Thrombolytic and Pharmacokinetic Properties of a Conjugate of Recombinant Single-Chain Urokinase-Type Plasminogen Activator With a Monoclonal Antibody Specific for Cross-Linked Fibrin in a Baboon Venous Thrombosis Model

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Chemical conjugates between recombinant single-chain urokinase-type plasminogen activator (rscu-PA) and a murine monoclonal antibody directed against fragment D-dimer of cross-linked human fibrin (MA-15C5), rscu-PA/MA-15C5, and between rscu-PA and a control monoclonal antibody (MA-1C8), rscu-PA/MA-1C8, were produced by cross-linking with N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). In an in vitro system composed of a $[^{125}I]$fibrin-labeled baboon plasma clot immersed in autologous citrated plasma, dose- and time-dependent lysis was obtained with a ratio of the potencies of free and conjugated rscu-PA similar to that in human plasma: 50% lysis in 2 hours required 4.3 µg/ml rscu-PA, 1.0 µg/ml urokinase-type plasminogen activator (u-PA) equivalent rscu-PA/MA-15C5, or 15 µg/ml u-PA equivalent rscu-PA/MA-1C8. The thrombolytic and pharmacokinetic properties of rscu-PA and of rscu-PA/MA-15C5 were compared in baboons with a 0.8-1.0 ml $[^{125}I]$fibrin-labeled autologous blood clot produced in a femoral vein. Continuous intravenous infusion of these compounds during a 2-hour period resulted in dose- and time-dependent lysis. The thrombolytic potency of rscu-PA/MA-15C5 was 3.0±0.5 times higher (50% lysis with 0.3±0.02 mg u-PA equivalent/kg body wt) than that of rscu-PA measured by ex vivo isotope recovery from the femoral vein segment ($p<0.001$) and was 2.7±0.5 times higher (50% lysis with 0.35±0.02 mg/kg rscu-PA/MA-15C5) by external radioisotope counting ($p<0.001$). A dose of 0.5 mg/kg of rscu-PA/MA-1C8 was much less active than rscu-PA. After the end of the infusion, u-PA-related antigen disappeared from plasma in a biphasic manner with an initial half-time of 2.7±0.5 for rscu-PA, 24±1.2 for rscu-PA/MA-15C5, and 21±0.5 minutes for rscu-PA/MA-1C8 with corresponding plasma clearances of 340±40, 20±3, and 24±2 ml/min, respectively. In conclusion, the increased thrombolytic potency of rscu-PA/MA-15C5 is the result of a reduction of the thrombolytic potency due to coupling of rscu-PA to the antibody molecule, which is counterbalanced by an enhancement of the thrombolytic potency due to fibrin targeting by the specific idiotype. (Circulation 1990;82:1744-1753)

Urokinase-type plasminogen activator (u-PA) has been purified from several sources as a single-chain molecule (scu-PA), which is converted to a two-chain derivative (tcu-PA) by cleavage of the Lys$^{158}$Ile$^{159}$ peptide bond after limited digestion with plasmin.¹ scu-PA induces relatively fibrin-specific clot lysis in human plasma in vitro and in animal models of thrombosis,²⁻⁵ although no direct binding to fibrin has been demonstrated.²

See p 1874

scu-PA has been used successfully for the treatment of patients with acute myocardial infarction.⁶,⁷ However, high doses are required for coronary thrombol-
ysis, resulting in extensive systemic activation of the fibrinolytic system and fibrinogen breakdown.6,7

One approach to improve the fibrin specificity of scu-PA consists of targeting the molecule to the fibrin clot by means of conjugation with fibrin-specific monoclonal antibodies.8 Conjugates of monoclonal antibodies, directed against the NH2-terminal region of the β-chain of fibrin, and scu-PA have been made by chemical cross-linking and have been shown to enhance the thrombolytic potency of scu-PA in vitro.9 The specific epitope in the NH2-terminus of the β-chain of fibrin may, however, be partially masked during fibrin polymerization and lost relatively early during thrombolysis.10 Targeting to specific epitopes in fragment D-dimer of fibrin, which remains associated with the core of the fibrin clot until complete lysis,10 may be associated with more efficient targeting.

