Sustained Thrombolysis With DNA-Recombinant Tissue Type Plasminogen Activator in Rabbits

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Tissue type plasminogen activator (t-PA) is an effective thrombolytic agent in experimental animals. The duration of the thrombolytic effect of infused t-PA is unknown. We compared the duration of the thrombolytic effect of t-PA with streptokinase by measuring the lysis of ^{125}I-fibrin–labeled thrombi in rabbit jugular veins at different times after a bolus injection of the fibrinolytic agents. The pharmacodynamics of both thrombolytic agents were determined in rabbits using a sensitive ex vivo fibrinolytic assay. Streptokinase and t-PA were given as a bolus dose of 15,000 U/kg. There was no detectable circulating fibrinolytic activity 30 minutes after the bolus dose of t-PA and 120 minutes after the bolus dose of streptokinase. The t-PA injection produced 34% thrombolysis at 30 minutes, 90% thrombolysis at 120 minutes, and 96% thrombolysis at 240 minutes. The streptokinase injection produced 17% thrombolysis at 30 minutes, 34% at 120 minutes, and 34% at 240 minutes. These observations indicate that the thrombolytic effect of t-PA is sustained beyond its time of clearance from the circulation whereas the thrombolytic effect of streptokinase closely parallels its activity in the circulation.

EXTRINSIC tissue-type plasminogen activator (t-PA) is a recently developed fibrinolytic enzyme that, in experimental animals, can produce thrombolysis without inducing a generalized hemostatic defect. A serine protease, t-PA binds to fibrin with a greater affinity than either streptokinase or urokinase, and, when bound to fibrin, it converts plasminogen to plasmin several hundredfold more efficiently than in the absence of fibrin.[1] Originally, t-PA was isolated and purified from a human melanoma cell line (Bowes melanoma) culture supernatant,[2] and more recently, the human t-PA gene has been cloned and expressed in *E coli* through recombinant DNA technology.[3] Investigations in animals and in humans have demonstrated that the infusion of melanoma cell-derived t-PA results in thrombolysis.[4-11] These findings have recently been confirmed with DNA-recombinant t-PA.[12-14] We have recently reported that equivalent thrombolytic doses of t-PA produce less hemorrhage than streptokinase.[11]

Although t-PA has a short half-life in plasma,[15] the duration of its thrombolytic effect is unknown. Since t-PA binds to fibrin, it is possible that its thrombolytic effect may persist after it is cleared from the circulation. We have recently reported that ^{125}I-fibrin–labeled thrombi in jugular veins of rabbits are significantly smaller at three hours after a one-hour infusion of t-PA than immediately after the infusion.[16] Knowledge of the duration of the thrombolytic effect of t-PA is important for planning optimal therapy. We have therefore performed a study in rabbits to investigate the duration of the thrombolytic effect of a bolus dose of t-PA and to compare the time course of thrombolysis of t-PA with thrombolysis obtained via streptokinase.

MATERIALS AND METHODS

Materials. DNA-recombinant t-PA (100,000 U/mg), which was produced by the cloning and expression of human t-PA in *E coli*,[3] was obtained from Genetech, South San Francisco (lot No. BH 011 DA). One unit of t-PA activity, according to the manufacturer's specifications, is equal to 5 IU (according to the International Committee on Thrombosis and Haemostasis, Miami, November 1984.) Streptokinase (Streptase, lot No. 0180 A) was obtained from Behringwerke AG, Marburg, FRG. The chromogenic substrate (S2251, lot No. 8569451) and the human plasmin (25 casein units per bottle, lot No. 6728951) used in the α_2-antiplasmin assays were obtained from Kabi Diagnostica, Stockholm. ^{125}I ($Na^{125}I$, 610 mCi/mL, carrier-free) was obtained from New England Nuclear, Boston. Sodium pentobarbital was obtained from MTC Pharmaceuticals, Hamilton, Ontario. Aprotinin (Trasylol) was obtained from Bayer, Leverkusen, FRG. Fibrinogen (>90% clottable) was prepared from pooled rabbit plasma and labeled with ^{125}I by the iodine monochloride method of Regoeczi.[17]

