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In vivo kinetics of ^{99m}Tc labeled recombinant tissue plasminogen activator in rabbits

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Our previous studies demonstrated that ^{99m}Tc labeled recombinant tissue plasminogen activator (rt-PA) retained high affinity with fibrin *in vitro* but showed unexpectedly low uptake in fresh thrombi *in vivo*. The present study was performed to determine the *in vivo* kinetics of radiolabeled t-PA in the rabbit.

Sequential images and blood samples after the intravenous administration of ^{99m}Tc labeled rt-PA in thrombus-bearing rabbits were taken. The radioactivity and immunological level of t-PA and PAI-1 in the solution eluted to each fraction by gel permeation chromatography were measured by means of a well scintillation counter and enzyme-linked immunosorbent assay (ELISA). Most of the radioactivity was eluted in the fraction (Fr. 7) of larger molecular weight than that (Fr. 9) of intact t-PA. The level of intact rt-PA was increased with a regimen involving the preadministration of cold rt-PA which was followed by the administration of hot rt-PA. The level of PAI-1 in plasma showed an increased rebound 15 minutes after the intravenous injection. These results suggest two possible reasons why rt-PA retains high affinity with fibrin *in vitro*, once radiolabeled, but was ineffective in delineating fresh thrombi with a gamma camera: 1) some plasma components such as PAI-1 combine with circulating radiolabeled rt-PA and form a larger molecule immediately and/or 2) radiolabeled rt-PA is modulated as a consequence of the radiolabeling and forms a larger molecule than intact rt-PA.

Key words: recombinant tissue plasminogen activator, radiolabeling, pharmacokinetics, animal study

INTRODUCTION

THROMBUS FORMATION has become a major cause of ischemic manifestations in organ function and death in aged persons. Although many methods for the detection of thrombi have been developed, noninvasive imaging diagnosis is still a challenging problem.[1] A scintigraphic method with radionuclide labeled agents that concentrate active thrombi has been considered to be a promising method for localizing their active sites.[2–4]

Recombinant tissue plasminogen activator (rt-PA) has very strong fibrinolytic activity as well as affinity with fibrin. The latter biological property is promising for the positive delineation of thrombi by means of scintigraphy. We have studied the *ex vivo* and *in vivo* affinities of Technetium-99m (^{99m}Tc) labeled rt-PA with the fibrin and venous thrombi formed in rats and cats.[5–7] ^{99m}Tc-rt-PA showed a very high affinity with fibrin *ex vivo* but showed an unexpectedly low concentration at the sites of fresh thrombi formed in animals. Two reasons were considered for the discrepancy between *ex vivo* and *in vivo* results: 1) rt-PA may concentrate in active thrombi but leave immediately after resultant fibrinolytic activity[8]; 2) only a very small amount of extrinsic rt-PA may accumulate in *in vivo* thrombi due to interaction with the fast-acting inhibitor of the plasminogen activator (PAI-1) which may be systemically and locally augmented by thrombus formation.[9–12] We confirmed that the former mechanism significantly prolonged ^{99m}Tc-rt-PA accumulation in thrombi in rats when aprotinin was used.[6] Combined dose injection of aprotinin and hot rt-PA, however, was not enough to

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clearly visualize the clots.

We extended the study to identify *in vivo* behavior of intravenously administered hot rt-PA in relation to interaction with PAI-1. The molecular weight of rt-PA and PAI-1 is estimated to be 67,000 and approximately 50,000, respectively. The complex of rt-PA with PAI-1 in plasma may have an even greater molecular weight. It is proposed that these three molecules are able to be separated from the complex by gel permeation chromatography, at least in the case of rt-PA and PAI-1. The final goal of our study is to determine the clinical usefulness of ^{99m}Tc-rt-PA as a promising thrombus imaging agent.

MATERIALS AND METHODS

Preparation of ^{99m}Tc labeled rt-PA

The rt-PA (SM-9527, Sumitomo Chemical Co., Osaka, Japan) consisted of a double polypeptide chain. The molecular weight was estimated to be 67,000. The rt-PA was dissolved in 0.7% normal saline containing only 0.01% Tween 80. It was adjusted to pH 3 and was frozen for storage. The specific activity was 7.82×10^6 IU/mg.