A murine monoclonal antibody (MA-15C5) with a more than 1,000-fold higher affinity for fragment D-dimer of human cross-linked fibrin than for fibrinogen has been developed11,12 and has been used successfully for venous thrombus imaging in animal models.12,13 Chemical conjugation of rscu-PA to MA-15C5 (rscu-PA/MA-15C5) increased its thrombolytic potency in a human plasma system in vitro by about sixfold14 and in rabbits with a human plasma clot in the jugular vein by about eightfold.15 The kinetic constants for plasminogen activation were comparable for rscu-PA and rscu-PA/MA-15C5, whereas binding of the conjugate to fibrin fragment D-dimer in vitro did not affect its catalytic efficiency for plasminogen activation.14 The enhanced thrombolytic potency and fibrin specificity of conjugates of rscu-PA with MA-15C5, demonstrated in vivo in rabbits, cannot, however, as such be extrapolated to humans. Indeed, interference of endogenous fibrin or fibrinogen with clot lysis by the conjugate was avoided in the rabbit because of the species specificity of MA-15C5. Although fibrin fragments do not trigger activation of the fibrinolytic system by the conjugate in plasma in vitro,14 they might well reduce its thrombolytic efficacy by blocking the antigen binding site of the antibody.

The purpose of the present study was, after demonstrating cross-reactivity of MA-15C5 with cross-linked baboon fibrin, to investigate the thrombolytic and pharmacokinetic properties of rscu-PA and its conjugate with MA-15C5 or with a control antibody MA-1C8 in baboons with autologous [125I]fibrin-labeled femoral vein clots.

Methods

Proteins and Reagents

Recombinant scu-PA (rscu-PA), prepared by expression of cDNA encoding scu-PA in Escherichia coli, was a kind gift from Grüenthal AG (Aachen, FRG). Murine monoclonal antibody MA-15C5, which reacts with fragment D-dimer of human fibrin, was produced and characterized as described.11,12 Murine monoclonal antibody MA-1C8, which is directed against an epitope covering the fibrin-binding site of human tissue-type plasminogen activator, was characterized previously.16 Human fibrinogen labeled with iodine-125 was purchased from Amersham Research Products (Amersham, Buckinghamshire, UK).

Normal human plasma was obtained from pooled, fresh frozen, citrated blood bank plasma from at least five healthy blood donors. Normal baboon plasma was fresh, frozen citrated plasma from individual animals.

Cross-reactivity of MA-15C5 with human or baboon plasma was evaluated as follows. Plasma was allowed to clot with CaCl2 (final concentration, 25 mM) and bovine thrombin (final concentration, 4 National Institutes of Health [NIH] units/ml) for 2 hours at 37°C. The clots were then digested by overnight incubation at 37°C with recombinant tissue-type PA (final concentration, 1 μg/ml; Actiase, Genentech, Inc., South San Francisco, Calif.) followed by addition of aprotinin (final concentration, 200 kallikrein inhibitor units [KIU]/ml; TrasyloL, Bayer, Leverkusen, FRG). Immunoreactivity was evaluated by a solid-phase enzyme-linked immunosorbent assay with microtiter plates coated with MA-15C5 and horseradish peroxidase–conjugated MA-6C1, a monoclonal antibody raised with human fibrin fragment D-dimer, which on immunoblotting cross-reacts with baboon fibrin fragment D-dimer. In this way, the immunoreactivity of MA-15C5 with human or baboon fibrin was found to be nearly identical (results not shown).

Preparation of Conjugates of rscu-PA With Monoclonal Antibodies

MA-15C5 and rscu-PA were conjugated with the heterobifunctional cross-linking reagent N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) with a three-step procedure and were purified by chromatography on the insolubilized u-PA–specific monoclonal antibody MA-4DIE8 and on insolubilized fibrin fragment D-dimer essentially as described previously.14 Alternatively, rscu-PA was conjugated to the control antibody MA-1C8 with the same procedure, and the conjugate was purified by chromatography on insolubilized MA-4DIE8 followed by gel filtration as described elsewhere.14

