Annals of Nuclear Medicine Vol. 5, No. 2, 59–64, 1991

Tc-99m labeled tissue-type plasminogen activator: Preparation, stability and preliminary imaging of thrombus-bearing rats

Kazuo ITOH,* Eriko TSUKAMOTO,* Toshiya NISHIBE,** Shoki Sakurama,*** Masahiro IEKO,*** Tatsuzo TANABE** and Masayori FURUDATE*

*Department of Nuclaer Medicine, **Second Department of Surgery, and ***Second Department of Internal Medicine, Hokkaido University School of Medicine

Tissue-type plasminogen activator (t-PA) is a thrombolytic agent that directly binds to fibrin formed in clots. In terms of radiolabeling and nuclear imaging, t-PA has several advantages in Tc-99m labeling: it is stable in acidic solution at pH 3, which is suitable for labeling Tc-99m by a method of stannous reduction and blood disappearance after administration is rapid, which is desirable for imaging targets using short-lived radionuclides.

Recombinant t-PA was labeled with Tc-99m by a method of stannous reduction without significant degradation of biochemical activity, over 95% of which was retained after the labeling procedure. Labeling efficiency in paper chromatography was over 98%. The moiety of hydrolyzed Tc-99m that was not eluted through the Sephadex column was estimated to be less than 10%. Tc-99m labeled t-PA, however, appeared to become unstable when diluted with normal saline. Nevertheless, in *in vitro* fibrin binding, Tc-99m labeled t-PA showed high affinity with fibrin: 80% of 100 ng/m*l* of Tc-99m t-PA bound to 10^{-5} mol of the fibrinogen. Preliminary animal studies also showed a concentration of Tc-99m labeled t-PA at fresh thrombi formed in the inferior vena cava.

Tc-99m labeled t-PA appears to have potential for thrombus imaging and the preparation of an instant kit.

Key words: recombinant tissue-type plasminogen activator (t-PA), Tc-99m labeling, scintigraphy, thrombus imaging

INTRODUCTION

THE NON-INVASIVE diagnosis of thrombi formed in peripheral deep veins is still a challenging problem.[1] Various radiopharmaceuticals have been developed for defining vascular thrombi.[2,3] Anti-platelet antibodies[4,5] and anti-fibrin antibodies[6] have recently been shown to accumulate specifically in both fresh and old thrombi. However, radiolabeled antibodies have some limitations for routine clinical use becuase of their slow blood clearance and non-human protein origin.

Tissue-type plasminogen activator (t-PA) is a new type of thrombolytic agent.[1,7,8] Many experimental and clinical trials performed to evaluate the efficacy and safety of intravenous administration of t-PA have already confirmed that it promotes reperfusion of occluded vessels due to thromboemboli.[9–12] T-PA's biochemical properties seem to have desirable attributes for *in vivo* imaging of thrombi: 1) strong affinity with fibrin *in vitro* and 2) short blood disappearance *in vivo*. Hnatowich et al.[13] developed In-111 labeled t-PA and confirmed its affinity with artificially formed thrombi of the aorta in dogs. Recently Fry et al.[14] reported *in vitro* labeling of human clots with I-125 labeled mutant t-PA, which is thrombolytically inactive but has the same affinity with clots as thrombolytically active t-PA.

We have been interested in another biochemical property of t-PA for Tc-99m labeling. It is very

Received January 23, 1991, revision accepted March 8, 1991.

For reprint contact: Kazuo Itoh, Department of Nuclear Medicine, Hokkaido University School of Medicine, Kita-15, Nishi-7, Kita-ku, Sapporo 060, JAPAN.

stable in acidic solution, and it was therefore postulated that t-PA could be labeled with Tc-99m by a method of stannous reduction without affecting its biochemical activity. In this report we will discuss the labeling procedure, its biochemical and radiopharmaceutical stability, affinity with fibrin *in vitro*, and preliminary imaging studies.

