Localization of Tissue Plasminogen Activator on Experimental Thrombi in Rats

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The localization of tissue plasminogen activator (t-PA) or urokinase plasminogen activator (u-PA) on thrombi was investigated in disseminated intravascular coagulation rats (DIC rats) induced by thrombin.

One hour after the intravenous infusion of thrombin to rats, the plasma fibrinogen level decreased, while the plasminogen activator activity in the plasma euglobulin fraction increased. The whole body autoradiography was studied after an injection of [125 I]fibrinogen in DIC rats. The high radioactivity which indicated the presence of microthrombi was observed in the renal cortex, liver, spleen and lung. Furthermore, a large venous thrombus with higher radioactivity was observed in the abdominal vena cava. These results show that the thrombin-treated animal is one of the best DIC models. After the intravenous administration of [125 I]t-PA, the autoradiograms of DIC rats showed a radioactivity in the blood and much higher radioactivities in the renal cortex, spleen and lung in comparison with the normal rat. However, there was no difference in the distribution of [125 I]u-PA between normal and DIC rats at all. The strong radioactivity of [125 I]t-PA but not [125 I]u-PA was observed on the surface of large thrombus in the vena cava. These results suggest that t-PA localizes more preferentially on microthrombi than u-PA.

The ratio of the radioactivity in the tissue to that in the blood was calculated to compare quantitatively the localization of [125 I]t-PA and [125 I]u-PA on microthrombi formed in organs. The ratios of the [125 I]t-PA radioactivities in the kidney, lung and liver showed 1.6 to 2.5 times higher amounts in DIC rats as compared with normal rats. On the other hand, the ratios of the [125 I]u-PA radioactivities did not show a significant difference between normal and DIC rats. These results further support the preferential localization of t-PA on microthrombi formed in organs.

Keywords — tissue plasminogen activator; urokinase; thrombosis model; disseminated intravascular coagulation; thrombus affinity; autoradiography; rat

Introduction

Plasminogen activators are proteins that promote fibrinolysis by converting the proenzyme, plasminogen, to the enzyme, plasmin. The well known mammalian plasminogen activators are urokinase plasminogen activator (u-PA) found in urine¹⁾ and tissue plasminogen activator (t-PA) found in several tissue extracts.²⁾ The plasminogen activator in the vascular wall (endothelium), which is released into the circulating blood, is similar to t-PA and probably represents the same molecule.³⁾ The t-PA is a unique enzyme which activates plasminogen through binding to fibrin clot as a substrate of plasmin.⁴⁾ u-PA weakly binds to fibrin clot⁵⁾ and does not activate fibrin-associated plasminogen.⁶⁾ In the case of thrombolysis therapy, u-PA causes a systemic decrease in fibrinogen (Fbg) and induces

a systemic lytic state associated with bleeding complications.⁷⁾ As the clot selectivity of t-PA is attributed to its high affinity for fibrin, it has been assumed that the adsorption of t-PA to thrombi can bring about the selective thrombolysis.⁸⁾ Although there have been a few *in vitro* experiments such as the binding of t-PA to fibrin-Sepharose column⁹⁾ and fibrin clots,¹⁰⁾ an *in vivo* study with regard to the localization of t-PA on thrombi has not been reported. We have previously reported that t-PA has a high affinity for blood clots of human and various species, and that the bound t-PA produces extensive blood clot lysis.¹¹⁾

The present study is devised to investigate autoradiographically the localization of [125 I]t-PA on thrombi in experimental disseminated intravascular coagulation (DIC) rats.

Materials and Methods

Materials — The t-PA (single chain form, M_r ca. 63000) was purified from confluent culture of human diploid fibroblasts as previously described.¹²⁾ The u-PA (two chain form, M_r ca. 54000) purified from human urine was obtained from Kowa Co., Japan. Human serum albumin (HSA) and (plasminogen free human) fibrinogen (Fbg) were purchased from Sigma Chemical Co., St. Louis. Bovine thrombin (Mochida Co., Japan) was used for preparation of DIC rats. Chemicals administered to the animals were dissolved in saline.