After melting frozen rt-PA at room temperature, a few hundred μl of rt-PA was pipetted into a plastic tube, and thereafter a commercially available stannous solution (containing 1 μmol/m*l* of stannous ion at pH 3, Nihon Medi-Physics Co., Osaka, Japan) used in the ^{99m}Tc liver image kit was added. After gently stirring for 5 minutes, freshly eluted ^{99m}Tc-pertechnetate was added and followed by incubation for 20 minutes at room temperature with occasional gentle agitation. The amounts of rt-PA, stannous ion and ^{99m}Tc-pertechnetate were 1.38 mg, 0.001 μmol and 0.1 m*l*, respectively. The process was described in more detail in our previous report.[5]

Imaging of thrombus-bearing rabbits

Female house rabbits of approximately 3 kg in body weight were anesthetized with ketamine hydrochloride (10 mg/kg). A wire-guide for angiography (0.9 mm in diameter), which was coated with fibrin (Beliplast, Behringwerke Ag, Hoechst Japan limited), was set at the inferior vena cava through the femoral vein. A venula needle was also inserted into the inspilateral vein for further blood sampling.

The anesthetized rabbit was fixed on a board in a supine position and was put under a digital gamma camera. The rt-PA which was diluted to 0.1 mg/m*l* just prior to the intravenous injection was administered to two different groups of rabbits through the ear vein. The first group (hot group; n = 3) received ^{99m}Tc labeled rt-PA (hot rt-PA) alone as an i.v. bolus injection of 1 mg/kg given over 2 minutes. The second group (cold-hot group; n = 4) received two injections of intact (0.5 mg/kg) and hot (0.5 mg/kg) rt-PA at 5 minute intervals for a total dose of 1 mg/kg. Sequential images following the injection were obtained with a 64 by 64 matrix at 20 second intervals as storage data for the computer, and with a 128 by 128 matrix at 2 minute intervals for a film display. 3 m*l* venous blood samples were withdrawn at 0, 5, 10, 15 and 20 minutes after completing the injection. After completing an *in vivo* study, an image of the inserted wire-guide was taken by a gamma camera following sacrificing of the rabbit.

Table 1 Results of imaging of thrombus-bearing rabbits

Body weight	Dosing regimen	Dose of rt-PA	Thrombus formation and scintigraphy
3.5 kg	hot	3.5 mg	18 F-wire guide, negative image, no thrombus and no uptake in autopsy-material
3.1 kg	hot	3.0 mg	Lung emboli by Beliplast, negative image, no uptake in autopsy-lungs
3.1 kg	hot	3.0 mg	35 F-wire guide, negative image, positive thrombi and positive image in autopsy-material
3.1 kg	cold + hot	1.5 mg 1.5 mg	35 F-wire guide, negative image, no thrombus and no uptake in autopsy-material
3.1 kg	cold + hot	1.5 mg 1.5 mg	35 F wire-guide, negative image, no thrombus and no uptake in autopsy-material
3.25 kg	cold + hot	1.6 mg 1.6 mg	35 F-wire guide, negative image, penetration into peritoneal cavity, no thrombus and no uptake in autopsy-material
3.1 kg	cold + hot	1.6 mg 1.6 mg	35 F-wire guide, negative image, positive thrombus and positive uptake in autopsy-material

Measurements of radioactivity and immunological level of t-PA and PAI-1 in plasma

Plasma was separated from whole blood by sedimentation and was eluted to the fraction corresponding to the molecular weight by high performance liquid chromatography (HPLC): the column was a 1.4×31 cm Superose 12 (Pharmacia LKB, Uppsala, Sweden); elusion was at 1 m*l*/minute with 0.02 mol Tris buffer solution (pH 7.4); and each fraction collected was 2 m*l*. The radioactivity in the solution eluted in each fraction was counted in a well-scintillation counter. The radioactivity in each fraction was expressed as a percentage of the cumulative radioactivity eluted in all fractions. The immunological level of t-PA and PAI-1 in the solution of each fraction was determined with an enzyme-linked immunosorbent assay (ELISA) kit (Biopool, Umea, Sweden).

