Augmented and Sustained Plasma Concentrations After Intramuscular Injections of Molecular Variants and Deglycosylated Forms of Tissue-Type Plasminogen Activators

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We have previously explored induction of coronary thrombolysis with tissue-type plasminogen activator (t-PA) administered intramuscularly. Absorption-enhancing agents that rendered the approach feasible were identified, but large amounts of activator were required and initial elevations of concentrations in plasma could not be sustained. The present study was designed to determine whether more therapeutically favorable plasma concentrations could be induced by genetically engineering or chemically modifying t-PA to prolong its half-life based on the hypothesis that the ratio of absorption to clearance would be increased. Each of four genetically engineered variants (one variant with growth factor and kringle 1 domains deleted and kringle 2 duplicated, a second variant with a cysteine for Arg substitution in the growth factor domain, a third variant with an additional urokinase kringle inserted, and a fourth variant with the growth factor domain deleted) and enzymatically deglycosylated t-PA exhibited prolonged half-life after bolus intravenous injection in rabbits. Each elicited substantially higher and more sustained elevations in plasma after intramuscular injection in rabbits or dogs with absorption-enhancing agents as compared with wild-type t-PA that were not accompanied by a systemic lytic state. Thus, use of molecular variants of t-PA with prolonged half-lives in the circulation permits induction of augmented and sustained elevations of plasma concentrations after intramuscular injection with absorption-enhancing agents as compared with wild-type t-PA, rendering potentially therapeutic blood levels more attainable with relatively modest amounts of material. (Circulation 1990;81:1362–1373)

The efficacy of coronary thrombolysis in conferring benefit to the heart and ultimately to the patient appears to be critically dependent on the rapidity of its induction after the onset of antecedent ischemia.1 In experimental animals, the extent of salvage of myocardium induced by a lytic agent is directly proportional to the brevity of ischemia before coronary thrombolysis.2 Restoration of depressed regional ventricular function in jeopardized myocardium manifest by regression of regional wall motion abnormalities3 and preservation of global ventricular performance after coronary thrombolysis4–9 is more prominent among patients in whom treatment can be initiated early after the onset of ischemia. Even an end point as relatively insensitive as mortality reflects the more favorable impact of early as opposed to late coronary thrombolysis.10

In view of these considerations, we have been exploring the feasibility of more rapid implementation of coronary thrombolysis than that attainable conventionally in hospitalized patients.11,12 The possibility that appropriately preselected patients under medical surveillance and monitored with telephonic transmission of electrocardiograms at the time of acute episodes might ultimately benefit from self-administration of plasminogen activators or administration of the agents by appropriately trained lay
persons, such as spouses or paramedical personnel who could administer the agents intramuscularly, has been considered. The necessity to ensure appropriate selection of potentially benefited patients and to avoid administration of fibrinolytic drugs to patients with disorders simulating but differing remarkably from acute myocardial infarction obviously requires careful monitoring and precautions. As a first step, we have sought to determine whether administration of plasminogen activators by intramuscular injection can induce coronary thrombolysis in a timely manner.

In previous studies from our laboratory, we found that even massive doses of tissue-type plasminogen activator (t-PA)\textsuperscript{11} administered intramuscularly led to no appreciable augmentation of plasma concentrations of t-PA within the first 30 minutes although late sustained elevations were observed. Subsequently, we demonstrated that early absorption could be enhanced by addition of agents that we discovered increased permeability of capillaries at the injection site, particularly a combination of methylamine and hydroxylamine in concentrations that exhibited no systemic or local toxicity. The favorable plasma time-concentration curves were associated with coronary thrombolysis in dogs with experimentally induced coronary thrombosis. Studies with distributions of radiolabeled tracers and colloidal carbon characterized by light and electron microscopy demonstrated that the effect of the absorption-enhancing agents was an augmentation of prevenular capillary permeability and that intramuscular administration of t-PA with the enhancers was well tolerated. The rapidity of absorption led to an early peak of plasma t-PA (within approximately 5 minutes), followed by a trough and a secondary peak resembling the peak seen after intramuscular administration of t-PA without enhancers.\textsuperscript{12}

In the preliminary studies reported previously, it was found necessary to use doses of t-PA in the range of 2–10 mg/kg in rabbits to induce initial elevations of the concentration of t-PA in plasma similar to those associated with successful coronary thrombolysis in patients.\textsuperscript{11–13} Despite the favorable results obtained, the regimens used gave rise to a dromedary plasma time-concentration curve\textsuperscript{12} comprising the initial peak that reflected facilitated absorption attributable to the enhancing agents, the trough that might be therapeutically disadvantageous, and a secondary peak 30–60 minutes after injection, indicative of absorption of t-PA from intramuscular depots regardless of whether enhancer was present. The high doses required would probably not be economically feasible for clinical use and would be highly wasteful of material.

**Rationale for the Present Study**

We interpreted the trough after the initial peak of plasma t-PA after intramuscular injection with absorption-enhancing agents to be a reflection of the short half-life in the circulation of wild-type t-PA. Assuming that early absorption even when facilitated with absorption-enhancing agents is a relatively slow process, the magnitude and persistence of the early peak would be attenuated in proportion to the brevity of the circulating half-life of the absorbed agent. Although t-PA binds avidly and persists on the surfaces and in the interstices of clots for prolonged intervals despite rapid clearance from the circulation,\textsuperscript{14} the need for continual exposure to circulating t-PA of newly exposed binding sites in regions of clots undergoing progressive lysis is evident. Accordingly, increased therapeutic efficacy of intramuscular administration of fibrinolytic agents can be anticipated with elimination of the trough after the initial peak of plasma activity that occurs with intramuscular injections of wild-type t-PA with absorption-enhancing agents.

