Clearance of the heavy and light polypeptide chains of human tissue-type plasminogen activator in rats

Dingeman C. RIJKEN and Jef J. EMEIS

TNO Gaubius Institute for Cardiovascular Research, Herenstraat 5d, 2313 AD Leiden, The Netherlands

In order to assess which part of the tissue-type plasminogen activator (t-PA) molecule should be (genetically) modified to obtain more-slowly-clearing mutants, two-chain t-PA and its isolated heavy and light chains were radiolabelled and injected into rats. The vast majority of t-PA and the heavy chain disappeared from the blood circulation with half-lives of 2.3 and 1.0 min respectively. The clearance of the light chain was biphasic, owing to complex-formation with plasma proteinase inhibitors. The disappearance of di-isopropylphospho-light chain, which has a blocked active site, was nearly monophasic, with a half-life of 5.7 min. Organ distribution studies showed that hepatic clearance constituted the major pathway in all cases. These results strongly suggest that t-PA is recognized by the liver primarily through the heavy chain.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) catalyses the conversion of plasminogen into plasmin and plays a central role in the extrinsic fibrinolytic system (Bachmann & Kruihof, 1984; Emeis *et al.*, 1985a). It can be obtained from tissue extracts and, in larger quantities, from the culture medium of a melanoma cell line (Rijken & Collen, 1981) or by recombinant DNA technology (Pennica *et al.*, 1983). t-PA is a single-chain molecule (M_r 70000) and can be proteolytically converted into a two-chain form consisting of a heavy chain (M_r 38000) and a disulphide-linked light chain (M_r 31000), which contains the active site (Rijken *et al.*, 1979; Wallén *et al.*, 1983). Because of its fibrin-directed action, t-PA has recently been used as a specific thrombolytic agent (Matsuo *et al.*, 1981; Collen & Lijnen, 1984). A disadvantage of t-PA in thrombolytic therapy is that the protein is rapidly removed (half-life a few minutes) from the blood circulation by hepatic clearance (Korninger *et al.*, 1981; Nilsson *et al.*, 1984; Fuchs *et al.*, 1985; Nilsson *et al.*, 1985; Verstraete *et al.*, 1985; Emeis *et al.*, 1985b). Continuous infusions and relatively high amounts of t-PA are therefore required to achieve sufficiently high blood levels. It is anticipated that genetic mutants of t-PA, which are less rapidly cleared by the liver, will be more promising than normal t-PA. Specific mutagenesis is, however, still difficult, since it is not known which parts of the t-PA molecule are recognized by the liver. The carbohydrate part is probably not involved (Emeis *et al.*, 1985b), neither is the active site of the molecule (Korninger *et al.*, 1981; Fuchs *et al.*, 1985). The clearances of single-chain and two-chain t-PA are identical (Korninger *et al.*, 1981). Recently we isolated the heavy and light chains of human t-PA in a functionally active form (Rijken & Groeneveld, 1986). In the present study we localized its liver binding site(s) by measuring the liver clearance rates of the isolated chains in rats. The rat was chosen as a model since human t-PA and rat t-PA behave similarly in rats (Emeis *et al.*, 1985b). Results have been presented at the Tenth

International Congress on Thrombosis and Haemostasis held in San Diego in 1985 (Rijken *et al.*, 1985).

MATERIALS AND METHODS

DFP was purchased from Aldrich, Brussels, Belgium; Sephadex G-25, Sephacryl S-200 and S-300 were from Pharmacia, Uppsala, Sweden; heparin was from Leo, Ballerup, Denmark; Nembutal was from Sanofi, Paris, France; Na^{125}I (14.4 mCi/ μg of iodine) was from Amersham International, Amersham, Bucks., U.K., and Iodogen was from Pierce, Rockford, IL, U.S.A.

