Pharmacokinetics and Thrombolytic Properties of Deletion Mutants of Human Tissue-Type Plasminogen Activator in Rabbits

By Désiré Collen, Jean-Marie Stassen, and Glenn Larsen

The following mutants of human tissue-type plasminogen activator (t-PA) were constructed by deletion mutagenesis of t-PA cDNA, expressed in Chinese hamster ovary cells and purified to homogeneity: (a) t-PA−ΔFE:t-PA lacking both the fibronectin fingerlike (F) domain and the epidermal growth factor (E) domain, (b) t-PA−ΔFE1X:t-PA−ΔFE with the glycosylated 117Asn mutated to Gin, and (c) t-PA−ΔFE3X:t-PA−ΔFE with the three known glycosylated Asn residues replaced by Gin. The mutant and natural t-PA (Mel−t-PA obtained from melanoma cell culture) were infused intravenously for four hours into rabbits with jugular vein thrombosis at doses ranging between 0.12 and 0.75 mg/kg. Fifty percent thrombolysis, determined by intercalation, was obtained with 0.4 mg/kg Mel−t-PA, 0.37 mg/kg t-PA−ΔFE, 0.2 mg/kg t-PA−ΔFE1X, and 0.40 mg/kg t-PA−ΔFE3X. These infusion rates resulted in plateau levels of t-PA antigen in plasma of 0.055, 2.1, 0.6, and 0.5 µg/mL, respectively. At 50% lysis, the residual fibrinogen 30 minutes after the end of the infusion was 100%, 81%, 100% and 85% of baseline, and the residual α2-antiplasmin was 82%, 55%, 85%, and 90%, respectively. These results indicate that t-PA−ΔFE1X and t-PA−ΔFE3X have a specific thrombolytic activity and fibrin specificity comparable to that of Mel−t-PA. t-PA−ΔFE has a comparable specific thrombolytic activity but a lower fibrin specificity than Mel−t-PA. After the end of the infusion, t-PA-related antigen disappeared from plasma with an initial t½ of four minutes for Mel−t-PA, 25 minutes for t-PA−ΔFE, 42 minutes for t-PA−ΔFE1X, and 14 minutes for t-PA−ΔFE3X. It is concluded that t-PA can be modified by deletion mutagenesis to yield variants with a markedly longer half-life in the blood. Some of these variants have a specific thrombolytic activity and fibrin specificity similar to that of natural t-PA. These variants may be useful to identify the structures in t-PA responsible for its clearance, specific thrombolytic activity, and fibrin specificity in vivo.

HUMAN TISSUE-TYPE PLASMINOGEN activator (t-PA), produced by recombinant DNA technology (rt-PA) has been extensively investigated as a thrombolytic agent in patients with thromboembolic disease.1 Pharmacokinetically, t-PA is characterized by rapid clearance by the liver, which results in an initial half-life (t½) of only a few minutes.2 Therefore, to maintain a therapeutic plasma level, a continuous infusion of t-PA is required. The structures in t-PA responsible for this rapid clearance have not yet been defined. Evidence has been presented that carbohydrate side chains do not play a major role.3

Larsen et al have recently constructed a number of deletion mutants of t-PA with a much slower disposition rate in rats as compared with wild-type t-PA.4 In the present study we have determined, in a rabbit jugular vein thrombosis model,3 the thrombolytic properties and the plasma clearance of these deletion mutants of t-PA in an effort to correlate their disposition rates with their specific thrombolytic properties and with their fibrin specificity. The following deletion mutants were studied: t-PA lacking the fibronectinlike (F) domain and the epidermal growth factor (E) domain (t-PA−ΔFE), t-PA−ΔFE lacking the 117Asn glycosylation site (t-PA−ΔFE1X), and t-PA−ΔFE lacking the three known Asn-based glycosylation sites (t-PA−ΔFE3X).