Clearance of t-PA from the circulation seems to depend on the liver.\textsuperscript{15–17} Glycosylation sites, particularly the 117 Asn (N-linked), high mannose oligosaccharide, seem to be important recognition sites for endothelial and Kupffer cells that explain a substantial fraction of the $\alpha$-phase of the biexponential clearance seen after intravenous bolus injection of t-PA.\textsuperscript{17} Hepatocytes seem to contribute to clearance as well\textsuperscript{16} in a mechanism that might involve plasminogen activator inhibitor type 1 (PAI-1) and might explain a substantial fraction of the $\beta$-phase of biexponential clearance seen typically after bolus intravenous administration of t-PA. Other domains of the t-PA molecule such as the epidermal growth factor (EGF) homologous domain can be involved in recognition by endothelial cells, hepatocytes, or both, as judged from the prolonged half-life exhibited by deletion mutants devoid of EGF homologous domains.\textsuperscript{18} In vivo, clearance of functional activity has been shown to differ from clearance of total antigen (free t-PA plus t-PA complexed with inhibitors).\textsuperscript{19} Accordingly, kinetics of interaction with inhibitors of a given plasminogen activator in a given form (single-chain or two-chain), prevailing concentrations of inhibitors in plasma, rates of synthesis of inhibitors, and interactions of diverse cell types (e.g., parenchymal hepatocytes, Kupffer cells, and endothelium) and an extracellular matrix with diverse domains of free and complexed plasminogen activator can all influence clearance of parenterally administered plasminogen activators.

The present study was undertaken to determine whether intramuscular administration of enzymatically modified wild-type t-PA and diverse molecular variants of t-PA constructed such that the half-life in the circulation of each was prolonged, regardless of the mechanism or modification responsible, would lead to sustained plasma time-concentration curves with augmented early peaks presumably capable of inducing coronary thrombolysis rapidly and efficiently with relatively smaller amounts of the fibrinolytic agent than the amount of wild-type t-PA required. Because of the difficulties of transcribing and purifying large amounts of modified t-PAs from synthetic genes and the need to screen a large
number of compounds in this regard, the primary end point of this study was the early peak in plasma time-concentration curves inducible after intramuscular injection of the variants in small laboratory animals. As judged from results of experiments in several species and in clinical studies, prompt and sustained elevations of plasma fibrinolytic activity consistently induce coronary thrombolysis. Furthermore, it is possible to estimate with a high degree of certainty whether a particular plasma time-activity, and hence time-concentration curve if specific activity is known, can be anticipated to induce coronary thrombolysis rapidly and effectively.\textsuperscript{20}

Methods

Human t-PA produced by recombinant DNA technology and expressed in Chinese hamster ovary (CHO) cells was obtained from Genentech Incorporated (South San Francisco, California). The structure of this material is generally abbreviated as F, G, \(k_1\), \(k_2\), SP, a notation that symbolizes the following domains in sequence from the amino to the carboxy-terminal portion of the molecule: fibrin-binding finger domain (F), EGF-like domain (G), kringle 1 (\(k_1\)), kringle 2 (\(k_2\)), and Ser protease domains (SP). To determine whether selected deglycosylation of t-PA would prolong its clearance from the circulation without diminishing absorption and thereby lead to an increased magnitude of the initial peak after intramuscular injection with enhancers as well as a sustained concentration in plasma after the initial peak, 2.0 mg t-PA was exposed to 0.05 units endoglycosidase-H (Endo H) (Genzyme Corporation, Boston, Massachusetts) in 50 mM Tris HCl, pH 7.4, containing 150 mM NaCl and 0.01% (vol/vol) Tween-80 (TBS-Tween) for 12 hours at 37\(^\text{o}\) C. The reaction volume (2.125 ml) was loaded onto a Poly Prep column containing 1 ml Con-A sepharose equilibrated and eluted with TBS-Tween. Less than 1\% of t-PA treated identically in the absence of enzyme could be recovered after elution with the same volume of buffer, indicating that the column avidly bound nondeglycosylated t-PA. The eluted deglycosylated t-PA had intact primary structure, as judged from results of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and nonreducing conditions. Prolongation of its half-life in the circulation was demonstrated after bolus intravenous injections with plasma time-activity curves compared with those elicited after injections of corresponding amounts of wild-type t-PA in rabbits as previously described.\textsuperscript{17}

Additional experiments were performed with intravenous and intramuscular injection of type 1 (glycosylated at the Asn 117, 184, and 448 sites) and type 2 (glycosylated only at the 117 and 448 sites), separated and prepared from wild-type t-PA produced in CHO cells by Invitron Corporation (St. Louis, Missouri).

Additionally, experiments were performed with single-chain as compared with two-chain t-PA. To characterize clearance of each, studies were performed with intravenous injections of wild-type t-PA from CHO cells (>90\% single-chain t-PA, as judged from results of SDS-PAGE) from Genentech and two-chain material obtained by treating the material with Sepharose-plasmin formed from 1 mg/ml plasminogen in phosphate-buffered saline, 25\% glycerol. The plasminogen was bound to CNBr-activated Sepharose at 4\(^\circ\) C overnight and subsequently converted to plasmin-Sepharose with streptokinase (1,000 IU/mg plasminogen). Treatment of t-PA with this material yields virtually 100\% two-chain t-PA with a 1:5 M ratio of plasmin to t-PA.

