The Thrombolytic Effect of Human Tissue-type Plasminogen Activator on the Experimental Thrombosis in Rabbit

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(Received 11 June 1987/Accepted 20 August 1988)

ABSTRACT. The effects of human tissue-type plasminogen activator (t-PA) on thrombosis were investigated in rabbits. A venous thrombosis model was used for comparison of effects between 70000 IU/Kg of t-PA and 70000 IU/Kg of urokinase (UK). A thrombus was formed around a silk thread in jugular vein and then t-PA purified from the culture fluid of human fibroblast cell line (IMR-90) was infused locally and systemically. The local infusion of t-PA showed a greater thrombolysis than that of UK. Thrombolytic rates of t-PA and UK infusion group were 72.2% and 43.5%, respectively. UK caused the elevation of FDP and the decrease of fibrinogen, plasminogen and α 2-antiplasmin in the plasma. On the contrary, these hemostatic parameters of the animal infused with t-PA scarcely changed. These results indicate that t-PA can induce specific thrombolysis without disseminated activation of the fibrinolytic system as compared with UK. The disappearance of t-PA activity in euglobulin fraction was very rapid after the completion of infusion. T-PA has a broader safety margin and greater potential as a thrombolytic agent than UK. —KEY WORDS: IMR-90, rabbit, thrombolysis, t-PA.

Jpn. J. Vet. Sci. 50(6): 1232–1238, 1988

The concentration of tissue-type plasminogen activator (t-PA) appearing in most tissue has been determined in many animal tissues [1, 9]. Both vascular plasminogen activator (VPA) and t-PA have the same molecular weight, share the same antigenic properties, and bind strongly to fibrin and the inhibitors for t-PA. These plasminogen activators are different from urokinase (UK) with respect to their immunological properties as well as functional ones. Recently, t-PA production by human embryonic fibroblast (IMR-90) has been realized [6, 13].

The purpose of the present study is to estimate the therapeutic effects of purified t-PA derived from normal diploid fibroblast (IMR-90) on venous thrombosis using UK as a comparative agent.

MATERIALS AND METHODS

T-PA was purified from the culture fluid of human normal diploid fibroblast (IMR-90) [6, 13]. On SDS-gel electrophoresis, purified t-PA showed a single band with a molecular weight of 65,000–68,000. On the other hand, molecular weight of t-PA was 68,000–70,000 by gel filtration on Sephacryl S-200. UK was obtained from Mochida Pharmaceutical Co., Tokyo.

Activity of UK and t-PA used for this examination was separately compared with that of standard human UK and human t-PA from the World Health Organization. A specific activity of t-PA determined by the fibrin plate method was 370,000 IU/mg protein. An activity of UK determined by the two-step method of clot lysis was 168,000 IU/mg protein [14]. Protein concentration of t-PA was determined by amino

acid analysis or ELISA using melanoma t-PA as a standard.

Fibrin plates for all the experiments were prepared by pouring 12 ml of 1.3% low melting point (42°C) agarose (EPI-55, No. 00136) containing 120 mg of human fibrinogen enriched plasminogen (KabiVitrum, grade L: No. 250232) into petri dish containing 0.2 ml (25 IU/ml) of human thrombin solution (Green Cross Pharmaceutical Co., Osaka).

Procedure for the assay of thrombolysis: In order to examine the thrombolytic effects of t-PA and UK, a venous thrombus was formed in rabbit jugular vein. Forty three male Japanese white rabbits weighing 2.4 ± 0.1 kg (mean $\pm$ SD) were used for these experiments [7]. The surgical procedure was as follows. A rabbit was anesthetized by subcutaneous injection of 1.2 g/kg body weight of urethane and intubated. A femoral vein catheter was introduced for blood sampling. A 5 cm paramedial incision was made in the neck. The external jugular vein was separated from the muscle and its accessory veins were ligated. The vein filled with blood was clamped at both proximal and distal sites to isolate a vein segment and then a silk thread that had been soaked in thrombin solution (500 IU/ml) was introduced into the lumen of the isolated vein over a distance of 2 cm with a straight round needle. The clot was formed and allowed to age for 30 minutes before both clamps were removed [4].

One ml (10% of total volume) of 70000 IU/Kg of t-PA, UK, or physiological saline (control) was injected initially and 9 ml was infused at a constant rate using a infusion pump. A facial vein was ligated and the agent was infused locally through ipsilateral marginal ear vein for over 2 hours. A facial vein was not ligated to maintain venous return and the agent was infused systemically through contralateral marginal ear vein for over 2 hours.