In the present study, a large batch of rscu-PA/MA-15C5 was prepared starting from 300 mg total protein, containing approximately 80 mg rscu-PA and 220 mg MA-15C5. The complex was purified on columns containing 80 ml gel substituted with MA-4DIE8 (3 mg antibody/ml Sepharose) and 130 ml gel with insolubilized fragment D-dimer (3 mg/ml Sepharose). The final yield was 50% of the total protein (43% recovery of rscu-PA), and the conjugate contained on average 1.2 immunoglobulin G (IgG) molecules/rscu-PA molecule. The specific activity of the conjugate, measured by hydrolysis of the chromogenic substrate S-2444 (Pyroglu-Gly-Arg-p-nitroanilide) after conversion to two-chain urokinase by plasmin,14 was 110,000 IU/mg u-PA, by
comparison with the International Reference Preparation for Urokinase (batch 66/46) obtained from the National Institute for Biological Standards and Control (London). The specific activity of the rscu-PA used as starting material was 120,000 IU/mg.

A large batch of rscu-PA/MA-1C8 was prepared starting from 165 mg total protein, containing 45 mg rscu-PA and 120 mg MA-1C8. The complex was purified on columns containing 55 ml gel substituted with MA-4D1E8. The final yield was 45% of the total protein (34% recovery of rscu-PA), and the conjugate contained on average 1.5 lgG molecules per rscu-PA molecule. The specific activity of the conjugate, determined as described above, was 95,000 IU/mg u-PA.

\[ ^{125}\text{I}]\text{Fibrin-Labeled Plasma Clot Lysis in Vitro \]

Lysis of \[^{125}\text{I}]\text{fibrin-labeled human or baboon plasma clots, immersed in citrated autologous plasma (human plasma clot immersed in human plasma or baboon plasma clot immersed in baboon plasma), was monitored for 4 hours after addition of rscu-PA, rscu-PA/MA-15C5, or rscu-PA/MA-1C8 (final concentration, 0.05–5 \mu\text{g} \text{ u-PA/ml} \) as described elsewhere.\(^{14}\) Equine fibrinogen, containing 50% clot lysis in 2 hours, was obtained from plots of the percent lysis at 2 hours versus the concentration of u-PA–related antigen. Clot lysis experiments were performed with plasma from five individual baboons, and the results were compared with those obtained with pooled human plasma. Fibrinogen in plasma was determined by a coagulation rate assay.\(^{17,18}\)

\textit{Thrombolysis in a Baboon Femoral Vein Thrombosis Model}

The thrombolytic properties of rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8 were compared in baboons with femoral vein blood clots. Therefore, baboons (\textit{Papio hamadraeus}) of either sex, weighing 7–12 kg, were anesthetized with 10 mg/kg ketamine hydrochloride (Imalgene 500, Sanofi, Brussels, Belgium) and 0.06 mg/kg atropine i.m., after premedication with 5 mg diazepam i.m. (Valium, Hoffmann-LaRoche, Basel, Switzerland). After endotracheal intubation, the lungs were ventilated with a Bird Mark 7 respirator (Bird Mark Corp., Palm Springs, Calif.). Anesthesia was maintained with 30 mg pentobarbital i.v. (Nembutal, Abbott Laboratories, North Chicago, Ill.).

The surgical procedure for femoral vein clot formation was modified from a previously described canine thrombosis model.\(^{19}\) A femoral vein was exposed through a 5-cm incision of the inguinal region, and the vein was cleared from the entry of the femoral vein in the groin to 2 cm below the saphenofemoral junction. Small side branches and the deep femoral vein were ligated, except for the predominant musculoscutaneous branch, which was cannulated with a Portex “pin gauge” cannula (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 by the side branch catheter. The volume of the segment, measured by injection of saline from a volumetric syringe until the vessel was fully distended, was 0.8–1.0 ml.

The thrombus was then produced as follows. Approximately 10–20 \mu\text{l} \[^{125}\text{I}]\text{labeled human fibrinogen, containing 1 to } \times 10^6 \text{ cpm, was mixed with fresh baboon blood and aspirated in a 1.0-ml syringe to a volume 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 0.2 ml thrombin (100 NIH units/ml) solution containing 10 mM CaCl}_2 was injected, followed immediately by the mixture of blood and 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, after which it was allowed to age for 30 minutes. Then both vessel clamps were removed, and 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 from the original amount of radioactivity in the syringe.