RESULTS

Imaging of thrombus-bearing rabbits

^{99m}Tc-rt-PA concentrated in the liver immediately and the concentration in the kidney and the urinary bladder occurred later (Fig. 1). The fibrin coated wire-guide was not

Table 2 Blood clearance time ($T_{1/2}$) of ^{99m}Tc labeled rt-PA in rabbits

	Hot group		Cold + Hot group	
	Fast segment	Slow segment	Fast segment	Slow segment
Rabbit 1	1.01 min	33.47 min	0.80 min	16.62 min
Rabbit 2	1.34 min	13.28 min	1.29 min	20.73 min
Rabbit 3	1.68 min	33.99 min	1.94 min	39.32 min
mean	1.34 min	26.9 min	1.34 min	25.22 min
s.d.	0.33 min	11.8 min	0.57 min	11.52 min

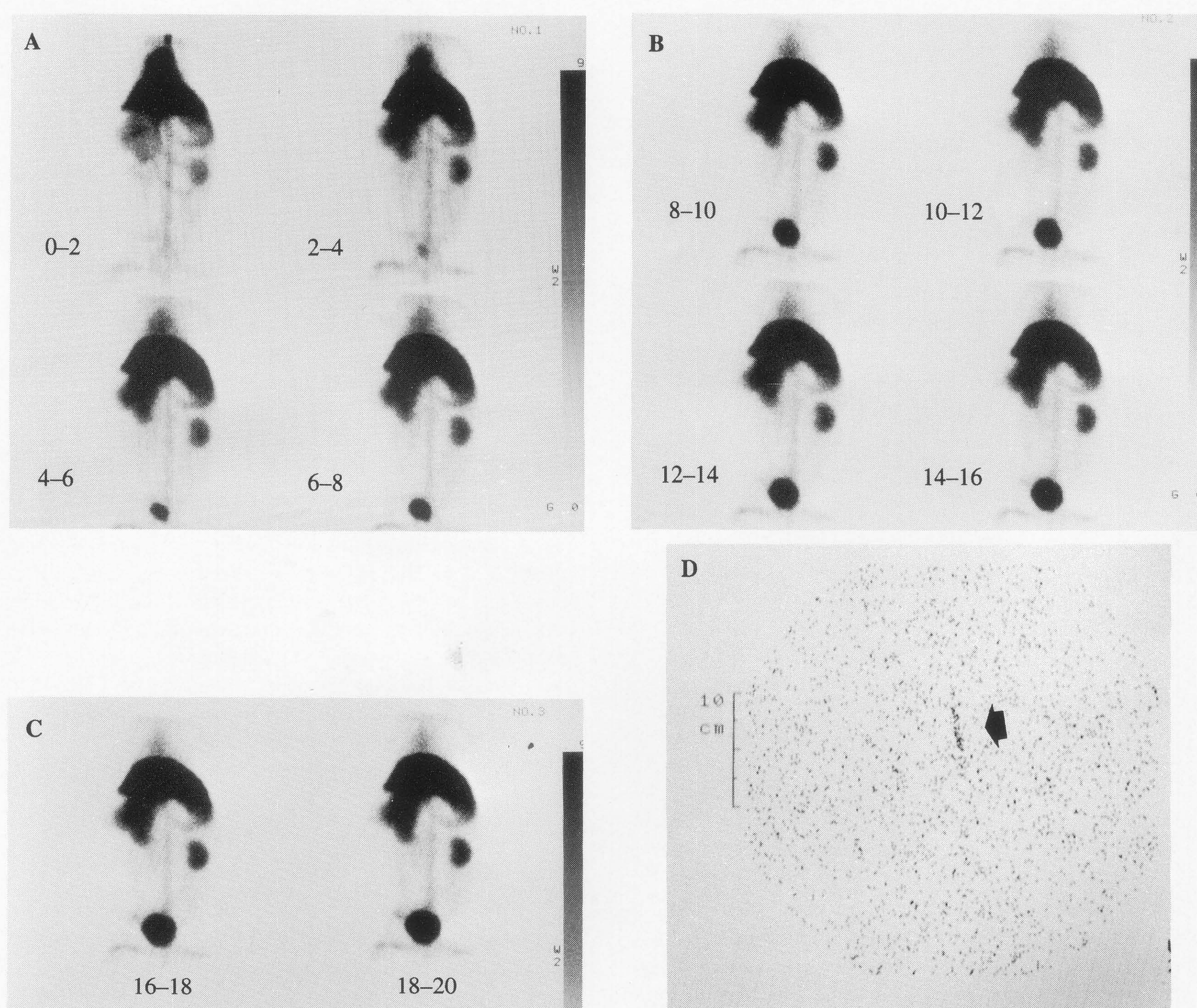

Fig. 1 Sequential images taken at 2 minute intervals following the intravenous injection of ^{99m}Tc labeled rt-PA into a thrombus-forming rabbit. A: 0–8 minute, B: 8–16 minute, C: 16–20 minute, D: image of a fibrin-coated wire-guide following sacrifice of the rabbit.

shown as a high uptake area in the sequential images, but was removed after completion of the study did show a minimal concentration of ^{99m}Tc-rt-PA (Fig. 1). Positive uptake in the wire-guide was dependent on the formation of fresh thrombi around the artificially made fibrin clot (Table 1).

