Thrombolysis with Human Extrinsic (Tissue-type) Plasminogen Activator in Dogs with Femoral Vein Thrombosis

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ABSTRACT Extrinsic (tissue-type) plasminogen activator (plasminogen activator) was isolated either as a single-chain or as a two-chain molecule from the culture medium of a human melanoma cell line. The thrombolytic activity of both molecular forms of activator was investigated in beagle dogs with an experimental femoral vein thrombosis and compared with that of urokinase. The ^{125}I-fibrinogen-labeled thrombus was formed in an isolated 4-cm segment of the vein, aged for 30 min, and the thrombolytic substances were infused over a 4-h period. The degree of thrombolysis was measured 2 h later as the difference between the injected and recovered ^{125}I.

In six control animals with a saline infusion the extent of thrombolysis was 16.3±3.8% (mean±SEM), in five dogs receiving 100,000 IU urokinase, 17.4±3.7% ($P > 0.4$) and in four dogs with 1,000,000 IU urokinase 40.6±4.8% ($P < 0.001$). Infusion of 100,000 IU single-chain plasminogen activator in five dogs resulted in 33.5±7.8% lysis ($P < 0.05$) and of 100,000 IU two-chain plasminogen activator in five dogs in 60.1±10.8% ($P < 0.001$). Infusion of 300,000 IU one-chain plasminogen activator yielded 57.5% lysis and of the same amount of two-chain plasminogen activator 72.9%.

Significant activation of plasminogen, consumption of α_2-antiplasmin, and fibrinogen breakdown in plasma was only observed in the animals receiving the high doses of urokinase but not in the saline, plasminogen activator, or the low-dose urokinase groups.

It is thus concluded that in this thrombosis model human extrinsic plasminogen activator has a higher specific thrombolytic effect than urokinase. Plasminogen activator also appears to induce thrombolysis without systemic fibrinolytic activation and fibrinogen breakdown.

INTRODUCTION

Pharmacological dissolution of thrombi has been attempted in several ways, e.g., by infusion of plasmin (1), urokinase (2), streptokinase (3), streptokinase-plasminogen complex (4), and the protease extracted from *Aspergillus oryzae* (5, 6). Extensive clinical experience has mainly been obtained with urokinase, a plasminogen activator of urinary origin and with streptokinase, a bacterial protein that activates the human fibrinolytic system indirectly (7). Thrombolysis with either of these substances is, however, associated with systemic activation of the fibrinolytic system and with hemostatic breakdown, which may occasionally lead to serious hemorrhages. The side effects of these thrombolytic agents apparently relate to the fact that they activate both circulating and fibrin-bound plasminogen relatively indiscriminately, leading to systemic fibrinolytic activation.

"Extrinsic" or "tissue-type" plasminogen activators extracted from blood (8, 9), vessel perfusates (10), or from tissues (11–13) have the property to preferentially activate plasminogen in the presence of fibrin (14–16), thereby confining the activation process to the immediate environment of a formed thrombus.

Recently, we have purified extrinsic (tissue-type) plasminogen activator from a human melanoma cell culture (17) in sufficiently large amounts to allow more detailed investigation of its biochemical and biological properties (16, 18, 19). In the present study this enzyme will be designated plasminogen activator. Plasminogen activator was isolated as a one-chain molecule, which probably represents the vascular or blood plasminogen activator, or as a two-chain molecule, which is most probably a proteolytic degradation product of the former (14, 17). This study was undertaken to further investigate the thrombolytic effect of plasminogen activator in vivo. Dogs with experimental femoral vein thrombosis were chosen because this

model has proved to be suitable for the evaluation of various thrombolytic agents (20–23).

Because the thrombolytic effect of plasminogen activator in vitro is ~10 times higher than that of urokinase (18), a dose of 100,000 IU of plasminogen activator (~1 mg purified activator) was compared to 100,000 and 1,000,000 IU of urokinase. Additionally, single experiments were performed in which 300,000 IU of one-chain or two-chain plasminogen activator were infused.

