Characterization of a Modified Human Tissue Plasminogen Activator Comprising a Kringle-2 and a Protease Domain*

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To study structure/function relationships of tissue plasminogen activator (t-PA) activity, one of the simplest modified t-PA structures to activate plasminogen in a fibrin-dependent manner was obtained by constructing an expression vector that deleted amino acid residues 4–175 from the full-length sequence of t-PA. The expression plasmid was introduced into a Syrian hamster cell line, and stable recombinant transformants, producing high levels of the modified plasminogen activator, were isolated. The resulting molecule, mt-PA-6, comprising the second kringle and serine protease domain of t-PA, produced a doublet of plasminogen activator having molecular masses of 40 and 42 kDa. The one-chain mt-PA-6 produced by cultured Syrian hamster cells was purified in high yield by affinity and size exclusion chromatography. The purified mt-PA-6 displayed the same two types of microheterogeneity observed for t-PA. NH2-terminal amino acid sequencing demonstrated that one-chain mt-PA-6 existed in both a GAR and a des-GAR form. Purified mt-PA-6 also existed in two glycosylation forms that accounted for the 40- and 42-kDa doublet of activity produced by the cultured Syrian hamster cells. Separation of these two forms by hydrophobic interaction chromatography and subsequent tryptic peptide mapping demonstrated that both forms contained N-linked glycosylation at Asn448; in addition, some mt-PA-6 molecules were also glycosylated at Asn164. Plasmin treatment of one-chain mt-PA-6 converted it to a two-chain molecule by cleavage of the Arg270-Ile271 bond. This two-chain mt-PA-6, like t-PA, had increased amidolytic activity. The fibrinolytic specific activities of the one- and two-chain forms of mt-PA-6 were similar and twice that of t-PA. The plasminogen activator activity of one-chain mt-PA-6 was enhanced >80-fold by CNBr fragments of fibrinogen, and the one-chain enzyme lysed human clots in vitro in a dose-dependent manner. The ability to produce and purify a structurally simple plasminogen activator with desirable fibrinolytic properties may aid in the development of a superior thrombolytic agent for the treatment of acute myocardial infarction.

Plasminogen activators are a unique class of enzymes that

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The abbreviations used are: t-PA, tissue plasminogen activator; mt-PA-6, recombinant modified tissue plasminogen activator-6; IU, international fibrinolytic unit; AU, amidolytic unit; KIU, kallikrein International Units; TPCK-trypsin; D, dihydrofolate reductase; HPLC, high performance liquid chromatography; HIC, hydrophobic interaction chromatography; TPCK-trypsin; t-1-tosylamide-2 phenylethyl chloromethyl ketone-treated trypsin.
PA that will still activate plasminogen in a fibrin-dependent manner. The characteristics of a modified t-PA lacking the two kringle domains but incorporating the fibrin-binding finger domain of t-PA have been described previously (13). The present study describes the characterization of another deletion mutant of t-PA expressed by cultured Syrian hamster cells. This recombinant activator comprises the serine protease domain and another fibrin binding domain of t-PA, the second kringle. Limited characterization of a similar t-PA mutant, produced by transient expression in transfected mouse Ltk- cells, has been described (10). The structural aspects and functional properties of a variety of point, deletion insertion, and substitution mutants of human t-PA have been reviewed recently (15).

EXPERIMENTAL PROCEDURES*  
RESULTS  
Cloning and Expression of mt-PA-6  
Construction of Domain-deleted t-PA—Observing exon boundaries, a synthetic oligonucleotide was used to delete the sequence coding for amino acids 4-175 of native t-PA (Fig. 1A). This deletion resulted in the generation of mt-PA-6 cDNA containing the sequences coding for the signal peptide, propeptide, second kringle, and serine protease domains of native t-PA (Fig. 1B). The mt-PA-6 cDNA was inserted into a multicistronic vector in which the expression of mt-PA-6 cDNA was driven by the ElA-responsive RI transcriptional unit (22). The resulting plasmid pT6-hd, also contained cistrons for the expression of the cDNAs for hygromycin phosphotransferase and DHFR, both driven by the SV40 early promoter/enhancer.