Human two-chain t-PA (variant I) was purified from Bowes melanoma cells, and its heavy and light chains were isolated in functionally active forms after reduction and reoxidation at low protein concentration, as described by Rijken & Groeneveld (1986). The proteins were radiolabelled with ^{125}I by using the Iodogen method (Fraker & Speck, 1978), centrifuged through a 2 ml Sephadex G-25 (coarse grade) column in 50 mM-Tris/HCl buffer, pH 8.0, containing 1 M-NaCl and 0.01% Tween 80, to remove excess free ^{125}I , and finally gel-filtered on a Sephacryl S-300 (t-PA) or S-200 (heavy and light chain) column (1.5 cm $\times$ 40 cm) in the aforementioned buffer to remove aggregated protein. The specific radioactivities were 8×10^6 c.p.m./ μg of t-PA and 1.2×10^6 c.p.m./ μg of light or heavy chain. DIP derivatives of ^{125}I -t-PA and ^{125}I -light chain were obtained after two successive incubations of the enzymes with 1 mM-DFP for 1 h at room temperature, followed by overnight dialysis against the Tris/HCl buffer.

Blood disappearance studies

Male Wistar rats (TNO Central Institute for the Breeding of Laboratory Animals, Zeist, The Netherlands) of 200–250 g were anaesthetized with pentobarbital (Nembutal; 6 mg/100 g intraperitoneally) and heparinized (12.5 units/100 g). ^{125}I -labelled forms of t-PA, DIP-t-PA (both 100 ng), heavy chain (200 ng), light chain and DIP-light chain (both 350 ng) were rapidly

injected through the vein of the penis. Serial blood samples of 150–300 μ l were drawn via a cannula in the carotid artery between 1 and 60 min after injection, mixed with citrate and counted for radioactivity. The trichloroacetic acid-precipitable radioactivities were determined by mixing plasma samples with 9 vol. of 11% (w/v) trichloroacetic acid and by counting the resulting precipitates. Blood radioactivity at zero time was calculated by dividing total injected radioactivity by the blood volume (7.3 ml/100 g, as determined in independent rats by using radiolabelled, biologically screened, fibrinogen). Disappearance curves were constructed (Figs. 1a–1c below) and manually resolved into two exponential components by the method of residuals (Gibaldi & Perrier, 1982) (results not shown). Each experiment was performed four times.

Organ distribution studies

Rats were treated as described above. At 10 min after injection of the radiolabelled proteins the rats were killed and the major body organs were partially or fully recovered and counted for radioactivity. For some organs (skin, muscle and adipose tissue) the total weight was taken from the literature (Munniksmas *et al.*, 1980).

Gel-filtration studies

Plasma samples containing radiolabelled light-chain derivatives (approx. 10^4 c.p.m.) were gel-filtered on a Sephacryl S-300 column (83 cm $\times$ 1.5 cm) in 50 mM-Tris/HCl, pH 8.0, containing 1 M-NaCl, 10 mM-citrate and 0.01% Tween 80. Fractions (2 ml) were collected at a flow rate of 8 ml/h. Plasma preparation and gel filtration were performed at 0–4 $^{\circ}$ C.

RESULTS AND DISCUSSION

Radiolabelled t-PA, its heavy chain or its light chain, were intravenously injected into rats, and disappearance curves were constructed from trichloroacetic acid-precipitable blood radioactivities measured during 1 h after injection. Fig. 1(a) shows the disappearance curve of 125 I-t-PA. The majority disappeared rapidly, with a half-life of 2.3 ± 0.2 min (mean $\pm$ S.D., $n = 4$), which is in close agreement with the literature (Korninger *et al.*,

1981; Nilsson *et al.*, 1984; Emeis *et al.*, 1985b; Fuchs *et al.*, 1985; Nilsson *et al.*, 1985; Verstraete *et al.*, 1985). A minor proportion of the 125 I-t-PA (approx. 6%) disappeared more slowly, with a half-life of about 30 min. This second phase was also observed with DIP- 125 I-t-PA (not shown), which cannot form covalent complexes with proteinase inhibitors. The presence of 6% denatured t-PA in the 125 I-t-PA preparation, which may explain the second phase, cannot be excluded. Similar disappearance curves were found in clearance studies of t-PA in human beings by Verstraete *et al.* (1985). They described the biphasic curves by a two-compartment mammillary model with peripheral (liver) elimination. Total blood radioactivity increased again after 15 min (Fig. 1a) as a result of the rapid appearance in blood of trichloroacetic acid-soluble degradation products.