MATERIALS AND METHODS

t-PA and t-PA mutants. Natural t-PA (Mel−t-PA) was purified from the conditioned medium of a human melanoma cell line.6,7 The recombinant deletion mutants lacking Cys-6 to Ile-86 were produced by Genetics Institute, Inc, Boston. They were constructed by deletion mutagenesis, were expressed and amplified in Chinese hamster ovary cells, and were purified from conditioned medium by affinity chromatography using erythrina trypsin inhibitor-agarose as previously described.8

Human fibrinogen was prepared according to Blomback and Blomback9 and labeled with 125I with the iodogen method.10 Human thrombin was purified as described by Fenton et al.11

Experimental procedure. The rabbit jugular vein thrombosis model is described in detail elsewhere.3 The present study was carried out with fresh thrombi (aged for 30 minutes). The infusions were given systemically through a marginal ear vein contralateral to the jugular vein thrombus over a time period of four hours after 10% of the thrombus over a time period of four hours after 10% of the thrombus was given as a bolus. The extent of thrombolysis was determined 30 minutes after the end of the infusion. In all other aspects the experiments were carried out exactly as previously described.

Blood samples of 4 mL were drawn into trisodium citrate (final concentration, 0.011 mol/L) every two hours and 30 minutes after the end of the infusion, and the plasma was used to measure radioactivity, fibrinogen,12 α2-antiplasmin,13 and t-PA-related antigen.14 In addition, blood samples were collected 1, 2, 5, 10, 15, 20, and 30 minutes after the end of the infusion to determine the disposition rate of t-PA-related antigen from plasma.14

Analysis of the data. The values reported in Table 1 and the figures represent mean values ± SEM; n represents the number of experiments in each group.

Laboratory procedures. Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis on 12% slab gels was performed as described by Laemmli.15 The concentration of the mutant proteins was determined with an enzyme-linked immunosorbent assay (ELISA)16 by calibration against the international reference preparation of t-PA (83/517) obtained from the National Institute for Biological Standards and Control, London, which was assumed to...
Table 1. Thrombolysis, Hemostasis Parameters, and Initial Disposition Rate of t-PA Deletion Mutants

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>Thrombolysis (%) (Percentage of Control)</th>
<th>α2-AP (Percentage of Control)</th>
<th>t-PA Ag (ng/mL)</th>
<th>t1/2 (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>6</td>
<td>8 ± 1</td>
<td>98 ± 3</td>
<td>90 ± 4</td>
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<tr>
<td>Mel-t-PA</td>
<td>0.13</td>
<td>4</td>
<td>24 ± 2</td>
<td>105 ± 5</td>
<td>91 ± 5</td>
<td>25 ± 6</td>
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<tr>
<td></td>
<td>0.25</td>
<td>6</td>
<td>32 ± 1</td>
<td>93 ± 1</td>
<td>85 ± 2</td>
<td>29 ± 6</td>
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<tr>
<td></td>
<td>0.50</td>
<td>9</td>
<td>55 ± 2</td>
<td>99 ± 3</td>
<td>87 ± 3</td>
<td>79 ± 6</td>
</tr>
<tr>
<td>t-PA−ΔFE</td>
<td>0.13</td>
<td>3</td>
<td>18 ± 1</td>
<td>95 ± 3</td>
<td>93 ± 6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>3</td>
<td>34 ± 5</td>
<td>92 ± 6</td>
<td>78 ± 7</td>
<td>850 ± 230</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>3</td>
<td>63 ± 10</td>
<td>69 ± 9</td>
<td>32 ± 12</td>
<td>3,400 ± 750</td>
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<tr>
<td>t-PA−ΔFE1X</td>
<td>0.13</td>
<td>3</td>
<td>22 ± 4</td>
<td>93 ± 10</td>
<td>90 ± 2</td>
<td>150 ± 15</td>
</tr>
<tr>
<td></td>
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<td>3</td>
<td>59 ± 8</td>
<td>98 ± 3</td>
<td>83 ± 11</td>
<td>780 ± 100</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
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<td>83 ± 8</td>
<td>89 ± 15</td>
<td>73 ± 10</td>
<td>1,300 ± 240</td>
</tr>
<tr>
<td>t-PA−ΔFE3X</td>
<td>0.19</td>
<td>3</td>
<td>30 ± 2</td>
<td>86 ± 5</td>
<td>86 ± 7</td>
<td>20 ± 30</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
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<td>87 ± 4</td>
<td>94 ± 3</td>
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<tr>
<td></td>
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<td>69 ± 5</td>
<td>73 ± 1</td>
<td>71 ± 13</td>
<td>1,400 ± 110</td>
</tr>
</tbody>
</table>

Abbreviation: α2-AP, α2-antiplasmin; Fg, fibrinogen.