Three ml of blood samples were drawn through the catheter introduced into the femoral vein before the start of the infusion and every one hour for 4 hours. One ml of blood sample was added to a mixture of 20 U of thrombin and epsilon aminocaproic acid (EACA) for fibrinogen degradation products (FDP) test. Two ml of blood sample was mixed with 3.8% trisodium citrate to isolate plasma.

The thrombus formed around silk thread was taken out from the vein and then weighed. The amount of protein in thrombus was measured by the Lowry's method [10].

Fibrinolytic activity in the euglobulin fraction prepared at pH 5.2 and the plasma was determined by the fibrin plate method. Ten μ l of the euglobulin fraction or the plasma was put into the hole ($\varnothing$ 3 mm) made up on fibrin plate in triplicate. The lysed area of fibrin was measured after 22 hours incubation at 37°C. Plasminogen was determined with the chromogenic substrate S-2251 (KabiVitrum) after the plasmin inhibitors were neutralized by acidification with 1N HCl and then activated with 1000 IU of UK to 5 μ l of plasma for 10 minutes at 37°C [3]. Alpha-2-antiplasmin was determined using chromogenic substrate S-2251 according to the method of Teger-Nilsson *et al.* [15]. Clottable fibrinogen in plasma was determined by the thrombin clotting time method using COAGACHEK Fibrinogen Test Kit (HYLAND). FDP was determined by staphylococcal clumping test (Sigma Chemical Co., U.S.A.) [5].

Measurement of t-PA activity in vivo: Three male rabbits weighing 2.4 ± 0.1 kg were used. Each catheter was introduced into both right and left facial veins to collect blood samples. Seventy thousands IU/kg of t-PA was infused for over 2 hours in the same way described above. During the examination, 1 ml of blood samples were collected before infusion and at 1 and 2

hours during infusion and at 1, 2, 3, 4, 5, 10, 15 and 30 minutes after the completion of infusion. T-PA activity in euglobulin fraction was measured by fibrin plate method and was calculated as IU/ml using the calibrated standard curve.

RESULTS

Thrombolytic effects of t-PA and UK: The changes of fibrinolytic activity in euglobulin fraction and plasma on the fibrin plate were summarized in Fig. 1. The lysed area formed by euglobulin fraction was 25 mm² (mean value) and did not significantly change throughout the experimental period. Infusion of t-PA and UK caused the increase of the lysed area formed by euglobulin fraction from 25 mm² to about 250 mm² and 180 mm², respectively. Within an hour after the completion of t-PA infusions, the lysed area by euglobulin fraction fell to about 35 mm². Fibrinolytic activity in plasma was not detected during the infusion of saline and UK but showed an increase of 40 mm² (mean value) during the infusion of t-PA.

The effect of the local infusion of 70000 IU/Kg t-PA on thrombus was shown in Table 1. Thrombus weights after infusion of

Fig. 1. The size of lysed area formed by euglobulin fraction on fibrin plate was shown. Saline control (●, n=23), 70000 IU/Kg of t-PA (○, n=10), 70000 IU/Kg of UK (△, n=10). And the size of lysed area formed by plasma on fibrin plate was shown as 70000 IU/Kg of the t-PA (⊙, n=10). The lysed area formed by plasma was not detected during infusion of saline (◆, n=23) and UK (▲, n=10). The data represent means and the vertical bars SE.

Table 1. Thrombus weight and thrombolysis after infusion

Local infusion	n	Thrombus weight (mg)	Protein in thrombus (mg)	Thrombolysis		
				Thrombolysis (%)	Thrombolysis ratio	Fibrinogenolysis
Control	12	113.8±12.0 ^{a)}	39.8±4.1	—	—	—
t-PA 70000IU/Kg	5	31.6± 9.4**	11.0±3.2**	72.2 ^{b)}	72.4 ^{c)}	7.2
Urokinase 70000IU/Kg	5	64.2±22.6*	22.7±7.8*	43.5	43.0	1.4
Systemic infusion	n	Thrombus weight (mg)	Protein in thrombus (mg)	Thrombolysis		
				Thrombolysis (%)	Thrombolysis ratio	Fibrinogenolysis
Control	11	80.8± 9.7	30.2±3.3	—	—	—
t-PA 70000IU/Kg	5	61.0±10.4	22.2±3.2*	24.5	26.5	2.5
Urokinase 70000IU/Kg	5	79.6±12.3	30.9±4.3	1.5	-2.3	0.05

a) mean±SE.

b) Thrombolysis were calculated by thrombus weights.

c) Thrombolysis were calculated by protein in thrombus.

