Imaging of Thrombi With Tissue-Type Plasminogen Activator Rendered Enzymatically Inactive and Conjugated to a Residualizing Label

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Background. Contemporary cardiovascular practice relies increasingly on thrombolysis as a therapeutic modality. Its optimal use requires prompt, noninvasive delineation of thrombotic occlusion in arterial beds and rapid detection of reocclusion after initially successful thrombolysis.

Methods and Results. We have been developing an approach to noninvasively image thrombi in which plasminogen-activating properties of tissue-type plasminogen activator (t-PA) are attenuated by treatment with D-Phe-L-Pro-L-Arg-chloromethyl ketone (PPACK) and have shown that the inactive t-PA avidly and promptly binds to clots in vitro. In the present study, we conjugated this material to a residualizing label, radioiodinated dilactitol tyramine (131I-DLT), and characterized the potential use of the inactivated, conjugated t-PA as a radiopharmaceutical for imaging thrombi in vivo. The approach developed requires not only avid binding of the tracer to thrombi but also rapid clearance from plasma and a lack of prompt release of radiolabeled degradation products from the liver. The rapid clearance of unaltered or PPACK-treated t-PA was not influenced by conjugation to 131I-DLT, but the release of radioiodinated degradation products into plasma after injection of 131I-DLT–conjugated t-PA was markedly less than release of degradation products of directly radioiodinated t-PA. When 131I-DLT–PPACK–t-PA was infused for 15 minutes intravenously after a bolus injection of 20% in dogs with coronary, pulmonary, or carotid artery thrombi, clearance was rapid. Mean±SEM thrombus-to-blood ratios of radioactivity were high, ranging from 37±9:1 and 2.8±0.6:1 with carotid thrombi formed concomitantly or approximately 30 minutes before infusion of tracer, respectively, to 35:1 for concomitantly formed coronary thrombi, 42±7:1 and 8.1±0.8:1 for concomitantly formed and preformed pulmonary thrombi, respectively, and 18:1 for a preformed femoral artery thrombus. Thrombi were detectable by planar gamma scintigraphy even though image quality was affected adversely by low concentrations of radioactivity that in aggregate composed a relatively large amount of radioactivity in underlying and overlying tissues. This limitation was overcome by tomographic imaging, which was used to detect both femoral and pulmonary thrombi.

Conclusions. Use of enzymatically inactivated t-PA coupled to a residualizing label permits rapid detection and localization of thrombi in vivo. (Circulation 1992;85:288–297)

Thrombolysis is being used increasingly as primary therapy for several thromboembolic disorders. Its widespread implementation combined with the relatively high incidence of early thrombotic reocclusion in a substantial minority of patients with acute myocardial infarction treated with thrombolytic drugs (≈15%) requires rapid noninvasive detection to facilitate decisions critical in management. Several radiopharmaceutical candidates have been evaluated as potential clot-imaging agents, including 125I-labeled fibrinogen, 99mTc-labeled plasminogen.
labeled urokinase, and $^{131}$I- and $^{99m}$Tc-labeled streptokinase. However, none has exhibited sufficient sensitivity and specificity. $^{111}$In-labeled platelets provide specificity but require isolation and labeling of autologous platelets in each case. Furthermore, the long residence time of platelets in blood requires use of a second tracer to correct for radioactivity associated with circulating platelets. Radiolabeled antibodies to platelet membrane proteins and to fibrin are being evaluated, but their practical usefulness remains to be determined.

Tissue-type plasminogen activator (t-PA) binds to thrombi in the fibrin domain in interstices and on luminal surfaces of thrombi. t-PA is cleared rapidly from plasma with an $\alpha$-phase half-life in dogs of approximately 2 minutes. Accordingly, its properties are attractive for imaging thrombi. Unfortunately, as was evident in studies with $^{111}$In-labeled t-PA, the serine protease activity leads to lysis of the thrombus being imaged and limits image quality obtainable with radiolabeled native t-PA. Furthermore, release of radiolabeled degradation products into the blood after rapid clearance of t-PA and hepatic catabolism degrades image quality by increasing the ratio of radioactivity in blood compared with that in the thrombus.