Expression of mt-PA-6 and Amplification of pT6 hd  
The expression plasmid pT6-hd was introduced into an adenovirus-induced Syrian hamster tumor line (AV12-664), and stable transformants were selected as described under "Methods." The average level of mt-PA-6 secreted from the stable cell lines was 220 IU/10^6 cells/day, with a range of 35-1100 IU/10^6 cells/day. Although the AV12-664 cell line is a dihydrofolate reductase-producing line, we were able to use the murine dihydrofolate reductase cDNA as a dominant selectable marker in this cell line by plating transfected cells in 500 nM MTX. The resulting MTX-resistant colonies were pooled and subjected to growth in stepwise increasing concentrations of MTX. With increasing resistance to MTX, we observed a concomitant increase in the level of secreted mt-PA-6, suggesting that coamplification of the dihydrofolate reductase and mt-PA-6 sequences had occurred. One clone, T65B5, isolated at 200 μM MTX, was chosen for further study of the secreted mt-PA-6. Additional gene-amplified clones, isolated at 500 μM MTX, produced 20,000-24,000 IU/10^6 cells/day. The clone T6A1 was selected for use in the fluidized bed reactor for production of the culture medium used for the purification of mt-PA-6.  

By Southern blot analysis, we determined that T65B5 contained approximately 35 copies of both DHFR and mt-PA-6 cDNAs and by Northern blot analysis produced approximately 30 times more mt-PA-6 than a transformant with a single copy of the mt-PA-6 gene (data not shown). Clone T65B5 produced approximately 12,000 IU/10^6 cells/day. In roller bottle culture (850 cm², 200-ml medium), this clone was capable of producing titers as high as 117 Kg/ml in serum-free growth medium. The amplicon in the gene-amplified T65B5 was found to be genetically stable; no decrease in mt-PA-6 gene dosage was observed in the absence of selection for at least 11 serial passages.

Characterization of the mt-PA-6 Activity Secreted from Clone T65B5—To characterize further the mt-PA-6 activity secreted by clone T65B5, serum-free conditioned culture medium was subjected to SDS-PAGE. Under reducing conditions (Fig. 2A, lane 2), two major proteins were detected in the conditioned medium, displaying apparent molecular masses of 40 and 42 kDa, which were the expected size for the mt-PA-6 construction. By Western blot analysis, using a poly-
clonal antibody against native t-PA (Fig. 2A, lanes 3 and 4), and by fibrin autography to detect fibrinolytic activity (Fig. 2B), the same doublet of bands at 40 and 42 kDa was observed in the conditioned medium, indicating that the mt-PA-6 molecule was secreted in the form of a one-chain doublet. To determine if the doublet represented variation in glycosylation, mt-PA-6 secreted from tunicamycin-treated T65B5 cells was compared to that from nontreated cells by Western blot analysis. As shown in Fig. 2C, a single form of mt-PA-6, migrating at an apparent molecular mass of approximately 37 kDa, was secreted from the tunicamycin-treated cells, suggesting that the doublet at 40 and 42 kDa represented glycosylation heterogeneity. This was confirmed in the biochemical analysis of the purified protein described below.