* P<0.05, ** P<0.01. Significant different as compared with the control group.

saline, 70000 IU/Kg of t-PA and UK were 113.8 ± 12.0 (mean $\pm$ SE), 31.6 ± 9.4 and 64.2 ± 22.6 mg, respectively. The thrombus weights after infusion of t-PA and UK were significantly different from that of control. Thrombolytic rates of t-PA and UK that were calculated from the average of each thrombus weight were 72.2% and 43.5%, respectively. The amounts of protein in thrombus after infusions of saline, t-PA, and UK were 39.8 ± 4.1 , 11.0 ± 3.2 and 22.7 ± 7.8 mg, respectively. Thrombolytic rate calculated from the average protein amount of thrombus was the same as that based on thrombus weight.

The effects of the systemic infusion of 70000 IU/Kg of t-PA on thrombus are shown in Table 1. Thrombus weights of control, t-PA and UK infusion group were 80.8 ± 9.7 , 61.0 ± 10.4 and 79.4 ± 12.3 mg, respectively. The protein amounts in the thrombus of control, t-PA and UK infusion group were 30.2 ± 3.3 , 22.2 ± 3.2 and 30.9 ± 4.3 mg, respectively. The value in t-PA group was significantly lower than the others. The thrombolytic rate in t-PA group was about 25%. The systemic infusion of UK did not cause any thrombolysis. In this dose, the thrombolysis after the systemic t-PA infusion was apparently less than that after the local infusion.

During and after infusion of each drug, the hemostatic changes in the local infusion were similar to those in the systemic infusion. Fig. 2 shows hemostatic changes in both the local and systemic infusions. During infusion of t-PA, slight decrease in the fibrinogen was noted (10% of preinfusion value). On the contrary, the fibrinogen level in UK group fell to 70% of preinfusion value (Fig. 2a). FDP resulted in a marked increase during UK infusion. It still remained 5 times of preinfusion value at 2 hours after the completion of infusion. The increasing degree of FDP in the t-PA group was less than that in the UK group (Fig. 2b).

In order to confirm the characteristic of specific fibrinolysis, the ratio thrombolysis to fibrinogenolysis was calculated (Table 1). On the local infusion of t-PA and UK, the ratios thrombolysis to fibrinogenolysis were 7.2 and 1.4. On systemic infusion of t-PA and UK, those were 2.5 and 0.05, respectively. Infusion of t-PA did not cause any decrease in both levels of plasminogen and $\alpha 2$ -antiplasmin as well as that of the control. On the other hand, infusion of UK made the plasminogen and $\alpha 2$ -antiplasmin levels fall to 75% and 40%, respectively (Fig. 2c, 2d).

T-PA activity and elimination in vivo: As shown in Fig. 3a, the plasminogen activator activity in the euglobulin fraction was calculated using standard t-PA from WHO. The local and systemical infusion of 70000 IU/Kg of t-PA raised the t-PA activities in blood to 200 and 40 IU/ml, respectively. T-PA activity in blood during the systemic infusion of 70000 IU/Kg of t-PA fell to 20% of that during the local infusion. The difference of t-PA activity in blood between the local and systemic infusions reflected the thrombolysis (Table 1).

The disappearance of t-PA activity in euglobulin fraction was very rapid as shown in Fig. 3b. T-PA activities in the local infusion decreased from 200 U/ml to 55, 40 and 8 U/ml at 1, 2 and 4 minutes after the completion of infusion, respectively. And those in the systemic infusion decreased from 40 U/ml to 19, 14 and 5 U/ml at 1, 2 and 4 minutes after the completion of infusion, respectively.

DISCUSSION

The local infusion of 70000 IU/Kg of t-PA indicated extremely higher thrombolytic activity (thrombolytic rate of 72%) than that of 70000 IU/Kg of UK. As well as the local infusion, the systemic infusion of t-PA also indicated higher activity than that of UK. However, the thrombolysis by the systemic

Fig. 2. Time-related effects of saline (●, $n=10$), 70000 IU/Kg of t-PA (○, $n=10$) and 70000 IU/Kg of UK (△, $n=10$) on the fibrinogen levels (a), the fibrinogen degradation products (FDP) (b), the plasminogen levels (c) and the levels of α_2 -antiplasmin (d) in rabbits. The data summarize the changes in both the local and systemic infusions and represent means and the vertical bars SE.

infusion (about 25%) was less than that by the local infusion.