To overcome these difficulties, we prepared enzymatically inactive t-PA and conjugated it to a residualizing radiolabel that remains entrapped at the site of catabolism. t-PA was inactivated irreversibly with D-Phe-L-Pro-L-Arginyl-chloromethyl ketone (PPACK). Enzymatically inactive t-PA was conjugated to radioiodinated dilactitol tyramine ($^{125}$I-DLT) to facilitate entrapment of radiolabeled degradation products in intracellular sites of catabolism as applied in our previous studies of metabolism of diverse plasma proteins, including albumin, lipoproteins, immunoglobulins, and asialofetuin. The enzymatically inactive, conjugated material was administered intravenously to rabbits and dogs to define its potential usefulness as an imaging agent for prompt, noninvasive detection of thrombi in vivo.

Methods

Materials

Recombinant t-PA was obtained from Genentech Corp., San Francisco, Calif.; Iodogen from Pierce Chemical Co., Rockford, Ill.; galactose oxidase from Worthington Biochemical Corp., Freehold, N.J.; and sodium cyanoborohydride, galactose oxidase, and all other reagent-grade chemicals from Sigma Chemical Co., St. Louis, Mo. Carrier-free $^{125}$I and $^{131}$I were obtained from ICN Radiochemicals, Irvine, Calif.; $^{125}$I from Nordion International Inc., Kanata, Canada; PPACK from Calbiochem, San Diego, Calif.; the synthetic tripeptide chromogenic substrate, S-2288, from Helena Laboratories, Beaumont, Tex., and Kabi Vitrum, Stockholm, Sweden; G-50 medium-grade Sephadex from Pharmacia; and G-25 Sephadex spin columns from Isolab, Akron, Ohio.

Concentrations of t-PA

Concentrations of t-PA were determined by both enzyme-linked immunosorbent assay (ELISA) and protein assay with a modified Lowry procedure. The ELISA (American Diagnostica, Inc., Greenwich, Conn.) was performed with specific, affinity-purified goat anti-human t-PA immunoglobulin G as the capture antibody.

Inactivation of t-PA With PPACK

Human recombinant t-PA (10–20 mg) produced in transfected Chinese hamster ovary cells was incubated with a 5:1 molar excess of PPACK for 1 hour at room temperature in arginine-phosphate buffer (0.2 M, pH 7.2) containing Tween-80 (0.01% vol/vol). The mixture was then dialyzed four or five times against a 1,000-fold volume excess of desired buffer (3 M imidazole, pH 7.8; or 1.6 M KSCN in 0.15 M NaCl, pH 7.8). Complete inactivation of the serine protease activity of t-PA was verified by amidolytic assay with S-2288 as described previously in assay medium containing Tris (0.1 M, pH 8.4), NaCl (0.1 M), and Triton X-100 (0.01% vol/vol).

Radioiodination of PPACK-t-PA

Direct radioiodinations of t-PA and PPACK-treated t-PA were performed with the use of the solid-phase oxidant, Iodogen. Radiolabeled protein was harvested with spin columns packed with either G-25 Sephadex or G-50 Sephadex columns (0.9 × 30 cm).

DLT was radioiodinated with iodogen as described previously. For studies with $^{125}$I and $^{131}$I, the radionuclide was supplied by ICN Radiochemicals in high concentration and in liquid form. $^{125}$I was obtained in dry form.

Vials of dry $^{131}$I (20–40 mCi) were resuspended in phosphate buffer (40 μl, 0.5 M, pH 7) and transferred to iodogen-coated tubes containing DLT (5–10 nmol). pH was adjusted to 6.5–7, and the reaction mixture was incubated at room temperature for 1 hour. The reaction mixture was transferred to a tube containing galactose oxidase (8 units), pH was adjusted to 7, and the mixture was incubated at 37°C for 1 hour. PPACK-t-PA (2–8 mg) dissolved in imidazole (3 M) was added to each tube, pH was adjusted to 8.0, and the reaction mixture was incubated at 37°C for 10 minutes. NaBH$_4$CN was added to a final concentration of 50 mM, and the reaction mixture was incubated for an additional 45 minutes. *125I-DLT–conjugated PPACK–t-PA was separated from unconjugated material with a G-50 medium Sephadex column (0.9 × 30 cm) and eluted with imidazole (1.5 M, pH 7.5), Tween 20 (0.01% vol/vol), and sodium azide (0.01% wt/vol).