The plasminogen activators were administered intravenously as a 10% bolus injection, followed by continuous infusion of the remaining 90% dose during 2 hours by a brachial vein. The animals were anticoagulated with heparin (300 units/kg as an intravenous bolus and a continuous infusion of 60 units/kg/hr until the end of the experiment). Thrombolysis was quantified 30 minutes after the end of the infusion, both by determination of the residual radioactivity in the femoral vein segment and by external gamma counting. For determination of the ex vivo clot recovery, the thrombosed segment of the femoral vein was removed after careful suturing of both ends, and the remaining radioactive material in the vein segment was measured. The degree of lysis was expressed as the difference between the radioactivity originally calculated to be incorporated in the clot and the residual radioactivity in the vein segment and was expressed in percent. The time course of clot lysis was monitored continuously by external gamma counting using a 3\times0.5-in sodium iodide/thallium crystal (Bicron, Newbury, Ohio) positioned over the thrombosed femoral vein segment. The rest of the body, and particularly the region over the urine bladder, was shielded using 5-mm thick lead plates. The scintillation crystal was connected to a dedicated Canberra-S100 system (Canberra-Packard, Meriden, Conn.), which allows direct data acquisition on an IBM compatible personal computer (Compaq Deskpro 386) and data analysis by an application manager (Canberra-Packard) into a spreadsheet (Excel v.2.1, Microsoft, Redmond, Wash.). Data were accumulated continuously in 30-second intervals, which
were processed by a 3-point smoothing at 5-minute intervals. The extent of clot lysis was determined as the difference between the initial and final isotope measurements.

Two-milliliter blood samples were drawn on citrate (final concentration, 0.01 M) before, at hourly intervals after the start of the infusion, and at the end of the experiment. The plasma samples were used for measurement of radioactivity, fibrinogen, and $\alpha_2$-antiplasmin. $\alpha_2$-Antiplasmin was measured with a specific ELISA.

To limit the number of primates used in the present study to an acceptable minimum, we performed 23 experiments in 13 baboons. When two experiments were performed in the same animal, both femoral veins were used once. The experiments were performed at an interval of 2–8 days. Solvent was infused once as a first and twice as a second experiment; rscu-PA was infused six times as a first and five times as a second experiment; rscu-PA/MA-15C5 was infused five times as a first and twice as a second experiment; and rscu-PA/MA-1C8 was infused once as a first and once as a second experiment. No correlation was found between the sequence of the experiments and the clot lysis results. For example, results obtained with the ex vivo isotope recovery method were, for solvent infusion, 8% when given first and 4% and 11% when given second; for 0.5 mg/kg rscu-PA, 10% and 15% when given first and 9% and 44% when given second; for 1 mg/kg rscu-PA, 31%, 36%, and 90% when given first and 70% when given second; for 2 mg/kg rscu-PA, 98% when given first and 66% and 97% when given second; for 0.25 mg/kg rscu-PA/MA-15C5, 28% and 47% when given first and 23% when given second; for 0.5 mg/kg rscu-PA/MA-15C5, 91% when given first and 94% when given second; and for 0.5 mg/kg rscu-PA/MA-1C8, 23% when given first and 27% when given second. All fibrinogen levels measured before the start of a second infusion were normal. These studies conformed to the guiding principles of the American Physiological Society.

Pharmacokinetic Properties

rscu-PA–related antigen was determined in blood samples taken before infusion and 1, 2, 5, 7, 10, 15, 20, and 30 minutes after cessation of infusion for rscu-PA and before infusion and 15, 30, 60, 90, and 120 minutes after the end of the infusion of the conjugates. The experimental data describing the disappearance of u-PA–related antigen from plasma were fitted with a sum of two exponential (exp) terms: $C(t) = R \exp(-\alpha t) + S \exp(-\beta t)$. The coefficients (R and S) and exponents ($\alpha$ and $\beta$) of this function were obtained from semilogarithmic plots by graphic curve peeling performed as follows. The curve for the terminal phase of the plasma antigen plotted against time was fitted with a straight line yielding the ordinate intercept S and the slope $-\beta$. The extrapolated values were subtracted from the values obtained during the initial phase, and these data were fitted with a straight line yielding the intercept R and the slope $-\alpha$. The disposition of u-PA–related antigen from plasma was represented by a two-compartment mammalian model composed of one central and one peripheral compartment with elimination occurring from the central compartment.