METHODS

Purified proteins. One-chain and two-chain plasminogen activator were highly purified from the culture fluid of a human melanoma cell line by a method described elsewhere (17). The activity was expressed in international units of urokinase by comparison of its fibrinolytic activity on plasminogen-enriched bovine fibrin films (17). Because the specific activity of plasminogen activator is ~90,000 IU/mg and its mol wt 72,000 (17), equal amounts of units of plasminogen activator and urokinase (M_r 56,000; sp act ~100,000 IU/mg) correspond to equal molar quantities. Urokinase was a gift from Institut Choay, Paris, France (courtesy of Dr. Toulemonde). Human fibrinogen was prepared according to Blombäck and Blombäck (24) and labeled with ^{125}I according to McFarlane (25). Human thrombin was purified as described by Fenton et al. (26).

Experimental procedure. The thrombolytic effect of one- or two-chain plasminogen activator, as compared with urokinase was evaluated in a dog femoral vein thrombosis model developed by Smith et al. (23, 27). Beagle dogs (9–11 kg) were premedicated with ketamine hydrochloride (10 mg in 1 ml, i.m.) and atropine sulfate (500 μg in 1 ml, i.m.). Anesthesia was induced with sodium pentobarbital (5 ml of a 6% solution, i.v.) and a tracheal tube and a urinary catheter were introduced. 21-g butterfly needles were introduced in superficial veins of both fore legs for infusion and blood sampling.

Additional pentobarbital (~1 ml every 2 h, i.v.) was administered to maintain anesthesia. Thyroidal uptake of radio iodide was blocked by administration of sodium iodide (5 ml of a 2% solution, i.v.).

The surgical procedure was as follows. A femoral vein was exposed through a 5-cm incision in the inguinal region. The vein was cleared from the entry of the femoral vein in the groin to the sapheno-femoral junction. Small side branches were ligated, except for the predominant musculocutaneous branch, which was canulated with a 5-cm length of a Portex "pink gauge" canulla (Portex, Hythe, England). A woolen thread was then introduced in the lumen of the vein over a distance of 4 cm. When bleeding had ceased, the vein was clamped both proximally and distally to isolate a vein segment that was emptied of all blood via the side branch catheter. The volume of the segment was measured by injection of saline from a volumetric syringe until the vessel was fully distended.

The thrombus was then produced as follows. Approximately 10–20 μl of ^{125}I-labeled human fibrinogen, containing ~3 × 10⁶ cpm, was aspirated in a 1.0-ml syringe followed by a volume of fresh blood precisely corresponding to the measured volume of the isolated vein segment. The segment was then emptied by withdrawal of the saline through the side branch catheter and 0.1 ml thrombin [100 National In-

stitutes of Health (NIH) U/ml] was quickly injected, followed at once by the volume of blood containing the radioactive fibrinogen. Care was taken to avoid injection of air bubbles. Cotton swabs were then placed over the vessel to absorb blood leaking from the vein segment. In most instances, the clot formed quickly and was allowed to age for 30 min. Then both vessel clamps were removed and venography was carried out using a standard portable x-ray unit. 5 ml of Urographin 60% (Schering Corp., Berlin, West Germany) contrast medium was infused over 15 s, through the ipsilateral dorsal pedal vein and the exposure was made towards the end of the infusion. After demonstration of a suitable thrombus the leg wound was temporarily closed. The cotton swabs were removed for radioisotope counting and the amount of radioactivity delivered to the clot was calculated by subtracting the swab losses, the radioactivity remaining in the syringe, and the total blood radioactivity after venography from the original amount of radioactivity in the syringe. 4-ml blood samples were drawn on citrate (final concentration 0.01 M) and 1-ml samples on mixtures of 40 NIH U thrombin and 1,000 U aprotinin (trasylol, the Bayer Co., Leverkusen, West Germany) immediately after venography and then at hourly intervals for 6 h. The plasma samples were used for measurement of radioactivity, fibrinogen (28), plasminogen (29), α_2-antiplasmin (30), and fibrin(ogen) degradation products (31).

The thrombolysis experiments were carried out under minimal heparinization once the initial x ray confirmed adequate thrombus formation. Heparin (100 U/kg) was injected intravenously, and during the experiment 50-U/kg amounts were given when the whole blood clotting time approached normal values. The bladder was emptied every hour, the urine volume was measured, and an aliquot was taken for radioisotope counting.