Purification of mt-PA-6 from Conditioned Cell Culture Medium

The recombinant plasminogen activator mt-PA-6 was purified in high yield from 75 to 100-liter batches of conditioned medium having enzyme titers of 15,000–30,000 IU/ml. An immobilized p-aminobenzamidine-agarose column was used to select for active enzyme in the large volumes of culture medium which were being processed. Recoveries from this column were high (90 ± 5% in 10 runs) with a concurrent 10 to 15-fold reduction in volume. Increased binding of mt-PA-6 to the immobilized p-aminobenzamidine column was observed if the column was equilibrated at pH 9.0; the mt-PA-6 was eluted from the column more efficiently, however, by pH 8.0 buffers. The observation that deletion mutants of t-PA that contained the second kringle domain bound to lysine-Sepharose (31) was exploited to purify mt-PA-6 further. Chromatography of the diaffiltered pool from the immobilized p-aminobenzamidine column on lysine-Sepharose gave a similar 90 ± 5% recovery of mt-PA-6 activity with a 6-fold volume reduction. The mt-PA-6 could be eluted from the lysine-Sepharose column with 6-amino-n-hexanoic acid, lysine, or arginine. Arginine was most frequently used, since it increased the solubility of the mt-PA-6 at pH 8.0. The final chromatography of the mt-PA-6 on a Sephadex G-75 column equilibrated at pH 3.0 separated the mt-PA-6 from both high molecular weight contaminants and the aprotinin used during the earlier affinity chromatography. The high recovery of activity from this step gave an overall yield of mt-PA-6 from the three-step purification procedure of 70%. The preparations of purified mt-PA-6 were very soluble in the pH 3.0 buffer (>5 mg/ml) and could be stored frozen at −20 °C for extended periods of time without losing enzymatic activity.

Characterization of mt-PA-6

The average specific activity of mt-PA-6 purified in the above manner was 1.2 ± 0.2 × 10^6 IU/mg (n = 10). Titration of the purified enzyme with dansyl-Glu-Gly-Arg-chloromethyl ketone determined a ratio of 0.90 ± 0.04 mol of active site/mol of mt-PA-6 (n = 10). SDS-PAGE analysis (Fig. 3) indicated that >90% of the mt-PA-6 was purified as a one-chain form and would remain in that form when stored for 24 h at 37 °C at pH 3.0 (lanes 2 and 12). However, when incubated under the same conditions at pH 8.5, multichain forms of the enzyme became apparent (lanes 3 and 13). This multichain conversion was inhibited if 0.2 M arginine was included in the incubation buffer (lanes 4 and 14). Limited plasmin digestion of one-chain mt-PA-6 (lanes 5 and 15) resulted in conversion of the enzyme to what appeared to be a two-chain form. This digestion by plasmin was not inhibited by 0.2 M arginine (lanes 6 and 16), but was partially blocked by aprotinin (lanes 7 and 17). The conversion of mt-PA-6 to multichain forms after incubation at pH 8.5 was not inhibited by a similar level of aprotinin (lanes 8 and 18).

When one-chain mt-PA-6 was subjected to NH2-terminal sequence analysis for 20 cycles (data not shown), two predominant amino acids were seen at each cycle. After assigning the amino acids at each cycle to one of two sequences based on the anticipated sequence from identity with t-PA, one NH2-terminal sequence, GARSYQQNSDXYFGNGSAYRGTH, corresponded to that anticipated from normal cellular processing of the NH2 terminus of the pro-form of mt-PA-6. The second sequence observed, SYQQNSDXYFGNGSAYR, lacked the GAR NH2-terminal sequence, indicating either a difference in NH2-terminal processing or further proteolytic digestion of the full-length mt-PA-6 in the culture medium. To determine if the AV12-664 cell line processed the mt-PA-6 molecule to yield both the GAR and the desGAR NH2-terminal sequence, mt-PA-6 producing cells were grown in serum-free medium with 10 KIU/ml of aprotinin, and the mt-PA-6 in the crude medium was isolated by PAGE, blotted to a polyvinylidene difluoride membrane, and the mt-PA-6 band subjected to NH2-terminal analysis. Only the mt-PA-6 with the GAR NH2-terminal sequence was detected in the crude medium at 6 and 18 h of culture, indicating the variation in
GAR NH₂ terminus content of the purified mt-PA-6 (20 to 80%) was not a result of different levels of processing of the proform by the AV12-664 cell machinery. The desGAR sequence of the mt-PA-6 in the culture medium was increased to a level of ~20% of the total following incubation of the crude medium for 48 h at 37°C. The appearance of desGAR mt-PA-6 in the culture medium of large-scale reactors could be minimized by chilling and rapid processing of the culture medium as well as by incorporating higher levels of aprotinin in the culture medium and pH 5.0 purification buffers. When plasmin-treated purified mt-PA-6 was subjected to NH₂-terminal sequence analysis for 19 cycles (data not shown), no GAR NH₂-terminal sequence was observed, indicating a plasmin-mediated removal of the GAR sequence. Moreover, in addition the anticipated SYQGNSDXYFGNGSAYRGT sequence, a second sequence, IKGLFDIASHPWQAIF, was observed. Based on sequence identity with t-PA, this indicated a plasmin-mediated cleavage at Arg²⁷⁵-Ile²⁷⁶, resulting in a two-chain form of mt-PA-6.