Pharmacokinetic parameters were calculated from these coefficients and exponents with standard formulas derived by Gibaldi and Perrier. The variables A and B were first calculated, assuming steady state at the end of the infusion, using the formulas $A = R X_0 / k_0$ and $B = S X_0 / k_0$, where $X_0$ is total administered dose, and $k_0$ is the rate of infusion. From these constants, the following drug disposition parameters were derived: 1) volume of the central compartment ($V_c$) = $X_0 / (A + B)$, 2) extrapolated area under the curve (AUC) = $A / \alpha + B / \beta$, and 3) plasma clearance ($Cl_p$) = $X_0 / AUC$. The clearance (ml/min) during the steady-state phase accompanying continuous intravenous infusion was also calculated from the ratio between the infusion rate ($\mu$g/min) and the plasma concentration ($\mu$g/ml).

Statistical Methods

Results are expressed as mean ± SEM. Statistical analysis of the data for comparison of different groups was performed by Student’s t test for paired or unpaired samples as appropriate. Curves of percent lysis versus dose (mg/kg u-PA equivalents) were fit by antilogit functions as described previously.

Results

Lysis of $[^{125}\text{I}]$Fibrin-Labeled Plasma Clots Immersed in Plasma

rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8 caused a time- and concentration-dependent lysis of a $[^{125}\text{I}]$fibrin-labeled autologous plasma clot immersed in citrated baboon or human plasma (not shown). Figure 1, panel IA, presents dose-response curves of plasma clot lysis using a baboon clot in the baboon system, as percent lysis after 2 hours plotted against the concentration of plasminogen activator: 50% clot lysis in 2 hours was obtained with a concentration (expressed in u-PA equivalents) of 4.3 rscu-PA, 1 rscu-PA/MA-15C5, and 15 $\mu$g/ml rscu-PA/MA-1C8. Figure 1, panel IB, presents residual fibrinogen levels at 2 hours plotted against the concentration of plasminogen activator in the baboon system. At concentrations yielding 50% clot lysis in 2 hours, residual fibrinogen levels were 85% for rscu-PA, 85% for rscu-PA/MA-15C5, and less than 25% for rscu-PA/MA-1C8. Corresponding values obtained in parallel experiments with a human clot in pooled human plasma were 0.6, 0.2, and 1.1 $\mu$g/ml for rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8, respectively (Figure 1, panel IIA), with residual fibrinogen levels of 85, 50, and less than 25% (Figure 1, panel IIB).
**Thrombolytic Properties of rscu-PA and Its Conjugates With MA-15C5 and MA-1C8 in Baboons With 125I-Fibrin-Labeled Femoral Vein Blood Clots**

Figure 2 summarizes the results of continuous measurements of clot lysis by external gamma counting over the thrombosed femoral vein segment. In animals infused with saline, the decrease in radioisotope content over the entire observation period was less than 10%. Infusion of rscu-PA at a dose of 0.5 mg/kg rscu-PA caused a progressive disappearance of radioisotope amounting to 25±7% during the 150-minute experimental period (Figure 2A). With 1.0 mg/kg rscu-PA, lysis was initially more rapid but then leveled, reaching a final value of 52±16%. With 2.0 mg/kg rscu-PA, lysis was progressive and reached a final value of 80±6%. From these results, a dose for 50% lysis of 0.9±0.13 mg/kg rscu-PA was calculated. With rscu-PA/MA-15C5 at a dose of 0.125 mg/kg u-PA, lysis did not exceed the background value obtained with solvent (Figure 2B). With 0.25 mg/kg u-PA, significant initial lysis occurred, leveling at a value of 19±6%. With 0.5 mg/kg u-PA, however, progressive lysis reached 82±6% during the entire observation period. Fifty percent lysis was obtained with 0.35±0.02 mg u-PA/kg rscu-PA/MA-15C5, which is a dose that is 2.7±0.5 times lower than that required for rscu-PA. In two baboons, rscu-PA/MA-1C8 was administered at a dose of 0.5 mg/kg u-PA, but this did not produce lysis greater than the background value (Figure 2B).