Immediately after the result of venography had been obtained (i.e., ~15 min after removal of the vessel clamps), intravenous infusion of saline, plasminogen activator, or urokinase was started. First 2.5 ml (10% of the total volume) was given as a bolus injection, followed by an infusion of 24 ml over 4 h, using a constant rate infusion pump (speed 6 ml/h). 6 h after the start of the infusion a control venography was performed. The thrombosed segment of the femoral vein was removed after careful suturing of both ends and the remaining radioactive material was measured. The degree of lysis was expressed as the difference between the radioactivity originally calculated to be incorporated in the clot and the radioactivity in the vein segment.

At the end of the experiment the surgical wound was carefully closed and the dogs were given an injection of 200,000 U of sodium-penicilline G. The dogs were used again for a second experiment (using the contralateral femoral vein) after an interval of 48 h. An isotope recovery balance was made by adding the radioactivity in the recovered thrombus, in the blood at the end of the experiment, (multiplied by a Factor 3 for extravascular distribution), and in the urine. Thyroidal uptake of radioiodide or accumulation of labeled material in the lungs was negligible.

In total, 33 experiments were performed in 19 dogs. From those, six experiments were excluded from the analysis because of the following reasons: two dogs died during the experiment, probably due to pentobarbital overdose, and four experiments were dropped because the initial total blood radioactivity exceeded 15% of that in the clot. In five dogs only one experiment was performed.

Analysis of the data. Statistical analysis of the data and determination of the level of significance was performed according to Student's t test (32).

TABLE I
Extent of Thrombolysis and Isotope Recovery

Group	Thrombolysis‡			Isotope recovery‡
	n*	%	P§	%
Controls	6	16.3±3.8		91.9±4.2
Urokinase, 100,000 IU	5	17.4±3.7	>0.4	92.4±2.0
Urokinase, 1,000,000 IU	4	40.6±4.8	<0.001	89.1±4.6
One-chain plasminogen activator, 100,000 IU	5	33.5±7.8	<0.05	84.5±5.9
Two-chain plasminogen activator, 100,000 IU	5	60.1±10.8	<0.001	79.3±4.6
One-chain plasminogen activator, 300,000 IU	1	57.5		85.9
Two-chain plasminogen activator, 300,000 IU	1	72.9		92.0

* n = number of experiments performed.
‡ The data represent means±SEM.
§ Level of significance.

RESULTS

The extent of thrombolysis and the isotope recovery balance are shown in Table I. In the control group the average degree of thrombolysis was 16.3±3.8%. The values ranged, however, between 9.1 and 33.1%. This relatively large variability might result from individual differences affecting the blood flow to the thrombus. The extent of thrombolysis in our control group is nearly identical to that obtained by Smith et al. (23).

Thrombolysis in the animals given 100,000 IU of urokinase was 17.4±3.7%, which is very similar to that of the control group. Infusion of 1,000,000 IU of urokinase resulted in 40.6±4.8% thrombolysis, which is significantly higher than the control value ($P < 0.001$).

Infusion of 100,000 IU of two-chain plasminogen activator yielded 60.1±10.8% lysis ($P < 0.001$). Surprisingly, the extent of thrombolysis following infusion of 100,000 IU of one-chain plasminogen activator was 33.5±7.8%, which is significantly higher than the control value ($P < 0.05$), but significantly lower than the value obtained with two-chain activator ($P < 0.05$). Because the same amounts of activity of plasminogen activator and urokinase correspond to similar molar quantities, these data indicate that plasminogen activator has a much higher specific thrombolytic effect than urokinase.

In one experiment infusion of 300,000 IU of two-chain plasminogen activator resulted in 72.9% lysis. A single infusion of 300,000 IU of one-chain activator yields 57.5% lysis.

Venography before the start of the infusion revealed an occlusive thrombus in ~60% of the experiments and a large thrombus with marginal flow of contrast medium in ~40% of the cases.

Comparison of venograms before and after the in-

fusions revealed, in most cases, a reasonably good correlation between the extent of thrombolysis, determined on the basis of radioactivity measurements and the degree of venographic clearance. Venography did however not permit a reliable quantitation of the extent of thrombolysis. As an example, Fig. 1 shows the venogram of a dog before and after infusion of saline (thrombolysis 9.6%) and Fig. 2 of a dog before and after infusion of two-chain plasminogen activator (thrombolysis 70.2%).

As isotope recovery balance was made as described in Methods to ensure that no significant parts of the thrombus were lost. The recovery was ~90% in the control and urokinase groups, and ~80% in both plasminogen activator groups (Table I). It can be assumed that in cases of extensive thrombolysis the ^{125}I-labeled fibrin degradation products are distributed in a larger volume of body fluids than three times the blood volume.

