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In vivo distribution of Tc-99m labeled recombinant tissue-type plasminogen activator in control and thrombus-bearing rats

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In vivo distribution of Tc-99m labeled recombinant tissue-type plasminogen activator (Tc-99m-rt-PA) was studied in control rats and thrombus-bearing rats. To compare fibrin binding *in vivo* with that *in vitro*, Tc-99m-rt-PA binding to fibrin gel *in vitro* was also imaged.

Rapid blood clearance and accumulation into the liver and kidneys were observed in both control and thrombus-bearing rats. Accumulation in the stomach, which indicates instability of labeled rt-PA *in vivo*, was very low until two hours after injection. Tc-99m-rt-PA accumulation in the clots was higher than that in skeletal and heart muscles, although it was lower than in blood, liver, and kidneys. Administration of aprotinin, an antifibrinolytic agent, significantly prolonged clot accumulation of Tc-99m-rt-PA at 30 minutes after injection. These results suggest that fibrinolysis is responsible for the low rt-PA concentration in the clots. A scintigram of a thrombus-bearing rat demonstrated increased radioactivity at the clot forming site. On the other hand, Tc-99m-labeled human albumin, which was used as a control, was not accumulated in the clot. Tc-99m-rt-PA binding to fibrin gel *in vitro* was clearly imaged.

By comparison, *in vivo* fibrin binding of Tc-99m-rt-PA was much lower than *in vitro*. The reasons for low thrombus uptake *in vivo* may be: 1. biochemical inactivation of extrinsically administered rt-PA by t-PA inhibitor. 2. fibrinolysis by rt-PA activated plasminogen. Overcoming these limitations will enable Tc-99m-rt-PA to reach the stage of clinical trials.

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

INTRODUCTION

THROMBOTIC DISEASE is one of the main causes of death in Japan, since many people die from myocardial infarction and cerebral infarction every year.[1] To give proper treatment to these patients, including anticoagulant therapy, it is necessary to know the exact location of the thrombi. Because thrombotic disease needs repeated studies, noninvasive methods are desirable instead of contrast angiography which has been the main method used in the diagnosis of thrombosis.[2] The scintigraphic method has been proposed for the diagnosis of thrombosis, and suitable radiopharmaceuticals with high thrombus affinity and specificity have been sought.[3,4]

Several radiopharmaceuticals have been developed, and some of them such as In-111 labeled platelets,[5,6] radiolabeled antifibrin antibodies[7,8] and antiplatelet antibodies[9] have shown good sensitivity in locating thrombi. However, In-111 labeled platelets have high blood pool activity and considerable time is needed to obtain a clear thrombus image. Radiolabeled mouse antibodies have the problem of human antimurine antibodies when studies are repeated. Tissue-type plasminogen activator, a native human protein with high affinity with fibrin and little immunogenic risk,[10] seems the ideal compound to overcome these problems.

My coworkers and I previously reported a simple

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labeling procedure with Tc-99m-rt-PA, the high thrombus affinity of Tc-99m-rt-PA and the possibility of thrombus imaging.[11] In this paper, I will show rt-PA distribution in control and thrombus-bearing rats, and discuss the clinical utility of Tc-99m-rt-PA as a thrombus imaging agent.

MATERIALS AND METHODS

Preparation of Tc-99m labeled recombinant tissue-type plasminogen activator

Recombinant t-PA (SM-9527, lot No. p-113) was generously donated by Sumitomo Chemical Co., Osaka, Japan. The protein concentration was 18 mg/m*l*, and its specific activity was found to be 7.82×10^6 IU/mg. Other precise qualities of the rt-PA were described in the previous paper.[11]

The rt-PA was labelled by previously described procedures:[11] 0.078 m*l* (1.38 mg) of rt-PA was pipetted into a plastic tube, and thereafter 0.01 m*l* of a commercially available stannous solution (Nihon Medi-Physics Co., Osaka, Japan) was added. After stirring for 5 minutes, 0.1 m*l* of Tc-99m pertechnetate was added and incubated for 15 minutes at room temperature. The stability of the labeled materials was described in the previous paper.[11] Radiolabeling efficiency was checked at the time of each *in vivo* and *in vitro* use by paper chromatography.