The activation of plasminogen by one-chain mt-PA-6 was enhanced by fibrin mimics such as CNBr fragments of fibrinogen (Fig. 4). In the absence of the fibrin mimic, little activation of plasminogen was observed when the activity of liberated plasmin was measured with S2251 over a 6-h period. However, when the CNBr fragments of fibrinogen were included in the plasminogen activation mixture, a >80-fold increase in plasmin activity was observed over the final linear phase of the activation.

The fibrinolytic and amidolytic activities of the one- and two-chain forms of both t-PA and mt-PA-6 are shown in Table I. Like t-PA, mt-PA-6 lost little fibrinolytic activity when it was converted with plasmin from a one- to a two-chain form by cleavage at Arg²⁷⁵-Ile²⁷⁶ but the amidolytic activity of the two-chain enzyme using S2288 was enhanced about 4-fold. The stimulatory effect of CNBr fragments of fibrinogen on the ability of both enzymes to activate plasminogen was also decreased when the one-chain forms were converted to two-chain forms. This resulted from an increase in the activation of plasminogen by the two-chain forms in the absence of the fibrin mimic, whereas the activity in the presence of the CNBr fibrinogen fragments was the same for both the one- and two-chain forms of t-PA and mt-PA-6 (data not shown).

When one-chain mt-PA-6 was chromatographed on a hydrophobic interaction chromatography column, two peaks of enzyme activity were eluted from the column by the isocratic elution with 0.05 M potassium phosphate, pH 7.0 (Fig. 5). These two peaks were collected, concentrated, and analyzed by SDS-PAGE. Visualizing with either staining or fibrin autography, the two active pools were found to correspond to two-chain forms of both the one- and two-chain forms of t-PA and mt-PA-6 (data not shown).

**Table I**

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<thead>
<tr>
<th>Enzymatic activity of plasminogen activators</th>
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<td>IU/mg, AU/mg, and fibrin dependence (F*/F) were determined as outlined under &quot;Methods.&quot; The one-chain t-PA and mt-PA-6 contained ≤10% two-chain enzyme as determined by NH₂-terminal amino acid analysis. The two-chain forms were generated by plasmin digestion and contained &lt;5% of the one-chain enzyme.</td>
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<td><strong>Enzyme</strong></td>
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The ability of mt-PA-6 to lyse clots formed from freshly drawn human blood containing ¹²⁵I-labeled fibrinogen was determined. In Fig. 7 the in vitro lysis of ¹²⁵I-labeled clots by t-PA (Fig. 'A) and mt-PA-6 (Fig. 'B) is shown as a percent of initial thrombus radiolabel released into the surrounding human plasma by the lytic process. Only 80% of the human clot could be dissolved by either enzyme with increasing dose levels. At the two lower dose levels of mt-PA-6, a lag was observed over the first hour of the in vitro fibrinolysis before a linear dissolution of the clot occurred over the final 5-h
period. This lag was not observed at the two higher dose levels of mt-PA-6. Comparing the dissolution of the human clot caused by 200 IU of t-PA with that caused by 400 IU of mt-PA-6, it appears that mt-PA-6 has about 50% of the in vitro fibrinolytic activity of t-PA in this clot lysis system. However, since the specific activity of mt-PA-6 is approximately twice that of the t-PA used in these studies, their clot-lysis activity is equivalent on a mass basis.