Figs. 3 and 4 represent the evolution of the relevant blood parameters during the thrombolytic experiments. All parameters were expressed as a fraction of the values obtained before the start of the infusion in order to normalize the data. After restoration of the blood flow (sample 0), the radioactivity in the blood rose to ~5.7% (ranging from 1.5 to 12.8%) of the injected radioactivity, probably due to washing out of some nonclotted labeled fibrinogen, and to a lesser extent of free [125]I. In both the control group and the low-dose urokinase group, blood radioactivity thereafter slowly declined, to ~60% of the initial value after 6 h (Fig. 3, upper half). These results are in agreement with the expected disappearance rate of labeled fibrinogen from the blood (33).

In contrast, infusion of two-chain plasminogen activator resulted in a threefold increase of the blood
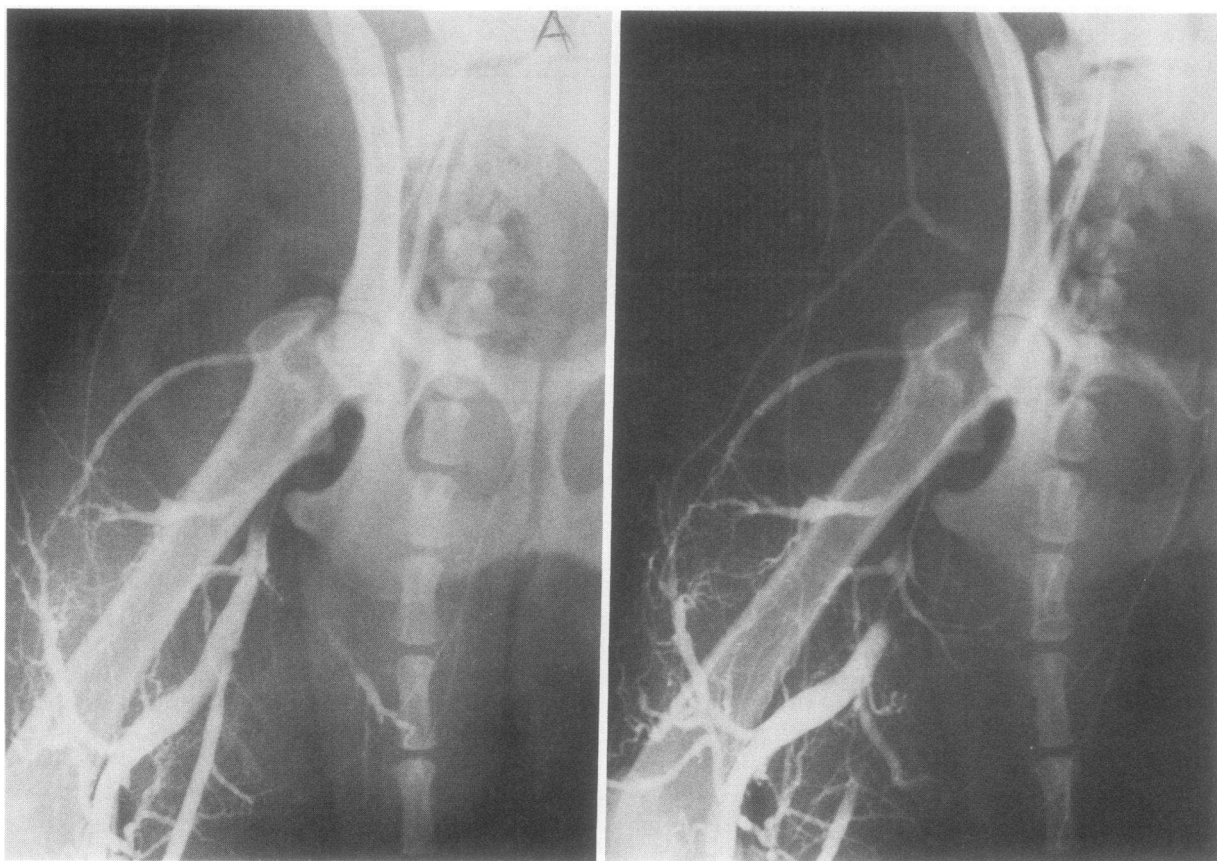
FIGURE 1 Venograms of a dog with femoral vein thrombosis before (left side) and after (right side) infusion of saline (extent of thrombolysis 9.6%).

radioactivity, whereas infusion of 1,000,000 IU of urokinase resulted in a twofold increase. During infusion of one-chain plasminogen activator, only a transient slight increase of the blood radioactivity was observed, whereafter it declined to reach preinfusion values at the end of the experiments.

