Catabolism of Human Tissue Plasminogen Activator in Mice

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The catabolism of human tissue plasminogen activator (t-PA) was studied in mice. The clearance of t-PA labeled with iodine 125 was rapid (t1/2). The clearance of phenylmethylsulfonyl-125I-t-PA, which is active site-inhibited, was identical to the active enzyme. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated that the vast majority of 125I-t-PA injected into the circulation was present as free enzyme and not in a complex with inhibitors. The clearance of 125I-t-PA was unaltered by large molar excesses of several ligands of known clearance specificities, including macroalbumin, asialoorosomucoid, and diisopropylphosphorylthrombin and was also not altered in the presence of a 1,000-fold molar excess of unlabeled t-PA. Organ distribution studies demonstrated that the early rapid clearance of 125I-t-PA occurred in hepatocytes, followed by a later renal phase of clearance. The clearance of 125I-urokinase (UK) also was studied and was very similar in all aspects to the clearance of 125I-t-PA. These results suggest that both t-PA and UK are cleared from the circulation by unique nonsaturable processes localized in the liver that are independent of the proteinase active site.

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The activation of plasminogen to plasmin by tissue plasminogen activator (t-PA) is the central reaction in the extrinsic fibrinolytic system. t-PA has been isolated from numerous tissue extracts and human melanoma cell culture. It appears that this enzyme is identical to the vascular plasminogen activator that is found in endothelial cells and released into the blood following several stimuli. Fibronolysis may thus be regulated by the release of t-PA into the blood, removal from the blood, and modulation of t-PA activity by specific molecular interactions with other components of the fibrinolytic system, including potential inhibitors.

The presence of specific inhibitors of t-PA in blood is a matter of dispute. Early studies indicated that there was no specific inhibitor of t-PA, although α2-antiplasmin, α2-macroglobulin, and α,1-proteinase inhibitor inactivate t-PA at extremely slow rates. Recently, a fast-acting inhibitor present at very low concentrations in plasma has been described.

The in vivo clearance of t-PA from the blood is rapid (t1/2 approximately two minutes) with accumulation of t-PA in the liver. The mechanism of this clearance is poorly understood. Proteinase complexes of the plasma proteinase inhibitors clear from the circulation through hepatic receptors. In addition, several carbohydrate-mediated hepatic clearance mechanisms for glycoproteins have been described. We have previously reported altered proteinase inhibitor-proteinase interactions in vivo as compared to results obtained in vitro. Therefore, we chose to study the catabolism of t-PA and the role of the plasma proteinase inhibitors and carbohydrate receptors in this catabolism using our previously described mouse model.

MATERIALS AND METHODS

The human melanoma G-361 cell line was obtained from American Type Culture Collection, Rockville, Md. p-Aminobenzamidine-agarose was obtained from Pierce Chemical Company, Rockford, Illinois; phosphocellulose P-11 was obtained from Whatman Inc., Clifton, N.J. Bolton-Hunter reagent labeled with iodine 125 was purchased from New England Nuclear, Boston, and the colorimetric substrate (H-n-valyl-l-leucyl-l-lysine-p-nitroanilide - H2O HCl (S-2251) from Helena Laboratories, Beaumont, Tex. Disopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were obtained from the Sigma Chemical Company, St. Louis, Missouri. CD-1 female mice were obtained from Charles River Laboratory, Wilmington, Mass, and high molecular weight (mol wt) urokinase (UK) was obtained from Calbiochem-Behring, San Diego.

Human α1-proteinase inhibitor and α2-macroglobulin were purified from plasma, and their trypsin complexes were prepared as previously described. Human α-thrombin (specific activity, 2,700 U/mg) and diisopropylphosphoryl-(DIP)-thrombin were prepared as previously described. DIP-Urokinase was prepared similarly. Asialoorosomucoid (ASOR) and macroalbumin were prepared as previously described. All other reagents were of the best commercial grade available.