MATERIALS AND METHODS

Tissue-type plasminogen activator

The recombinant t-PA (SM-9527, lot No. 6103), which was generously supplied by Sumitomo Chemical Co. Osaka, Japan, consisted of a double polypeptide chain that had a molecular weight of about 57,000 as determined by electrophoresis. Although commercially available t-PA contains human serum albumin as a stabilizer, the t-PA used for radiolabeling was pure and was dissolved in 0.7% normal saline containing only 0.01% Tween 80. It was adjusted to pH 3 with hydrochloric acid and was frozen for storage. The protein concentration of t-PA was 6.9 mg per m*l*, and its specific activity was found to be 7.7×10^5 IU/mg. It has been confirmed to be very stable for over one year in the condition described above.

Tc-99m labeling

After melting frozen t-PA at room temperature, 200 μl (1.38 mg) of t-PA was pipetted into a plastic tube, and thereafter 10 μl (containing 0.001 μmol of stannous ion) of a commercially available stannous solution (containing 1 μmol/m*l* of stannous ion at at pH 3, Nihon Medi-Physics Co., Osaka, Japan) used in the Tc-99m liver imaging kit was added. After gently stirring for 5 minutes, 0.1 m*l* of freshly eluted Tc-99m pertechnetate was added followed by incubation for 15 minutes at room temperature with occasional gentle agitation.

Analysis of labeling yields and stability

Gel Chromatography (GCG)

For separation of Tc-99m labeled t-PA from free Tc-99m pertechnetate and hydrolyzed Tc-99m, gel chromatography was performed. Commercially available disposable gel columns (Sephadex G-25M, column PD-10, Pharmacia LKB, Uppsala, Sweden) were used for this purpose. Labeled materials were eluted at a speed of 1 m*l*/minute/fraction with 0.7% normal saline buffer solution containing 0.1% Tween 80 (Kanto Chemical Co., Inc, Tokyo, Japan) adjusted to pH 3 with 0.1 normal hydrochloric acid (Kanto Chemical Co., Inc, Tokyo, Japan).

Paper Chromatography (PCG)

Tc-99m labeled materials before and after the purification by GCG and free Tc-99m were independently developed on filter papers (Advantec 51B, Toyo Roshi Kaisha Ltd., Tokyo, Japan) in a solvent of 60% acetonitrile (Kanto Chemical Co., Inc, Tokyo, Japan) st a distance of 12 cm. These papers were cut into 1-cm length and thereafter each piece was counted in a well scintillation counter.

Stability of the labeled t-PA of a specified concentration after dilution by adding normal saline was tested by PCG.

Assays of biochemical activity and fibrin binding

Biochemical activity of the t-PA before and after the processing by radiolabeling and separation through gel chromatography was measured by parabolic rate assay modified by Ransy et al.[15]

The affinity of labeled t-PA with the fibrin was tested according to the method described by Johannessen et al.[16] Fibrinogen and thrombin from human plasma (Sigma Chemical Co., St Louis, USA) were used for this assay. One hundred ng/m*l* of Tc-99m labeled t-PA and a specified concentration of fibrinogen were dissolved in a buffer containing 0.1 mol sodium chloride, 0.05 mol sodium phosphate, and 0.01% Tween 20, at pH 7.2. Fibrin polymerization was initiated by the addition of 1 NIH unit/m*l* of thrombin. After incubation with agitation at 37°C for 1 hour the samples were centrifuged for 20 min at $2,500 \times$ g. The radioactivity of Tc-99m of a precipitate after removing the supernatant was counted in a well scintillation counter. Fibrin binding of Tc-99m labeled t-PA was calculated from the radioactivity of a precipitate per total radioactivity of 100 ng/m*l* of Tc-99m t-PA by 100%.