In studies of molecular variants of t-PA, each of which was constructed to prolong the half-life of the modified material in the circulation albeit through alteration of diverse determinants, several variants were used. Their structures are shown in Table 1. The first, variant 1, was a genetically engineered variant of t-PA (provided by Monsanto Company, St. Louis, Missouri) transcribed from a synthetic gene constructed such that both the G and \(k_1\) domains were deleted and an additional \(k_2\) domain was substituted for the \(k_1\) domain. The structure of this variant conformed to F, \(k_2\), SP. The first \(k_2\) had a substitution, point mutation such that amino acid residue 184 was mutated (Asn to Ser) to preclude glycosylation (which might prolong clearance) at this site. A second variant, variant 2, was genetically engineered and transcribed from a synthetic gene constructed such that the protein comprised F, G, \(k_1\), \(k_2\), SP, with a substitution of Cys for Arg in position 73 in the growth factor domain (Monsanto Company). A third molecular variant, variant 3, provided by Wyeth Corporation (Philadelphia, Pennsylvania) (F, G, \(k_3\), \(k_2\), SP, containing a kringle from urokinase [\(k_{uk}\)], inserted into the t-PA molecule), was used in additional experiments.\textsuperscript{21} A fourth plasminogen activator with prolonged half-life in the circulation, variant 4, was transcribed from a synthetic gene constructed by Monsanto and comprised F, \(k_3\), \(k_3\) SP, representing a plasminogen activator from which the growth factor domain had been deleted.

These four molecular variants were selected because they exhibited prolonged half-lives in the circulation after intravenous injection. Additionally, in control experiments, another molecular variant was used, that is, human t-PA produced in mouse C127 cells (provided by Invitron Corporation). This material has the same primary structure as that of wild-type human t-PA produced by recombinant DNA technology.\textsuperscript{22} It exhibits, however, a glycosylation pattern differing from that of the wild-type t-PA used, which is produced in CHO cells. It exhibits a half-life in the circulation similar to that exhibited by the wild-type t-PA.

Biochemical Reagents

Reagents used in enzyme-linked immunosorbent assays (ELISAs) for t-PA antigen concentrations in plasma were purchased from American Diagnostica.
TABLE 1. Structures of the Plasminogen Activators Used

<table>
<thead>
<tr>
<th>Plasminogen activator</th>
<th>Amino acid structure</th>
<th>Domains*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human t-PA</td>
<td>Ser 1-Pro 527</td>
<td>FG k₁k₂ SP</td>
</tr>
<tr>
<td>Human recombinant t-PA (Activase)</td>
<td>Same</td>
<td>Same</td>
</tr>
<tr>
<td>Variant 1</td>
<td>Ser 1–Ser 50</td>
<td>F k₁k₂ SP</td>
</tr>
<tr>
<td></td>
<td>Gly 176–Ser 262</td>
<td>(k₁) ASN 184→Ser</td>
</tr>
<tr>
<td></td>
<td>Glu 175–Pro 527</td>
<td>(k₁+SP)</td>
</tr>
<tr>
<td>Variant 2</td>
<td>Cys 573→Arg</td>
<td>FG k₁k₂ SP</td>
</tr>
<tr>
<td>Variant 3</td>
<td>Ser 1–Ser 50</td>
<td>(F) FG (k₅₁)k₅₃ SP</td>
</tr>
<tr>
<td></td>
<td>Cys 51–Asp 87</td>
<td>(G)</td>
</tr>
<tr>
<td></td>
<td>Thr 88–Thr 91</td>
<td>(k₆) (ku-PA)</td>
</tr>
<tr>
<td></td>
<td>Cys 92–Pro 527</td>
<td>(k₆₃+SP)</td>
</tr>
<tr>
<td>Variant 4</td>
<td>Ser 1–Ser 50</td>
<td>(F) F k₁k₂ SP</td>
</tr>
</tbody>
</table>

Deglycosylated human recombinant t-PA (Endo H-treated) | Asn 117 deglycosylated |
Endo H control | Asn 117 glycosylated as in human recombinant t-PA |

Variants 1, 2, and 4 were transcribed from synthetic genes based on exonic sequences only that do not contain intronic regions. All of exons conform to nucleotide boundaries encoding functional domains of mature protein described in second footnote. Amino acid protein structure of variants is shown. Variant 3 was constructed as described previously (Reference 21). Urokinase kringle (k₅₆) coding sequence was inserted at bp 462–463 of t-PA cDNA encoding amino acid residues 91 and 92.

*A symbol

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Amino acid (AA) residues</th>
<th>Domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>AA 1–50</td>
<td>Fibrinectin finger domain</td>
</tr>
<tr>
<td>G</td>
<td>AA 51–87</td>
<td>Epidermal growth factor domain</td>
</tr>
<tr>
<td>k₁</td>
<td>AA 88–175</td>
<td>Kringle 1</td>
</tr>
<tr>
<td>k₂</td>
<td>AA 176–263</td>
<td>Kringle 2</td>
</tr>
<tr>
<td>SP</td>
<td>AA 264–527</td>
<td>Ser protease domain</td>
</tr>
</tbody>
</table>

AA 1 corresponds to serine (Reference 22); k₅₆ represents 244 nucleic acid residues encoding urokinase kringle domain.