Purification of t-PA

t-PA was purified from serum-free conditioned medium from human melanoma G-361 cells as described by Rijken and Collen, with the following modification. t-PA activity from 4 L of conditioned medium was adsorbed on a p-aminobenzamidine-agarose column (0.9 x 1.5 cm) instead of a zinc-chelate column. After washing the column with 0.05 mol/L Tris-HCl, 0.4 mol/L NaCl, 0.001 mol/L EDTA, 0.1% Triton X-100, 0.5 mol/L t-PA activity was eluted in the same buffer containing 0.5 mol/L benzamidine, 4 mol/L urea. This material was dialyzed overnight into 0.01 mol/L sodium phosphate, 1.0 mol/L NaCl, pH 7.4. Subsequent steps were performed as described. The final preparation was judged to be...
>95% pure, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and staining with Coomassie blue.

**Assay of t-PA Activity**

Because no standard presently exists for t-PA, activity is stated in reference to an international unit (IU) of M, = 33,000 urokinase (UK) using a fibrin 125 l plate assay as described previously. The purified t-PA had a specific activity of 230,000 IU/mg.

**125I-Labeling of t-PA**

An aliquot (10 μL) of t-PA containing 3 μg protein in 0.02 mol/L sodium phosphate, 1 mol/L NaCl, pH 8.0 was labeled with 500 μCi of Bolton-Hunter reagent 125I (4,400 Ci/mmol) for one hour at 0 °C. Enzyme labeled with iodine 125 was separated from unincorporated label by dialyzing the sample with 0.5 mL 0.1 mol/L Tris-HCl, 0.5 mol/L urea, 0.001 mol/L EDTA, and 0.1% Triton X-100, pH 6.5 and adsorbing the enzyme on a 0.2-mL phosphocellulose column. The column was washed with the dialution buffer, and the labeled t-PA was eluted in 0.5 mL 0.1 mol/L Tris-HCl, 1 mol/L NaCl, 4 mol/L urea, 0.001 mol/L EDTA, 0.1% Triton X-100, pH 8.0. The preparation labeled with iodine 125 retained full enzymatic activity as assayed by the S-2251 chromogenic assay, with a specific radioactivity of 3 x 10^6 cpm/μg.

**Inactivation of 125I-t-PA**

Inactivation of 125I-t-PA was performed by incubating an aliquot of 125I-t-PA with 0.01 mol/L PMSF in 0.025 mol/L Tris-HCl, 0.15 mol/L NaCl, 5% isopropanol, pH 8.8, overnight at 0 °C. The preparation was dialyzed for eight hours against the same buffer without PMSF, followed by overnight dialysis into 0.02 mol/L sodium phosphate, 0.5 mol/L NaCl, pH 7.40. By use of this technique, approximately 90% inhibition of the 125I-t-PA was achieved, as assessed by an S-2251 chromogenic assay.

**Mouse Plasma**

Mouse plasma for in vitro studies was obtained by incising anesthetized mice in the midline and cannulating the inferior vena cava. Blood was drawn into syringes with 5% sodium citrate and centrifuged immediately. The plasma was drawn off and used for experiments within one hour of bleeding.

**In Vitro Plasma Studies**

Citrated human or mouse plasma (500 μL) was incubated with 0.5 to 1.0 μg of 125I-t-PA at 37 °C. At various times, 25-μL aliquots were removed, denatured in 40 μL 10% SDS sample buffer, and subjected to SDS-PAGE as described by Weber and Osborn. Autoradiograms were prepared from dried gels as previously described.

**Plasma Elimination Studies**

Plasma elimination studies of 125I-t-PA or UK 125I (0.3 to 1.0 μg) alone or in the presence of large molar excesses of unlabeled proteins were performed in CD-1 female mice as previously described. In some studies, blood samples were precipitated in 1 mL 5% trichloroacetic acid (TCA), and the precipitates were then counted for radioactivity. In other studies, blood samples were denatured in SDS and subjected to SDS-PAGE and autoradiography as described above. All experiments were performed at least in quadruplicate, except for competition experiments, which were performed in duplicate.

**Tissue Distribution Studies**

Organ distribution studies were performed as previously described after the injection of 1.0 μg 125I-t-PA or UK 125I.