The blood disappearance curve was different in each rabbit (Table 2). The half times for the blood clearance curves for the two groups, calculated by a computer program 2 compartment model, were similar.

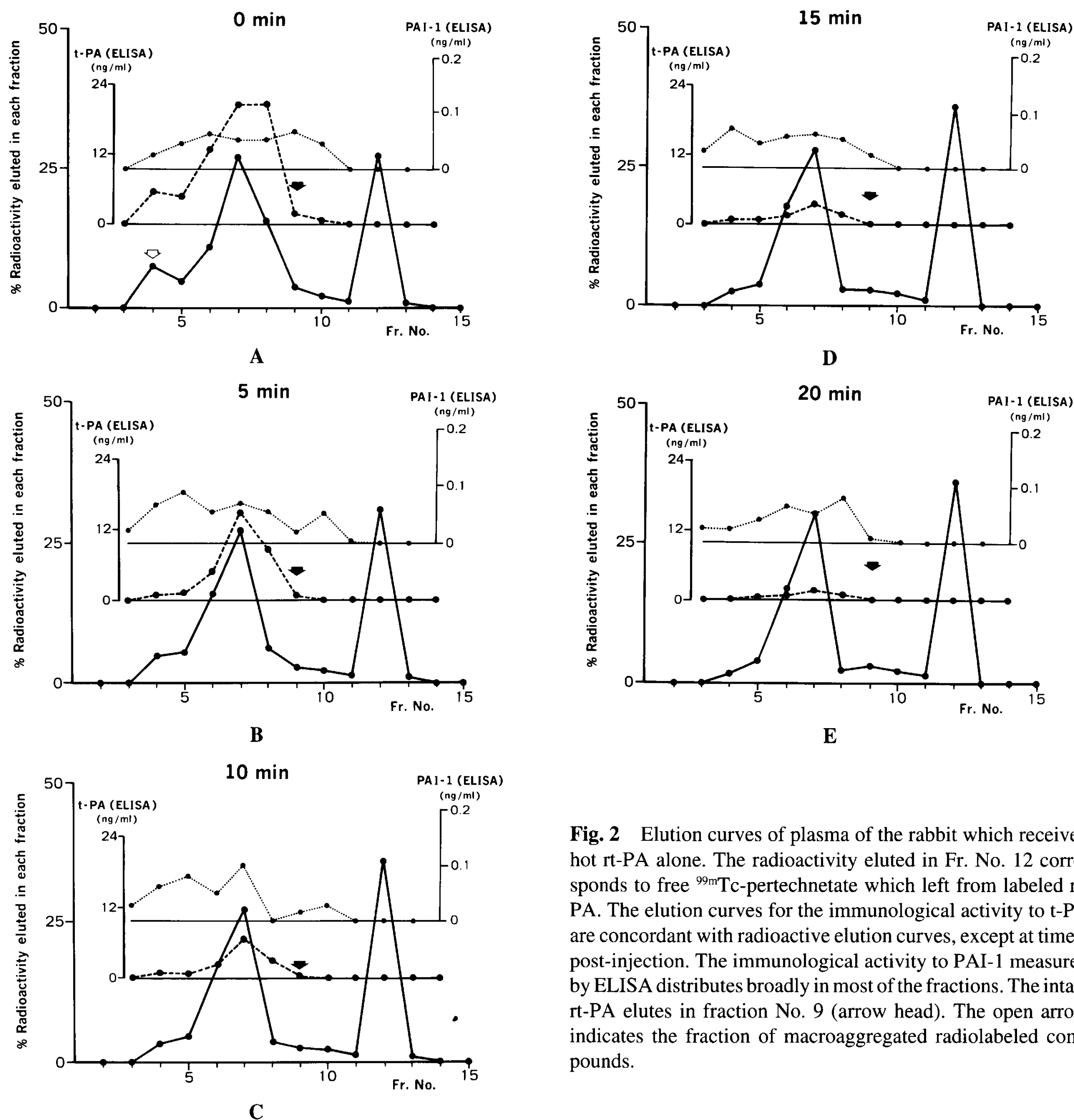

Fig. 2 Elution curves of plasma of the rabbit which received hot rt-PA alone. The radioactivity eluted in Fr. No. 12 corresponds to free ^{99m}Tc-pertechnetate which left from labeled rt-PA. The elution curves for the immunological activity to t-PA are concordant with radioactive elution curves, except at time 0 post-injection. The immunological activity to PAI-1 measured by ELISA distributes broadly in most of the fractions. The intact rt-PA elutes in fraction No. 9 (arrow head). The open arrow indicates the fraction of macroaggregated radiolabeled compounds.

Elution curves for plasma sample by HPLC

The radioactive peak was observed in two fractions: the first peak in fraction No. 7 and the second peak in fraction No. 12 (Fig. 2). The latter peak corresponded to free pertechnetate which was separated from labeled rt-PA during the storage of plasma and gel permeation procedure. The elution pattern of radioactivity was similar for both hot (Fig. 2) and cold-hot groups (Fig. 3). In contrast, the elution pattern for the immunological level of t-PA in each fraction was different in hot and cold-hot groups. In the former group, the peak level of t-PA was observed in fraction No. 8 at time 0. This peak level immediately disappeared