Inc. (New York, New York) and used as previously described.¹⁹ Activated microspheres used for immunoprecipitation were purchased from Kirkegaard and Sperry Laboratories, Inc. (Gaithersburg, Maryland), D-phenylalanyl-propyl-arginyl-chloromethyl ketone (PPACK) from Cal-Biochem (San Diego, California), and aprotinin from Sigma Chemical Company (St. Louis, Missouri). A Protean II Mini gel system, Transblot system, Poly Prep columns, acrylamide, and SDS-PAGE molecular weight standards were acquired from BioRad (Richmond, California).

Determination of Concentrations of t-PA Antigen in Plasma

An ELISA system developed by Bergsdorf et al²³ was used to determine concentrations of t-PA antigen in plasma samples as previously described.¹⁹,²²,²³ Standardization was performed originally with Bowes melanoma cell t-PA, kindly provided by D. Collen and maintained by cross-standardization with human t-PA from American Diagnostica Inc. Slopes of curves constructed from serial dilutions for assays of wild-type and molecular variant t-PA species were identical.

Determination of t-PA Activity

Activities of each of the t-PA variants studied and deglycosylated t-PA were assayed spectrophotometrically with a solid-phase assay system (SOFIA) in citrated platelet-poor plasma samples as previously described.²⁴,²⁵ The microtiter assay system used wells coated with fibrin monomer and incubations for 2 hours at 37°C. Determination of t-PA activity was performed with 0.2 μM Glu plasminogen (American Diagnostica Inc.) and 1.0 mM S-2251 chromogenic substrate (Helena Laboratories, Beaumont, Texas). Results were expressed as international units per milliliter (IU/ml) based on comparisons with activity of concomitantly assayed standards calibrated with reference to the International Reference Preparation of t-PA (83-517) obtained from the National Institute for Biological Standards and Control (London, England). Fibrinogen (sulfite precipitation method), plasminogen, and α₂-antiplasmin (spectrophotometric chromogenic substrate procedure) were assayed as previously described.²⁶

Procedures in Experimental Animals

Care and handling of experimental animals conformed to the standards established by the Washington University Committee for Humane Care of Laboratory Animals, which are consistent with those of the Helsinki declaration. Studies were performed in rabbits anesthetized with 0.1 ml Innovar-Vet (0.4 mg/ml fentanyl and 20 mg/ml droperidol) and ketamine (20 mg/kg) in doses sufficient to maintain anesthesia for approximately 1.5 hours. When more prolonged analgesia and anesthesia were required, additional intravenous injections of 0.5-ml doses of
pentobarbital (25 mg/ml) were administered as needed. For studies of circulatory half-life of intravenously injected plasminogen activator, 0.2 mg/kg of deglycosylated human recombinant t-PA in 10 ml of NaCl 0.15 M, Tris 0.05 M, pH 7.4, Tween-80, 0.01%; wild-type t-PA in 1 ml of 0.2 M Arg phosphate, pH 7.2; or variant t-PA in 1 ml of 1 M NH4HCO3 was injected intravenously through a femoral venous catheter. For studies of intramuscular administration of plasminogen activator unless stated otherwise, injections were made percutaneously without exposure of underlying quadriceps muscle with concentrations of t-PA in the injection medium adjusted to be approximately 5 mg/ml. Injection media comprised components in the following concentrations: methylamine hydrochloride (0.63 M), hydroxyamine (0.079 M), sodium acetate (pH 3.0), Arg phosphate (0.2 M), and the selected concentration of t-PA in a final volume of 2 ml. Each animal was given two simultaneous 1-ml injections intramuscularly.

**Acquisition of Plasma Samples**

Whole blood samples were collected from a femoral arterial catheter with a three-syringe procedure and transferred promptly into tubes maintained at 0–4°C containing sodium citrate (12.9 mM, final concentration). Catheters were flushed with 3 ml sterile saline. The same catheter was never used for administration of agent and acquisition of blood samples. For rabbits given plasminogen activators intravenously through a femoral venous catheter, samples were drawn from an ipsilateral femoral arterial catheter. For those given intramuscular injections, samples were drawn through a femoral arterial catheter. Plasma was separated by centrifugation at 4°C at 2,000g for 15 minutes and stored at −70°C before subsequent assay. Samples to be used for assay of fibrinogen were collected in EDTA Vacutainer tubes (2 ml) with 2 μM PPACK.26 Samples to be used for SDS-PAGE were collected in tubes containing final concentrations of 4 μM PPACK and 400 KIU/ml aprotinin to prevent activation of plasminogen in vitro.24,27,28

**Immunoblots and SDS-PAGE Electrophoresis**

Blots of immunoprecipitates from plasma samples were performed as previously described.24 SDS-PAGE gels were equilibrated in transfer buffer (0.025 M Tris HCl, 0.192 M Gly, and 10% methanol) for 30 minutes. Electrostatic transfer to nitrocellulose was performed at 4°C with a Transblot system for 40 minutes at 100 V. SDS-PAGE gels were stained with 0.02% Coomassie blue in 40% methanol and 10% acetic acid to monitor efficiency of transfer. For immunoblotting at 25°C, membranes were blocked with 1% (wt/vol) bovine hemoglobin in 0.05 M Tris HCl, 0.15 M NaCl, pH 7.5 (TBS) for 1 hour, incubated with 10 μg/ml goat polyclonal antibody to t-PA in TBS with 1% bovine hemoglobin for 1 hour. After additional washing three times with TBS-Tween for 10 minutes each, the membranes were exposed to Kodak XAR film for 24–48 hours with two sheets of Cronk intensifying screens at −70°C. Autoradiograms were analyzed by laser densitometry within the linear range defined experimentally.