Iodination of Proteins — Each of t-PA, u-PA, HSA and Fbg was labelled with Na[¹²⁵I] (17.4 Ci/mg, NEN products, Boston) using the solid state lactoperoxidase and glucose oxidase (Enzymobeads®, Bio-Rad Co., Richmond) according to the method described by David and Reisfeld.¹³⁾ The specific radioactivities of [¹²⁵I]t-PA, [¹²⁵I]u-PA and [¹²⁵I]HSA were approximately 1×10^8 cpm/nmol. The [¹²⁵I]Fbg showed a 0.5×10^8 cpm/nmol specific radioactivity and approximately 90% of the clottability. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) migrations of [¹²⁵I]-labelled proteins were identical to the corresponding unlabelled proteins. The enzymatic activities of [¹²⁵I]t-PA and [¹²⁵I]u-PA were determined with the fibrin plate assay¹⁴⁾ and showed the same activities as those before labelling.

Preliminary Test for Determination of an Optimal Dose of Thrombin to Produce DIC Rats

— Thrombin in the doses of 150–750 U/kg was infused for 1 h through a polyethylene tube (cannulated to the abdominal vena cava) in male Wistar rats. Blood samples were taken from the jugular vein before and just after, and 1 h after the end of the thrombin infusion. A part of blood (0.3 ml) was mixed with ethylenediaminetetraacetic acid (EDTA) in a plastic test tube and submitted to the measurement of platelet counts by Automatic Hematology Analyzer, ELT8/ds (Ortho). The rest of the blood samples (9 volumes) were collected in plastic tubes containing 3.8% trisodium citrate (1 volume) and immediately centrifuged at 4 °C to obtain plas-

ma. Plasma was frozen and stored at –70 °C for assay.

For the measurement of Fbg level, aprotinin was added to the plasma samples to prevent proteolysis. Fibrinogen plasma levels were determined by the method of Quick.¹⁵⁾ Plasminogen activator activity in the euglobulin fraction of plasma was determined by [¹²⁵I]fibrin solid phase method of Moroz and Gilmore.¹⁶⁾

Preparation of Experimental DIC Rats — Experimental DIC rats induced by thrombin were prepared as follows. Male Wistar rats weighing ca. 250 g were anesthetized with an intraperitoneal injection of sodium pentobarbital. A polyethylene cannula was inserted to the abdominal vena cava from the exposed femoral vein for the administration of agents. Five minutes after the injection of [¹²⁵I]Fbg (7×10^6 cpm/0.25 ml/rat), thrombin was infused at a rate of 500 U/kg/h by an infusion pump (Truth A-II). Just after and 1 h after the end of the thrombin infusion, animals were frozen immediately in *n*-hexane cooled to –78 °C with solid carbon dioxide. Thereafter, the whole body autoradiography (ARG) of these DIC rats and normal rats (control) was carried out.

Distribution of [¹²⁵I]t-PA in DIC Rats — [¹²⁵I]t-PA (1×10^7 cpm/12.5 µg/rat) or [¹²⁵I]u-PA (1×10^7 cpm/1400 IU/rat) was administered to normal rats (control) and DIC rats through the caudal vein. The whole body ARG was performed 2 min after the administration of [¹²⁵I]t-PA or [¹²⁵I]u-PA. The distribution and localization of microthrombi in the lung, liver and kidney were quantitatively determined by the levels of radioactivity. [¹²⁵I]HSA (1×10^7 cpm/10 µg/rat) was administered for the measurement of blood distribution in normal and DIC rats. In order to study an effect of t-PA or u-PA on the blood distribution in organs, [¹²⁵I]HSA was administered with t-PA (12.5 µg/rat) or u-PA (1400 IU/rat) to the normal rats, and the radioactivity in the organs was determined.