Biodistribution of Tc-99m-rt-PA in rats

«Control rats»

The rats (inbred WKM-Hkm rats, weighing 200–350 g) were anesthetized with pentobarbital (40–50 mg/kg). Two m*l* of Tc-99m-rt-PA, which was adjusted to 0.1 mg/m*l* by dilution with normal saline just before injection, was administered through the tail vein. The animals were sacrificed to determine the tissue concentration of Tc-99m-rt-PA at 15, 30, 60, and 120 minutes. Samples of blood, muscle, lung, heart, liver, spleen, kidney, and stomach were weighed and counted in a well-type gamma counter. The results were also expressed as a percent of the injected dose per gram, and normalized for a 1 kg animal as well.

«Thrombus-bearing rats»

The thrombus-bearing rats were prepared by ligation of the inferior vena cava (IVC) just below the communication of the left renal vein. The rats were divided into two groups: the first group did not receive aprotinin (Trasylol, Bayer), the second received 20,000 KIE of aprotinin just before the injection of Tc-99m-rt-PA. Five m*l* of Tc-99m-rt-PA was then administered to the rats in both groups through a fine venula needle that had already been positioned in the central portion of the IVC ligation, and followed by flushing with normal saline. The rats were sacrificed to determine biodistribution and Tc-99m-rt-PA accumulation in clots at 15 and 30 minutes. The organs and clots were weighed and counted in the same way as in the control rats. The results were expressed in the same way as in the control rats.

In vivo *imaging of control and thrombus-bearing rats*

Tc-99m-rt-PA (0.1 mg/m*l* of rt-PA, 35 MBq/m*l* of Tc-99m) at a dose of 20 m*l*/kg was administered for imaging through the tail vein of control rats and through a fine venula needle positioned at the IVC of thrombus-bearing rats. Two of four thrombus-bearing rats were given an injection of 20,000 KIE of aprotinin just before the administration of Tc-99m-rt-PA. Images at 0, 5, 10, 15, and 120 minutes after injection were taken and recorded in a computer.

Thrombus imaging with Tc-99m-labeled human albumin (Tc-99m-HSA) was also performed as a control image. Images were obtained in the same way as those of Tc-99m-rt-PA.

Binding of Tc-99m-rt-PA to fibrin gel

Beriplast P (Behringwerke AG. Hoechst Japan Limited), a kit containing human fibrinogen, aprotinin, $CaCl_2$, and thrombin, was used for forming fibrin gel blocks. 120 mg of fibrinogen powder was mixed with 1,500 KIE of aprotinin. 450 NIHU of thrombin with 22 mg of $CaCl_2$ was then added. The fibrin block was formed in a $1\times1\times5$ cm box. After incubation for 24 hours at 37 degrees centigrade, the fibrin block was cut into five pieces. Each piece was put into a plastic box containing 3 m*l* of Tc-99m-rt-PA (0.1 mg/m*l* of t-PA, 35 MBq of Tc-99m) and incubated for 1 hour at 37 degrees centigrade. Thereafter, imaging and counting of fibrin blocks were performed after several rinses with normal saline. As controls, Tc-99m-HSA and pertechnetate, which were also reacted with fibrin gel blocks and rinsed in a same way as described above, were imaged and counted.

RESULTS

Stability of labeled rt-PA

Labeling stability of diluted Tc-99m-rt-PA at the time of injection was 81.9%–89.3% (mean±sd being 84.6%±3.4%).

Biodistribution

«Control rats»

Organ biodistribution of Tc-99m-rt-PA in control rats is shown in Table 1. Blood clearance was rapid. The radioactivity in the liver showed the highest concentration at 15 minutes after injection and decreased as a function of time. On the other hand,