Method of preparation of radioactive jugular vein thrombi. Standard-sized preformed ^{125}I-labeled thrombi were produced in the external jugular veins of New Zealand white rabbits (2.3 to 3.5 kg). Briefly, both external jugular veins were exposed through a paramedial incision in the neck. Each vein was cleared over a distance of 2 cm and small side branches were ligated. A 3-0 Ti-Cron braided polyester thread, presoaked in a collagen solution, was then introduced lengthwise in the lumen of the jugular vein to prevent the embolization of preformed thrombi. After 30 minutes, the vein was clamped both proximally and distally to isolate the vein segment. A 150-μL aliquot of homologous rabbit blood containing 5 μL of ^{125}I-labeled rabbit fibrinogen (approximately 500,000 cpm) was aspirated in a 1-mL syringe containing 1 unit of thrombin and 10 μL of $CaCl_2$ (0.25 mol/L), and the clotting blood quickly injected in the isolated jugular vein segment. In all instances, the thrombus formed quickly and was allowed to age for 30 minutes before both vessel clamps were removed and blood flow was restored.

Urea solubility and release of radioactivity during in vitro thrombolysis. Radioactive jugular vein thrombi, prepared as described above, were removed at 30 minutes, washed in saline, and then incubated in 5 mL of 5 mol/L urea. Aliquots of the supernatant were collected and assayed for radioactivity at 0, ½, 1, 2, 4, 6, and 24 hours. Less than 1% of the radioactivity appeared in the supernatant over the 24-hour incubation period.

To confrm that the thrombi were uniformly labeled with ^{125}I, jugular vein thrombi were recovered and incubated in rabbit platelet-poor plasma containing streptokinase in a final concentration of 300 U/mL. Two thrombi were removed at 1, 2, 3, and 4 hours, respectively. Each thrombus was weighed and its total radioactivity

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Supported in part by the Ontario Heart and Stroke Foundation.

Submitted June 8, 1984; accepted Feb 18, 1985.

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0006-4971/85/6602-0028$03.00/0

was measured. There was a parallel decrease in weight and total radioactivity over the four-hour period ($r = .998$, $P < .001$).

Assessment of thrombolysis in vivo. Rabbits were injected through the marginal ear vein with a bolus of 15,000 U/kg of streptokinase or t-PA, or an equivolume of suspending vehicle (saline), given over one minute. Thirty, 120, and 240 minutes after the bolus, the thrombi remaining in the vessel of different rabbits were removed, and thrombolysis was determined by measuring the amount of residual ^{125}I-labeled fibrin remaining in the thrombi and comparing it to the residual ^{125}I-labeled fibrin remaining in thrombi of saline-treated animals.

Streptokinase and t-PA pharmacodynamics. Rabbits were infused through the marginal ear vein with a bolus of 15,000 U/kg of streptokinase or t-PA. Serial blood samples were collected into heparin (final concentration, 10 U/mL) for up to one hour for t-PA and for up to four hours for streptokinase. One-milliliter aliquots of each blood sample were incubated for five minutes in tubes precoated with ^{125}I-fibrin prepared as described by Moroz and Gilmore.[18] The fibrinolytic activity of each blood sample was then determined by measuring the lysis of the ^{125}I-fibrin. This was expressed as a percent of the total initial radiaoctivity of the tube.