Whole Body ARG — According to the method of Ullberg,¹⁷⁾ the animals frozen were embedded with an aqueous gel of carboxymethyl cellulose and immersed into *n*-hexane cooled to –78 °C with solid carbon dioxide. A whole body

Fig. 1. Changes in Hematological Parameters of Rats Treated with Thrombin Infusion

Each point represents the mean of two rats. (A) Fibrinogen levels in plasma. (B) Fibrinolytic response in euglobulin. (C) Platelet counts in blood. Doses of thrombin: ▲, 150 U/kg; △, 300 U/kg; ●, 500 U/kg; □, 750 U/kg; ○, 0.9% saline.

was sagittally cut in the thickness of 30 μ m by a cryomicrotome (LKB-2250). The sections were dry-frozen and then exposed to ARG films (Konica MARG film, ³H-type) for 4 weeks.

Measurement of Radioactivity in Organs

After weighing a whole or part of blood, lung, liver and kidney isolated from frozen animals, the radioactivity per unit of weight (cpm/g) was measured with γ -ray scintillation detector (Aloka ARC-251).

Results

Optimal Dose of Thrombin to Produce DIC rats

Figure 1 shows changes in hematological parameters of the experimental DIC rats produced by various doses of thrombin. After the infusion of thrombin into the abdominal vena cava in the doses up to 750 U/kg, the plasma level of Fbg dose-dependently decreased. Thrombin in the dose of more than 500 U/kg reduced the plasma Fbg level to zero. Plasminogen activator activity in the plasma euglobulin fraction was unchanged until 1 h after the start of thrombin infusion in all animals. One hour after the end of infusion, plasminogen activator activity dose-dependently increased in the range of 50–340 mU/ml. Platelet counts in blood dose-dependently decreased at 1 h and showed

(A)

(B)

Fig. 2. ARGMs Showing the Distribution of Radioactivity in a Normal Rat (A) and a Thrombin-Induced DIC Rat (B) 1 h after Intravenous Injection of [¹²⁵I]Fbg

Fig. 3. ARGM Showing the Distribution of Radioactivity into the Abdominal Vena Cava Thrombus in a Thrombin-Induced DIC Rat 1 h after Intravenous Injection of [125 I]Fbg

a tendency to recover at 2 h.

From the results given above, an optimal dose of thrombin to induce the significant consumption of Fbg, which was one of the symptoms of DIC, was determined as 500 U/kg for 1 h. Based on this experimental condition (a dose of thrombin : 500 U/kg), the plasma Fbg level in DIC rats ($n = 10$) was further confirmed with a good reproducibility that the preinfusion level (196 ± 5 mg/dl) decreased to 3.2 ± 2.3 mg/dl (mean $\pm$ S.E.) after the infusion of thrombin.

Thrombus Formation Sites in DIC rats

Whole body autoradiograms (ARGMs) of a normal rat (control) and a thrombin-induced DIC rat are shown in Fig. 2. The white areas in the illustrations represent both the localization and intensity of radioactivity. In the normal rat at 1 h after [125 I]Fbg injection, the highest radioactivity was observed in the thyroid (data not shown) and contents of the stomach. The blood was found to have a higher concentration of radioactive material than were presented in the lung, liver and kidney. The distribution patterns at 2 h were similar to that at 1 h (data not shown). On the other hand, the DIC rat just after thrombin infusion (1 h) showed higher dot-like radioactivity in the renal cortex, lung, and liver. Similarly, high radioactivity was noted in the boundary between the white pulp and red pulp of spleen. Furthermore, the highest radioactivity was observed in a macrothrombus in the abdominal vena cava (Fig. 3). The radioactivity in the blood was qualitatively much lower than that of a normal rat. In addition, the distribution of

radioactivity in the renal cortex, lung and liver markedly decreased 2 h after [125 I]Fbg injection, while high radioactivity was noted in the bladder. The highest radioactivity was still observed in the macrothrombus present in the abdominal vena cava 2 h after the injection. The radioac-

Fig. 4. ARGMs Showing the Distribution of Radioactivity 2 min after Intravenous Injection of [125 I]t-PA in a Normal Rat (A) and a Thrombin-Induced DIC Rat (B)

Fig. 5. ARGM Showing the Distribution of Radioactivity into the Abdominal Vena Cava Thrombus 2 min after Intravenous Injection of [125 I]t-PA in a Thrombin-Induced DIC Rat

tivity in the blood also was similar to the level just after thrombin infusion (data not shown).