Preliminary imaging studies

The thrombus-bearing rats (inbred WKM-Hkm rat, n=4) were prepared by ligation of the inferior vena cava (IVC) just below the communication of the left renal vein. Two mg/kg of Tc-99m labeled t-PA, which was adjusted to 0.1 mg/m*l* by dilution with normal saline just before injection, was administered intravenously 1.5 hours to 2 hours after the ligation through a fine venula needle that had already been positioned at the central portion of the IVC ligation. The dilute Tc-99m t-PA was slowly injected for 4 to 5 minutes and was followed by a flush of normal saline. A clot that was actually formed at the site of the IVC ligation was confirmed by post-mortem examination of the vessel after the imaging studies.

RESULTS

Labeling yields and stability

The elution curve of Tc-99m labeled materials through GCG is shown in Fig. 1. Most of its radioactivity was eluted in void volumes (fractions No. 4,

5, and 6). Tc-99m pertechnetate was eluted in fractions between No. 11 and No. 15. Both elution peaks for Tc-99m labeled t-PA and free Tc-99m were clearly separated. The sum of the radioactivity recovered in all 25 elution fractions was calculated to be from 87% to 90%. Accordingly, hydrolyzed Tc-99m that was not eluted through the column was estimated to be about 10% to 13%. The elution curve for the biochemical activity of t-PA contained in an aliquot of each eluted fraction was consistent with the radioactivity elution curve for Tc-99m labeled t-PA (Fig. 2). The biochemical activity of pre-labeling cold t-PA was estimated to be 6.0×10^5 IU/mg by our parabolic rate assay. This biochemical activity of Tc-99m labeled t-PA was more than 97% of cold t-PA after labeling procedures.

Tc-99m labeled material and free Tc-99m pertechnetate showed clearly different migration patterns in PCG with 60% acetonitrile buffer solution (Fig. 3). Tc-99m labeled t-PA and hydrolyzed Tc-99m did not move from the origin and only free Tc-99m developed a front. Tc-99m labeled t-PA before GCG separation showed very high labeling efficiency and was stable until 2 hours after the labeling. However, separated Tc-99m labeled t-PA eluted in Faction No. 4 or No. 5 was unstable, contrary to our expectations. This radiolabeling property was proved to be due to radiolabeling instability of diluted Tc-99m t-PA (Fig. 4). The labeling yields of Tc-99m t-PA appeared to be significantly decreased by dilution to more than 100 times with normal saline (Fig. 5).

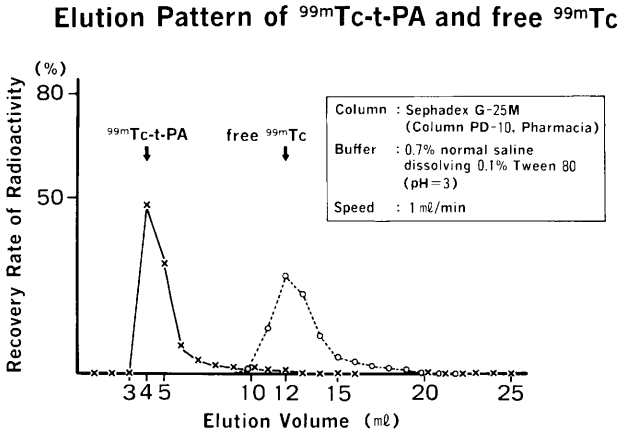

Fig. 1 Elution curve of Tc-99m labeled t-PA. The Tc-99m elution curve was independently determined and superimposed on the former.