RESULTS

Partial characterization of the mutants. On SDS–gel electrophoresis after reduction with dithiothreitol, the t-PA mutants as well as natural t-PA migrated essentially as one main doublet band, except for t-PA−ΔFE3X, which is totally unglycosylated (Fig 1). The molecular weights (mol wts) contain 2 μg t-PA per vial. Fibrinolytic activity was measured on bovine fibrin plates and expressed in international units by comparison with the reference preparation.

Binding to purified fibrin. Human fibrinogen (final concentration, 0 to 3.3 mg/mL) in 0.038 mol/L NaCl and 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 1 mg/mL bovine serum albumin was mixed with the t-PA variants to a final concentration of 50 to 100 ng/mL. The mixture was clotted by the addition of thrombin to a final concentration of 20 National Institutes of Health U/mL. After one-minute incubation, the samples were centrifuged for one minute at 10,000 g. The concentrations of t-PA–related antigen in the supernatants were determined with an ELISA.

Fig 1. Dodecyl sulfate polyacrylamide gel electrophoresis of t-PA mutants after reduction on 12% gels. (1) Protein in calibration mixture (phosphorylase b, 94,000 mol wt; bovine serum albumin, 67,000 mol wt; ovalbumin, 43,000 mol wt; carbonic anhydrase, 30,000 mol wt; soybean trypsin inhibitor, 20,000 mol wt. (2) t-PA–ΔFE. (3) Mel-t-PA. (4) t-PA–ΔFE1X. (5) t-PA–ΔFE3X.

Fig 2. Binding of the t-PA mutants to a fibrin clot. O, Mel-t-PA; □, t-PA–ΔFE; ■, t-PA–ΔFE1X; ●, t-PA–ΔFE3X.
ranged between 50,000 and 70,000, as anticipated from the structures of the molecules. The specific activities on fibrin plates were 640,000 IU/mg for wild-type t-PA, 160,000 IU/mg for t-PA–ΔFE, 440,000 IU/mg for t-PA–ΔFE1X, and 470,000 IU/mg for t-PA–ΔFE3X. The relative affinity of the t-PA mutants for fibrin is summarized in Fig 2. Whereas t-PA–ΔFE3X has maintained significant fibrin affinity as compared with natural t-PA, this was markedly decreased in t-PA–ΔFE and in t-PA–ΔFE1X.

Thrombolysis. The extent of thrombolysis 30 minutes after the end of the infusion is shown in Table 1. In the control group infused with solvent (0.3 mol/L NaCl containing 0.01% Tween 80), the degree of “thrombolysis” was 8% ± 1% (n = 6).

Systemic infusion of the t-PA variants resulted in significant thrombolysis at doses ranging between 0.13 and 0.75 mg/kg. The isotope recovery balance was above 90% (not shown) in each group, thereby confirming that no significant loss of thrombus by embolization had occurred. The dose-response curves for thrombolysis with each variant of t-PA were linear (Fig 3). Fifty percent clot lysis, as determined by interpolation, was obtained with 0.4 mg/kg Mel-t-PA, 0.37 mg/kg t-PA–ΔFE, 0.2 mg/kg t-PA–ΔFE1X, and 0.4 mg/kg t-PA–ΔFE3X. This was associated toward the end of the experiment with residual fibrinogen levels (interpolated values) of 100%, 81%, 100%, and 85% of baseline and residual α2-antiplasmin levels of 82%, 55%, 85%, and 90% of baseline respectively. The significantly greater decrease of the α2-antiplasmin levels with t-PA–ΔFE suggests that this variant, when infused at a dose required to produce 50% clot lysis, is somewhat less fibrin specific than Mel-t-PA, t-PA–ΔFE1X, or t-PA–ΔFE3X.

The plasma disappearance rate of t-PA–related antigen, which was measured for 30 minutes after the end of the infusion of the t-PA variants, is summarized in Fig 4. All mutants are cleared much more slowly from the circulation (initial t½ of 15 to 40 minutes) as compared with natural t-PA (initial t½ less than five minutes).

DISCUSSION

The present study was undertaken to compare the thrombolytic properties and disposition rate from plasma of deletion mutants of t-PA in a simple and quantitative thrombolysis model consisting of a radiolabeled jugular vein thrombus in rabbits. This model was previously developed and used to study thrombolysis by natural t-PA and urokinase and by natural and recombinant t-PA.