The disappearance curve of 125 I-heavy chain of t-PA was similar to that of the parent molecule (Fig. 1b). The half-life of the initial phase was 1.0 ± 0.1 min (mean $\pm$ S.D., $n = 4$).

The clearance of 125 I-light chains of t-PA (Fig. 1c) was clearly different from that of intact t-PA: the half-life of the initial phase was approx. 5 min and a much greater fraction of the total blood radioactivity disappeared in the second phase (approx. 30%). To check if the light chain had remained in its original form, plasma samples were gel-filtered on Sephacryl S-300. Control experiments (Fig. 2a) showed that DIP- 125 I-light chain, incubated in citrated rat plasma for 10 min at 37 $^{\circ}$ C, was eluted in a single radioactivity peak after the main protein peak (albumin) of rat plasma, which accords with M_r of approx. 31 000. 125 I-labelled components in blood taken 10 min after injection were eluted in several radioactivity peaks with M_r values above 31 000 (Fig. 2b; peaks at fraction nos. 29, 34, 38 and 43). Only a small fraction, visible as a shoulder at fraction 46, was eluted in the original position (this shoulder was not present in blood taken 60 min after injection; results not shown). These experiments strongly suggested that the light chain *in vivo* rapidly formed complexes with plasma proteinase inhibitors and that these complexes were relatively slowly cleared.

In order to study the half-life of 125 I-light chain

Fig. 1. Blood disappearance curves of 125 I-t-PA (a), 125 I-heavy chain (b), 125 I-light chain and 125 I-DIP-light chain (c) after intravenous injection into rats (typical experiment)

Both total blood radioactivity ($\blacktriangle$) and trichloroacetic acid-precipitable radioactivity ($\bullet$) are shown to indicate the appearance of trichloroacetic acid soluble degradation products.

Fig. 2. Gel-filtration studies on Sephacryl S-300

(a) Control experiment with ¹²⁵I-DIP-light chain (10⁴ c.p.m.) incubated for 10 min at 37 °C in 275 µl of citrated rat plasma. (b) A 1 ml portion of citrated rat plasma from blood taken 10 min after injection of ¹²⁵I-light chain. The pattern of the plasma proteins in (a) shows the separation capacity of the column.

without interference by complex-formation with plasma proteinase inhibitors, its DIP derivative was injected. Fig. 1(c) shows that the main portion of DIP-¹²⁵I-light chain disappeared with a half-life of 5.7 ± 1.1 min (mean $\pm$ S.D., $n = 4$).

Table 1 summarizes the organ distribution of radioactivity 10 min after injection of the radiolabelled proteins. The radiolabel of ¹²⁵I-t-PA, eliminated from blood, was found mainly (86.4%) back in the liver, a finding that is in agreement with those of other studies (Korninger *et al.*, 1981; T. Nilsson *et al.*, 1984; Fuchs *et al.*, 1985; S. Nilsson *et al.*, 1985). The radiolabels of the heavy and light chains were also recovered mainly in the liver (61.0 and 52.4% respectively), indicating that

hepatic clearance was involved. The latter values were somewhat lower than that for ¹²⁵I-t-PA, probably because the chains with M_r values (38000 and 31000, respectively) lower than that of intact t-PA (70000), disappeared from the circulation by renal clearance and extravascular distribution in addition to liver clearance (see Table 1).

The results indicate that the heavy chain of t-PA ($t_{1/2} = 1.0$ min) is cleared almost six times faster than the DIP-light chain ($t_{1/2} = 5.7$ min). This strongly suggests that intact t-PA is recognized by the liver primarily through the heavy chain and much less through the light chain. This concept is supported by our recent finding that complexes of t-PA with plasma proteinase inhibitors prepared *in vitro* were cleared as rapidly as free t-PA (Rijken & Emeis, 1986), whereas complexes of the light chain with proteinase inhibitors were cleared apparently much more slowly than was the free light chain (the present study). In other words, if the light chain is involved significantly in the clearance of t-PA, then t-PA proteinase inhibitor complexes would be expected to disappear from the circulation less rapidly than free t-PA.