Binding of t-PA to Clots In Vitro

Clots were formed from whole human blood in vitro as described previously. Venous blood was...
collected without anticoagulants from normal volunteers and immediately transferred to Chandler tubes (27-cm length of Tygon tubing; o.d., 3/16 in.; i.d., 1/8 in.; 1 ml blood per tube). The ends of each tube were brought together to form a loop, secured with a collar of tubing (o.d., 5/16 in.; i.d., 3/16 in.), and rotated at a 60° angle on a tube rotator (Scientific Equipment Co., Baltimore, Md.) for specified intervals at 37°C. Clots were approximately 2 mm in diameter, 4–8 mm long, 5–15 mg dry weight, and similar morphologically to arterial thrombi formed in vivo containing a platelet head and fibrin tail. At specified intervals, radiolabeled t-PA (125I-DLT–PPACK–t-PA or 125I–PPACK–t-PA) was introduced at selected concentrations (250–10,000 ng/ml). Tubes were closed and rotated for 60 minutes and then reopened. Clots were recovered from each tube onto preweighed polyethylene mesh filters (Spectrum Medical Ind., Inc., Los Angeles) and washed thoroughly with NaCl (0.15 M) containing Tween-80 (0.01% vol/vol). Radioactivity bound to washed clots was measured with a gamma counter (Beckman LS 3801). Filters and clots were dried to a constant weight at 60°C overnight. The amount of t-PA bound per milligram of clot was calculated by dividing the radioactivity of each clot by the product of dry weight and specific radioactivity of 125I–t-PA. The comparison of binding of 125I–PPACK–t-PA to that of 125I–DLT–PPACK–t-PA was performed with clots formed 60 minutes before the introduction of radiolabeled PPACK–t-PA in a concentration of 250–5,000 ng/ml.

Studies in Rabbits to Define Pharmacokinetics of Radiolabeled t-PA

Rabbits weighing 1.8–2.2 kg were fasted overnight. Radiiodinated t-PA and related t-PA analogues were injected into the marginal vein of the ear of restrained conscious rabbits. Blood was drawn at selected intervals from the marginal vein of the opposite ear into syringes containing EDTA (final concentration, 1.5 mg/ml). Plasma was separated from whole blood by centrifugation, and aliquots were placed into tubes for determination of radioactivity. Additional aliquots of plasma were precipitated with trichloroacetic acid (TCA, 10% wt/vol), and radioactivity was determined in pelleted and soluble fractions.

Kinetics of clearance of radiolabeled t-PA and radiolabeled t-PA derivatives from plasma were calculated with respect to concentrations present at zero time (i.e., theoretical maximal concentration assuming dispersal in plasma volume at the time of injection). Initial concentrations of radioactivity in plasma were determined based on knowledge of the total amount of radiolabeled material injected (total counts per minute) and an estimated plasma volume of 33 ml/kg body wt.28

Studies in Dogs to Define Pharmacokinetics of Radiopharmaceutical Studied, Clot-to-Blood Ratios of Radioactivity, and Suitability of Tracer for Imaging

Adult fasted, conditioned dogs weighing 20–25 kg were premedicated with 1 mg/kg morphine s.c. and anesthetized 30–60 minutes later with 12.5 mg/kg thiopental i.v. and 72 mg/kg α-chloralose i.v. Dogs were intubated and ventilated with room air, and femoral arteries and veins were catheterized. Positioning of the catheter in the desired location (coronary, pulmonary, or carotid artery) was guided and confirmed by fluoroscopy. Guide wires were placed in the target vessel, and copper coils of appropriate size and diameter were advanced over the guide wire via a Forma Cath. 123I-DLT–PPACK–t-PA (0.4–1.8 mCi) was diluted to 20 ml with saline. The tracer was injected as a bolus (20% of total radioactivity) followed by the remainder given as a continuous infusion (5% of total radioactivity per minute). Aliquots of blood were obtained at selected intervals after bolus injection to monitor clearance of radioactivity from the circulation. Lidocaine (1 mg/kg) was administered as a bolus followed by a continuous infusion of 50 μg/kg/min to prevent malignant ventricular arrhythmia after placement of copper coils in coronary arteries. In some experiments, clots were formed with cotton threads inserted into the target vessel with a guide wire. The presence of total or partial occlusion was determined by angiography at repeated intervals.

Imaging. Images comprising 50,000–250,000 counts were obtained with a large-field planar gamma camera fitted with a low- or medium-energy parallel-hole collimator for 123I and 131I, respectively. Energy discriminators were set at the peak of the energy spectrum for each isotope with a 20% window. For tomographic studies, single-photon emission computed tomography (SPECT) was performed with a low-energy collimator for detection of 123I. 99mTc-labeled red blood cells were subsequently injected in some studies to permit visualization of the vasculature. Images were interpreted by a nuclear medicine physician at the Mallinckrodt Institute of Radiology (Dr. Henry Royal).