**RESULTS**

**Plasma Elimination of t-PA**

The clearance of 125I-t-PA was rapid, with a t1/2 of approximately three minutes (Fig 1A). Studies performed with TCA precipitation of blood samples yielded identical results throughout a 15-minute period, after which the TCA-precipitable counts continued to decrease with time, whereas the total counts in the blood samples reached a plateau and began to increase (data not shown). These results are consistent with the reappearance of degradation peptides from 125I-t-PA in the circulation 15 minutes after injection of 125I-t-PA. The clearance of 125I-t-PA that had been inactivated with PMSF was identical to the clearance of active 125I-t-PA (Fig 1A). This is similar to the results obtained with thrombin, factor Xa, and factor IXa, which bind to the vessel wall. In order to determine whether t-PA was binding to the endothelial binding sites described for these proteinases, PMSF-125I-t-PA was injected into mice along with a 1,000-fold molar excess of DIP-thrombin. The clearance of PMSF-125I-t-PA under these conditions (Fig 1A) was unaltered, indicating that this endothelial binding site was not involved in the rapid removal of t-PA from blood, since this molar excess of DIP-thrombin blocked the clearance of thrombin, factor Xa, and factor IXa, as described above.

![Fig 1. Clearance from the circulation of intravenously injected 125I-t-PA or PMSF-125I-t-PA. Protein labeled with 125I (0.3 to 1.0 μg) was injected into mice and blood samples were collected at intervals. (A) 125I-t-PA (●); PMSF-125I-t-PA (○); clearance of 125I-t-PA in the presence of a 1,000-fold molar excess of DIP-thrombin (△). (B) Clearance of 125I-t-PA in the presence of: 3 mg macroalbumin (●); 1 mg ASOR (○), or 1,000-fold excess of unlabeled t-PA (□); clearance of 125I-t-PA in nephrectomized mice (△). Clearance of t-PA in normal mice is shown for comparison, solid line.](www.bloodjournal.org)
SDS-Polyacrylamide Gel Electrophoresis of t-PA

The possible role of the plasma proteinase inhibitors in the catabolism of t-PA was examined, using SDS-polyacrylamide gel electrophoresis and autoradiography of samples obtained from the incubation of $^{125}$I-t-PA with human or mouse plasma in vitro or on blood samples obtained 1 minute after injection of $^{125}$I-t-PA. In human and mouse plasma in vitro, these studies demonstrated the rapid appearance of a minor component of M, approximately 110,000 (Fig 2, lanes A through H), which corresponds to a complex with the fast-acting t-PA inhibitor recently identified in plasma. These studies demonstrate the presence of an inhibitor in mouse plasma with very similar properties to those of the human protein. Assaying mouse plasma for the rapid inhibitor as described for the human protein yielded an inhibitor value in t-PA units of $0.89 \pm 0.33 \text{IU/mL}$ or $4.5 \pm 1.6 \text{ng/mL}$, assuming similar properties for the mouse and human proteins. These are comparable to the results obtained in human plasma. The in vivo sample demonstrated an essentially identical result (Fig 2, lane i) indicating that although a small amount of t-PA may be present in a complex with this fast-acting inhibitor, the greater part of the t-PA was rapidly cleared from the circulation as free t-PA. At all times during the study, t-PA remained in excess of the inhibitor. It must be emphasized that the amount of t-PA injected into the mice was far in excess of the available inhibitor and in a range relevant to thrombolytic therapy.

Plasma Elimination of t-PA in the Presence of Other Ligands of Known Clearance Specificities

In an attempt to determine the mechanism for the rapid clearance of free t-PA from the circulation, clearance studies were performed with t-PA in the presence of large molar excesses of unlabeled ligands whose clearance pathways are well characterized. The possibility that $^{125}$I-t-PA was cleared by a nonspecific reticuloendothelial system clearance mechanism was examined by injecting 0.4 µg $^{125}$I-t-PA with 3 mg macroalbumin, a nonspecific reticuloendothelial system blocking agent (Fig 1B). These studies demonstrated no alteration in the clearance of $^{125}$I-t-PA, indicating that $^{125}$I-t-PA was cleared by a different mechanism. The possibility that the $^{125}$I-t-PA was cleared by a carbohydrate-mediated mechanism as described by Ashwell and Morell was examined by injecting 0.4 µg $^{125}$I-t-PA together with 1 mg ASOR. These studies demonstrated no alteration in the clearance of $^{125}$I-t-PA and are consistent with our findings that the melanoma-derived t-PA contains complete carbohydrate chains, including terminal sialic acid residues. These results indicated that the rapid clearance of $^{125}$I-t-PA was not carbohydrate-mediated.