Distribution of [125 I]t-PA in DIC Rats

The ARGMs of animals were prepared 2 min after an intravenous injection of [125 I]t-PA (Fig. 4). In the normal rat, the radioactivity was highest in the liver. The radioactivity in the blood was found to have a higher concentration of [125 I]t-PA than those in the lung, kidney and spleen. In the DIC rat, the distribution of highest dot-like radioactivity was noted in the renal cortex and lung, and highest radioactivity was also observed in the liver, while the radioactivity in the blood was relatively lower than that of the

Fig. 6. ARGMs Showing the Distribution of Radioactivity 2 min after Intravenous Injection of [125 I]u-PA in a Normal Rat (A) and a Thrombin-Induced DIC Rat (B)

normal rat. The distribution of radioactivity in the spleen was obviously different from that of the normal rat. The high radioactivity was shown in the boundary between the white pulp and red pulp in the DIC rat. The distribution of radioactivity to a macrothrombus existed in the abdomi-

Fig. 7. ARGMs Showing the Distribution of Radioactivity into the Abdominal Vena Cava Thrombus 2 min after Intravenous Injection of [125 I]u-PA (A) and [125 I]HSA (B) in Thrombin-Induced DIC Rats

nal vena cava was significantly noted on its surface but not within the thrombus (Fig. 5).

Distribution of [125 I]u-PA and [125 I]HSA in DIC Rat

The ARGMs (Fig. 6) of animals were prepared 2 min after the intravenous injected [125 I]u-PA. In the normal rat, the distribution of highest radioactivity was noted in the renal cortex. The radioactivities in the liver and blood were higher than those in the lung and spleen. In the DIC rat, the observation on the ARGMs were similar to those of the normal rat described above, except that the distribution of radioactivity was noted a little on the surface of macrothrombus in the abdominal vena cava (Fig. 7A).

The normal and DIC rat received an injection of [125 I]HSA. There was no difference in the distribution of radioactivity between both rats, namely, the high radioactivity was noted in the blood and subsequently the liver and kidney (Fig. 8). [125 I]HSA did not distribute to the macrothrombus (Fig. 7B).

Quantitative Analysis of Distribution of [125 I]t-PA in DIC Rats

Two minutes after an intravenous injection of [125 I]t-PA, [125 I]u-PA or [125 I]HSA to normal rats and DIC rats, the radioactivity was quantitatively determined in major organs (lung, liver and kidney). Table I shows the ratios of tissue to blood concentration of the two [125 I]-labelled thrombolytic agents and [125 I]HSA, in DIC rats (B) and those in normal rats (A). The

Fig. 8. ARGMs Showing the Distribution of Radioactivity 2 min after Intravenous Injection [125 I]HSA in a Normal Rat (A) and a Thrombin-Induced DIC Rat (B)

parameters, B/A, indicates changes of the accumulation of [125 I]-labelled proteins.

After the administration of [125 I]HSA, the values of B/A are approximated 1 in every or-

TABLE I. Distribution of Radioactivity 2 min after Intravenous Injection of [125 I]HSA, [125 I]t-PA and [125 I]u-PA in Normal or Thrombin-Induced DIC Rats

Drug	Organ	Ratio of tissue to blood concentration		B/A
		Normal rats (A)	DIC rats (B)	
[125 I]HSA	Lung	0.66 ± 0.02	0.63 ± 0.03	0.95
	Liver	0.32 ± 0.01	0.32 ± 0.01	1.00
	Kidney	0.29 ± 0.01	0.30 ± 0.02	1.03
[125 I]t-PA	Lung	0.78 ± 0.04	1.61 ± 0.09	2.06
	Liver	1.46 ± 0.22	2.34 ± 0.21	1.60
	Kidney	0.46 ± 0.01	1.13 ± 0.05	2.46
[125 I]u-PA	Lung	0.73 ± 0.02	0.65 ± 0.01	0.89
	Liver	0.83 ± 0.05	0.98 ± 0.04	1.18
	Kidney	1.12 ± 0.14	0.97 ± 0.19	0.87

Values represent the mean $\pm$ S.E. of three rats.

gan. The ratios of tissue to blood concentration of [125 I]t-PA in normal rats were 0.78 in the lung and 0.46 in the kidney. On the other hand, the ratio of [125 I]t-PA in the liver was 4-times higher than that of [125 I]HSA.