Assay of radioactivity in tissue and blood. Radioactivity in aliquots of whole blood, plasma, and TCA precipitates of plasma was determined with an LKB 1282 gamma counter. Plasma was obtained by centrifugation and precipitated with TCA. After completion of imaging studies, dogs were injected with thiopental and potassium chloride to induce cardiac arrest. Clots and selected tissues were removed for assay of radioactivity. Arteries containing clots were excised, and the thrombi were removed, weighed, rinsed with saline, and placed into a gamma counter for assay of radioactivity. Samples of myocardium, liver, and lung and skeletal muscle were obtained for assay of the distribution of radioactivity in regions near the thrombus. Clot-to-blood ratios of radioactivity were expressed as the quotient of counts per minute per milligram of thrombus divided by counts per minute per microliter of whole blood in the last sample obtained. Clot-to-tissue ratios of radioactivity were expressed as counts per minute per milligram of thrombus divided by counts per minute per milligram of tissue.
Results

Conjugation of Radioiodinated DLT to PPACK-t-PA

*I-DLT was conjugated to proteins via reductive amination, a process that exhibits a predilection for primary amines. Stock solutions of the t-PA were in buffer containing high concentrations of arginine to enhance solubility. Accordingly, conjugation of *I-DLT to the unprocessed material initially led to unacceptable efficiencies of less than 1%. To circumvent this difficulty, t-PA was dialyzed against either imidazole or KSCN under conditions that maintained t-PA in solution in a concentration as high as 50 mg/ml and permitted efficient radiolabeling with *I-DLT.

Binding of t-PA to Clots

Reductive amination of *I-DLT to proteins results in formation of adducts to ε-amino groups of lysine residues. Because specific lysine residues of t-PA may participate in the binding of the protein to fibrin, the binding of the radiolabeled material to clots formed in vitro was characterized to determine whether radiolabeling impaired binding. As illustrated in Figure 1, conjugation of 125I-DLT to PPACK-t-PA did not result in any significant change in the binding affinity to preformed clots compared with that of the directly radioiodinated PPACK-t-PA.18

To determine whether binding of 125I-DLT-PPACK-t-PA to clots was influenced markedly by the interval between formation of the clot and exposure to the radiolabeled t-PA derivative, additional clot-binding assays were performed in vitro. The interval between the initiation of clot formation and the introduction of 125I-DLT-PPACK-t-PA was varied from 0 to 4 hours. Binding was greatest when 125I-DLT-PPACK-t-PA was introduced at the time of clot formation, undoubtedly because of optimal access of the tracer to fibrin throughout the evolving clot. However, binding remained considerable even when the radiolabel was introduced 30 minutes after onset of clot formation and for longer intervals throughout the 4-hour interval studied (Figure 2).

Clearance of *I-DLT–PPACK–t-PA in Rabbits

Kinetics of clearance from plasma of radioiodinated t-PA were characterized in rabbits to determine whether the conjugation of *I-DLT to t-PA influenced removal of t-PA from the vascular compartment. Intravenous administration of directly radioiodinated t-PA was followed by initial, rapid clearance with the concentration of radioactivity in plasma reaching a nadir in 15 minutes. Thereafter, concentrations of radioactivity in plasma increased slightly before reaching a plateau (Figure 3A). In contrast to the concentration of radioactivity in aliquots of plasma, radioactivity in the fractions precipitable by TCA continued to decline. Conjugation of PPACK to directly radioiodinated t-PA did not appreciably alter clearance or the appearance of TCA-soluble radioactivity (Figure 3B). In contrast to either 125I–t-PA or 125I–PPACK–t-PA, radioactivity in aliquots of plasma after intravenous injection of 125I-DLT-PPACK-t-PA declined continuously over the 2-hour interval of observation. In addition, differences between the amount of radioactivity present in plasma compared with that in the TCA-precipitable fractions were minimal (Figure 3C). Thus, clearance of 125I-DLT-PPACK–t-PA from plasma in vivo was considerably less distorted by release into the plasma of radiolabeled degradation products compared with the use of 125I–t-PA and 125I–PPACK–t-PA.