Table 1 Biodistribution of control rats

Organ	Tissue concentration after injection*1			
	15 min (n=6)	30 min (n=6)	60 min (n=5)	120 min (n=6)
Blood	0.192±0.027	0.111±0.010	0.080±0.001	0.100±0.037
Heart	0.035±0.006	0.024±0.004	0.019±0.003	0.023±0.010
Lung	0.108±0.022	0.064±0.011	0.042±0.005	0.052±0.021
Liver	2.306±0.261	2.007±0.142	1.456±0.120	1.237±0.170
Spleen	0.559±0.038	0.537±0.110	0.519±0.117	0.328±0.060
Kidney	1.060±0.226	1.038±0.143	1.251±0.162	1.383±0.060
Muscle	0.020±0.003	0.010±0.001	0.008±0.001	0.013±0.005
Stomach*2	0.328±0.063	0.282±0.040	0.393±0.233	0.339±0.155

*1 expressed as %dose/g tissue normalized for a 1 kg animal (mean±sd)
*2 expressed as %dose/whole organ (mean±sd)

Table 2 Biodistribution of thrombus-bearing rats

Organ	Tissue concentration after injection*1			
	Without aprotinin		with aprotinin	
	15 min (n=7)	30 min (n=4)	15 min (n=5)	30 min (n=6)
Clot	0.067±0.028*	0.036±0.009**	0.104±0.021*	0.049±0.003**
Blood	0.305±0.041	0.159±0.043	0.300±0.105	0.215±0.615
Heart	0.040±0.007	0.027±0.009	0.069±0.029	0.034±0.006
Lung	0.163±0.019	0.040±0.007	0.231±0.037	0.151±0.073
Liver	1.089±0.154	1.035±0.168	0.822±0.163	0.850±0.270
Spleen	0.328±0.016	0.238±0.045	0.523±0.304	0.277±0.035
Kidney	0.511±0.061	0.580±0.101	0.540±0.091	0.710±0.054
Muscle	0.017±0.030	0.014±0.005	0.020±0.003	0.013±0.073
Stomach*2	0.183±0.043	0.128±0.025	0.291±0.113	0.197±0.35

*1 expressed as %dose/g tissue normalized for a 1 kg animal (mean±sd)
*2 expressed as %dose/whole organ (mean±sd)
*,**: statistically significant ($p<0.05$)

the radioactivity in the kidney increased until 120 minutes after injection. Radioactivity in the whole stomach was 0.282%–0.393% per administered dose, and it did not increase as a function of time.

«Thrombus-bearing rats»

Biodistribution of Tc-99m-rt-PA in thrombus-bearing rats was almost the same as in control rats (Table 2). Tc-99m-rt-PA accumulation in clots was lower than that in blood, but higher than that in skeletal and heart muscles. Radioactivity in the clot was significantly higher in the group with aprotinin than in the group without aprotinin ($p<0.05$).

In vivo imaging

Serial images of the control rat, the thrombus-bearing rat, and the thrombus-bearing rat which received aprotinin are shown in Figs. 1, 2, and 3, respectively. They all received an injection of Tc-99m-rt-PA. Thrombus-bearing rat with Tc-99m-HSA is shown in Fig. 4. The radioactivity in the cardiac region was rapidly decreased and the liver and kidney were clearly visualized in both control and thrombus-bearing rats. Blood clearance was calculated by a two-component biexponential analysis of the time activity curve of the region of interest, which was drawn over the heart. This calculation was done in all rats except those which received an injection of Tc-99m-HSA. No significant difference was obtained between the blood-clearance half-time in control rats and that in thrombus-bearing rats. The mean half-time value for the fast component was 2.6±0.26 minutes (mean±SD), and for the slow component it was 24.2±6.4 minutes (mean±SD). Increased radioactivity in the bladder with time suggested rt-PA excretion through the urinary system. The stomach was not visualized till 2 hours after injection. However, an image at 24 hours (Fig. 1) after injection showed a high concentration in the stomach.

Thrombus-bearing rats which received an injection of Tc-99m-rt-PA with and without aprotinin had slightly increased radioactivity at the position of