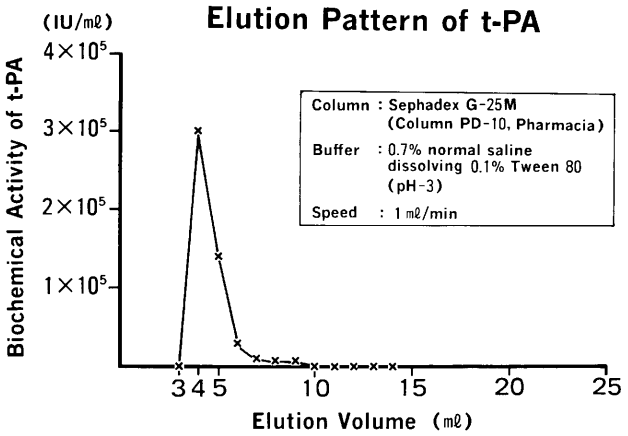

Fig. 2 Biochemical activity curve of solution eluted through gel chromatography of Tc-99m labeled t-PA. Biochemical activity was assayed by the parabolic rate method.

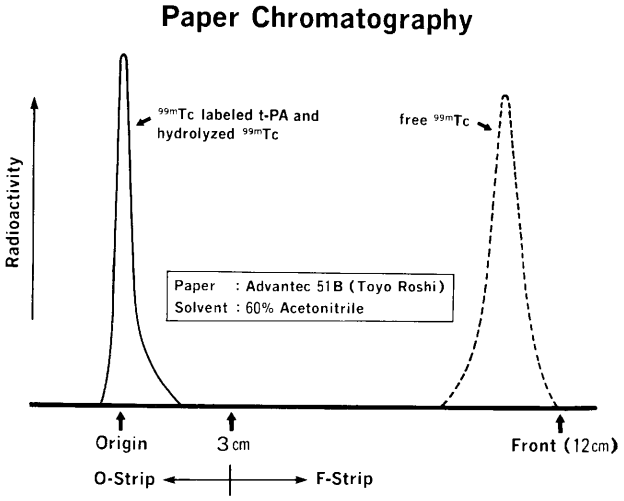

Fig. 3 Development patterns of Tc-99m labeled t-PA and free Tc-99m in buffer solution of 60% acetonitrile. Labeling yields and stability of Tc-99m labeled t-PA distance of 3 cm from the origin.

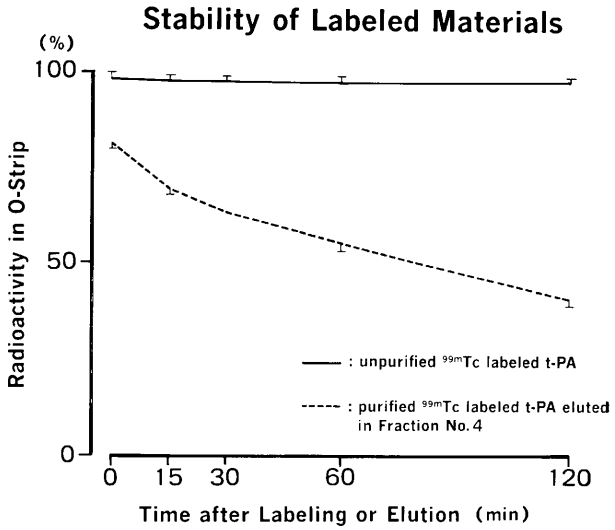

Fig. 4 Labeling yields and stability of Tc-99m labeled t-PA before (a solid line) and after (a dotted line) gel chromatography.

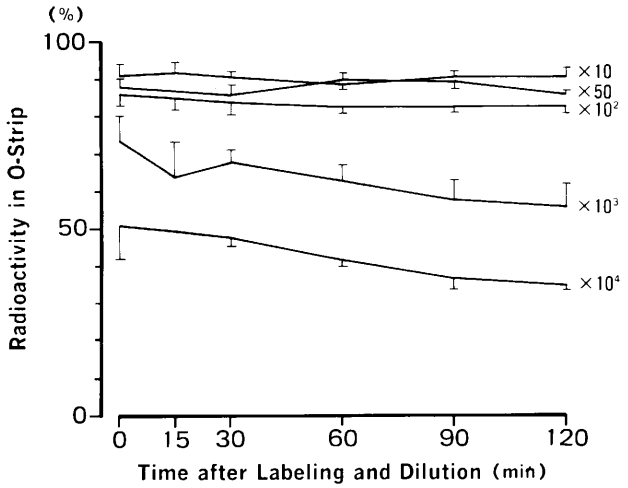

Fig. 5 Effect of dilution on stability of Tc-99m labeled t-PA. The Tc-99m t-PA maintains 80% to 85% of radiolabeling yields when diluted 100 times with normal saline solution.