The results of thrombolysis and fibrin specificity of rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8 are summarized in Table 1. Determination of clot lysis by ex vivo isotope recovery, which was measured 30 minutes after the end of the infusion, gave the following results. Infusion of solvent into three control animals was associated with 8±2% lysis. rscu-PA/MA-15C5 at a dose of 0.25 mg/kg u-PA induced 33±8% lysis in three animals, and at a dose of 0.5 mg/kg u-PA, it induced 91±3% lysis in two animals. The results correspond to a dose of 0.3±0.02 mg/kg u-PA for 50% lysis. Fifty percent lysis required a dose of rscu-PA approximately 3.0±0.5 times higher (0.9±0.13 mg/kg u-PA for 50% lysis). rscu-PA/MA-1C8 at a dose of 0.50 mg/kg u-PA induced only 25±2% lysis. Figure 3 illustrates the dose-response curves of percent femoral vein clot lysis, determined by ex vivo isotope recovery (panel A) and by external gamma counting (panel B), plotted against dose, expressed in milligrams of u-PA equivalent per kilogram of body weight.

All single-chain u-PA moieties produced thrombolysis in the absence of extensive systemic fibrinolytic activation as evidenced by normal or only slightly decreased fibrinogen and α2-antiplasmin levels (Table 1).

**Pharmacokinetics**

Steady-state plasma concentrations of u-PA–related antigen obtained during infusion of the conjugates increased proportionally according to the infusion rate.
and ranged from 430±100 to 1,900±300 ng/ml for doses of rscu-PA/MA-15C5 between 0.125 and 0.5 mg/kg (Table 1). For unconjugated rscu-PA, steady-state plasma concentrations were 110±14, 260±23, and 180±10 ng/ml for doses of rscu-PA of 0.5, 1.0, and 2.0 mg/kg, respectively. From these steady-state plasma levels, plasma clearances of 17–30 ml/min were determined for rscu-PA/MA-15C5 and of 370–790 ml/min for unconjugated rscu-PA. Steady-state plasma levels of u-PA–related antigen after infusion of 0.5 mg/kg of u-PA, urokinase-type plasminogen activator; Clp, plasma clearance; rscu-PA, recombinant single-chain u-PA.

Expressed in u-PA equivalent amounts.
*Expressed at the end of the experiment.
†Measured toward the end of the infusion.
§Measured at the end of the experiment.
FIGURE 3. Dose-response curves of femoral vein clot lysis. Lysis at 150 minutes (percent) is plotted against the dose (mg u-PA equivalent/kg body wt). Curves are derived from mean±SEM of the data reported in Table 1. Panel A: Lysis measured by ex vivo isotope recovery. Panel B: Lysis measured by external radioisotope counting. ○, rscu-PA; ■, rscu-PA/MA-15C5; ●, rscu-PA/MA-1C8. u-PA, urokinase-type plasminogen activator.

rscu-PA/MA-1C8 were 2,100±270 ng/ml, corresponding to a plasma clearance of 24±2 ml/min.

The disappearance rate of rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8 from plasma after the end of the infusion, as determined by quantification of u-PA-related antigen, was biphasic (Figure 4). The experimental data were fitted with a sum of two exponential (exp) terms: C(t)=R exp (-αt)+S exp (-βt) by graphical curve peeling as detailed in the “Methods,” yielding the variables summarized in Table 2.

The pharmacokinetic parameters of the disposition of rscu-PA, rscu-PA/MA-15C5, and rscu-PA/MA-1C8 from plasma, derived from these values, are summarized in Table 3. Results were 1) rscu-PA: volume of the central compartment (Vc)=1.7±0.17 l; initial half-life (t½α)=2.7±0.5 minutes; and plasma clearance (Clp)=340±40 ml/min; 2) rscu-PA/MA-15C5: Vc=1.6±0.23 l; t½α=24±1.2 minutes; and Clp=20±3 ml/min; 3) rscu-PA/MA-1C8: Vc=2.0±0.37 l; t½α=21±0.5 minutes; and Clp=24±2 ml/min.