Only approximately 5% of the injected dose of either 125I–t-PA or 125I–PPACK–t-PA was present in the liver after 2 hours. In contrast, 73±3% of the injected...
radioactivity with $^{125}$I-DLT-PPACK–t-PA was present in the liver at corresponding intervals as judged from assay of radioactivity in excised samples of liver (Figure 4). This observation is consistent with retention in the liver of a large fraction of the residualizing label after hepatic uptake of $^{125}$I-DLT-PPACK–t-PA.

**Figure 3.** Plots of clearance from plasma of (panel A) $^{125}$I–tissue-type plasminogen activator ($^{125}$I–t-PA), (panel B) $^{125}$I–D-Phe-L-Arg-chloromethyl ketone–t-PA ($^{125}$I–PPACK–t-PA), and (panel C) $^{125}$I–dilactitol tyramine–PPACK–t-PA ($^{125}$I-DLT-PPACK–t-PA) in rabbits. Triangles represent concentrations of radioactivity in aliquots of plasma, and circles represent values in precipitable fractions after exposure to trichloroacetic acid (10% wt/vol). Concordance between the two variables is evident in rabbits given $^{125}$I-DLT-PPACK–t-PA. Points represent mean values of four observations, and bars represent SEM values.

**Figure 4.** Bar graphs of percentages of injected radioactivity accumulated in liver 2 hours after intravenous administration of $^{125}$I–tissue-type plasminogen activator ($^{125}$I–t-PA) (open bar), $^{125}$I–D-Phe-L-Arg-chloromethyl ketone–t-PA ($^{125}$I–PPACK–t-PA) (solid bar), or $^{125}$I–dilactitol tyramine–PPACK–t-PA ($^{125}$I-DLT-PPACK–t-PA) (cross-hatched bar) in rabbits. Points represent mean values of four observations, and bars represent SEM values.

**Figure 5.** Scatterplots of clearance of $^{131}$I from plasma after a 15-minute intravenous infusion of $^{131}$I–dilactitol tyramine–D-Phe-L-Arg-chloromethyl ketone–tissue-type plasminogen activator ($^{131}$I-DLT-PPACK–t-PA) in dogs. Infusion interval is designated by solid bar. A total of 2–6 mg t-PA coupled to 0.1–2.1 mCi $^{131}$I-DLT was infused in each case. Points represent all data acquired from each of 13 dogs. Results from individual animals are represented by specific symbols. Curve represents best fit of biexponential clearance curve after termination of infusion.

**Clearance of *I-DLT-PPACK–t-PA in Dogs**

*I-DLT-PPACK–t-PA was injected as a bolus followed by a 15-minute infusion into the femoral vein of anesthetized dogs. The initial bolus contained 20% of the total mass of protein injected. Concentrations of radioactivity in plasma decreased rapidly after cessation of the infusion. The concentration in plasma had declined within 1 hour to only 19% of the initial, maximal concentration (Figure 5). Accordingly, imaging commenced 36–74 minutes after the onset of infusion of the tracer. As in studies in rabbits, clearance was biexponential in dogs.

**Clot-to-Blood and Clot-to-Tissue Ratios of Radioactivity**

Clot-to-blood ratios of radioactivity were determined 1–3 hours after completion of *I-DLT-PPACK–t-PA infusions in dogs. Clot-to-blood ratios of radioactivity were consistently high with clots formed while tracer was being infused, as shown in Table 1. Thrombi in coronary arteries exhibited the highest values, probably related in part to their diminutive size. Clots formed in the pulmonary and carotid arteries were consistently larger than those formed in the coronary arteries. In clots formed before infusion of tracer, the clot-to-blood average ratios ranged from 2.8:1 to 18:1 (Table 2). Higher values were induced systematically as our technique for producing an optimal amount of chemically homogenous tracer improved with experience and with subtle modifications, leading to the final protocol described in "Methods."

In addition to the high ratios of radioactivity in clots compared with that in blood, ratios of radioactivity in clots compared with that in adjacent and surrounding tissue were also high, as shown in studies.
of concomitantly formed clots in carotid arteries (Table 3) and in preformed clots in pulmonary, carotid, and femoral arteries (Table 4). Concomitantly formed clot-to-tissue ratios of radioactivity were high when comparisons were made with respect to vascular tissue (analysis of the contralateral carotid artery) or to surrounding skeletal muscle. Preformed clot-to-tissue ratios of radioactivity were also high with lung and femoral artery clots.