Because we were unable to inhibit the clearance of $^{125}$I-t-PA with ligands of known specificities, attempts were made to have t-PA compete with itself. In the presence of a 1,000-fold molar excess of unlabeled t-PA (Fig 1B), the clearance of $^{125}$I-t-PA was unaltered, indicating that t-PA is cleared from the circulation by a nonsaturable process since with all ligands studied to date, a 1,000-fold molar excess has been more than sufficient to block the clearance of labeled ligand. One mechanism for rapid, nonsaturable clearance is glomerular filtration. To examine this possibility, functional nephrectomies were performed in mice by placing ligatures around the renal vascular pedicles bilaterally and then performing clearance studies as described above. The extent of exclusion of the kidneys from the circulation was examined by comparing tissue radioactivity distributions from autopsy studies in mice with and without nephrectomy (see below). In general, the radioactivity recovered in kidney was decreased almost 100-fold by nephrectomy. The clearance of t-PA was unaltered by this procedure, indicating that the kidneys were not involved in the rapid clearance of t-PA from the circulation.

Organ Distribution Studies With t-PA

The distribution of injected $^{125}$I-t-PA among various organs at several times is shown in Table 1. These studies indicated that the early uptake of $^{125}$I-t-PA occurred in the liver and that, at later times, as TCA nonprecipitable material reappears in the circulation, this material was cleared by the kidney, with radioactivity appearing in the urine. These results also demonstrated the effectiveness of the functional nephrecto-
Fig 3. Plasma elimination of $^{125}$I-UK, DIP-$^{125}$I-UK, and $^{125}$I-t-PA. Clearance studies as described in Fig 1. (A) Clearance of UK $^{125}$I (●); DIP-UK $^{125}$I (○); and clearance of DIP-UK $^{125}$I in the presence of a 10,000-fold molar excess of unlabeled DIP-UK (□). (B) Clearance of $^{125}$I-t-PA in the presence of a 10,000-fold molar excess of unlabeled DIP-UK (●); clearance of $^{125}$I-t-PA is shown for comparison (○).

Table 1. Organ Distribution of $^{125}$I-t-PA

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<th>Percentage of Recovered Dose</th>
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<tr>
<td></td>
<td>(5 Min)</td>
<td>(30 Min)</td>
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<tr>
<td>Heart</td>
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<tr>
<td>Lung</td>
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<td>Spleen</td>
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<tr>
<td>Kidneys and urine</td>
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<td>78.4</td>
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<tr>
<td>Liver</td>
<td>76.2</td>
<td>19.7</td>
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<tr>
<td>Aorta and vena cava</td>
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Table 2. Organ Distribution of $^{125}$I-t-PA or DIP-UK $^{125}$I

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<th></th>
<th>Percentage of Recovered Dose</th>
<th>125I-t-PA</th>
<th>DIP-UK $^{125}$I</th>
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<tbody>
<tr>
<td></td>
<td>(5 Min)</td>
<td>(30 Min)</td>
<td>(30 Min)</td>
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<tr>
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DISCUSSION

There has been considerable interest recently in the clinical use of t-PA to treat patients with evolving myocardial infarction.33,34 Before t-PA is widely used as a drug in humans, the catabolism of injected t-PA must be elucidated, and the kinetics of plasminogen activity must be studied. Toward this end, we studied the catabolism of t-PA in our well-characterized mouse model of in vivo regulation of proteinase inhibitors and proteinases.11,14,20,22,30,32 $^{125}$I-t-PA retained full enzymatic activity and cleared rapidly from the murine circulation ($t_{1/2}$ approximately three minutes). This is consistent with previous studies in rabbit10 and with a recent report in humans.35 The clearance of PMSF-$^{125}$I-t-PA that has a blocked active site, and therefore cannot react with any of the plasma proteinase inhibitors, including the rapid t-PA inhibitor present in plasma,3.9 was identical to the clearance of the active enzyme. These results were confirmed by SDS-polyacrylamide gel electrophoresis of $^{125}$I-t-PA incubated with human or mouse plasma in vitro, or blood samples obtained from in vivo clearance studies. These studies demonstrated a minor band of radioactivity at $M_r$ approximately 110,000, corresponding to the reaction of a small fraction of the $^{125}$I-t-PA with the rapid inhibitor from plasma,3.9 but the vast majority of the $^{125}$I-t-PA was present as free enzyme. This suggests that t-PA is rapidly cleared from the circulation as free t-PA and not in complex with an inhibitor. This is expected, since the dose of t-PA injected is far in excess of the amount required to neutralize the inhibitor capacity of mouse plasma completely.