Fig. 3 (lower part) represents the evolution of fibrinogen and fibrinogen degradation products (FDP)[1] in blood during and 2 h after the infusion. The fibrinogen level remained essentially unchanged in the experimental groups receiving saline, the low dose of urokinase, or both the low or high dose of plasminogen activator and no fibrinogen degradation products were generated in these groups. Infusion of 1,000,000 IU of urokinase, however, was associated with complete defibrinogenation, evidenced by a depletion of plasma fibrinogen and the generation of very high concentrations of degradation products.

In line with these observations infusion of 1,000,000

IU of urokinase provoked extensive systemic fibrinolytic activation with a drop of plasminogen to ~30% of the preinfusion value and a complete exhaustion of α_2-antiplasmin (Fig. 4). No evidence for systemic activation of the fibrinolytic system was observed in the other experimental groups.

Administration of the large dose of urokinase invariably resulted in profuse bleeding from the surgical wounds and puncture sites, whereas these problems were not encountered in the other experimental groups.

DISCUSSION

Arterial thrombosis and venous thromboembolism are a major cause of morbidity and mortality (34). Despite the undoubtable achievements of prophylaxis, thrombosis remains a major medical problem. In the past, surgical thrombectomy has been the treatment of choice, but it involves certain risks and is limited to the larger vessels. Alternatively, pharmacological thrombolysis has been extensively investigated in the 1950s and 1960s, when streptokinase and urokinase

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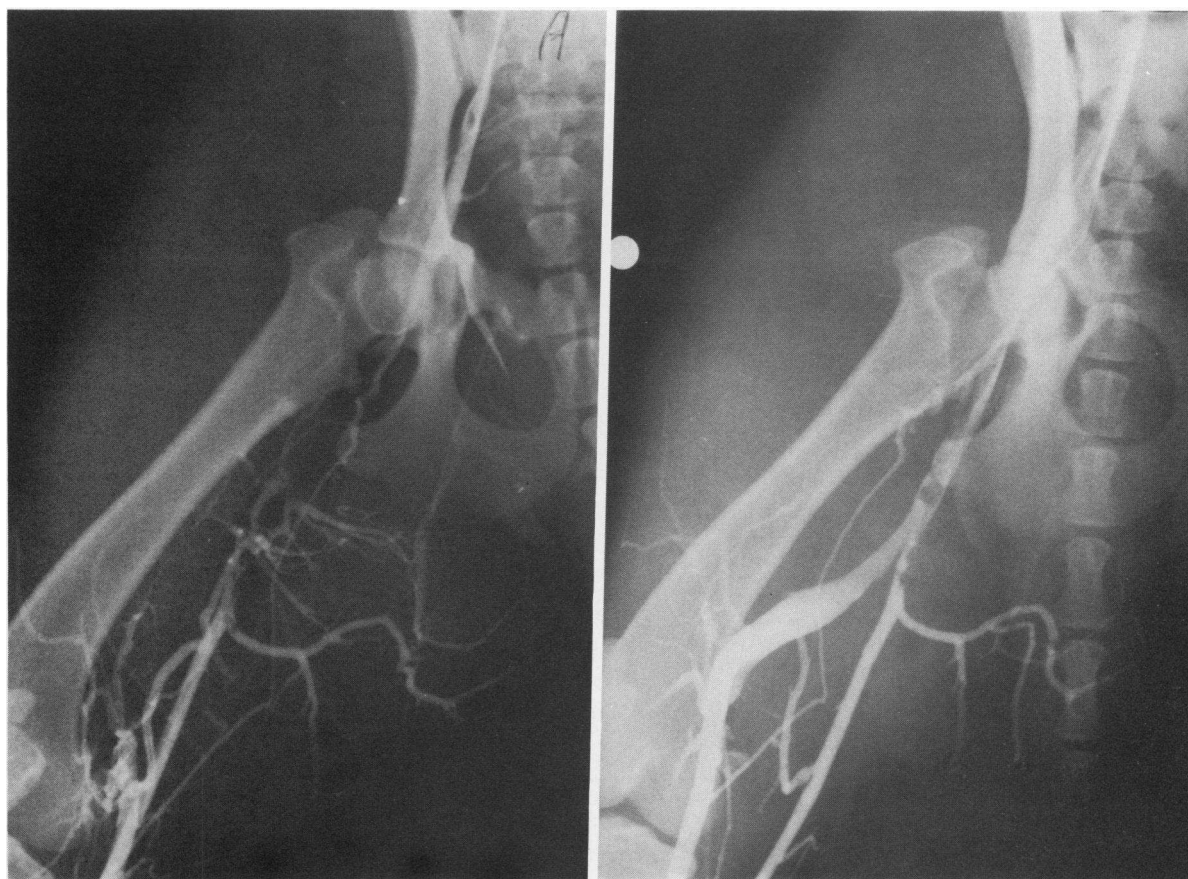[1] *Abbreviation used in this paper*: FDP, fibrin(ogen) degradation products.