in plasma obtained 5 minutes after the injection. In the latter group, the peak level of t-PA was observed in fractions No. 8 and 9 at 0 minutes post-injection. The intact rt-PA was eluted in the fraction No. 9. The peak level of t-PA in fraction Nos. 8 and 9 was also immediately disappeared 5 minutes following the intravenous administration. The level of rt-PA in each fraction of each plasma sampled 10 minutes after the injection of ^{99m}Tc-rt-PA appeared to be parallel to the radioactive elution curve. The elution curve of the PAI-1 level in the fractions showed no specific peak (Fig. 2). This result was due to a very low level of PAI-1 in fractions once eluted and represented a non-specific immunological reaction to PAI-1 at such a plasma level. When the level of PAI-1 was measured in plasma before HPLC, it showed an increased rebound at 15 minutes in the hot group and a somewhat delayed increase in the cold-hot group following the administration of hot rt-PA (Fig. 4).

Fig. 3 Elution curves of plasma of the rabbit which received cold rt-PA prior to the administration of hot rt-PA. The elution curves for the immunological activity to t-PA is only different at time 0 post-injection. Fraction No. 9 (arrow head) corresponds to the fraction eluting intact rt-PA. The open arrow indicates the fraction of macroaggregated radiolabeled compound.

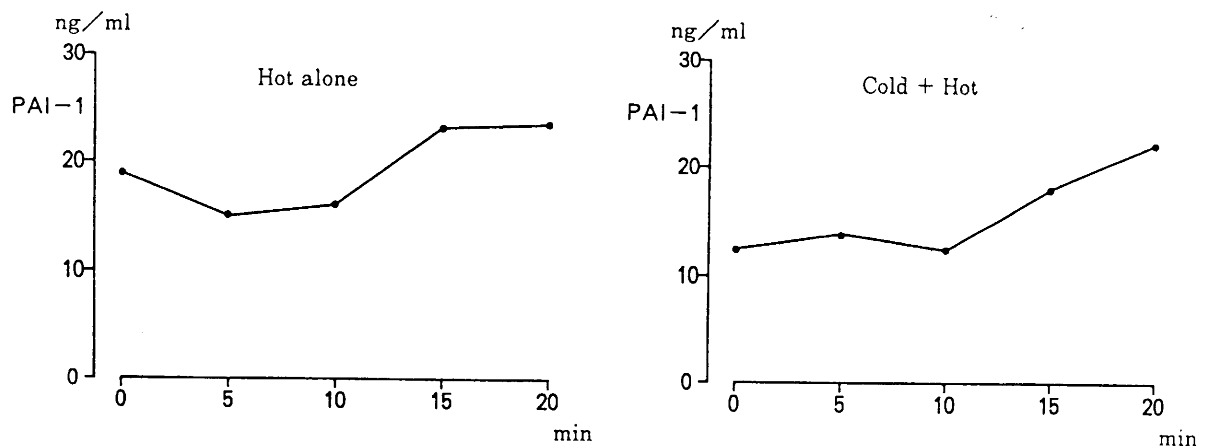

Fig. 4 Change of immunological activity to PAI-1 in sampled plasma over time.