This study suggests that the heavy chain (kringles, growth factor or fingerdomain) should be modified to obtain more-slowly-clearing mutants. One may speculate that mutants with a heavy chain that is not recognized by the liver will be cleared through the light chain with a significantly lengthened half-life.

We thank Dr. T. Kooistra for helpful discussions. This work was supported by the 'Praeventiefonds' (Grant no. 28-813).

REFERENCES

- Bachmann, F. & Kruithof, E. K. O. (1984) *Semin. Thromb. Haemostasis* **10**, 6-17
- Collen, D. & Lijnen, H. R. (1984) *Arteriosclerosis* **4**, 579-585
- Emeis, J. J., Brommer, E. J. P., Kluft, C. & Brakman, P. (1985a) in *Recent Advances in Blood Coagulation* (Poller, L., ed.), vol. 4, pp. 11-33, Churchill-Livingstone, Edinburgh, London, Melbourne and New York
- Emeis, J. J., Van den Hoogen, C. M. & Jense, D. (1985b) *Thromb. Haemostasis* **54**, 661-664
- Fraker, P. J. & Speck, J. C. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849-857

Table 1. Organ distribution of radioactivity 10 min after injection of ¹²⁵I-labelled t-PA, heavy chain and light chain

The values represent means for two separate experiments. The total recovery (including the blood radioactivity) was 106 ± 12 (mean $\pm$ S.D.) % of the injected dose.

Organ	Percentage of recovered dose		
	t-PA	Heavy chain	Light chain
Liver	86.4	61.0	52.4
Kidney	1.0	5.1	15.4
Bladder + urine	< 0.1	2.5	0.6
Spleen	1.6	1.1	1.0
Heart	0.1	0.2	0.3
Lung	0.3	0.4	1.0
Stomach + intestines	2.2	14.0	5.1
Skin	2.0	5.9	9.7
Muscle	5.2	8.4	12.8
Adipose tissue	1.1	1.4	1.8

- Fuchs, H. E., Berger, H. & Pizzo, S. V. (1985) *Blood* **65**, 539–544
- Gibaldi, M. & Perrier, D. (1982) *Pharmacokinetics*, 2nd edn., pp. 433, Marcel Dekker, New York
- Korninger, C., Stassen, J. M. & Collen, D. (1981) *Thromb. Haemostasis* **46**, 658–661
- Matsuo, O., Rijken, D. C. & Collen, D. (1981) *Nature (London)* **291**, 590–591
- Munniksmas, J., Noteborn, M., Kooistra, T., Stienstra, S., Bouma, J. M. W., Gruber, M., Brouwer, A., Praaning-van Dalen, D. & Knook, D. L. (1980) *Biochem. J.* **192**, 613–621
- Nilsson, S., Einarsson, M., Ekvärn, S., Häggroth, L. & Mattsson, Ch. (1985) *Thromb. Res.* **39**, 511–521
- Nilsson, T., Wallén, P. & Mellbring, G. (1984) *Scand. J. Haematol.* **33**, 49–53
- 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. (1983) *Nature (London)* **301**, 214–221
- Rijken, D. C. & Collen, D. (1981) *J. Biol. Chem.* **256**, 7035–7041
- Rijken, D. C. & Emeis, J. J. (1986) *Eur. J. Clin. Invest.* **16**, A18 (abstr.)
- Rijken, D. C. & Groeneveld, E. (1986) *J. Biol. Chem.* **261**, 3098–3102
- Rijken, D. C., Wijngaards, G., Zaal-de Jong, M. & Welbergen, J. (1979) *Biochim. Biophys. Acta* **580**, 140–153
- Rijken, D. C. Emeis, J. J. & Groeneveld, E. (1985) *Thromb. Haemostasis* **54**, 61 (abstr. 0358)
- Verstraete, M., Bounameaux, H., De Cock, F., Van de Werf, F. & Collen, D. (1985) *J. Pharmacol. Exp. Ther.* **235**, 506–512
- Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M., Ny, T. & Jörnvall, H. (1983) *Eur. J. Biochem.* **132**, 681–686

Received 10 March 1986/8 May 1986; accepted 13 May 1986