**DISCUSSION**

The enzymatic properties of t-PA have been extensively investigated in both in vitro and in vivo systems. Since the molecule contains four distinct structural domains in the heavy chain in addition to the serine protease domain in the light chain, the study of the contribution of these individual domains to the unique fibrinolytic properties of this plasminogen activator has led investigators to construct deletion mutants of t-PA by linking the sequences coding for the signal peptide and various combinations of the four different domains of the heavy chain with the light chain serine protease domain. A number of such mutants have been expressed transiently in mouse Ltk- cells (10). The deletion mutants present in the culture medium were characterized for their enzymatic activity as well as their ability to bind to fibrin. From these studies it was apparent that the fibrin binding properties of t-PA, which distinguish it from the plasminogen activator urokinase, are mediated by the finger and second kringle domains present in the heavy chain of the t-PA molecule. However, in another study (9) in which specific domains were deleted from full-length t-PA, the mutants lacking either the first or second kringle were equally stimulated by fibrin when their ability to activate Lys-plasminogen was measured, whereas the mutant lacking both kringles was not stimulated. Such differences in ascribing function to specific domains must be interpreted with caution since fibrin binding may be greatly affected by changes in the conformation of the kringles in the deletion mutants as compared with their conformation in full-length t-PA. In addition, both of these studies were done with crude t-PA derivatives in culture medium, rather than with the purified molecules.

To begin an evaluation of purified mutant tissue plasminogen activators as potential in vivo clot lysis agents, we constructed some of the simpler molecules which we felt would activate plasminogen in a fibrin-dependent manner. In the present study, we have evaluated a mutant t-PA comprising the second kringle and protease domains of the native molecule. This construction was similar but not identical to the previously described LK2 variant (10) that lacked amino acids 1–3 present in mt-PA-6 at its NH2 terminus and included the last 11 amino acids of the first kringle domain, including 2 unpaired cysteine residues.

Following the construction of the plasmid for mt-PA-6 and its expression in Syrian hamster cells, the enzyme was purified to homogeneity on a large scale to provide sufficient quantities for further characterization. This purified mt-PA-6 was both homogeneous and predominantly in the one-chain form as assessed by SDS-PAGE. However, two forms of structural heterogeneity similar to those observed for t-PA were found when mt-PA-6 was further characterized. Like t-PA (33), different preparations of mt-PA-6 had varying levels of NH2-terminal amino acid sequence heterogeneity. One form had a GAR NH2 terminus, whereas the second form lacked this NH2-terminal sequence of amino acids. No differences were observed in the in vitro activity parameters of these two NH2 terminus forms of mt-PA-6 (data not shown). In addition to the two forms of mt-PA-6 resulting from NH2-terminal amino acid sequence heterogeneity, mt-PA-6 was found to exist in two glycosylation forms. The mt-PA-6 molecule, like t-PA (34), was found to be fully glycosylated at Asn117 in the serine protease domain, but only 10–20% of the molecules were also glycosylated at Asn134. Another similarity to t-PA was observed when mt-PA-6 was subjected to limited plasmid digestion. The plasmin cleavage site, Arg2–Ile3, of the two-chain form of mt-PA-6 was identical to that observed for t-PA (34), and the amidolytic activity of the two-chain form of mt-PA-6 was increased significantly (35).