Discussion

scu-PA is a relatively fibrin-specific thrombolytic agent both in vitro and in vivo.1 Its fibrin specificity is, however, not absolute, and treatment of patients with acute myocardial infarction with scu-PA has been associated with extensive systemic activation of the fibrinolytic system and fibrinogen breakdown.6,7 Several approaches to improve the thrombolytic potency and fibrin specificity of scu-PA have been evaluated. One approach consisted of the construction of recombinant chimeric molecules containing the protease part of scu-PA and the fibrin-binding structures

FIGURE 4. Plot of disappearance rate of u-PA–related antigen from plasma after the end of the infusion of rscu-PA, rscu-PA/MA-15C5, or rscu-PA/MA-1C8. Data are mean values, and the vertical bars are SEM, expressed as a fraction of the first sample, to allow pooling of data obtained with different doses. ○, rscu-PA; ●, rscu-PA/MA-15C5; △, rscu-PA/MA-1C8. rscu-PA, recombinant single-chain urokinase-type plasminogen activator.
of t-PA, but such chimeras did not display a significantly increased fibrinolytic potency in a rabbit jugular vein thrombosis model. However, conjugation of scu-PA to monoclonal antibodies directed against the NH₂-terminus of the β-chain of fibrin and construction of bifunctional antibody complexes containing a fibrin-binding site and a u-PA-binding site have resulted in increased fibrinolytic potency in vitro.

Alternatively, chemical conjugates of scu-PA with a monoclonal antibody directed against fibrin fragment D-dimer have been shown not only to have a sixfold higher fibrinolytic potency than unconjugated scu-PA in vitro but also an eightfold higher thrombolytic potency associated with a fourfold reduced clearance in an in vivo model consisting of rabbits with a fibrin-labeled human plasma clot introduced in the jugular vein. Consequently, provided that the in vivo thrombolytic potency of the scu-PA/MA-15C5 in the rabbits was not an artifact due to species specificity of the monoclonal antibody in the heterologous plasma clot model, such conjugates may represent potentially useful thrombolytic agents.

To investigate the extent and the mechanism of antibody targeting of scu-PA to fibrin by MA-15C5 in an autologous thrombosis model, we performed the present study in baboons. Complexes of scu-PA with the fibrin-specific antibody MA-15C5 or of scu-PA with a control antibody MA-1C8 were prepared with yields and characteristics comparable to those previously described. Initial experiments in an in vitro plasma clot lysis system indicated that although the baboon system was less sensitive to scu-PA than the human system (50% clot lysis in 2 hours required 4.3 µg/ml and 0.6 µg/ml of scu-PA, respectively), the enhancement of the thrombolytic potency by conjugation of scu-PA to MA-15C5 was proportional to the fibrinolytic activity of scu-PA/MA-15C5 in the baboon system (0.2 µg/ml in the human system).

The fibrin specificity of scu-PA and scu-PA/MA-15C5 were comparable in the baboon system. At the equieffective dose causing 50% clot lysis, residual fibrinogen was approximately 85% in both systems. Furthermore, the dose-response values obtained with plasma of five different animals were similar. The results obtained with scu-PA/MA-1C8 revealed both a lower thrombolytic potency (50% lysis in 2 hours in the baboon system required 15 µg/kg scu-PA/MA-1C8) and a reduced fibrin specificity (residual fibrinogen level of less than 25% at the dose causing 50% clot lysis).

To evaluate the thrombolytic potency of scu-PA and its conjugate with the fibrin-specific antibody in vivo, a femoral vein thrombosis model was developed by modification of a previously described venous thrombosis model in the dog. A venous thrombus was produced by formation of a radioactive blood clot in a superficial femoral vein, allowing continuous monitoring of the thrombolytic process by external isotope scanning and quantification of the degree of thrombolysis both by external scanning and by recovery of the residual clot ex vivo. The woolen thread introduced in the lumen to anchor the labeled clot, efficiently prevented embolization as evidenced by the gradual decrease of radioactivity in the femoral vein segment.

