Comparison of Intravenous Bolus Injection or Continuous Infusion of Recombinant Single Chain Urokinase-Type Plasminogen Activator (Saruplase) for Thrombolysis. A Canine Model of Combined Coronary Arterial and Femoral Venous Thrombosis

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The thrombolytic efficacy of recombinant unglycosylated full length single chain urokinase-type plasminogen activator (rscu-PA, saruplase), applied either as single intravenous bolus or as a continuous infusion over 60 minutes, was studied in 5 randomized blinded groups of 5 dogs with combined copper coil induced coronary artery thrombosis and fibrin-labeled femoral vein clots. Infusion of 0.5 mg/kg rscu-PA (group I) induced coronary lysis in 4 of 5 dogs and 98 ± 1% (mean ± SEM) venous clot lysis. Bolus injection of 1 mg/kg rscu-PA (group II) caused reflow in 3 of 5 dogs and 88 ± 5% venous clot lysis. Infusion of 0.5 mg/kg rscu-PA (group II) caused reflow in 3 of 5 dogs and 82 ± 6% venous clot lysis. Bolus injection of 0.5 mg/kg rscu-PA (group IV) produced clot lysis in 4 of 5 dogs and 48 ± 12% venous clot lysis. Placebo infusion (group V) was associated with late recanalization in 1 of 5 dogs and 18 ± 8% venous clot lysis. Coronary artery reocclusion after reflow was not observed in groups I and II, but occurred in 2 of 3 animals in group III and in 3 of 4 animals in group IV (P = .02). The time to reflow in responsive animals was 22 ± 8 minutes with infusion of 0.5 or 1 mg/kg rscu-PA and 14 ± 1 minute with bolus injection of 0.5 or 1 mg/kg (P = .14). Depletion of fibrinogen and α2-antiplasmin to <25% of baseline levels was observed in the 5 dogs given 1 mg/kg rscu-PA by bolus and in 3 of the 5 dogs given 1 mg/kg rscu-PA via infusion, but in none of the dogs that received 0.5 mg/kg rscu-PA (P < .001). Plasma clearance rates were 170 ± 44 and 230 ± 30 mL/minute after bolus injection and 190 ± 47 and 310 ± 56 mL/minute during infusion of rscu-PA for the 1 mg/kg and 0.5 mg/kg doses respectively. Thus, intravenous bolus injection of rscu-PA (saruplase) appears to be equipotent to an infusion over 60 minutes for both coronary and venous thrombolysis. This animal model of combined arterial and venous thrombolysis may be useful for the evaluation of new thrombolytic strategies.

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SINGLE CHAIN urokinase-type plasminogen activator (scu-PA) obtained from conditioned cell culture media1-4 or by recombinant DNA technology,5-7 has been shown to induce fibrin-specific clot lysis in animal models of arterial9-11 or venous12-13 thrombosis. Arterial and venous thrombi differ with regard to platelet content; this may require a different thrombolytic strategy, as recently demonstrated in experimental animal models.9 At present no animal model is available for the direct comparison of pharmacologic approaches to dissolve arterial and venous thrombi. In the first randomized trial in patients with acute myocardial infarction, rscu-PA obtained by expression in Escherichia coli (saruplase) was shown to produce more rapid coronary artery reflow than streptokinase.10 However, systemic fibrinolytic activation with saruplase was very extensive.

The benefits of thrombolytic therapy in acute myocardial infarction appear to be directly related to the time interval between onset of symptoms and coronary artery recanalization.11 Bolus injection of thrombolytic agents, provided it achieves comparable efficiency without excessive bleeding complications, might not only simplify treatment but also induce more rapid recanalization. The feasibility12 and efficacy13 of bolus administration has been demonstrated for acylated plasminogen streptokinase activator complex (APSAC), and more recently for rt-PA.14,15 The protracted activity of APSAC is most likely related to its slow clearance,16 whereas for the short-lived rt-PA it may be due, at least in part, to its affinity for fibrin.17 The relative efficacy of bolus injection versus infusion of rscu-PA, which has no significant affinity for fibrin,18,19 is unclear.