In DIC rats, the ratios of tissue to blood concentration of [125 I]t-PA significantly increased in comparison with normal rats, and the values of B/A in the kidney, lung and liver consequently were greater than 1, respectively.

After administration of [125 I]u-PA to normal rats, the ratio of lung to blood concentration was 0.73 which was almost same as that of [125 I]HSA. However, those values in the liver and kidney were 0.83 and 1.12 which were 2.5 and 4-times higher than those of [125 I]HSA, respectively. Every ratio of [125 I]u-PA in DIC rat was scarcely different from that in normal rats, and the values of B/A were approximated about 1 in all the organs.

In order to study an effect of t-PA or u-PA on the blood distribution in organs, [125 I]HSA was administered with or without non-labelled t-PA and u-PA to the normal rats. The ratios of tissue to blood concentration of [125 I]HSA were not affected with t-PA and u-PA except that in the liver which the combination with u-PA increased about 20% more than that of [125 I]HSA alone (Table II).

Discussion

t-PA specifically binds to fibrin and induces fibrinolysis through activating plasminogen.¹⁸⁾ We have previously reported that t-PA has more

specific affinity to blood clots than u-PA *in vitro*.¹¹⁾ In the present study, we demonstrated the affinity of t-PA to thrombi *in vivo*. Although the demonstration of the accumulation of t-PA on the surface of thrombi is difficult because of the spontaneous thrombolysis, the accumulation of t-PA into a lot of microthrombi in organs was revealed by the whole body ARG technique in DIC rats.

The DIC animal model was prepared with the intravenous infusion of thrombin at a dosage of 500 U/kg for 1 h resulting in the significant consumption of Fbg. After the bolus injection of [125 I]Fbg to DIC rats, [125 I]Fbg completely disappeared from the plasma, and consequently the radioactivities of the ARGMs were observed in systemic organs such as the kidney, lung and liver indicating the formation of a lot of microthrombi. These thrombotic rats were recognized as DIC rats.^{19,20)} One of the marked characteristics of this DIC model was formation of a macrothrombus near the cannulated site for thrombin infusion in the abdominal vena cava. The dot-like distribution of radioactivity in the renal cortex suggested microthrombi formation in the glomeruli. The macrothrombus kept the original form for 1 h after the end of thrombin infusion, while the microthrombi persisted in the renal cortex tended to disappear. The prompt disappearance of the microthrombi was possibly due to the release of plasminogen activator from the vascular endothelium since the activity of plasminogen activator in the plasma increased 1 h after the end of thrombin infusion. This phenomenon might be the endogenous defensive fibrinolysis to eliminate microthrombi. It appears that the increase in endogenous t-PA was not enough to dissolve the macrothrombi in large vessels.

Since we established the thrombin-induced DIC model in rats, the ARGMs studies were then carried out after the intravenous injection of [125 I]t-PA. The radioactivity of [125 I]t-PA significantly increased in the lung, kidney and spleen. Particularly, the dot-like distribution of the radioactivity was observed in the renal cortex. These observations of the ARGMs were similar to that of the DIC rat after [125 I]Fbg injection. It was clear that [125 I]t-PA was accumu-

TABLE II. Effects of t-PA or u-PA on Tissue Distribution of [125 I]HSA in Normal Rats 2 min after Intravenous Injection

Organ	Ratio of tissue to blood concentration		
	Control	t-PA	u-PA
Lung	0.69 $\pm$ 0.03	0.68 $\pm$ 0.01	0.71 $\pm$ 0.01
Liver	0.34 $\pm$ 0.02	0.37 $\pm$ 0.02	0.42 $\pm$ 0.02 ^{a)}
Kidney	0.32 $\pm$ 0.02	0.34 $\pm$ 0.02	0.35 $\pm$ 0.01