In agreement with results of the earlier studies performed in rabbits, more than 90% of the injected radioactivity was present in liver at the completion of experiments. Other tissues such as skeletal muscle, heart, and lung contained minimal percentages of the total amount of injected radioactivity as determined at autopsy.

**Imaging**

In the initial experiments, $^{131}$I-DLT-PPACK-t-PA failed to delineate coronary, pulmonary, or carotid thrombi with conventional planar gamma scintigraphy despite favorable clot-to-blood ratios of radioactivity. Coronary thrombi with the highest clot-to-blood ratios were particularly difficult to image because of scatter from high uptake of tracer in the adjacent liver. However, after injection of $^{131}$I-DLT-PPACK-t-PA, coronary and pulmonary thrombi were imaged readily ex vivo (data not shown). It appeared likely that an additional difficulty was the confounding effect of low concentrations of radioactivity in large amounts of overlying and underlying tissue relative to the highly localized radioactivity present in clots and that of scatter attributable to the high energy of emission by $^{131}$I. To overcome these difficulties, we performed additional experiments with $^{131}$I as the tracer and tomographic imaging with SPECT. As can be seen in Figure 6, carotid thrombi with high clot-to-blood ratios (27:1) could be readily delineated with this approach. Carotid artery clots were imaged in each of three dogs in which thrombosis was initiated at approximately the same time as the onset of tracer infusion.

To determine whether imaging was possible with introduction of tracer after clots had been formed, additional experiments were performed with induction of carotid and femoral thrombosis 15-70 minutes before infusion of tracer. As can be seen in Figure 7, good visualization was accomplished even though clot-to-blood ratios of radioactivity were lower (18:1) with the preformed clot, presumably because of a decreased amount of fibrin surface being available for interaction with plasma as the clot coalesces and contracts as well as because of decreased delivery of label to clot.

**Table 1. Clot-to-Blood Ratios of Radioactivity in Thrombi Being Formed at Diverse Loci While Tracer Was Being Infused**

<table>
<thead>
<tr>
<th>Location of thrombi (artery)</th>
<th>No. of dogs studied</th>
<th>No. of thrombi induced</th>
<th>Weight of thrombi (mean±SEM mg)</th>
<th>Clot-to-blood ratios of radioactivity after 2 hours (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td>5</td>
<td>8</td>
<td>12±3</td>
<td>137±35:1</td>
</tr>
<tr>
<td>Pulmonary</td>
<td>2</td>
<td>4</td>
<td>101±13</td>
<td>42±7:1</td>
</tr>
<tr>
<td>Carotid</td>
<td>8</td>
<td>15</td>
<td>70±12</td>
<td>37±9:1</td>
</tr>
</tbody>
</table>

In several individual animals, more than one clot was induced and characterized. Among animals included in table, three were imaged by single-photon emission computed tomography. Among nine carotid clots induced in these animals, eight were visualized.

**Table 2. Clot-to-Blood Ratios of Radioactivity in Thrombi of Diverse Ages and Loci at Autopsy**

<table>
<thead>
<tr>
<th>Location of thrombi (artery)</th>
<th>Age of thrombi (mean±SEM minutes)</th>
<th>Weight of thrombi (mean±SEM mg)</th>
<th>Clot-to-blood ratios of radioactivity at 2 hours after infusion of tracer (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary</td>
<td>31±4</td>
<td>16±32</td>
<td>8.1±0.8:1</td>
</tr>
<tr>
<td>Carotid</td>
<td>48±2.4</td>
<td>156±32.3</td>
<td>2.8±0.6:1</td>
</tr>
<tr>
<td>Femoral</td>
<td>15</td>
<td>109</td>
<td>18:1</td>
</tr>
</tbody>
</table>

Pulmonary artery thrombi were initially much larger than carotid thrombi as evident from angiography. However, because of well-appreciated high fibrinolytic activity of canine blood in vivo, considerable dissolution of pulmonary thrombi occurred during the course of imaging. The magnitude of dissolution was generally the same, regardless of whether enzymatically inactive radiolabeled dilactitol tyramine-D-Phe-I-Pro-I-Arg-chloromethyl ketone–tissue-type plasminogen activator (I-DLT-PPACK-t-PA) was infused. In additional experiments in vitro (data not shown), I-DLT-PPACK-t-PA did not inhibit lysis of Chandler tube human whole blood clots by exogenous t-PA.

In several animals, more than one clot was induced and characterized.

With the final protocol developed as described in the text, two clots were imaged by single-photon emission computed tomography. In both of these, the induced clot was visualized.