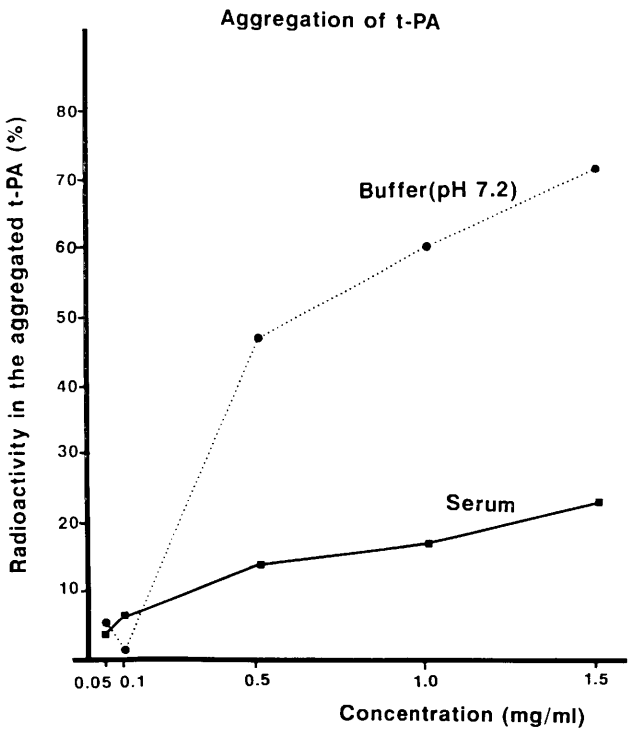

Fig. 6 Relationship between aggregation and concentration of Tc-99m labeled t-PA in phosphate buffer solution at pH 7.2 and murine plasma. The rate of the aggregated t-PA was measured from the radioactivity of the precipitates after centifugation.

Fibrin binding

When Tc-99m labeled t-PA was suddenly exposed to a buffer solution at pH 7.2, it tended to aggregate in the solution. Then we examined the relationship between the concentration of Tc-99m t-PA and the aggregation in a buffer solution used for fibrin

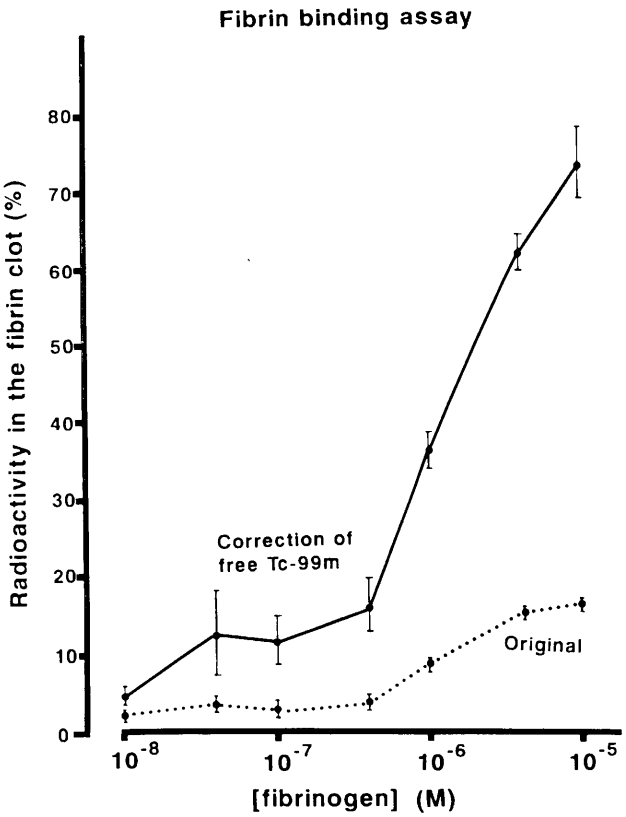

Fig. 7 Binding of Tc-99m labeled t-PA to fibrin. The solid line indicates a crude fibrin binding rate, and the dotted line is a binding curve after the correction of the labeling yields of the dilute Tc-99m t-PA in the medium.