The purpose of the present study was to evaluate the pharmacokinetics, thrombolytic potency and fibrinogen breakdown of rscu-PA, given either as a single bolus or a 25% bolus followed by an infusion of 75% of the dose over 60 minutes, in a combined coronary artery and femoral venous thrombosis model in the dog.

MATERIALS AND METHODS
rscu-PA (saruplase). Full length rscu-PA was obtained by expression of the cDNA encoding human scu-PA in E coli20 and was provided by Grünenthal GmbH, Aachen, FRG. It was devoid (<0.1%) of urokinase activity and of pyrogens. Its specific activity as measured on chromogenic substrate (S-2244, Kabi Diagnostica, Stockholm, Sweden) after activation with plasmin was 113,000 IU/mg by comparison with the International Reference Preparation for Urokinase (batch 66/46, Institute for Biological Standards and Control, London, UK), which corresponds to 200,000 CTA U/mg as defined by the supplier of the substrate.

Coronary arterial and femoral venous thrombosis models. Shepherd dogs, weighing 20 ± 1 kg, were sedated with 0.25 mg/kg fluanisone, anesthetized with 15 mg/kg sodium pentobarbital, intubated and artificially ventilated. Catheters were placed in the left jugular and brachial veins for infusion of agents and withdrawal of blood samples. The right femoral artery was used for monitoring blood pressure and both carotid arteries for coronary angiography and copper coil positioning.

Coronary thrombosis was induced by placing a 3 to 5-mm long copper coil over an intracoronary wire into the left anterior descending coronary artery distal to the first main diagonal branch, as previously described.21,22 An occlusive thrombus formed within 5 to 10 minutes and was confirmed angiographically. All animals develope-
oped electrocardiographic evidence of transmural ischemia. The coronary thrombus was allowed to age for one hour before heparin was administered and the infusion protocol started. Recanalization and reocclusion were assessed by angiography, performed at 15 minute intervals, and whenever arrhythmias or electrocardiographic changes suggestive of recanalization or reocclusion occurred. Reflow was defined as TIMI grade 2 or 3 and occlusion as TIMI grade 0 or 1.24

The femoral venous thrombosis model has been described elsewhere.24-26 Briefly, the left femoral vein was exposed in the inguinal region and all side branches ligated, except for a predominant musculocutaneous branch that was cannulated. After introduction of a woolen thread in the lumen to prevent clot embolization, the vein was clamped in order to isolate a segment of 4 cm, which was emptied via the side branch catheter. The segment was then filled with 0.1 mL thrombin solution (6 to 8 units) followed by a mixture of 0.6 to 0.8 mL of fresh blood and a trace amount of 125I-labeled human fibrinogen (approximately 10^9 cpm). The timing of the experiment was designed to allow the venous clot to age for approximately 30 minutes before the clamps were removed and the infusion protocol started. The radiisotope content of the venous clot was calculated by subtracting, from the original amount of 125I aspirated in the syringe, the sum of the radioactivity which remained in the syringe, the radioisotope that was adsorbed on cotton swabs placed around the vein segment, and the radiiodine that was washed out from the thrombus into the blood stream after removal of the clamps. At the end of the experiment (one hour after the end of the infusion), the thrombosed segment of the femoral vein was ligated at both ends, removed, and its residual radioisotope content measured. The degree of lysis was defined as the residual radioactivity in the vein segment and was expressed as percent of the radioactivity originally incorporated in the clot. An isotope recovery balance was made by comparing the sum of the total blood radioactivity at the end of the experiment (multiplied by 3 for extravascular distribution) and the radioactivity in the recovered thrombus with that originally present in the clot.

