of all species with antibodies raised against either native t-PA (principally the M_r = 63,000 species) or the B-chain of native t-PA (17) was examined. Fig. 5 shows that all species cross-reacted with both antibodies. The M_r = 65,000 species is likely to be the Type I variant of native t-PA (9). Specific identification of the two smaller M_r species was carried out by N-terminal sequencing studies. The M_r = 38,000 species was sequenced without separation of the two chains (Fig. 6). By accounting for the known N-terminal sequence of the t-PA B-chain (9) it was possible to identify the M_r = 38,000 species as alanine$_{160}$t-PA. It is likely that the isolated M_r = 38,000 species

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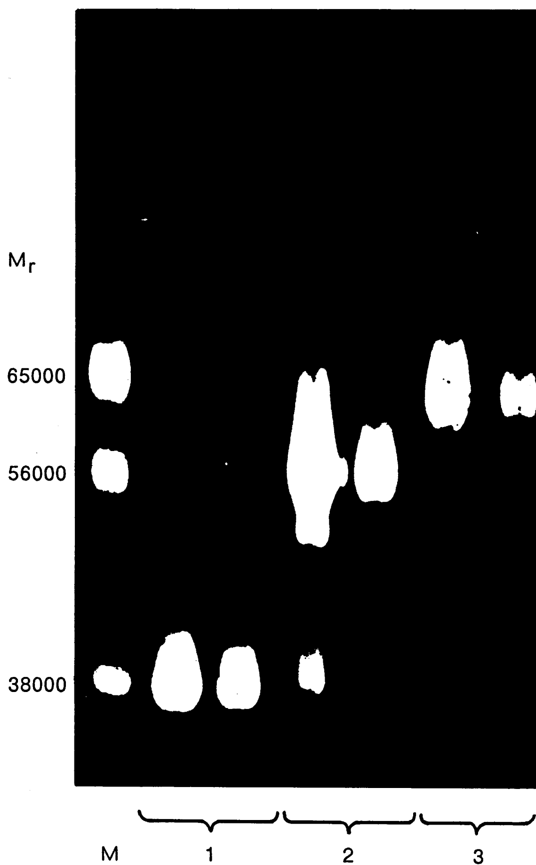

Fig. 4 SDS PAGE followed by fibrin zymography of purified t-PA species. The individual tracks are: M, markers of all three species, 1. M_r = 38,000 species, 2. M_r = 56,000 species and 3. M_r = 65,000 species. For lanes 1 to 3 the left-hand track contains 2 IU, the right-hand track contains 0.5 IU. The stained fibrin zymogram is shown

was the Type II equivalent variant, i.e. not glycosylated at asparagine 184 (10), because (i) evidence was obtained from other preparations for a species with apparent M_r = 41,000, (ii) the ratio of 38,000 to 41,000 species was the same as that of 63,000 and 65,000 (63,000≫65,000), and (iii) the 38,000 species showed a greater affinity than the 41,000 species for lysine Sepharose, similar to native 63,000 (Type II) and 65,000 (Type I) t-PA variants (10). The deduced molecular weight of alanine$_{160}$t-PA agreed with the sum of the M_r of the individual polypeptides determined by SDS PAGE under reducing conditions, i.e. 51,000. Determination of the N-terminus of the M_r = 56,000 A-

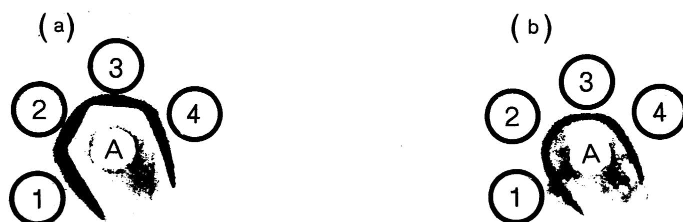

Fig. 5 Double immunodiffusion studies of purified t-PA species: cross-reaction with anti-t-PA IgG (a) or anti t-PA B-chain IgG (b) (ref. 17). Antibody (500 µg/ml, 15 µl) is in the centre well (A). The outer wells contain the antigen (50 µg/ml, 15 µl): 1,4, M_r = 38,000 species; 2, M_r = 56,000 species; 3, M_r = 65,000 species. The agarose gel stained for precipitin lines using PAGE Blue 83 is shown