**Nature of Comparative Studies**

All experiments characterizing the half-life of intravenously administered material and plasma time-concentration or time-activity curves of administered plasminogen activators were performed at least twice for each set of conditions reported (16 times for wild-type t-PA, five times for variant 1, three times for variant 2, twice for variant 3, twice for variant 4, and four times for deglycosylated t-PA). Because of limitations of the amount of material available and the amount that could be produced conveniently by enzymatic deglycosylation, the amounts of modified t-PA or molecular variants used were not the same in all experiments. Each comparison shown between wild-type and modified t-PA, however, is presented based on results of experiments performed concomitantly in which the same amounts of the two types of t-PA were used under identical conditions. Thus, differences between wild-type and modified t-PAs are compared by evaluating results from experiments in which the same amount of material of each type was administered to animals under the same conditions and in the same buffers. The primary end points in all experiments with intramuscular injections were plasma time-concentration values. In all cases, however, specific fibrinolytic activity of each of the variant plasminogen activators used was measured (Table 2). Concordance between specific activity in plasma samples (Table 3) obtained at selected intervals after intramuscular injections and specific activity of the variants evaluated was consistent with changes in specific activity measured in purified systems and in plasma and with the decline of specific activity of plasminogen activators in the circulation reflecting interactions with inhibitors and inactivation.19

**Studies in Dogs**

In some instances, when sufficiently large amounts of the plasminogen activators to be characterized could be harvested, studies were performed in dogs weighing approximately 20 kg. Anesthesia was induced with 12.5 mg/kg pentoxybenon plus 60 mg/kg a-chloralose after allogenesia with 1 mg/kg morphine sulfate subcutaneously. Ventilation was maintained with room air with a Harvard Respirator (Harvard Apparatus, South Natick, Massachusetts) and endotracheal tube. For studies of intramuscular absorption of plasminogen activators, two injections of 2 ml each (containing 0.75–10 mg/kg body wt) were given intramuscularly to each animal through the two sartorius muscles into the vastus medialis manually and percutaneously through a 21-gauge stainless-steel needle.
TABLE 2. Activation of Plasminogen by Plasminogen Activators

<table>
<thead>
<tr>
<th>Plasminogen activator</th>
<th>Assays of PA in buffer</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PA antigen (ng/ml) (ELISA)</td>
<td>PA activity (IU/ml) (SOFIA)</td>
<td>Specific activity (IU/ng)</td>
</tr>
<tr>
<td>Human recombinant t-PA</td>
<td>2.72x10^6</td>
<td>9.89x10^5</td>
<td>0.36</td>
</tr>
<tr>
<td>Deglycosylated, control*</td>
<td>6.94x10^4</td>
<td>1.37x10^6</td>
<td>1.98</td>
</tr>
<tr>
<td>Deglycosylated, human recombinant t-PA</td>
<td>6.92x10^6</td>
<td>1.17x10^6</td>
<td>1.68</td>
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<tr>
<td>Variant 1</td>
<td>1.63x10^6</td>
<td>2.37x10^6</td>
<td>1.45</td>
</tr>
<tr>
<td>Variant 2</td>
<td>1.73x10^6</td>
<td>3.07x10^6</td>
<td>1.78</td>
</tr>
<tr>
<td>Variant 3</td>
<td>1.79x10^6</td>
<td>1.24x10^6</td>
<td>0.69</td>
</tr>
<tr>
<td>Variant 4</td>
<td>3.60x10^5</td>
<td>3.46x10^5</td>
<td>0.96</td>
</tr>
</tbody>
</table>

Both antigen assay and functional activity assay results are averages of assays in duplicate of each of two samples in each case. PA, plasminogen activator.*Human recombinant t-PA exposed to same conditions as those used for enzymatic deglycosylation with Endo H except for lack of exposure to glycosidase.

Serial venous blood samples were obtained through an indwelling catheter in the inferior vena cava.

Results
Specific Activity of the Plasminogen Activators Studied
As shown in Table 2, each of the variants studied in vivo had specific, plasminogen-activating activity exceeding that of wild-type human recombinant t-PA synthesized in CHO cells. Thrombolysis in vivo depends on numerous factors besides specific activity measured with any given assay system in vitro.29 These include clearance of circulating protein (mass), rates of disappearance of plasminogen-activating activity in vivo reflected by changes of specific activity in plasma attributable to both interactions with inhibitors and denaturation, relative activity in fluid and fibrin domains (fibrin stimulation), penetration and persistence of binding of the activator to thrombi, interactions of the activator with endothelial cells and extracellular matrix and with factors associated with and elaborated from them, persistence of single- as opposed to two-chain forms in the vicinity of thrombi, and interactions of the activator with platelets and platelet-secretory products including inhibitors and other determinants. Nevertheless, consistent relations exist between functional activity measured in vitro and specific activity measured in vivo such that fibrinolytic potential can be inferred reasonably accurately from knowledge of plasma time-concentration curves and specific activity.20 However, anticipated behavior of specific activators in vivo must be confirmed ultimately by studies of clot lysis per se. With the activators tested in this study, specific plasminogen-activating activity in vivo early after intramuscular injection was generally consistent with specific activity in vitro measured with the same functional assay (Table 3).