Values represent the mean $\pm$ S.E. of six rats. a) $p < 0.01$: significant difference from control ([125 I]HSA alone) by Student's *t*-test.

lated to the microthrombi formed in various organs. [^{125}I]t-PA was also located on the surface of a macrothrombus in the abdominal vena cava and a penetration of [^{125}I]t-PA into the thrombus was not observed. Therefore, it was suggested that the localization of t-PA on the thrombi can be achieved in a very short time (within 2 min after administration), and the thrombolysis on macrothrombus occurs around the surface of the thrombus. However, when [^{125}I]u-PA was intravenously administered to the DIC rats, the distribution of radioactivity in organs was not different from that of normal rats, indicating that u-PA does not preferentially accumulate to microthrombi. The radioactivity around the macrothrombus was also much lower than that of [^{125}I]t-PA. [^{125}I]HSA, a major plasma protein, did not distribute around the macrothrombus, therefore, the localization of u-PA around the macrothrombus was not an artifact.

In order to analyze quantitatively the accumulation of thrombolytic agents to thrombi, the [^{125}I]-labelled protein was administered to normal and DIC rats and its distribution to major organs was determined by the concentration ratio of radioactivity in tissue to blood. Since [^{125}I]HSA distributes uniformly in the blood just after the administration,²¹⁾ the ratio of tissue to blood concentration (normal rat: A, DIC rat: B) in the organ was the value representing the blood volume in those. Therefore, these ratios (0.29—0.66) were considered one of the characteristic figures in every organ. The ratios of tissue to blood concentration after administration of [^{125}I]HSA also were compared with those after administration of [^{125}I]thrombolytic agents to study the organ affinity of each protein. The ratio of B/A can be determined as a parameter showing the accumulation to thrombi, unless t-PA and u-PA change the blood volume in the organs. The administration of t-PA mixed up with [^{125}I]HSA did not effect the blood volume of organs tested. u-PA also did not change the blood volume of organs, except for the liver which increased slightly. Since the physiological role of intrinsic u-PA is not established, the mechanism of extrinsic u-PA in the change of the blood volume in liver is not clear. The

study with administration of [^{125}I]HSA showed that the ratio of tissue to blood concentration in each organ was similar between normal and DIC rats, and the values of B/A of all organs were approximately one. It is concluded that there is no difference in the blood volume in major organs between the two groups of normal and DIC rats.

In the liver of normal rats, the administration of [^{125}I]t-PA increased the ratio of tissue to blood concentration more than 4-times as compared to that of [^{125}I]HSA, showing that [^{125}I]t-PA rapidly distributed in the liver soon after injection. As described previously in several reports,^{22–24)} t-PA intravenously administered rapidly disappears from the blood and is catabolized (metabolized) in the liver. This was supported by the present results. However, in DIC rats, the distribution of [^{125}I]t-PA considerably increased not only in the liver but also in the lung and kidney, suggesting that t-PA is quickly accumulated to microthrombi formed in these major organs. Particularly, the finding that the ratios of B/A of kidney and lung reached to 2.5 and 2.1, respectively, is in agreement with the results of ARG.

The amount of [^{125}I]u-PA distributed in the kidney and liver was high in normal rats, especially in the kidney, where the radioactivity of [^{125}I]u-PA was about 4-times higher than that of [^{125}I]HSA. This corresponded with the report of Tajima *et al.*²⁵⁾ which demonstrated a high organ affinity of u-PA in the kidney. Although [^{125}I]u-PA showed the high organ affinity in the kidney of DIC rats, the ratio of tissue to blood concentration was not different from that of normal rats. Furthermore, since every value of B/A in other organs was approximately one, it is considered that the accumulation of [^{125}I]u-PA to the microthrombi is very little.

In conclusion, we proved the more preferable accumulation of t-PA to thrombi as compared to u-PA in the thrombin-induced DIC model together with the ARG.

A histological study on micro-levels is now undergoing to determine the more accurate accumulation of t-PA to microthrombi in organs.

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