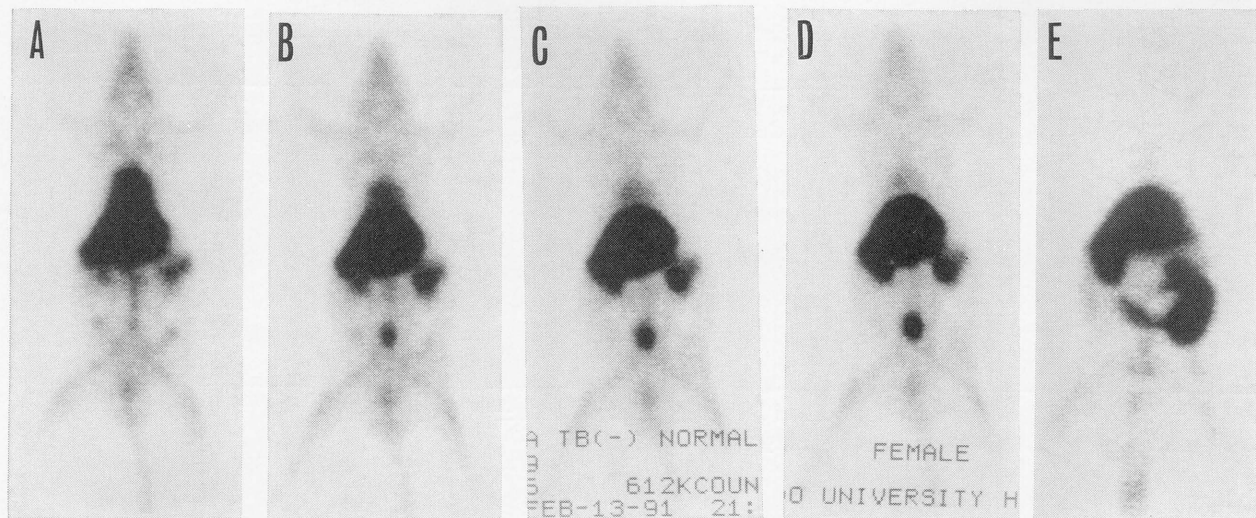

Fig. 1 Sequential images of a control rat every five minutes and 24 hrs after a single administration of 2.0 mg/kg of Tc-99m-rt-PA. Rapid clearance from the heart and serial increases of radioactivity in the liver, kidney, and urinary bladder are noted. Marked accumulation of Tc-99m is noted in the stomach at 24 hr. A: 0–5 min, B: 6–10 min, C: 11–15 min, D: 16–20 min, E: 24 hr.

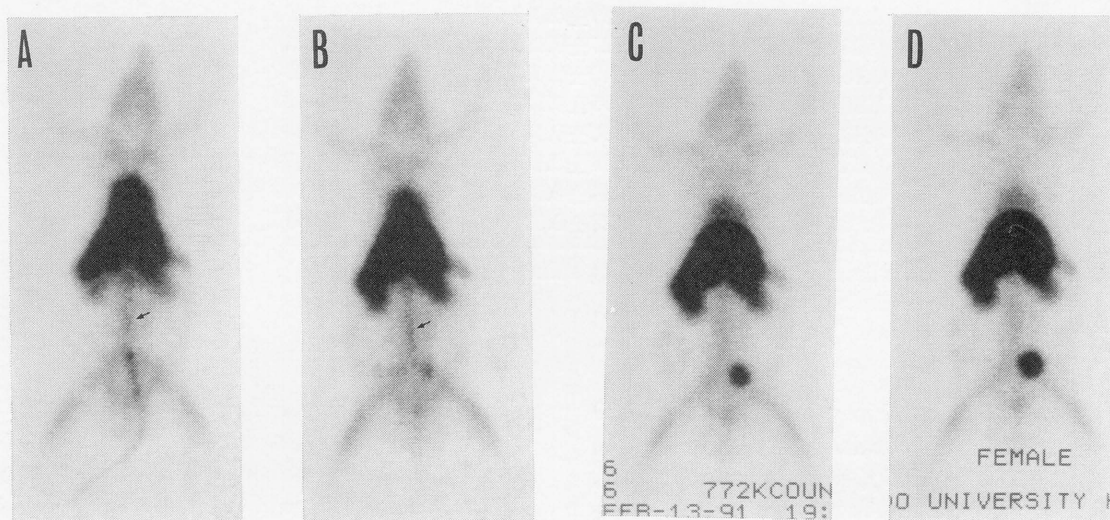

Fig. 2 Sequential images of a thrombus-bearing rat every five minutes after a single administration of 2.0 mg/kg of Tc-99m-rt-PA. Positive uptake (arrow) corresponding to the clot is noted in the abdomen. A: 0–5 min, B: 6–10 min, C: 11–15 min, D: 16–20 min.

ligation. The findings suggested an accumulation of the radioagent to the clot. Radioactivity in the clot disappeared as a function of time by 30 minutes after injection.