Time-Concentration Curves After Intramuscular Injections of Plasminogen Activators
The typical plasma time-concentration curve of intramuscularly administered t-PA without absorption-enhancing agents is shown in Figure 1, which depicts results after administration of a large dose of wild-type t-PA (10 mg/kg) in two simultaneous 2-ml injections in a dog in the absence of absorption-enhancing agents. As seen in the figure, plasma levels do not rise appreciably until 30–60 minutes after injection and reach a peak after only 2–3 hours. In contrast, as shown in Figure 2, addition of absorption-enhancing agents to the injection medium results in a remarkably different pattern with an early peak evident within 10–15 minutes, followed by a trough and a subsequent peak resembling the peak seen in the absence of absorption-enhancing agents. These results were obtained with injections of t-PA in 0.2 M Arg phosphate, pH 7.2, without (Figure 1) or with (Figure 2) 0.63 M methylamine and 0.079 M hydroxylamine.

TABLE 3. Activation of Plasminogen by Plasminogen Activators

<table>
<thead>
<tr>
<th>Plasminogen activator</th>
<th>Dose (mg/kg)</th>
<th>Time of acquisition of blood sample after dose (min)</th>
<th>PA antigen (ng/ml) (ELISA)</th>
<th>PA activity (IU/ml) (SOFIA)</th>
<th>Specific activity (IU/ng)</th>
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</thead>
<tbody>
<tr>
<td>Human recombinant t-PA</td>
<td>2</td>
<td>45</td>
<td>40</td>
<td>18</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>0.86</td>
<td>5</td>
<td>11</td>
<td>3</td>
<td>0.27</td>
</tr>
<tr>
<td>Deglycosylated, control*</td>
<td>0.86</td>
<td>5</td>
<td>55</td>
<td>44</td>
<td>0.80</td>
</tr>
<tr>
<td>Deglycosylated, human recombinant t-PA</td>
<td>0.86</td>
<td>5</td>
<td>36</td>
<td>36</td>
<td>1.00</td>
</tr>
<tr>
<td>Variant 1</td>
<td>2</td>
<td>45</td>
<td>301</td>
<td>200</td>
<td>0.66</td>
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<tr>
<td>Variant 2</td>
<td>2</td>
<td>45</td>
<td>231</td>
<td>174</td>
<td>0.75</td>
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</table>

Both antigen assay and functional activity assay results are averages of assays in duplicate of each of two samples in each case. Assays of blood samples obtained at times specified after intramuscular injection in rabbits of specified doses of PAs. PA, plasminogen activator.
In the present study, results after intramuscular injections of wild-type t-PA in rabbits were consistent with those we previously reported. Thus, after doses of 2, 4, and 12 mg/kg i.m. of wild-type t-PA with absorption-enhancing agents, peak plasma t-PA values occurred in 5 minutes and averaged 140±39 (± SD) (n=9), 233±113 (n=10), and 794±247 (n=2) in the present study.

Clearance of Intravenously Administered Plasminogen Activators

The biexponential clearance after intravenous administration of a bolus injection of 1 ml of wild-type t-PA (0.2 mg/kg) in 0.2 M Arg phosphate, pH 7.2, is evident from Figure 3, which depicts changes in plasma t-PA as a function of time after intravenous bolus injection. As reported previously, the half-life attributable exclusively to the α-phase averages 1.16±0.28 and that attributable to the β-phase averages 10.54±2.28 in rabbits (n=16). Effects of deglycosylation on clearance of intravenously administered t-PA are evident in Figure 4. As shown in the figure, t-PA that had been treated with Endo H to remove the 117 high mannose N-linked oligosaccharide exhibited prolonged α-phase and β-phase clearance as compared with wild-type t-PA processed similarly except for lack of exposure to the deglycosylating enzyme. Virtually identical results were obtained in each of four experiments. The apparent modest prolongation of α-phase clearance of the wild-type t-PA in experiments of this type probably reflects some denaturation induced by the conditions necessary for enzymatic deglycosylation.

The clearance of two-chain wild-type t-PA was indistinguishable from that of single-chain t-PA. Clearance of type 2 (with no glycosylation at the 184 Asn site) t-PA was only slightly more rapid than that of type 1. The α-phase rate constants for type 1 averaged 1.6 as compared with 1.2 minutes for type 2 (n=2, for each). The corresponding β-phase rate constants averaged 18.9 and 14.2 minutes. In contrast, clearance of several of the molecular variants used in this study was noticeably prolonged as compared with that of wild-type t-PA. For example, clearance of intravenously administered variant I was virtually monoexponential with an average half-life of disappearance of 23.6 minutes (range, 22.7–25.2 minutes; n=3). An example after injection of 0.2 mg/kg in 1 ml is shown in Figure 5.

The clearance of variant 2 was distinctly prolonged as well. The α-phase rate constant averaged 2.2 minutes, and the β-phase rate constant averaged 29.7 minutes.
(n=3). Clearance of variant 3 t-PA was 3.5 minutes (α-phase) and 31.0 minutes (β-phase). These results are averages of duplicate experiments. Variant 4 exhibited an α-phase rate constant of 2.7 minutes and a β-phase rate constant value of 45.7 minutes (n=2). Thus, it too exhibited remarkably prolonged clearance.

Because these four variants and the deglycosylated t-PA exhibited qualitatively similar and prolonged circulating half-lives with respect to the circulating half-life of wild-type t-PA despite exhibiting diverse structural modifications, the similarity of results obtained after intramuscular administration of each seems to be attributable to prolongation of the half-life of each in the circulation rather than to a specific structural alteration.