binding and in murine plasma by counting the radioactivity of the precipitates after centrifugation (2,500 × g for 20 minutes) and removing the supernatant. The higher the concentration of Tc-99m t-PA was, the more proportionately increased the aggregation of t-PA in the buffer and plasma was, but no significant radioactivity of precipitates was detected at a concentration of 0.1 mg/m*l* of t-PA (Fig. 6). The fibrin binding of Tc-99m t-PA was estimated to be very low from data of crude counts of the precipitate. At a concentration of 100 ng/m*l* of the Tc-99m labeled t-PA, the radiolabeling rate was decreased to about 27%, and the net fibrin binding rate of the actually labeled t-PA was obtained by correcting the labeling rate in the medium. From the results of the correction, about 80% of 100 ng/m*l* of Tc-99m labeled t-PA was estimated to bind to 10^{-5} mol of fibrinogen (Fig. 7).

Preliminary imaging studies

Figure 8 is an image obtained in a thrombus-bearing rat. Imaging studies showed a short blood disappearance of injected materials, a high concentration in the liver and kidney, and increased radioactivity at the ligation site, which was suggestive of an ac-

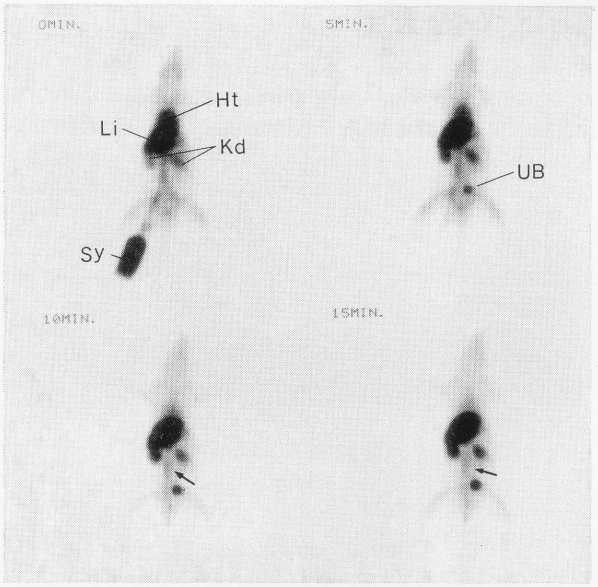

Fig. 8 Sequential images of a thrombus model rat every five minutes after a single slow injection of 2.0 mg/kg of Tc-99m labeled t-PA, which was adjusted to 0.1 mg/m*l* in the concentration just before injection. Most radioactivity is demonstrated in the heart (Ht), liver (Li), kidney (Kd), and urinary bladder. A thrombus formation, which corresponds to the positive uptake (black arrow) in the abdomen, was confirmed by post-mortem examination. The abbreviation Sy indicates the syringe containing the dilute Tc-99m t-PA.

cumulation of Tc-99m t-PA in clots. This positive delineation of clots persisted until 25 minutes after injection. No radioactivity was observed in the regions of the thyroid and stomach.