In thrombus bearing rats which received an injection Tc-99m-HSA, no radioactivity was noted at the position of ligation.

Fibrin gel imaging in vitro

Fibrin blocks incubated in Tc-99m-rt-PA were clearly imaged (Fig. 5A). Faint activity was detected in the blocks which were incubated in Tc-99m-HSA and pertechnetate (Fig. 5B). Fibrin binding was 13.25% per incubated dose for Tc-99m-rt-PA and 0.45% per incubated dose for Tc-99m-HSA and 0.25% per incubated dose for pertechnetate.

DISCUSSION

Tissue plasminogen activator is known to be an effective thrombolytic agent that activates plasminogen by binding fibrin in a thrombus.[12] Its high affinity with fibrin seems suitable for specific detection of thrombi. Several radioisotopes have been

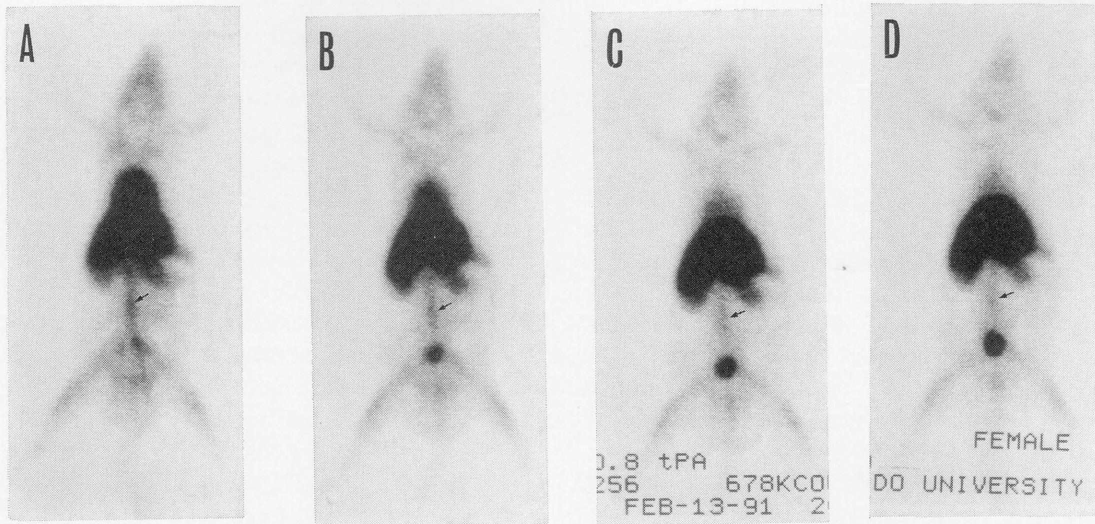

Fig. 3 Sequential images of a thrombus-bearing rat every five minutes after administration of 20,000 U of aprotinin and 2.0 mg/kg of Tc-99m-rt-PA. Positive uptake (arrow) corresponding to the clot is noted in the abdomen. A: 0–5 min, B: 6–10 min, C: 11–15 min, D: 16–20 min.