During infusion of 70000 IU/Kg of t-PA, the increase of fibrinolytic activity in euglobulin fraction and plasma was observed. On the other hand, infusion of 70000 IU/Kg of UK induced less increase of fibrinolytic activity in euglobulin fraction than that of t-PA, and did not show any fibrinolytic activity in plasma. Higher level of fibrinolytic

activity in the euglobulin fraction than in the corresponding plasma sample indicates the existence of plasmin inhibitors. It is considered that t-PA activates plasminogen that exists in fibrin plate while UK scarcely does when each plasma sample is placed on it. It is reasonable to presume that UK causes a production of plasmin in plasma but these plasmin are rapidly inactivated by α_2 -antiplasmin. Plasma inhibitors are great-

Fig. 3. A comparison of the t-PA activity during and after the local infusion (■) and the systemic infusion (□) of 70000 IU/Kg of t-PA (a) (b). The data represent means (n=3) and the vertical bars SE.

er importance in UK treatment and a great deal of α_2 -antiplasmin has to be consumed before any thrombolytic effect is obtained.

It has been found that t-PA had binding affinity to fibrin clot and required fibrin in order to express its activity. Therefore, t-PA binds to fibrin clot and activates the plasminogen which is included in fibrin clot and induces thrombolysis on fibrin clot. But t-PA does not generate plasmin in blood.

As compared with t-PA, UK activates mainly the plasminogen in plasma but has a poor effect in activating the plasminogen which is attached to fibrin [12]. UK has less binding affinity to clot. Fig. 2 shows that infusion of UK causes the elevation of FDP and the decrease of fibrinogen, plasminogen and α_2 -antiplasmin in plasma. It seemed that UK induced both fibrinolysis and fibrinogenolysis and activated plasmin was neutralized by α_2 -antiplasmin as soon as plasminogen was generally activated by the infusion of UK in plasma [11]. On the contrary, these hemostatic parameters of the animal infused with 70000 IU/Kg of t-PA scarcely

changed. These results indicate t-PA has the higher fibrin-specificity as compared with UK. T-PA can induce specific thrombolysis without disseminated activation of the fibrinolytic system.

The rapid disappearance of activity after infusion of t-PA seemed to be due to the dilution in systemic circulation, neutralization by plasma inhibitors, and the hepatic capture [2, 8]. During the local infusion, the levels of t-PA in flowing blood in jugular vein corresponded to be 200 IU/ml of standard t-PA. On the other hand, during the systemic infusion, the levels of t-PA corresponded to be 40 IU/ml of standard t-PA (Fig. 3a). These facts were reflected on the thrombolytic effect (Table 1).

The differences of t-PA activity between the local and systemic infusions suggested that the levels of t-PA in blood apparently decreased according to the distance from the position of infusion of t-PA. T-PA had a possibility to reduce hemorrhage as a side effect in the therapy of thrombosis, because t-PA scarcely changed the relevant hemostatic parameters in plasma. T-PA had more specific fibrinolytic and greater thrombolytic activity than UK had.

Furthermore, the local infusion of t-PA was proved to be the best clinical usage for the treatment of thrombosis, offering a great potency of thrombolysis. Local and continuous infusion of t-PA might be necessary for obtaining the best clinical effect.

ACKNOWLEDGEMENTS. The authors wish to thank Dr. K. Higashio for supplying of purified t-PA.

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要 約

家兎の実験的血栓症に対するヒト組織プラスミノゲンアクチベータの血栓溶解効果：升永博明・有沢広彦・片山純男（雪印乳業（株）生物科学研究所）——家兎の頸静脈に自家血による血栓を作製した。ヒト胎児正常線維芽細胞（IMR-90）の培養上清から精製した組織プラスミノゲンアクチベータ（t-PA）とウロキーゼ（UK）を、この家兎の同側または対側の耳介静脈からどちらも体重1 kg あたり7 万単位の割合で持続注入した。同側耳静脈から注入したときの血栓溶解率は、t-PA, UK ともに対側から注入したときよりも高く、t-PA で70%とUK (40%) を上廻った。またこの際、血漿中のフィブリノゲン、プラスミノゲン、 α_2 -アンチプラスミンの減少は、UK にくらべ明らかに少なかった。血栓溶解特性の目安として血栓溶解率と血漿中のフィブリノゲン分解率の比をとると、同側または対側、いずれの耳静脈内注入時にも t-PA が UK を上廻った。ヒト正常細胞（IMR-90）由来の t-PA は、家兎自家血栓に対して高い特異性を示した。フィブリン平板を用いて測定したユーグロブリン分画中のプラスミノゲンアクチベータ活性は、t-PA 投与終了後、血中からすみやかに消失した。以上のごとく、t-PA は、血漿中での血液凝固線溶バランスに対する影響が軽微であり、活性消失も速いので、UK 大量投与時にみられる出血、止血不全などの副作用が軽減される可能性が示唆された。