The ability of purified mt-PA-6 to activate plasminogen was stimulated over 80 x by the inclusion of CNBr fragments of fibrinogen. This agrees with the earlier observation that the plasminogen activation of the similar K2P mutant from transient cell expression (10) was stimulated by a similar fibrin mimic. One explanation for the lag in the generation of plasmin observed during the first 2 h of plasminogen activation by mt-PA-6 is the two-phase mechanism of fibrinolysis proposed by van Zonneveld et al. (31). The binding of mt-PA-6 to fibrin or to fibrin mimics, such as CNBr fragments of fibrinogen, is mediated only by the second kringle domain and may be initially weak, since mt-PA-6 lacks the high fibrin-affinity finger domain. As plasmin is initially generated, however, newly formed lysine binding sites are generated in the CNBr fibrinogen fragments that then bind the second kringle domain of mt-PA-6 with greater avidity, resulting in the higher rate of plasminogen activation observed during the final linear phase of the reaction.

When the fibrinolytic activity of mt-PA-6 was compared with that of t-PA using freshly formed human clots suspended in plasma, the mass of mt-PA-6 needed to achieve a comparable rate of fibrin clot lysis was the same as that for t-PA. However, a lag in the lysis of the clot by the lower doses of mt-PA-6 was observed during the early phase of the experiment. One explanation for this lag may again be the need to generate newly formed cleavage sites in the fibrin clot by the plasmin first generated by plasminogen activation. Once a sufficient number of newly formed lysine COOH-terminal residues appear in the partially degraded fibrin, more binding of the second kringle domain of mt-PA-6 to the fibrin will occur, resulting in a faster rate of plasminogen activation.

The present study with a highly purified preparation of a modified t-PA comprising the second kringle and serine protease domains of the full-length molecule extends the initial observation (10) that a transiently expressed mutant of similar structure will activate plasminogen in a fibrin-dependent manner. The fibrinolytic activities of the purified one and two-chain forms of mt-PA-6 are similar, and the amidolytic activity of the two-chain form, like t-PA, is greater than that of the one-chain form. The fibrinolytic specific activity, however, of mt-PA-6 is nearly twice that of t-PA. Finally, it has been demonstrated that purified mt-PA-6 will effect the in vitro dissolution of a clot of human fibrin in a dose-dependent manner.

The deletion from mt-PA-6 of both the finger/epidermal growth factor domains as well as the first kringle domain with the requisite glycosylation site at Asn117 would predict that mt-PA-6 should have a longer in vivo half-life than t-PA, since either mutation of the high mannose glycosylation site at Asn117 (37) or deletion of the finger/epidermal growth factor domains of t-PA (38) has resulted in modified t-PA structures with prolonged plasma half-lives. Indeed, early experiments (39) have indicated that the plasma half-life of mt-PA-6 in dogs was almost 20 times longer than that of t-PA. Moreover, when the clot lysis activity of mt-PA-6 and t-PA was compared in a canine model of coronary artery thrombosis (40),
the time to reperfusion was shorter with mt-PA-6, and better maintenance of flow after reperfusion was achieved. Thus, it appears that mt-PA-6 may have fibrinolytic properties that make it a potential thrombolytic agent for the clinical treatment of acute myocardial infarctions.

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REFERENCES

20. Continued on next page.
Materials

The modified PAGE for p-PA was used to determine the molecular weight of the fusion protein, 64, 43, or 31 kDa. The modified PAGE was developed according to the procedure of Miller and Sambrook (17). The gel composition was 20% acrylamide, 0.375 M Tris borate, and 0.01 M sodium lauryl sulfate. The modified PAGE was stained with Coomassie Blue and the samples applied to the gel as indicated in the figure caption. The modified PAGE was run at 200 V for 2 h, and the protein bands were visualized by Coomassie Blue staining. The modified PAGE was run at 200 V for 2 h, and the protein bands were visualized by Coomassie Blue staining. The modified PAGE was run at 200 V for 2 h, and the protein bands were visualized by Coomassie Blue staining.