*A total of 10 dogs were studied. In some, clots were induced in more than one vascular bed.
### Table 3. Ratios of Radioactivity in Carotid Artery Thrombi Formed While Tracer Was Being Infused Compared With That in Surrounding Tissue in Samples Taken After Imaging of Clots

<table>
<thead>
<tr>
<th>Surrounding tissue</th>
<th>No. of experiments*</th>
<th>No. of tissue samples</th>
<th>No. of thrombi</th>
<th>Ratio of radioactivity in concomitantly formed thrombi to that in surrounding tissue (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contralateral carotid</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>117±23:1</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>4</td>
<td>7</td>
<td>4</td>
<td>39.7±12.1:1</td>
</tr>
</tbody>
</table>

*A total of four dogs were studied, each with thrombus induced in more than one vascular bed.

### Discussion

Thrombosis is the precipitating cause of many instances of acute myocardial infarction, pulmonary embolism, and cerebrovascular accident. Treatment of thromboemboli with thromolytic agents is, however, not devoid of hazard. In patients with infarction, clinical criteria may suggest that thrombi are present but are often not definitive. Angiography is the gold standard but is not immediately and universally available or free from risk or high cost. Availability of a noninvasive method permitting prompt detection of thrombi would markedly facilitate selection of patients who are most likely to benefit from thrombolytic drugs.

Because t-PA has a high and specific affinity for binding to fibrin and exhibits rapid clearance from the vascular compartment, we considered it to be a promising candidate as a radiopharmaceutical for imaging thrombi. However, the potential usefulness of t-PA as an imaging agent is compromised by its serine protease activity, which leads to digestion of the clot binding the tracer during imaging. In addition, rapid release of radiolabeled degradation products of its metabolism leads to accumulation of radioactivity in plasma that lowers the ratio of radioactivity in clot compared with that in blood.

To overcome these limitations, we inactivated t-PA with PPACK, an irreversible inhibitor of serine proteases that has been demonstrated previously to suppress degradation of fibrin by t-PA. Conjugation of t-PA with PPACK leads to an apparent increase in its fibrin binding because of lack of degradation of components of clot to which the conjugate binds, in contrast to the use of t-PA. Subsequently, PPACK-t-PA was coupled to *I-DLT to diminish release of radioiodinated degradation products from the liver into blood during the imaging interval. The results we obtained demonstrate that the coupling of enzymatically inactivated t-PA to *I-DLT was advantageous. We found that *I-DLT coupled to t-PA did not impair binding of the tracer to fibrin or alter its kinetics of clearance from blood. In addition, conjugation to *I-DLT facilitated entrapment of the label in the liver, known to be the principal site of catabolism of t-PA. The half-life of retention within tissues of degradation products of proteins radiolabeled with *I-DLT is approximately 2 days. Accordingly, leakage of radiolabeled protein degradation products would be negligible over the time course of imaging (approximately the zero hour required as shown in the present study).

Although conjugation with *I-DLT has been performed with many proteins, t-PA presented unusual difficulties because of its sparse solubility, which is generally overcome with high concentrations of arginine. Because conjugation of *I-DLT to proteins via reductive amination requires a primary amine on the protein, principally lysine, the presence of arginine greatly decreases the efficiency of radiolabeling of proteins by this method. Among a plethora of buffer systems tested, we found that only those containing imidazole or potassium thiocyanate maintained sufficiently high concentrations of the protein in solution while permitting acceptable efficiency of conjugation of *I-DLT to PPACK-t-PA.

The use of 131I-DLT-PPACK-t-PA did not clearly delineate thrombi by planar scintigraphy because of the high energy of the isotope that limits effective collimation. Although carotid thrombi could be identified by planar gamma camera scintigraphy, delinea-

### Table 4. Ratios of Radioactivity in Preformed Pulmonary, Carotid, and Femoral Artery Thrombi Compared With That in Surrounding Tissues as Judged From Samples Obtained at Autopsy

<table>
<thead>
<tr>
<th>Location of thrombi (artery)</th>
<th>Surrounding tissue</th>
<th>No. of dogs*</th>
<th>No. of tissue samples</th>
<th>No. of thrombi</th>
<th>Ratio of radioactivity in preformed thrombi to that in surrounding tissue (mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary</td>
<td>Lung (infarcted and healthy)</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>26.1±11.8</td>
</tr>
<tr>
<td>Carotid</td>
<td>Uninvolved carotid artery</td>
<td>3</td>
<td>4</td>
<td>8</td>
<td>3.1±0.7</td>
</tr>
<tr>
<td>Femoral</td>
<td>Uninvolved femoral artery</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>23.6±12.4</td>
</tr>
</tbody>
</table>