Infusion protocols. Twenty-five dogs were randomly assigned to one of the following treatment groups: I) intravenous bolus injection of 0.25 mg/kg rscu-PA (saruplase), followed by continuous infusion of 0.75 mg/kg rscu-PA over 60 minutes (total dose: 1 mg/kg over 60 minutes); II) intravenous bolus injection of 1 mg/kg rscu-PA, followed by a placebo (saline) infusion; III) intravenous bolus injection of 0.125 mg/kg rscu-PA, followed by continuous infusion of 0.375 mg/kg over 60 minutes (total dose: 0.5 mg/kg over 60 minutes); IV) intravenous bolus injection of 0.5 mg/kg rscu-PA, followed by placebo infusion; and V) intravenous bolus injection of placebo, followed by placebo infusion. The experiments were prospectively performed in two blocks with an interim analysis: groups I and II with 2 placebo dogs were randomized in the first block, groups III and IV with 3 placebo dogs in the second block. The rscu-PA used in both blocks was from the same batch. Heparin was given to all animals as an intravenous bolus of 100 U/kg before the start of the protocol and was repeated after 1 hour. The animals were observed for one hour after the end of the infusion. Angiographic interpretation of coronary patency and quantitation of venous clot lysis were performed by investigators unaware of the treatment group allocation.

Plasma levels of rscu-PA, fibrinogen and α2-antiplasmin. Blood samples were collected on citrate before and 1, 2, 5, 10, 20, 30, 45, 60, 90 and 120 minutes after the initial bolus injection. Samples were cooled on ice and centrifuged for immediate analysis of fibrinogen.23,24 α2-antiplasmin activity was determined from frozen plasma samples.27 Circulating levels of rscu-PA were measured with an enzyme-linked immunosorbent assay.24

Pharmacokinetics. The pharmacokinetics of rscu-PA were determined by measuring the rscu-PA antigen levels in plasma at different time intervals after intravenous bolus injections of 1 mg/kg or 0.5 mg/kg rscu-PA in groups II and IV. The data describing the disposition of rscu-PA from plasma were plotted on semiilogarithmic paper and fitted with a sum of two exponential terms, C(t) = Ae^-<1/d> + Be^-<1/2>, by graphical curve peeling. Therefore, the linear terminal portion of the antigen versus time curve was extrapolated to yield the ordinate intercept B; this line has a slope -β. The extrapolated values were subtracted from the initial values yielding a line with an ordinate intercept A and a slope -α. Using standard formulas derived by Gibaldi and Perrier for bolus turnovers,29 the following drug disposition parameters were calculated from the coefficients (A and B) and exponents (α and β): volume of the central compartment Vc = dose/(A + B), total volume of distribution Vd = dose/B, extrapolated area under the curve AUC = A/α + B/β, plasma clearance Clp = dose/AUC, t1/2 = ln2/α and t1/2β = ln2/β.

For groups I and III, given an infusion regimen with an initial bolus of 25% of the total dose, steady state plasma antigen levels were determined as the mean values of 30, 45 and 60 minute values: plasma clearance was calculated as the ratio between the infusion rate (μg/minute) and the steady state plasma level (in μg/mL).

The disappearance rate of rscu-PA related fibrinolytic activity in plasma was determined in a clot lysis assay using a mixture of 800 μL fibrinogen solution (2 mg/mL, bovine fibrinogen, Oragnon, Oss, the Netherlands), 20 μL purified plasminogen (0.5 mg/mL) and 100 μL dog plasma containing rscu-PA or urokinase standard, which was clotted with 80 μL thrombin solution (30 U/mL, Topostasin, Hoffman-La Roche, Basel, Switzerland). The mixture was incubated at 37°C and its lysis time determined. The lysis time was converted to activity by comparison with the International Reference Preparation for Urokinase that was diluted in normal dog plasma. The sensitive range of the clot lysis assay ranged between 40 IU/mL (lysis time 6,000 seconds) and 1,200 IU/mL (lysis time 260 seconds).

Statistical analysis. The results are expressed as mean ± SEM. The means between two groups were compared using a Fisher's exact test for discrete variables and an unpaired Student's t-test for continuous variables.