Another molecular variant used for comparative purposes was one produced in mouse C/127 cells. This material was used because its half-life in the circulation was found to be similar to that of wild-type human t-PA (1.4 minutes for the α-phase and 16.4 minutes for the β-phase rate constant) (n=1).

**Plasma Time-Concentration Curves After Intramuscular Injections of Wild-Type Deglycosylated t-PA and Molecular Variants of t-PA in Rabbits**

As seen in Figure 6, intramuscular injection in rabbits of 2 mg/kg (two simultaneous 1-ml injections) of wild-type t-PA or human t-PA produced in C/127 mouse cells (which exhibits a different glycosylation pattern but has a comparably short half-life in the circulation) in 400 mM NaAc, 0.2 M Arg phosphate, pH 3.0, with absorption-enhancing agents leads to a prompt peak with a rapid decline of circulating activator. The very modest elevation above baseline that persists for 4–6 hours probably reflects continuing absorption of small amounts of t-PA. This pattern is typical of that seen in studies of more than 150 rabbits given diverse doses (0.86–12 mg/kg) of t-PA intramuscularly (data not shown). It was seen also with 2 mg/kg i.m. injections of type 1 (n=3) or type 2 (n=4) t-PA in two 1-ml aliquots of the generally used injection medium and was not affected by conversion of single-chain to two-chain t-PA. In contrast, intramuscular administration of very small doses of t-PA (0.86 mg/kg) that had been deglycosylated with Endo H resulted in a higher and more prolonged initial peak, as shown in Figure 7. This pattern was compatible with the possibility that the prolonged half-life of t-PA in the circulation was being reflected by a more pronounced elevation of concentration in plasma early after intramuscular injection and a more sustained elevation than is the case with wild-type t-PA. To confirm this hypothesis, experiments were performed with four molecular variants of t-PA with prolonged half-lives.

As shown in Figure 8, peak plasma t-PA concentrations of 2 mg/kg of variant 1 were substantially higher than those seen with wild-type t-PA at the same dose and under the same conditions. Furthermore, the peak was sustained throughout the 60 minutes of observation in comparison with results with wild-type t-PA. Similarly, corresponding doses of variant 2 t-PA...
exhibited a greater peak and more sustained elevation of the concentration of t-PA in plasma (n=2) as compared with wild-type t-PA (Figure 9).

Results with variant 3 in buffer containing 0.2 M Arg phosphate, 0.01% Tween-80, 0.1 M H₃PO₄, pH 7.4, as compared with wild-type t-PA, were analogous with peak concentrations 70% higher on average with the variant (n=2) as compared with wild-type t-PA (n=2) (data not shown). Furthermore, elevations were sustained for the 60 minutes of observation with the variant but not with wild-type t-PA.

Results with variant 4 as compared with wild-type t-PA are shown in Figure 10. A substantially higher initial peak concentration in plasma and sustained elevations were seen with the variant (n=2) at a dose of 4 mg/kg in two 1-mL injections in 0.2 M Arg phosphate, 400 mM NaAc, 0.63 M methylamine, 0.079 M hydroxylamine, pH 3.0.

These results indicate that plasma time-activity curves after intramuscular injection of four structurally different t-PAs with prolonged half-life in the circulation differ characteristically from plasma time-activity curves after intramuscular administration of wild-type t-PA with the same absorption-enhancing agents. Initial peaks are higher and elevations are more sustained when the injected t-PA has a prolonged half-life.

**Effects on the Fibrinolytic System**

Potential disadvantages of intramuscular administration of activators of the fibrinolytic system include delayed and potentially deleterious effects of conversion of plasminogen to plasmin in the circulation. However, depletion of circulating α₂-antiplasmin, barely detectable after intramuscular administration in rabbits of wild-type t-PA (7% depletion after 1 hour), however, was only modestly greater after intramuscular administration of variant 1 at the same dose (22% depletion after 60 minutes). Similar results were obtained in each of three rabbits studied. Depletion was maximal in 15 minutes, consistent with the timing of the initial elevation of plasma t-PA activity with variant 1. Analogous results were seen in dogs in which variant 1 was administered intramuscularly at a dose of 4 mg/kg. α₂-Antiplasmin depletion was maximal after 7 hours, reaching a nadir of 13% of baseline after intramuscular administration of wild-type t-PA in a dose of 10 mg/kg with absorption-enhancing agents. Twelve hours after injection, circulating α₂-antiplasminogen had risen to 24% of baseline. During this interval, fibrinogen concentrations were depressed only modestly with the value 12 hours after injection being 85% of baseline.

Serial determinations of specific activity (fibrinolytic activity in the microtiter assay system used per nanogram of immunoreactive protein in plasma) showed a consistent pattern with each of the t-PAs tested in this manner (i.e., wild-type, variant 1, and variant 2). During the first 15–30 minutes after intramuscular injection in the generally used buffer with absorption-enhancing agents but no arginine phosphate, specific activity was quite comparable with that in the injectate. Subsequently, specific activity declined despite persistent elevations of circulating antigen. For example, in one dog followed for 12 hours after intramuscular injection of wild-type t-PA (10 mg/kg) with absorption-enhancing agents...
agents, specific activity at 1–15 minutes after injection averaged 0.26 IU/ng. Between 60 minutes and 2 hours after injection, similar values were obtained; however, subsequently, specific activity declined to 0.16 (3 hours), 0.08 (4 hours), 0.01 (8 hours), and 0.05 (12 hours) after injection. Factors responsible for the decline of specific activity have not been fully elucidated, but the consistency of the phenomenon with diverse plasminogen activators might explain the relatively modest effects on degradation of fibrinogen and consumption of α-antiplasmin after intramuscular injection of even large amounts of t-PA.