Results

Construction and Expression of p-PA

The cDNA for p-PA was inserted into the expression vector pgEM-4, which was then transformed into Escherichia coli strain DH5a. The resulting plasmid, p-PA, was linearized with restriction enzymes and used for transfection. The resulting plasmid was then used to transform E. coli strain DH5a, and the transformants were selected on LB agar plates containing ampicillin.

Construction of expression vectors for p-PA

The expression vectors for p-PA were constructed by inserting the p-PA cDNA into the expression vectors described above. The resulting plasmids were then used to transform E. coli strain DH5a, and the transformants were selected on LB agar plates containing ampicillin.

Secondary structure

The secondary structure of the mature p-PA was determined by circular dichroism (CD) spectroscopy. The CD spectra were recorded at 20°C in 10 mM Tris-Cl, pH 7.5, using a Jasco J-720 CD spectropolarimeter. The CD spectra were recorded at 20°C in 10 mM Tris-Cl, pH 7.5, using a Jasco J-720 CD spectropolarimeter. The CD spectra were recorded at 20°C in 10 mM Tris-Cl, pH 7.5, using a Jasco J-720 CD spectropolarimeter.

Cell lines, RNA Transfection, and Drug Selection

The p-PA cDNA was transfected into the HEK293 cell line. The HEK293 cell line was transfected with p-PA cDNA using the Lipofectamine 2000 reagent (Invitrogen). The transfected HEK293 cells were selected for proliferation in the presence of 1 μg/ml puromycin. The transfected HEK293 cells were selected for proliferation in the presence of 1 μg/ml puromycin. The transfected HEK293 cells were selected for proliferation in the presence of 1 μg/ml puromycin.

Pharmacological Analysis

The pharmacological activity of p-PA was determined by measuring the inhibitory activity of p-PA on the plasminogen activator inhibitor (PAI-1) activity. The PAI-1 activity was measured in a plasminogen activator inhibition assay using human plasminogen. The PAI-1 activity was measured in a plasminogen activator inhibition assay using human plasminogen. The PAI-1 activity was measured in a plasminogen activator inhibition assay using human plasminogen.

Figure 3. Zymosan A-induced and inhibited platelet aggregation

The figure shows the effects of p-PA on platelet aggregation. The platelet aggregation was measured using a light transmission aggregometer. The aggregation was measured using a light transmission aggregometer. The aggregation was measured using a light transmission aggregometer.

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A Modified t-PA Comprising Kringle-2 and Protease Domains

Figure 4. Plasminogen activation by ml-PA-6 in the presence and absence of a fibrin stimulator. The activation of glu-plasminogen is determined by the ability of the liberated plasmin to cleave S2251 in this coupled assay using the conditions described in Methods. (A) S2251 activity in the absence of CNBr fibrinogen fragments, (B) S2251 activity in the presence of the fibrin mimic.

Figure 6. Tryptic peptide maps of the two glycosylation forms of ml-PA-6. A) HPLC profiles of tryptic peptides of M) monoglycosylated and D) diglycosylated ml-PA. B) HPLC profiles of tryptic peptides of M) monoglycosylated and D) diglycosylated ml-PA-6 after treatment with glycopeptidase F. The conditions for the HPLC are described in Methods.

Figure 7. In vitro fibrinolysis of human thrombi by A) t-PA and B) ml-PA-6. Preformed 125I labeled human plasma clots were incubated in the presence of A) (C) 200 IU, (D) 400 IU, (E) 800 IU, and (F) 2000 IU t-PA, B) (G) 400 IU, (H) 800 IU, (I) 2000 IU, and (J) 4000 IU ml-PA-6. The doses indicated are the final cumulative dose obtained by adding either ml-PA-6 or t-PA in equal doses at hourly intervals from 0 to 5 hours. Aliquots were taken at the indicated time intervals, and the progress of clot lysis determined by measuring the release of 125I into the plasma. Four determinations were made at each of the four levels of ml-PA-6 and t-PA.
Characterization of a modified human tissue plasminogen activator comprising a kringel-2 and a protease domain.

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