RESULTS

Coronary artery recanalization and reocclusion. The angiographic results of coronary thrombolysis are summarized in Fig 1. The higher dose of rscu-PA (1 mg/kg) induced rapid reflow (within 45 minutes) in 7 of 10 dogs, with an comparable patency rate produced with both infusion over 60 minutes (group I) and bolus injection (group II). No reocclusion occurred with 1 mg/kg rscu-PA. The lower dose (0.5 mg/kg) induced early reperfusion in 7 of 10 dogs, with comparable patency rate produced with both infusion over 60 minutes (group I) and bolus injection (group II). No reocclusion occurred in 2 of 3 dogs given the 60 minutes infusion (group III) and in 3 of 4 dogs allocated to the single bolus injection (group IV). Thus, coronary patency at the end of the experiment was obtained in only 2 of 10 dogs. Placebo infusion (group V) resulted in late reflow (at 75 minutes) in 1 of 5 dogs. The difference in patency rate at 120 minutes between the 0.5 and 1 mg/kg dose groups (2 of 10 versus 7 of 10) was borderline significant (P = .07); the difference in frequency of reocclusion (0 of 7 with 1 mg/kg and 5 of 7 with 0.5 mg/kg rscu-PA) was significant (P = .02). The time to recanalization by bolus injection of 0.5 or 1 mg/kg rscu-PA (14 ± 1 minute, n = 7) was comparable to that with infusion of 0.5 or 1 mg/kg rscu-PA over 60 minutes (22 ± 5 minutes, n = 7) (P = .14).
**Femoral venous thrombolysis.** Fig 2 summarizes the results of femoral vein clot lysis obtained with bolus injection or infusion. In the placebo-treated animals (group V), clot lysis was 18 ± 8%, as determined in 3 animals. The result of one animal with an estimated lysis of 98% (6 standard deviations above the mean value of the other results) was discarded from this analysis. It is possible that defective clotting had occurred in this animal, as suggested by the high blood radioactivity after release of the vessel clamps, although this was not experimentally confirmed. The higher dose of rscu-PA (1 mg/kg) resulted in virtually complete venous clot lysis in all animals (93 ± 3%, n = 8), without a significant difference between the infusion protocol in group I (98 ± 1%, n = 4) and the bolus protocol in group II (88 ± 5%, n = 4). The lower rscu-PA dose (0.5 mg/kg) induced significantly less lysis (50 ± 6%, n = 9, P < .001) both with the infusion protocol in group III (52 ± 6%, n = 5) and with the bolus administration in group IV (48 ± 12%, n = 4). The calculated isotope recovery balance (see Methods) was 86 ± 7%, 88 ± 5%, 93 ± 3%, 87 ± 2% and 98 ± 2% in groups I to V respectively, indicating that no significant parts of the labeled thrombus had been lost by embolization.

**Hemostasis analyses.** The results of hemostasis analyses are summarized in Table 1. The higher dose of rscu-PA (1 mg/kg) caused extensive fibrinogen and α2-antiplasmin consumption, with a somewhat more pronounced decrease in the

| Table 1. Hemostasis Parameters Following Administration of rscu-PA |  |
|---|---|---|---|---|---|---|---|
| Groups | rscu-PA Dose (mg/kg) | n | Residual Fibrinogen % | Residual α2-antiplasmin % | Steady-State rscu-PA Antigen (µg/mL) | Plasma Clearance* (mL/min) |
| I | 1.0 | 5 | 49 ± 21 | 33 ± 16 | 1.7 ± 0.5 | 190 ± 47 |
| II | 1.0 | 5 | 10 ± 3 | 9 ± 3 | — | — |
| III | 0.5 | 5 | 101 ± 7 | 92 ± 6 | 0.5 ± 0.1 | 310 ± 39 |
| IV | 0.5 | 5 | 93 ± 8 | 89 ± 2 | — | — |
| V | — | 5 | 110 ± 11 | 104 ± 4 | — | — |

The results represent mean ± SD and n the number of dogs. Residual fibrinogen and α2-antiplasmin levels are means of values obtained 60 and 120 minutes after the start of the infusion protocols.