**Discussion**

We have previously shown that intramuscular administration of t-PA with absorption-enhancing agents elicits substantial elevations of plasma concentrations of t-PA soon after injection without untoward local effects. The magnitude of the early peak, however, presumably an important determinant of the efficacy of early thrombolysis under conditions in which t-PA might ultimately be administered therapeutically by intramuscular injection, was modest despite administration of large amounts of the protein. Furthermore, the persistence of the peak was brief and followed by a trough before a sustained later peak compatible with prolonged absorption at a modest rate. The present study was undertaken to determine whether intramuscular administration of molecularly modified t-PA exhibiting persistence in the circulation results in plasma time-concentration curves with potentially more favorable therapeutic properties. We hypothesized that the prolongation of half-life would be associated with an increased amplitude of the initial plasma peak (because of accumulation of initially absorbed t-PA exceeding removal through clearance mechanisms). It was anticipated that even modest prolongation of early persistence of activator in the circulation (α-phase rate constant of disappearance) would alter the early time-concentration curve after intramuscular injection because of the impact of two competing exponential functions (absorption and disappearance). It was anticipated also that initial elevations in plasma would be more sustained because of reduced clearance. Later phenomena manifested in the plasma time-concentration curve would be anticipated to reflect the β-phase rate constant of disappearance and the impact of interactions between the activator and circulating inhibitors affecting clearance in vivo as well. As judged from comparisons of plasma time-concentration curves of wild-type t-PA and five different species of t-PA (deglycosylated wild-type t-PA and genetically engineered variants 1–4), prolongation of the α-phase of clearance was associated with alterations of plasma time-concentration curves after intramuscular injections resulting in higher and more sustained early peaks.

The likelihood that the changes in plasma time-concentration curves were attributable to prolongation of the half-life of modified t-PA in the circulation is supported by results with the C/127 t-PA, which is glycosylated differently as compared with the CHO cell wild-type t-PA used for reference, yet exhibits similar rapid clearance from the circulation. Plasma time-concentration curves after intramuscular injection of C/127 t-PA were indistinguishable from those seen with the wild-type t-PA produced in CHO cells. The lack of alteration of plasma time-concentration curves associated with intramuscular injection of either wild-type t-PA type 1 (glycosylated at three sites) as compared with type 2 (glycosylated only at two sites), or with single-chain as compared with two-chain wild-type t-PA, supports this interpretation in view of the similarity of clearance after intravenous bolus injection of type 1 and type 2 t-PA, and of single-chain and two-chain t-PA, respectively.

This study was not performed to define dose regimens for thrombolysis. We have previously shown that thrombolysis after intramuscular injection of wild-type t-PA occurs when plasma levels are above 100 ng/ml within the first few minutes after injection. These observations are consistent with results of early clinical studies in which coronary thrombolysis was promptly induced with intravenous doses of t-PA harvested from melanoma cell-conditioned media as low as 0.15 mg/kg. As judged from results of numerous experiments in our laboratory with intravenously administered t-PA, thrombolysis can be anticipated consistently with plasma concentrations of t-PA in the range of 100–200 ng/ml, particularly when lysis is induced early after clot formation. The present study was designed to determine whether diverse modifications of t-PA, each of which results in prolongation of the half-life of the modified material in the circulation, would give rise to plasma time-concentration curves after intramuscular injection with characteristics that are potentially more favorable therapeutically than those of curves seen with wild-type t-PA administered under the same conditions.

A divergence between functional activity and persistence of t-PA antigen in the circulation after intravenous administration has been recognized. Analogous divergence was evident in results of the present study in which specific activity of t-PA declined, beginning 30–60 minutes after appearance of intramuscularly administered t-PA in the circulation. The dichotomy might facilitate therapeutic use of wild-type or modified t-PA administered intramuscularly because it seems likely to preclude the induction of pronounced or sustained elevations of fibrinolytic activity long after intramuscular injection that would result in consumption of circulating α2-antiplasmin, plasminemia, and degradation of circulating proteins similar to that seen with first-generation thrombolytic agents administered intravenously. Serial measurements of components of the fibrinolytic system in plasma in the present study are consistent with the lack of induction of a systemic lytic state despite elevations of t-PA induced in plasma soon after intramuscular injections.
Facilitation of absorption of proteins with the absorption-enhancing agents we have identified offers potential therapeutic advantages. Development of the approach must be judicious, however, particularly with proteins such as t-PA that could induce deleterious as well as therapeutic effects. Any use of intramuscular t-PA under emergency conditions by lay personnel, paramedical personnel, or carefully schooled patients themselves would require concomitant medical surveillance and, almost certainly, telephonic transmission of electrocardiographic signals used to confirm the diagnosis of evolving infarction and to establish appropriate indications for emergency use of the material. The present study does not address the numerous precautions that will have to be taken as this concept is explored. It does address the potential molecular modifications in t-PA that can be particularly useful in conferring properties rendering the modified material more suitable for use intramuscularly and more capable of inducing therapeutically favorable plasma time-concentration curves as compared with those inducible with wild-type t-PA.

References


**KEY WORDS** • coronary thrombolysis • fibrinolysis • t-PA • plasminogen activators
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