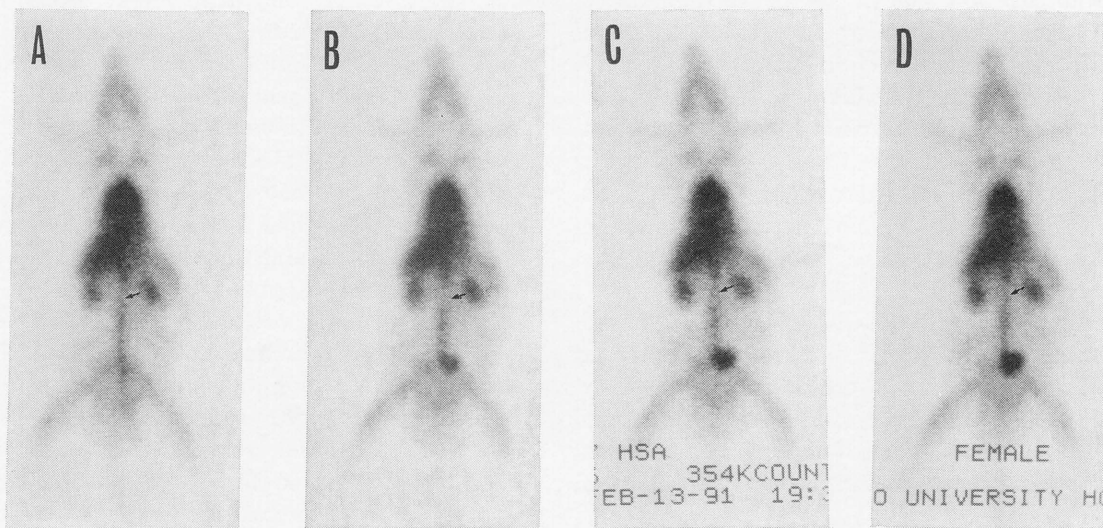

Fig. 4 Sequential image of a thrombus-bearing rat every five minutes after a single injection of Tc-99m-HSA. A photon deficient area (arrow) corresponding to the clot is noted in the abdomen. A: 0–5 min, B: 6–10 min, C: 11–15 min, D: 16–20 min.

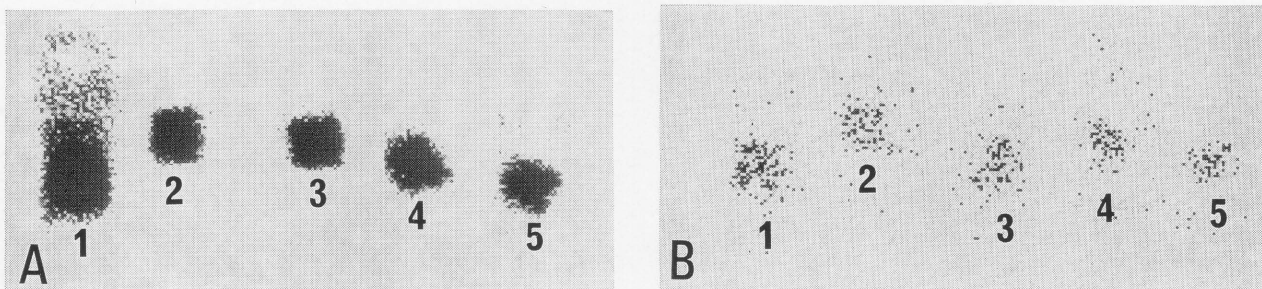

Fig. 5 A: Images of fibrin gel blocks (1–5) rinsed with normal saline after incubation in Tc-99m-rt-PA. B: Images of fibrin gel blocks rinsed with normal saline after incubation in Tc-99m-HSA (three from the left, 1–3) and in pertechnatate (two from the right 4–5).

used for labeling, including Tc-99m,[13] I-131,[14] and In-111.[15,16] Above all, as for the radioisotope itself, Tc-99m has several advantages, such as high photon flux, easy availability, and a relatively low radiation dose.

Tc-99m labeled t-PA was first reported in 1983 by DeBoer et al.[13] No further study was done thereafter. They tried thrombus imaging in patients and rabbits. Though it was a brief report, they concluded that thrombi were successfully visualized when Tc-99m labeled t-PA was injected locally. Because their methods and results were not described in detail, it is difficult to compare their results with the present study. However, they showed the possibility of thrombus imaging with Tc-99m labeled t-PA.

Blood clearance calculated by dynamic images in the present study was consistent with that obtained by blood sampling in previous literature.[15–20] Rapid concentration of Tc-99m-rt-PA in the liver, which indicates rapid clearance of Tc-99m-rt-PA from the circulation, was noted. Later increased uptake in the kidney, which indicates slow clearance of Tc-99m-rt-PA was also observed in the study. However, a second peak of radioactivity in the blood and a rapid drop in radioactivity in the liver within 60 minutes after injection, which were described in reports using radioiodinated t-PA,[17–19] were not observed. This phenomenon was not observed in the study using In-111 labeled t-PA,[15,16] either. It is accounted for by the deiodination of the tracer in the liver and the release of radioiodine into the circulation.