* Determined from steady state plasma antigen level during infusion.
bolus group (residual fibrinogen 10 ± 3% and α₁-antiplasmin 9 ± 2%) than in the infusion group (fibrinogen 49 ± 21% and α₁-antiplasmin 33 ± 16%) (P = .1 each). The lower rscu-PA dose (0.5 mg/kg) did not cause systemic fibrinolytic activation; in both the bolus group and the infusion group the residual fibrinogen and α₁-antiplasmin levels remained in excess of 85% of baseline (P < .001 versus 1 mg/kg dose).

All values of the activated partial thromboplastin time were at least twice the baseline value, when measured 60 minutes after the heparin bolus administration (results not shown).

**Pharmacokinetics of rscu-PA.** Fig 3 represents antigen versus time curves for the different rscu-PA regimens in groups I to IV. Peak plasma levels of rscu-PA (Co) were 9.3 ± 1.8 μg/mL after bolus injection of 1 mg/kg and 4.8 ± 0.6 μg/mL after bolus injection of 0.5 mg/kg rscu-PA. Bolus injection of 25% of the total dose in the infusion protocols (group I with a total dose of 1 mg/kg and group III with a total dose of 0.5 mg/kg) yielded peak plasma levels of 5.5 ± 0.5 μg/mL and 1.3 ± 0.1 μg/mL respectively, whereas the infusion of 75% of the dose produced steady state plasma levels of 1.7 ± 0.5 μg/mL and 0.5 ± 0.1 μg/mL respectively. Thirty minutes after the end of infusion, plasma levels of 13% and 4% of the respective steady state levels were measured. In group III and IV, given 0.5 mg/kg rscu-PA, antigen levels at 30 minutes tended to be lower in animals with early (30 minutes) reocclusion (0.2 ± 0.08 μg/mL, n = 3) as compared to animals with a persistently patent coronary artery (0.37 ± 0.2 μg/mL, n = 4), P = .23. Plasma clearance during infusion, calculated from steady state levels, were 190 ± 23 mL/minute for the 1 mg/kg group and 310 ± 39 mL/minute for the 0.5 mg/kg group.

The relevant pharmacokinetic parameters describing the disposition of rscu-PA from blood following intravenous bolus injection of 1 mg/kg and 0.5 mg/kg in two groups of 5 dogs are summarized in Table 2. The disappearance rate of rscu-PA related antigen was adequately described by a sum of two exponential terms by graphical curve peeling (see Materials and Methods), with initial and terminal half-lives of 4 to 5 and 13 to 16 minutes respectively. Plasma clearances determined from the drug disposition rate were 170 ± 44 mL/minute for the 1 mg/kg group and 230 ± 30 mL/minute for the 0.5 mg/kg group.

The results of plasma rscu-PA activity, determined by clot lysis assay, calibrated against the urokinase standard and expressed in percent of the initial value are represented in Fig 4. After bolus injection of 1 mg/kg or 0.5 mg/kg rscu-PA both the antigen levels and the activity levels disappeared in a biphasic and parallel manner, with 15% or less of the initial values measured at 30 minutes. After 30 minutes a slower disappearance of rscu-PA activity as compared with rscu-PA related antigen was observed.

**DISCUSSION**

The results of this randomized and blind study in dogs suggest that recombinant single chain urokinase-type plasminogen activator (rscu-PA, saruplase) is equipotent for coronary and venous thrombolysis, whether applied as single intravenous bolus or as an infusion over 60 minutes. Coronary reflow and reocclusion rates as well as the extent of venous clot lysis were dose-related. With a dose of 1 mg/kg, venous clot lysis was maximal, and coronary recanalization, which occurred in 7 of 10 animals, was persistent. At half this dose, venous clot lysis was approximately 50%, whereas coronary recanalization, which still was achieved in 7 of 10 dogs, was followed by reocclusion in 5 of these dogs.

Previous studies in dogs have shown similar coronary reperfusion rates with 30 minutes infusions of 0.6 mg/kg recombinant scu-PA and with natural scu-PA obtained from a transformed human kidney cell line. The thrombolytic efficacy of scu-PA given as a bolus has been tested in a
administration of 1 mg/kg (A) and 0.5 mg/kg (B) rscu-PA. The results, expressed in percent of the initial value, represent mean ± SEM of 5 experiments in each group.