DISCUSSION

Several experimental trials related to radiolabeling of t-PA with different radionuclides, I-131,[17] In-111,[13] and I-125[14] have been reported so far. Uehara et al.,[17] who used I-131 labeled t-PA for detecting thrombi formed after de-endothelialization of the aorta in the rabbit, suggested that radiolabeled t-PA may be inappropriate as a radiopharmaceutical for the scintigraphic detection of pre-existing thrombotic lesions. At the same time, Hnatowich et al.[13] demonstrated that the concentration of In-111-labeled t-PA at the site of thrombi formed in the descending aorta in dogs. Fry et al.[14] showed that I-125 labeled mutant recombinant t-PA bound to fibrin *in vitro*, and thereby showed promise as an imaging agent.

The radiolabeling of many proteins with radioiodine has already been established, and radioiodinated t-PA has been used for *in vitro* assay as well as analysis of *in vivo* distribution.[14,17–19] The radioiodine-labeled t-PA was stable *in vitro* even when diluted, but it was rapidly dehalogenated in the liver *in vivo*.[18,19] This property may be a factor in restricting further clinical application of radioiodine-labeled t-PA, even if it is not dehalogenated at the site of thrombi. In-111 labeling by the method of the bifunctional chelate DTPA seems to be preferable for *in vivo* use due to stable binding of the radiotracer to t-PA.[13] In-111 has a relatively long half-life and is not suitable as a labeling agent for materials with a short blood disappearance, because the radiation dose for critical organs may limit the administration of a large amount of labeled materials. In addition, the labeling method with bifunctional chelate DTPA is somewhat complicated. On the other hand, there are several advantages in Tc-99m labeling of t-PA: a high photon flux for relatively good quality images is obtained and the radionuclide used for labeling is easily available, which is essential for immediate diagnosis of thrombi. Our method employed for labeling t-PA with Tc-99m was very simple and very reproducible. Preparation of an instant kit is possible in the future.

One problem with the Tc-99m labeled t-PA that was obtained by the method of stannous reduction is radiolabeling instability. This radiolabeling property may become a critical factor in clinical applications, because t-PA tends to aggregate over a concentration of 0.1 mg/m*l* in plasma, and a dilution process for administration is essential. However, preliminary imaging studies indicate that the limitation of Tc-99m labeled t-PA due to radiolabeling instability could be overcome by preparing the dilute Tc-99m t-PA just before injection and by administering it immediately after dilution.

There are still other points to be discussed before Tc-99m labeled t-PA is used clinically. The biological activity of externally administered t-PA may be offset by internal t-PA inhibitor in the circulation.[20] Therefore, it is postulated that an overwhelming dose of extrinsic t-PA is required for positive delineation of the clot.[3] Another point is the short retention of intravenously administered t-PA after binding to fibrin clots.[21,22] Preliminary imaging studies suggest that Tc-99m t-PA accumulated immediately at the clot site, but the period of its positive delineation did not seem to exceed for only 10 to 15 minutes. Prolonged persistence of the test agents at the clot site is a desirable feature from the standpoint of the logistics of patient throughout and whole body imaging. In this context, mutant t-PA, which retains high affinity with fibrin but is not degraded after binding,[14] may be a better potential agent than biologically active t-PA for clinical application. These problems remains to be investigated further with *in vitro* and *in vivo* thrombus models for clinical application.

ACKNOWLEDGMENTS

We thank Teruo Sakurama, Ph.D. of Sumitomo Pharmacy Co., and Mr. Yoshinori Kurami of Nihon Medi-Physics Co., for their notable technical comments as to handling t-PA and labeling Tc-99m, and Leonard Rosenthall, M.D., of the Montreal General Hospital for his comments and advice.