FIGURE 2   Venograms of a dog with femoral vein thrombosis before (left side) and after (right side) infusion of 100,000 IU of two-chain extrinsic plasminogen activator (extent of thrombolysis 70.2%).

became available for clinical application. Both streptokinase and urokinase can induce significant thrombolysis in man, but their administration is accompanied by hemostatic breakdown and a bleeding tendency. Consequently, these "thrombolytics of the first generation" have not found their way to widespread clinical application.

During the last few years, our understanding of the mechanism of physiological fibrinolysis has been improved as the result of a better insight in the molecular interactions involved (35). It appeared that physiological fibrinolysis is mainly triggered by and directed towards fibrin through specific interactions between extrinsic plasminogen activator and fibrin.

This knowledge suggested a new, more rational approach to the problem of pharmacological thrombolysis, taking advantage of the physiological regulatory mechanisms.

Recently, plasminogen activator became available in sufficient amounts to allow more detailed in vitro and in vivo investigations. Using a human blood clot hanging in circulating human plasma, we demonstrated that plasminogen activator has a higher specific fibrinolytic and a much lower fibrinogenolytic activity than urokinase (17). These findings were in essence confirmed in vivo in rabbits with a pulmonary embolus (18), although only a limited degree of thrombolysis was obtained.

The present study was undertaken to further investigate the thrombolytic activity of plasminogen activator as compared with urokinase in vivo. Experimental thrombosis in dogs was chosen as a model, because various thrombolytic agents have already been evaluated in these animals (20–23). Acute venous thrombosis was induced by formation of a radioactive blood clot in a superficial femoral vein. The model allowed continuous monitoring of the thrombolytic process (by measuring the radioactivity released into the bloodstream), as well as exact quantitation of the degree of thrombolysis, because the remaining thrombus was recovered in toto.
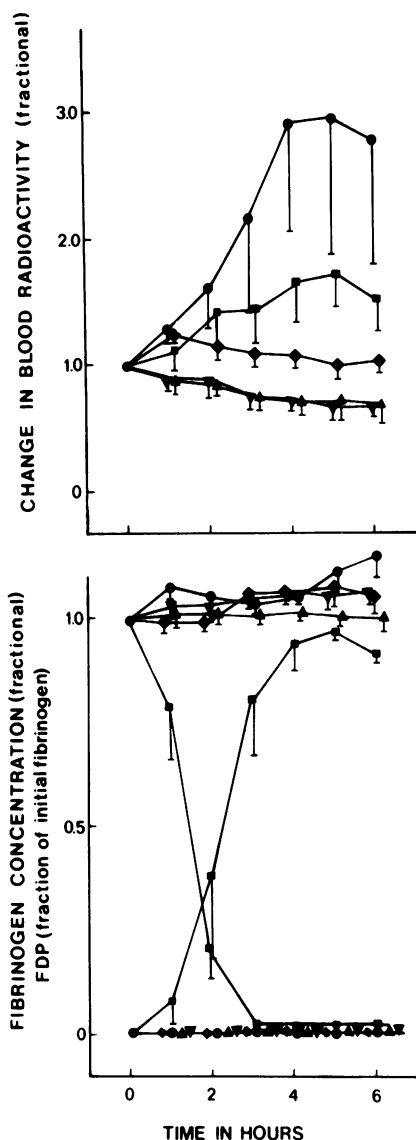
Thrombolysis in the dog model was carried out un-