The materials used in the present study were highly purified single-chain components as evidenced by their behavior on SDS–gel electrophoresis. The specific activity of t-PA–ΔFE1X and t-PA–ΔFE3X has remained largely intact, whereas both the specific activity and fibrin affinity of t-PA–ΔFE are significantly decreased. Our deletion mutants are similar to the LK1-2 mutant of van Zonneveld et al. These authors studied the fibrin binding of conditioned transfected cell culture media and also found a residual but decreased fibrin affinity of the mutant lacking the finger and growth factor domains.

The specific thrombolytic activity of t-PA–ΔFE and t-PA–ΔFE3X, expressed as the dose required to obtain 50% thrombolysis, is comparable to that of natural t-PA, whereas that of t-PA–ΔFE1X is increased approximately twofold. The specific activity, expressed as the ratio between the steady-state plasma level of t-PA–related antigen and the thrombolytic potency, is, however, significantly reduced. Indeed, interpolation of the results of Table 1 shows that 50% lysis is obtained at t-PA antigen levels of approximately 500 ng/mL for t-PA–ΔFE1X and t-PA–ΔFE3X and above 1 μg/mL for t-PA–ΔFE but with less than 80 mg/mL of Mel–t-PA. There are several potential explanations for this discrepancy. One would be that the t-PA mutants are inactivated in plasma, perhaps by proteinase inhibitors. This is probably not a major contributing factor because of the relatively high recoveries of functional activities in the globulin precipitates. Alternatively there may be an intrinsic reduction in the specific fibrinolytic activity of the mutants in a plasma environment. Using an in vitro clot lysis system composed of a fibrin–labeled plasma clot.
THROMBOLYSIS WITH T-PA MUTANTS

immersed in human plasma, we have observed that a two- to fourfold higher concentration is needed of the deletion mutants to induce a comparable degree of thrombolysis relative to Mel-t-PA (unpublished). Thus the dynamics of clot lysis in a plasma milieu do not strictly parallel the activity measured in fibrin plates. Furthermore, in vivo thrombolysis does not parallel in vitro thrombolysis, as demonstrated by the differential reactivity of t-PA in the presence of high PAI-1 (plasminogen activator inhibitor 1) levels19 or the marked difference in the synergistic effect of t-PA and scu-PA (single chain urokinase-type plasminogen activator) in in vitro and in vivo systems.20

1-PA-ΔFE1X and t-PA-ΔFE3X have largely maintained their fibrin specificity of thrombolysis in vivo. Indeed, 50% lysis is obtained at infusion rates that do not result in significant depletion of α2-antiplasmin or fibrinogen breakdown. t-PA-ΔFE, however, appears to induce less fibrin-specific thrombolysis in vivo because 50% lysis is only obtained at infusion rates that are associated with a significant α2-antiplasmin decrease and some fibrinogen breakdown. All mutants investigated in the present study are characterized by a much slower disposition rate from plasma as compared with natural t-PA. Although the plasma disappearance of t-PA–related antigen was not followed long enough to accurately determine the plasma clearance, the marked differences in initial t1/2 of the mutants as compared with natural t-PA indicate that their clearance from plasma is probably around ten times slower than that of natural t-PA. This explains their much higher plateau levels in plasma that are obtained with comparable infusion rates.

In conclusion, the present study suggests that the structures responsible for the clearance of t-PA, for its fibrin-affinity, and for its fibrin specificity are not localized in the same structures: thus t-PA–ΔFE has lost its fibrin affinity and fibrin specificity to a significant extent but has acquired a long in vivo t1/2. t-PA–ΔFE3X, on the other hand, has a markedly prolonged t1/2 but has retained significant fibrin affinity and fibrin specificity of thrombolysis. Consequently it appears to be possible to alter specific functions of t-PA that are relevant for its therapeutic application by deletion mutagenesis of the natural molecule. The pharmacokinetic and thrombolytic properties of t-PA–ΔFE3X, especially its high specific thrombolytic properties in the absence of markedly altered fibrin specificity, indicate that it may constitute a potentially useful alternative to wild-type t-PA for thrombolytic therapy in patients with thromboembolic disease.

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