Fig 4. Disappearance of fibrinolytic activity (diamonds) and rscu-PA related antigen plasma levels (circles) following bolus administration of 1 mg/kg (A) and 0.5 mg/kg (B) rscu-PA. The results, expressed in percent of the initial value, represent mean ± SEM of 5 experiments in each group.

Femoral artery thrombosis model, with reperfusion rates comparable to ours. However, reocclusion was not monitored in those studies, and rscu-PA has not been studied for venous thrombosis so far.

α2-antiplasmin consumption and fibrinogen breakdown tended to be more extensive with bolus application than with infusion for 1 mg/kg rscu-PA, but the difference was not significant. However, with a limited number of experiments in each group, our study is subjected to some beta error. Thus, with a larger number of experiments, significant differences in systemic activation of the fibrinolytic system with bolus rscu-PA versus continuous infusion might be observed. It is possible that the higher peak plasma level of 9.3 ± 1.8 μg/mL obtained by bolus injection of 1 mg/kg rscu-PA is associated with more rapid plasmin generation and more extensive conversion of the fibrin selective single chain u-PA to the non fibrin-selective two chain derivative. Indeed, fibrinogen breakdown in plasma following addition of rscu-PA only occurs above a threshold level of several μg/mL.31

Both the bolus injection and infusion regimen were associated with comparable plasma clearances of rscu-PA antigen of 170 to 300 mL/minute. Furthermore, a parallel disappearance-rate from plasma was observed of rscu-PA related antigen and plasminogen activator activity. The residual plasma levels of both antigen and activity 30 minutes after the bolus injection were between 5 and 15% of the peak levels. Thus, plasma levels may have been sufficiently high to explain the time course of the rscu-PA induced coronary recanalization and venous clot lysis without having to invoke its binding to the thrombus or to cell receptors. However, it is possible that extensive venous clots or coronary occlusions in the setting of high-grade stenosis might be differently sensitive to bolus injections versus infusions.

The present animal model of simultaneous arterial and venous thrombosis offers the possibility of convenient quantification of dose response characteristics of thrombolytic agents towards venous thrombosis. However, whether the copper coil induced coronary thrombosis indeed constitutes a representative model of coronary occlusion and reocclusion in humans may be questioned. Animal preparations designed to study the prevention of reocclusion, such as thrombosis on a high grade stenosis, thermal damage of endothelium with exposure of basement-membrane collagen by anodal current or erosion grafts produce platelet rich thrombi that are more resistant to lysis with rt-PA than the relatively fibrin-rich thrombi induced by a copper coil.33 Reocclusion can be prevented in those models by antplatelet agents, whereas antithrombin agents may be needed to prevent reocclusion by fibrin-rich thrombi. However, heparin did not prevent reocclusion after low-dose rscu-PA in our study.

These findings further indicate that the evaluation of new thrombolytic strategies in animal models and in patients should take the thrombus composition into account. Furthermore, any extrapolation of results obtained in animal models to patients with acute myocardial infarction or deep vein thrombosis is hampered by the large interspecies variability of response to thrombolytic agents and by the age and extent of thrombosis. Extrapolation of the results of the present study to patients with thromboembolic disease should therefore be done cautiously.

Recent clinical pilot-trials in acute myocardial infarction have shown that rt-PA may be effective as IV bolus in spite of its short plasma half-life. However, coronary patency rates were not higher than in trials based on the commonly used infusion regimen, thus questioning the hypothesis that high initial drug levels might improve reperfusion. Bolus application of thrombolytic agents, as demonstrated with APSAC, is nevertheless a convenient possibility of early treatment before hospital admission, provided such regimens prove to be safe.

In conclusion, rscu-PA applied as an intravenous bolus appears to be equipotent to infusion for coronary and venous thrombolysis in the dog, in spite of its short plasma half-life and its lack of fibrin-binding. The combined animal model for arterial and venous thrombosis used in the present study may be useful for the evaluation of new thrombolytic strategies.

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