In a previous report,[11] Tc-99m-rt-PA was observed to be unstable *in vitro* after dilution. In the present study, Tc-99m-rt-PA was confirmed to be fairly stable *in vivo* until two hours after injection, although the reason for this is unknown. Two hours is long enough for imaging, even for positive delineation of the active clot.

In vivo accumulation of Tc-99m-rt-PA in the clots was not as high as expected from the result of *in vitro* fibrin binding with fibrin gel blocks. Nevertheless, the clots were delineated by serial imaging of thrombus-bearing rats despite the lower concentration in tissue than in blood. This may be accounted for by the fact that rt-PA can bind to only the surface of the clots[12] and cannot infiltrate into the clots. As the result, tissue concentration of Tc-99m-rt-PA per given weight of the clots was lower than that in the blood, even if radioactivity in the surface of the clot was higher than that in the blood.

There may be several reasons for the unexpected low accumulation of the clots *in vivo* compared to that *in vitro*. Extrinsically administered t-PA immediately binds to the clots and thereafter is released to the blood from bound fibrin by thrombolysis. Since fibrin gel, which we used *in vitro*, was plasminogen free, it was free from fibrinolysis. Aprotinin was used to avoid *in vivo* fibrinolysis in this tudy. Aprotinin is an antifibrinolytic agent, and it stops thrombolysis immediately.[21,22] The administration of aprotinin prolonged clot accumulation significantly. It suggested that fibrinolysis was responsible for the prompt decrease in positive accumulation of Tc-99m-rt-PA in the clots. Even with aprotinin, the accumulation was not as high as *in vitro*, and the clot image was not clear enough for clinical use. Low accumulation of Tc-99m-rt-PA in clots cannot be explained only by a mechanism of fibrinolysis.

Another mechanism for low accumulation of Tc-99m-rt-PA in the clots *in vivo* might be the formation of a complex between rt-PA and the inhibitor. Verheijen et al.[23] showed that this complex did not bind to a fibrin clot. Extrinsic t-PA added to pooled normal plasma was immediately inhibited, and the inhibited t-PA level was almost identical before and after clotting in his study. In this context, fast-acting inhibitor type 1 (PAI-1)[23–25] might play a pivotal role in preventing very high uptake of extrinsically administered rt-PA by the clot which is expected from the results of *in vitro* fibrin binding.

Recently, many genetically engineered or chemically modified variants of t-PA have been produced.[26–28] Above all, active-site inhibited variants (genetically or chemically) abolish the plasminogen activation activity and formation of stable complex with PAI-1 to various degrees.[29–31] By using these variants, several attempts to overcome the limitations of t-PA have been made. Butler et al.[16] labeled the active-site inhibited rt-PA with In-111 and demonstrated a high thrombus-blood ratio in inhibited rt-PA binding to fibrin without fibrinolysis. Fry et al.[26] reported labeling an active-site mutant substituting threonine for 478 serine, and suggested its usefulness for clinical imaging *in vivo*. However, it is still unclear whether the administration of variant t-PA with only fibrin affinity and without fibrinolytic activity may influence to the affinity of therapeutic wild t-PA with clots.

There are some other points we have to recognize before we apply the results of this study to a human study. One is the fibrinolytic activity of human extrinsic t-PA in rats, and the other is the rate and extent of complex formation between the inhibitor present in rat plasma and human extrinsic t-PA. Korninger et al.[32] showed that the degree of fibrinolysis in rat plasma was only 10 percent of that in human plasma, and Haggroth et al.[33] showed that the rate of inhibition was faster in rat plasma than in human plasma. These results mean that the effect of fibrinolytic ability is higher and the effect of inhibitor is lower in a human study than in a rat study.

Tc-99m-rt-PA may be useful as diagnostic and prognostic indicator for anticoagulant therapy with extrinsic rt-PA at present. This study showed that Tc-99m-rt-PA demonstrated positive delineation of artificially made clots in rats. Further attempts to get a clearer image must be made to reach the stage of clinical application.

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