The mechanism of this rapid clearance of free proteinase was investigated by competition experiments with large molar excesses of unlabeled ligands of
CATABOLISM OF PLASMINOGEN ACTIVATOR

543

known clearance specificity. Thrombin, factor Xa, and factor IXa clear rapidly from the circulation via a shared endothelial binding site, independent of the proteinase active site.21,22,28 The possibility that this site may be involved in the clearance of t-PA was investigated by injecting 125I-t-PA with a large molar excess of unlabeled DIP-thrombin. The clearance of 125I-t-PA was unaltered, suggesting that this thrombin-binding site is not involved in the clearance of t-PA. Similarly, macroalbumin and ASOR failed to inhibit the clearance of t-PA, suggesting that the clearance of t-PA does not occur through a nonspecific reticuloendothelial system mechanism or through the carbohydrate-mediated clearance pathway described by Ashwell and Morell.15 Competition studies with large molar excesses of unlabeled t-PA failed to alter the clearance of 125I-t-PA, indicating that the mechanism for the rapid clearance of t-PA was not saturable. This is a novel result, as all proteinases studied to date, including trypsin, plasmin, thrombin, factor Xa, and factor IXa clear by saturable processes.21,22,28,36 It is conceivable that the system would be saturable at some higher competing concentration of t-PA. However, the amount injected is equivalent to a dose of 15 mg/kg body weight in mouse (or 1 g in humans) and the point appears moot. Moreover, nonsaturable uptake of t-PA has been observed in tissue culture studies with fibroblasts.37

In order to determine the site of clearance of t-PA, autopsy studies were performed, which demonstrated that the early rapid removal from the blood of t-PA occurred in the liver with a later renal phase of clearance probably involving degradation peptides released into the circulation by the liver. These results are consistent with the results obtained in humans.35 Autoradiographic studies demonstrated that the hepatocyte was the cell-type responsible for the rapid clearance of t-PA from the circulation. Organ distribution studies were consistent with these conclusions since nephrectomy did not influence the early, rapid clearance phase.

The clearance of t-PA was also compared to the clearance of another plasminogen activator, human UK. The clearances of UK 125I or DIP–UK 125I were identical, again mediating against the involvement of proteinase inhibitors in the clearance of UK. In addition, the clearance of UK 125I was not altered by large molar excesses of unlabeled DIP–UK, suggesting that UK also is cleared from the circulation by a nonsaturable process. Autopsy studies with UK 125I also demonstrated an early hepatic phase of clearance, followed by a later renal phase. The clearance of 125I-t-PA was not altered by a 10,000-fold molar excess of DIP–UK. The behavior of both t-PA and urokinase are quite different from our previous observations with the other therapeutic fibrinolytic agent, streptokinase.30 Streptokinase–plasmin catabolism is regulated by transfer of the plasmin moiety to α2-macroglobulin and rapid hepatic removal of a degraded form of streptokinase.30 This process is saturable by contrast to the data obtained with t-PA and UK.

These studies demonstrate that both t-PA and UK are cleared from the circulation by similar nonsaturable processes that do not require the proteinase active site, and that are located in liver. Subsequent to this hepatic phase of clearance, degraded material is released into the circulation to be excreted by the kidney. The details of the pathways by which t-PA and UK are catabolized still must be elucidated. Because these pathways are nonsaturable, however, this information may be extremely difficult to obtain. It must be emphasized that the conclusions reached in this study are most relevant to the situation that pertains in clinical use. The initial concentration of t-PA achieved in vivo in mice was 0.05 mg/kg body weight, which is comparable to the dose levels achieved in human studies of 0.07 to 2.0 mg/kg body weight.34 If t-PA and UK clears by a nonsaturable clearance mechanism in humans, it may be of importance in therapeutic situations. At any given infusion rate of activator, this hypothesis predicts that there will not be an accumulation of the agent in the circulation. It is conceivable that under physiological conditions the catabolism of t-PA may be mediated, in part, by the rapid-acting inhibitor. This question can only be addressed by study of the catabolism of purified inhibitor–t-PA complexes.

REFERENCES

7. Rijken DC, Juhan-Vague I, Collen D: Complexes between


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