Fig. 6 N-terminal sequence analysis of the M_r = 38,000 species. Reduced and carboxymethylated two chain M_r = 38,000 t-PA was sequenced as described in the Methods section. The four panels (A to D) show the HPLC analysis of the N-terminal PTH-amino acids determined in sequence: Panel A, residue 1; Panel B, residue 2, etc. For each panel the open arrow represents the amino acid attributable to the t-PA B-chain, the filled arrow that attributable to the A-chain moiety

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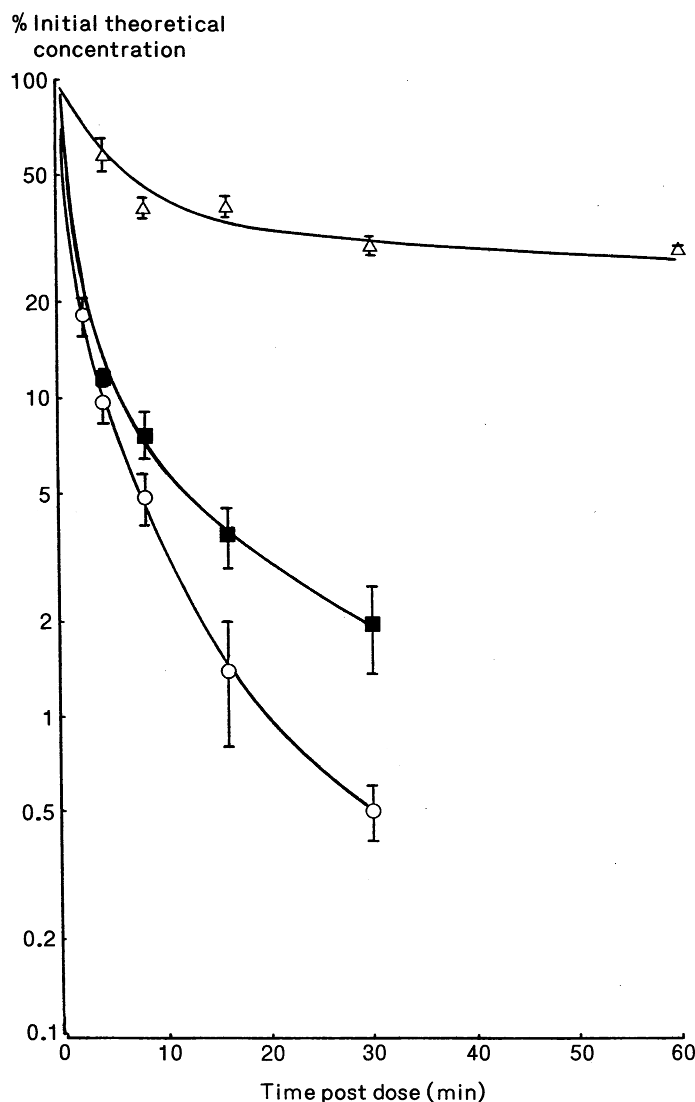

Fig. 7 Plasma disappearance profiles of acylated t-PA species in the guinea pig. Purified preparations of acylated t-PA were injected into a femoral vein of the guinea pig and samples of blood collected at intervals thereafterwards from a cannulated carotid artery. Euglobulin precipitates were prepared and were subsequently assayed for fibrinolytic activity. The t-PA species examined were: native t-PA, Type I, 2000 SU/kg (○), ΔF t-PA, 3500 SU/kg (■) and ΔFGKl t-PA, 3500 SU/kg (△). Full details are given in the Methods section

chain (Table 1) revealed that the species did not contain the fibronectin finger.

The three t-PA species were acylated for pharmacokinetic studies as described in Materials and Methods. The plasma disappearance results in the guinea pig are shown in Fig. 7. Both DAB → t-PA and DAB → ΔF t-PA were cleared rapidly, 80% or more being cleared within 2 to 3 min. The clearance of DAB → ΔFGKl tPA appeared to be much slower – less than 80% was cleared within the first 30 min. The two variants of native t-PA (Type I and Type II) were cleared at approximately the same rate (results not shown).