DISCUSSION

In *ex vivo* experiments which were performed prior to *in vivo* studies, ^{99m}Tc-rt-PA showed a high uptake in the fibrin coated segment of the wire-guide. However, no radioactive distribution was detected in a wire-guide in the *in vivo* study, although some wire-guides obtained following the sacrifice of the rabbits showed radioactive deposits in the fibrin-coated portion. These results were mainly due to minimal formation of fresh thrombi around the fibrin-coated wire-guide, which were confirmed by inspection after sacrifice of the rabbits. The positive delineation of fibrin-coated wire guide with ^{99m}Tc-rt-PA depended on intravascular formation of fresh red thrombi on the surface of the fibrin paste. It required 4 to 5 hours to form red thrombi around the wire-guide.

The present study provides *in vivo* kinetics of ^{99m}Tc labeled rt-PA. Most of the radiolabeled rt-PA administered collected had a greater molecular weight than that of the intact rt-PA in circulating plasma. This results suggest that extrinsic rt-PA interacts with fast-acting plasma protein, presumably PAI-1, immediately after the intravenous injection. It is well known that PAI-1 plays a pivotal role in preventing the concentration of externally administered rt-PA in active thrombi.[9–11] Thrombus formation also augments the introduction of PAI-1 at the site of the damaged vessel.[12] The level of free rt-PA, which can only act biologically as an effector of fibrin-binding *in vivo*, is therefore exhausted. Multiple injections of t-PA are more effective than a single dose injection on thrombolysis.[13] We were able to confirm that the second dose injection of the hot rt-PA extended the return to normal serum PAI-1 level. However, it was not demonstrated that pre-administration of the cold rt-PA results in a prolongation of exhaustion of the externally administered hot rt-PA in the circulation.

One finding in the present study is the different elution curves of radioactivity and the immunological level of rt-PA, particularly at 0 minutes after the injection in two different doses of hot and cold rt-PA. In the hot group, the radioactive peak was eluted in fraction No. 7 and the immunological peak of t-PA in fraction No. 8. In the cold-hot group, the radioactive peak was observed in fraction No. 7 and the immunological peaks in fractions 8 and 9. The radioactivity in the fraction corresponding to the intact rt-PA did not increase. These results suggest that most of the t-PA in plasma represents the pre-administered cold rt-PA rather than the later hot rt-PA. It is assumed that most of the radiolabeled rt-PA is modulated to a molecule with a weight larger than that of intact rt-PA in the circulation. This molecular modulation seems to be related to the interaction with circulating PAI-1 and/or radiolabeling on protein as an agent. The denaturation of radiolabeled protein influences its accumulation in the liver and elimination from the circulation. Modulation of the carbohydrate structure is reported to be important in the *in vivo* behavior of radiolabeled rt-PA.[14] We confirmed only the inherent affinity of ^{99m}Tc-labeled rt-PA with fibrin *in vitro* and did not investigate its influence on the denaturation of rt-PA in the molecular structure. If ^{99m}Tc-rt-PA, which still retains the biological activity of fibrin binding *in vitro*, is modulated to a larger molecular weight as consequence of radiolabeling without interaction with PAI-1 *in vivo*, then elimination from the circulation may be altered and thereby accumulation in intravascular thrombi may be affected. Further investigation will be required to clarify the *in vivo* behavior of ^{99m}Tc labeled rt-PA, particularly the relationship between the positive delineation of an active thrombus and the influence of PAI-1.

The wild-type t-PA has three different active domains: a fibrin-binding domain, fibrinolytic domain and PAI-1 binding domain.[15] The recombinant technique can produce mutant t-PA which has only a single domain.[15,16] From the pharmacological point of view, the rt-PA which retains only a fibrin-binding domain with a lack of properties such as fibrinolytic as well as PAI-1 binding domains is ideal for thrombus imaging agents.[16,17]

In conclusion, most of the radiolabeled rt-PA in rabbit plasma was collected by gel permeation chromatography as a complex with a molecular weight greater than that of intact rt-PA by gel permeation chromatography. This complex may be accounted for by the following: 1) interaction of ^{99m}Tc-rt-PA with PAI-1 during circulation and/or 2) modulation of rt-PA as a consequence of radiolabeling. This complex seems to be ineffective for the positive delineation of active thrombi. Clinical application of ^{99m}Tc-rt-PA is still far from being realized.

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