*A total of five dogs were studied. In several, thrombi were induced in more than one vascular bed.
tion was difficult, presumably because of low but diffuse background radioactivity in large amounts of tissue surrounding small clots (mean wet weight, 70 mg) and despite highly favorable clot-to-blood ratios of radioactivity. A component of the background tissue radioactivity may reflect binding of tracer to venous endothelium\(^{32}\) with a cumulative background radioactivity that degrades planar images of thrombi.

The detection of thrombi was enhanced with the use of a tomographic technique. Improved spatial resolution was obtained with substitution of \(^{123}\text{I}\) for \(^{131}\text{I}\). Although background radioactivity in surrounding tissue was not diminished, the use of SPECT circumvented its impact by virtue of tomographic delineation of the clot from multiple angles of view.

The conditions used in the present study were initially selected to be optimal in that the tracer was given at the time of clot formation. However, the binding of \(^{1}\text{I-DLT-PPACK-t-PA}\) to preformed clots was shown to be avid in vitro, and difficulties were not anticipated with imaging in vivo. Although clot-to-blood ratios were diminished in vivo with preformed clots, they remained high enough to permit successful imaging of clots in this portion of the study.

In our preparations both totally and partially occluding clots were imaged. Total occlusion would, of course, limit the amount of tracer that could be taken up by the clot because only the end surfaces would be sufficiently exposed to the tracer. Clinically, either partial occlusion or intermittent partial recanalization would be expected to expose a greater surface area of thrombus to the tracer, resulting in higher clot-to-blood ratios.

Our results suggest that detection of thrombi may be possible not only with SPECT but also with positron emission tomography with an \(^{18}\text{F}\)-containing residualizing label, synthesized previously by our group, that can be conjugated to proteins with a procedure similar to that used to produce \(^{1}\text{I-DLT}\) conjugates.\(^{33}\) Even though the radiation burden in the present study was well within acceptable limits (equal to approximately 0.8 rads to the liver in an analogous human study), the approach developed should permit further reduction with the use of a tracer with a short physical half-life such as \(^{18}\text{F}\) and highly sensitive tomographic detection equipment now becoming available.
Our results indicate that incorporation of the labeled congener of t-PA studied is particularly prominent in thrombi being formed while the tracer is being administered. Thus, particularly suitable targets for imaging with the approach developed may be the evaluation of deep venous thrombosis and detection of its presence after surgical procedures such as hip replacements in the elderly, imaging of evolving thrombi in patients with unstable angina in whom recurrent thrombosis appears to be an important pathogenetic mechanism, and characterization of thrombi undergoing dynamic modification with counterbalancing fibrinolysis and thrombosis such as coronary artery thrombi in patients treated with thrombolytic drugs. Clinical imaging of formed, compared with forming, thrombi is likely to be more difficult, not only because of the more modest incorporation of tracer in formed compared with forming thrombi but also because of the inhibition of accumulation of tracer by complete occlusion with consequent limitation of delivery of tracer to the clot that often accompanies fully developed intravascular thrombosis. In addition, the uptake of large amounts but only modest concentrations of tracer in tissues juxtaposed to vessels could compromise results. Future developments will be directed toward overcoming these limitations with several strategies, including further enhancement of clot-to-tissue ratios of accumulation of tracer by modification of the t-PA congener used, use of adjunctive agents to diminish uptake in specific tissues adjacent to clots, or both; use of positron emission tomography with \(^{18}\text{F}\)-t-PA residualizing labels and t-PA congeners; and use of enzymatically inactive molecular variants of t-PA such as the mutant we have synthesized that exhibits only a single amino acid substitution (478 serine to threonine)\(^{24}\) to avoid deleterious effects of an antigenic substance or introduction of active site inhibitors. Despite the difficulties inherent in detecting formed thrombi clinically, these and other approaches should facilitate their recognition in diverse vascular beds.

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**References**


**KEY WORDS** • tissue-type plasminogen activator • PPACK • radiiodinated dilactitol tyramine • thrombolysis • single-photon emission computed tomography
Imaging of thrombi with tissue-type plasminogen activator rendered enzymatically inactive and conjugated to a residualizing label.

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