Discussion

We have purified and identified two species of tissue-type plasminogen activator from the recombinant Bowes melanoma cell line TRBM6. Low molecular weight forms of t-PA have been described before from mammalian cells but none have been directly purified and identified. We believed that the M_r = 56,000 species might be similar to enzymes described by other workers (11, 12). If this were the case it should lack the fibronectin finger – the domain in t-PA homologous with the Type I structure in fibronectin (9). This fibronectin structure appears to contain a heparin binding site (18); therefore we attempted to separate the M_r = 56,000 and 65,000 species using Heparin Sepharose. This was successful. Subsequent N-terminal sequencing of the M_r = 56,000 species showed that the material was almost certainly identical with the fibronectin finger-lacking t-PA species identified from cDNA prepared from Detroit 562 mRNA (12). It is assumed that X_{51} in the M_r = 56,000 species is C_{51} of native t-PA. This species probably arises from alternative splicing of the t-PA pre-mRNA (12, 21), The origin of ΔFGKl t-PA (ala$_{160}$t-PA) is more obscure. It possibly arises from a trypsin-like cleavage of the native molecule at residues lysine$_{159}$/alanine$_{160}$. However, if the postulated tertiary structure of t-PA (9) is correct the gross conformation of t-PA should be essentially maintained due to the presence of two disulphides (cys$_{144}$-cys$_{168}$ and cys$_{92}$-cys$_{173}$) linking the polypeptides either side of the cleavage site. Unlike ΔF t-PA the origin of ala$_{160}$t-PA cannot be explained by alternative splicing of the pre-mRNA since no exon-intron junctions have been identified at the putative cleavage site (21). Thus the derivation of ala$_{160}$t-PA remains unknown. Both species of t-PA occurred in the harvest medium of TRBM6 and the original Bowes melanoma cell-line to approximately the same levels (results not shown). The amounts were not determined but were in the region of 5–20% of total plasminogen activator activity.

Although it is impossible to predict what effect removal of parts of the A-chain have on the overall conformation of the remainder of the molecule the double immunodiffusion studies carried out suggest it is minimal. This conclusion is supported by the observation that ala$_{160}$t-PA, in contrast to free t-PA B-chain, was adsorbed by an immobilised monoclonal antibody (ESP5; ref 22) directed at kringle 2 (results not shown). Thus ala$_{160}$t-PA is referred to as ΔFGKl t-PA as the only structural domains it contains are kringle 2 and the B-chain. Mutant, DNA-derived t-PA molecules lacking the F, G and Kl domains have been described (23, 24) but pharmacokinetic studies were not carried out.

The plasma disappearance profile of reversibly inactivated (acylated) forms of the various t-PA species were determined in the guinea pig. It is known that the activity of t-PA is lost in the bloodstream because of removal by the liver rather than inactivation by plasma inhibitors (19). However, inhibition could play a major role in the neutralization of plasminogen activators that are not susceptible to fast hepatic clearance mechanisms. In order to avoid such inhibition obscuring the pharmacokinetic behaviour of the activators, each was first reversibly inactivated by acylation of the active site (20). The acyl group selected (DAB) 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 the plasma samples. The possibility of artificially high apparent levels of plasminogen activator activity due to contamination of the plasma euglobulins by heparin is negated by the use of a fibrin plate assay. Fig. 7 shows that ΔFGKl t-PA had lost a major plasma disappearance recognition site whereas ΔF t-PA had not. It seems likely that the retarded clearance is not due to a conformational change elsewhere on the molecule as a result of removal of the FGKl region, because antibody cross-reactivity was not altered. Therefore the site(s) responsible for the rapid liver clearance probably resides in the G or Kl domains. The biphasic nature of the clearance might

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indicate that another, as yet unidentified, recognition site resides elsewhere on the molecule.

We have shown that species of t-PA identified in purified preparations from the recombinant cell line TRBM6 could be purified to near homogeneity and employed to analyse the region(s) of the molecule responsible for its rapid clearance. Further localisation of the clearance recognition site can be obtained probably only by the use of recombinant DNA techniques to dissect the molecule more specifically. The information provided here should facilitate this work and should lead to the more rapid development of a second generation t-PA molecule.