REFERENCES

1. Moser KM: Venous thromboembolism. *Am Rev Respir Dis* 141: 235–249, 1990
2. Krohn KA, Knight LC: Radiopharmaceuticals for thrombus detection: selection, preparation, and critical evaluation. *Semin Nucl Med* 12: 219–228, 1977
3. Knight LC: Radiopharmaceuticals for thrombus detection. *Semin Nucl Med* 20: 52–67, 1985
4. Som P, Oster ZH, Zamora PO, et al: Radioimmunoimaging of experimental thrombi in dogs using technetium-99m-labeled monoclonal antibody fragments reactive with human platelets. *J Nucl Med* 27: 1315–1320, 1986
5. Oster ZH, Som P: New perspectives in thrombus-specific imaging: radiolabeled monoclonal antibodies. *AJR* 152: 253–260, 1989
6. Knight LC, Maurer AH, Ammar IA, Shealy DJ, Mattis JA: Evaluation of indium-111-labeled antifibrin antibody for imaging vascular thrombi. *J Nucl Med* 29: 494–502, 1988
7. Matsuo O, Rijken DC, Collen R: Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. *Nature* 291: 590–591, 1981
8. Rijken DC, Hoylaerts M, Collen D: Fibrinolytic properties of one-chain and two-chain human extrinsic (tissue-type) plasminogen activator. *J Biol Chem* 257: 2920–2925, 1981
9. The TIMI study group, The thrombolysis in myocardial infarction (TIMI) trial: phase I findings. *New Eng J Med* 312: 932–936, 1985
10. Verstraete M, Bory M, Collen D, et al: Randomized trial of intravenous recombinant tissue-type plasminogen activator versus intravenous streptokinase in acute myocardial infarction. *Lancet* 13: 842–847, 1985
11. Williams DO, Borer J, Braunwald E, et al: Intravenous recombinant tissue-type plasminogen activator in patients with acute myocardial infarction: a report from the NHLBI thrombolysis in myocardial infarction trial. *Circulation* 73: 338–348, 1986
12. Topol EJ, George BS, Kereiakes DJ, et al: A randomized controlled trial of intravenous tissue plasminogen activator and early intravenous heparin in acute myocardial infarction. *Circulation* 79: 281–286, 1989
13. Hnatowich DJ, Virzi F, Doherty PW, et al: Characterization of indium-111 labeled recombinant tissue plasminogen activator for the imaging of thrombi. *Eur J Nucl Med* 13: 467–473, 1987
14. Fry ETA, Mack DL, Monge JC, et al: Labeling of human clots *in vitro* with an active-site mutant of t-PA. *J Nucl Med* 30: 187–191, 1990
15. Ranby M, Norrman B, Wallen P: A sensitive assay for tissue plasminogen activator. *Thromb Research* 27: 743–749, 1982
16. Johannessen M, Diness V, Pingel K, et al: Fibrin affinity and clearance of t-PA deletion and substitution analogues. *Throm Haemtostas* 63: 54–59, 1990
17. Uehara A, Isaka Y, Etani H, et al: Binding of ^{131}I-labeled tissue-type plasminogen activator on de-endothelialized lesions in rabbits. *Nuclearmedizin* 26: 224–228, 1987
18. Iida S, Iwamoto M, Suzuki M, et al: Metabolic fate of recombinant human tissue plasminogen activator (rt-PA): blood or plasma concentration, distribution, metabolism, excretion and accumulation in rats after single intravenous administration. *Yakubutsu-Doutai* 3: 309–329, 1988 (Japanese)
19. Komura S, Yamamoto R, Itoh M, et al: Metabolic fate of SM-9527 in rats-plasma levels, distribution, metabolism and excretion after single intravenous administration. *The clinical Report* 23: 6621–6638, 1989 (Japanese)
20. Wiman B, Cmielewska J, Ranby T: Inactivation of tissue plasminogen activator in plasma. *J Biol Chem* 259: 3644–3647, 1984
21. Hoylaerts M, Rijken DC, Lijnen HR, Collen D: Kinetics of the activation of plasminogen by human tissue plasminogen activator. role of Fibrin. *J Biol Chem* 257: 2912–2919, 1982
22. Eisenberg PR, Sherman LA, Tiefenbrunn AJ, et al: Sustained fibrinolysis after administration of t-PA despite its short half-life in the circulation. *Thromb Haemost* 57: 35–40, 1987