Assessment of systemic fibrinolysis. Blood samples were withdrawn from the rabbit carotid artery directly into 3.8% sodium citrate (9:1, vol/vol) before and 30, 60, 120, and 240 minutes after the t-PA, streptokinase, or saline bolus. All samples were immediately centrifuged at 8,000 g to obtain cell-free plasma, which was frozen at −70 °C until assayed. Aprotinin (250 U/mL) was added to all samples prepared for fibrinogen level measurements to prevent proteolysis in vitro. Fibrinogen plasma levels were determined by the method of van Clauss.[19] Levels of α_2-antiplasmin were evaluated by the chromogenic method of Teger-Nielsson et al[20] and the thrombin clotting time by the method of Fletcher et al.[21]

Statistical analysis. The data were analyzed using a one-way analysis of variance and a Neuman-Keuls test.[22,23]

RESULTS

The effects of t-PA and streptokinase on blood fibrinolysis and thrombolysis are shown in Fig 1. Circulating fibrinolytic activity was no longer detectable 30 minutes after the t-PA injection and was no longer detectable 120 minutes after the streptokinase injection. Thrombolysis with t-PA was 37% at 30 minutes, 90% at 120 minutes, and 96% at 240 minutes. Thrombolysis with streptokinase was 17% at 30 minutes, 34% at 120 minutes, and 34% at 240 minutes. Thus, there was no evidence of continuing thrombolysis with streptokinase after 120 minutes, $P > .40$ (at which time there was no detectable circulating fibrinolytic activity), whereas thrombolysis continued in the t-PA–treated animals long after the disappearance of circulating fibrinolytic activity ($P < .01$).

A slight but significant decrease of α_2-antiplasmin was observed only at 30 minutes after the injection with streptokinase (87.3% ± 4.7 of the basal value; $P < .05$) but not with t-PA or saline. No changes in fibrinogen level and thrombin clotting time were observed for any of the three treatment groups.

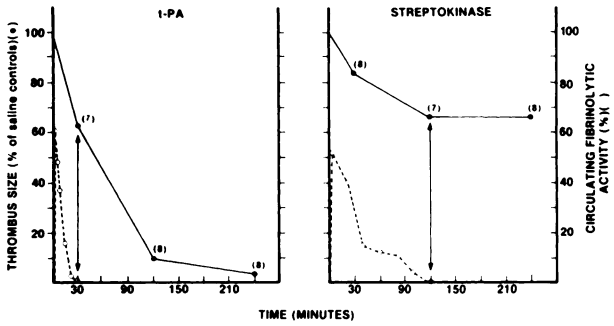

Fig 1. Effects of t-PA and streptokinase on thrombolysis (●—●) in vivo and fibrinolytic activity (○-----○) ex vivo. Fibrinolytic activity is expressed as the percent of ^{125}I-fibrin lysed from the tube. The arrows indicate the time when the fibrinolytic activity was no longer detectable. The thrombolytic data are expressed as the geometric means. The numbers in parentheses indicate the number of experiments performed.

DISCUSSION

The results of our study confirm other reports that t-PA is an effective thrombolytic agent and that it produces thrombolysis without inducing a plasma proteolytic state. The new and potentially important observation is that the thrombolytic effect of t-PA persisted for a number of hours after it was cleared from the circulation. In contrast to the effect of t-PA, thrombolytic activity of streptokinase paralleled its circulating plasma fibrinolytic activity. The enzyme t-PA has a number of unique properties that could explain the persistent thrombolytic effect that we observed; for example, it binds to fibrin where it is protected from inactivation by a recently described fast-acting inhibitor.[24] Plasminogen binds through its lysine binding site to the t-PA/fibrin complex where it is converted to plasmin. Plasmin formed on the fibrin surface has a longer half-life than circulating plasmin, probably because its lysine bindings site is protected from inactivation by α_2-antiplasmin.[25] Protection of both fibrin-bound t-PA and plasmin from their inhibitors could therefore explain the sustained thrombolytic activity of t-PA.

In conclusion, our findings indicate that the thrombolytic effect of t-PA persists after the drug is cleared from the circulation.

ACKNOWLEDGMENT

We wish to thank Ms G. Cross, Mrs A. Spriet, and Mrs C. den Hollender for their technical assistance; Mr R. Butt for the statistical analysis; and Ms T. Fick for typing the manuscript.

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