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References

1 Van de Werf F, Ludbrook P A, Bergmann S R, Tiefenbrunn A J, Fox K A A, De Geest H, Verstraete M, Collen D, Sobel B E. Coronary thrombolysis with tissue-typ plasminogen activator in patients with evolving myocardial infarction. N Engl J Med 1984; 310: 609-13.
2 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, Schartl 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.
3 Collen D, Topol E J, Tiefenbrunn A J, Gold H K, Weisfeldt M L, Sobel B E, Leinbach R C, Brinker J A, Ludbrook P A, Yasuda T, Bulkley B H, Robison A K, Hutter A M, Bell W R, Spadaro J J, Khaw B A, Grossbard E B. Coronary thrombolysis with recombinant human tissue-type plasminogen activator: a prospective, randomized, placebo-controlled trial. Circulation 1984; 70: 1012-7.
4 Klausner A. Researchers probe second-generation t-PA. Bio/Technology 1986; 4: 706-11.
5 Ezzell C. US patents in the balance. Nature 1987; 328: 189.
6 Nilsson S, Wallen P, Mellbring G. In vivo metabolism of human tissue-type plasminogen activator. Scand J Haematol 1984; 33: 49-53.
7 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.
8 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.
9 Pennica D, Holmes W E, Kohr W J, Harkins R N, Vehar G A, Ward C A, Bennett W F, Yelverton E, Seeburg P H, Heyneker H L, Goeddel D V, Collen D. Cloning and expression of human tissue-type plasminogen activator cDNA in E. coli. Nature 1983; 301: 214-21.
10 Pohl G, Kallstrom M, Bergsdorf N, Wallen P, Jornvall H. Tissue plasminogen activator: Peptide analyses confirm an indirectly derived amino acid sequence, identify the active site serine residue, establish glycosylation sites, and localize variant differences. Biochemistry 1984; 23: 3701-7.
11 Banyai L, Varadi A, Patthy L. Common evolutionary origin of the fibring-binding structures of fibronectin and tissue-type plasminogen activator. FEBS Lett 1983; 163: 37-41.
12 Kagitani H, Tagawa M, Hatanaka K, Ikari T, Saito A, Bando H, Okada K, Matsuo O. Expression in E. coli of finger-domain lacking tissue-type plasminogen activator with high fibrin affinity. FEBS Lett 1985; 189: 145-9.
13 Gilbert L C, Wachsmann J T. Characterisation and partial purification of the plasminogen activator from human neuroblastoma cell line, SK-N-SH. Biochim Biophys Acta 1982; 704: 450-60.
14 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.
15 Browne M J, Dodd I, Carey J E, Chapman C G, Robinson J H. Increased yield of human tissue-type plasminogen activator by means of recombinant DNA technology. Thromb Haemostas 1985; 54: 422-4.
16 Smith R A G. An active-site titrant for human tissue-type plasminogen activator. Biochem J 1986; 239: 477-9.
17 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.
18 Petersen T E, Thogersen H C, Skorstengaard K, Vibe-Pedersen K, Sahl P, Sottrup-Jensen L, Magnusson S. Partial primary structure of bovine plasma fibronectin: Three types of internal homology. Proc Natl Acad Sci 1983; 50: 137-41.
19 Korninger C, Stassen J M, Collen D. Turnover of human extrinsic (tissue-type) plasminogen activator in rabbits. Thromb Haemostas 1981; 46: 658-61.
20 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.
21 Ny T, Elgh F, Lund B. The structure of the human tissue-type plasminogen activator gene: Correlation of intron and exon structures to functional and structural domains. Proc Natl Acad Sci USA 1984; 81: 5355-9.
22 MacGregor I R, Micklem L R, James K, Pepper D S. Characterisation of epitopes on human tissue plasminogen activator recognized by a group of monoclonal antibodies. Thromb Haemostas 1985; 53: 45-50.
23 Verheijen J H, Caspers M P M, Chang G T G, de Munk G A W, Pouwels P H, Enger-Valk B E. Involvement of finger domain and kringle 2 domain of tissue-type plasminogen activator in fibrin binding and stimulation of activity by fibrin. Embo J 1986; 5: 3525-30.
24 Van Zonneveld A-J, Veerman H, Pannekoek H. Autonomous functions of structural domains on human tissue-type plasminogen activator. Proc Natl Acad Sci USA 1986; 83: 4670-4.

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