thrombosis model as compared with the rabbit pulmonary embolus model cannot be explained by inherent species differences nor by the effect of heparin. Indeed in a rabbit jugular vein thrombosis model with infusion of 1 mg plasminogen activator over 4 h in the absence of heparinization 70–80% lysis was obtained (unpublished results). Probably, accessibility of the clot, which was very poor in the pulmonary embolism model, is a major determining factor for the extent of
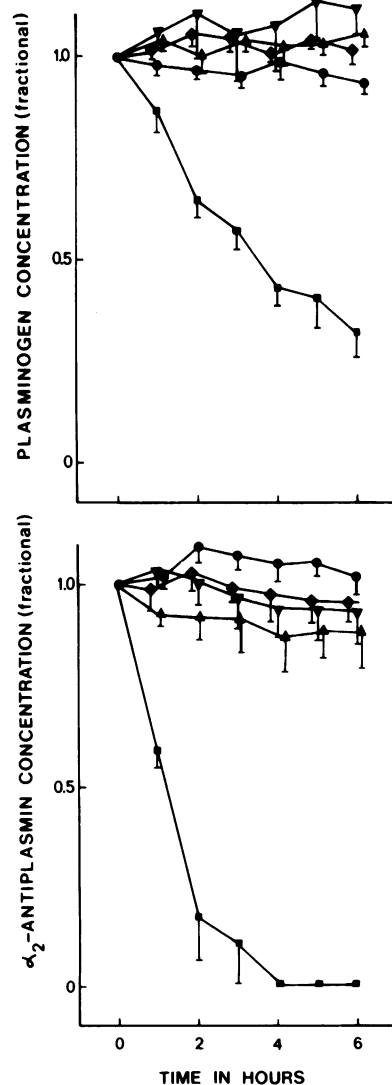
FIGURE 3 Evolution of the blood radioactivity and of plasma fibrinogen and fibrin(ogen) degradation products during infusion of extrinsic plasminogen activator or urokinase in dogs. ▼———▼, 6 control animals infused with saline; ▲———▲, 100,000 IU of urokinase in five dogs; ■———■, 1,000,000 IU of urokinase in four dogs; ◆———◆, 100,000 IU of one-chain plasminogen activator in five dogs; ●———●, 100,000 IU of two-chain plasminogen activator in five dogs. The concentration of FDP was expressed as a fraction of the initial fibrinogen concentration. The data represent mean values±SEM.

der minimal heparinization because in the absence of heparin, extension of the thrombosis frequently occurred in the control group. In the rabbit pulmonary embolism model such extension was never observed and heparinization was therefore omitted. The much higher degree of thrombolysis in the dog femoral vein

FIGURE 4 Evolution of plasminogen and α_2-antiplasmin in plasma during infusion of extrinsic plasminogen activator or urokinase in dogs. ▼———▼, 6 control animals infused with saline; ▲———▲, 100,000 IU of urokinase in five dogs; ■———■, 1,000,000 IU of urokinase in four dogs; ◆———◆, 100,000 IU of one-chain plasminogen activator in five dogs; ●———●, 100,000 IU of two-chain plasminogen activator in five dogs; The data represent mean values±SEM.

thrombolysis. The present and earlier (19) results indicate, however, that plasminogen activator is, on a molar basis, superior to urokinase both with respect to the extent and the specificity of its thrombolytic effect. Indeed, plasminogen activator induces extensive thrombolysis without systemic plasminogen activation, fibrinogen breakdown, and bleeding, which urokinase cannot. Surprisingly, the thrombolytic activity of two-chain plasminogen activator appeared to be higher than that of single-chain plasminogen activator. It is difficult to explain this difference at present because both molecular forms of plasminogen activator have very similar kinetic constants (36).

Because our study deals with artificial, acute, fresh thrombosis in an animal model, extrapolation to human thrombosis can only be done cautiously. Other in vitro experiments (37), however, indicate that plasminogen activator induces thrombolysis more readily in the human than in the rabbit or dog systems, and that 24-h aged clots are only slightly more resistant to lysis than fresh clots. All these findings encourage us to further investigate plasminogen activator with the aim to develop it into a new thrombolytic agent.

ACKNOWLEDGMENTS

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