### Table 2. Variables Describing the Disposition of u-PA-Related Antigen from Plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Infusion rate (µg/kg/min)</th>
<th>R (µg/ml)</th>
<th>α (min⁻¹)</th>
<th>S (µg/ml)</th>
<th>β (min⁻¹)</th>
<th>A (µg/ml)</th>
<th>B (µg/ml)</th>
<th>Cmin* (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>scu-PA</td>
<td>0.50</td>
<td>2</td>
<td>4.2</td>
<td>0.11±0.01</td>
<td>0.31±0.10</td>
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<td>1.0</td>
<td>3</td>
<td>8.4</td>
<td>0.18±0.02</td>
<td>0.24±0.02</td>
<td>0.081±0.03</td>
<td>0.043±0.008</td>
<td>5.0±0.2</td>
<td>0.37±0.12</td>
</tr>
<tr>
<td>scu-PA/MA-15C5</td>
<td>0.33†</td>
<td>3</td>
<td>2.8</td>
<td>0.61±0.10</td>
<td>0.029±0.001</td>
<td>0.94±0.12</td>
<td>0.004±0.0003</td>
<td>1.7±0.2</td>
<td>0.48±0.12</td>
</tr>
<tr>
<td>scu-PA/MA-1C8</td>
<td>0.50</td>
<td>2</td>
<td>4.2</td>
<td>0.48±0.05</td>
<td>0.034±0.001</td>
<td>1.63±0.01</td>
<td>0.006±0.001</td>
<td>1.9±0.2</td>
<td>1.2±0.19</td>
</tr>
</tbody>
</table>

Data are mean±SEM; n, number of experiments.

### Table 3. Pharmacokinetic Parameters of the Disposition From Plasma

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Half-time (α) (min)</th>
<th>Half-time (β) (min)</th>
<th>Value of central compartment (l)</th>
<th>Area under the curve (µg/min/ml)</th>
<th>Plasma clearance (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>scu-PA</td>
<td>0.50</td>
<td>2</td>
<td>2.5±0.8</td>
<td>14±0.5</td>
<td>1.4±0.17</td>
<td>17±3.2</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3</td>
<td>2.9±0.2</td>
<td>17±2.3</td>
<td>1.9±0.17</td>
<td>31±5.7</td>
</tr>
<tr>
<td>scu-PA/MA-15C5</td>
<td>0.33†</td>
<td>3</td>
<td>24±1.2</td>
<td>170±17</td>
<td>1.6±0.23</td>
<td>174±24</td>
</tr>
<tr>
<td>scu-PA/MA-1C8</td>
<td>0.50</td>
<td>2</td>
<td>21±0.5</td>
<td>110±18</td>
<td>2.0±0.37</td>
<td>252±7</td>
</tr>
</tbody>
</table>

Data are mean±SEM; n, number of experiments.

Half-time (α)=ln2/α; half-time (β)=ln2/β.

*Mean values of two experiments with 0.25 mg/kg and one experiment with 0.50 mg/kg.
recovery, with corresponding values of 0.94±0.17 and 0.35±0.02 mg/kg, respectively, as determined by external radioisotope counting. Thus, the thrombolytic potency of rscu-PA was increased 3.0±0.5 and 2.7±0.5 times by chemical conjugation to MA-15C5. A dose of 0.5 mg/kg u-PA conjugated to the control antibody MA-1C8 was practically inactive.

Pharmacokinetic analysis of the disposition of u-PA-related antigen from plasma after the end of the infusion of rscu-PA, rscu-PA/MA-15C5, or rscu-PA/MA-1C8 revealed that both conjugates have a plasma clearance that is reduced approximately 15-fold compared with unconjugated rscu-PA. Considered together, these data indicate that conjugation of rscu-PA to the fibrin-specific antibody MA-15C5 increases its thrombolytic potency much less than it reduces its clearance. Consequently, the specific thrombolytic activity (potency at comparable steady-state plasma levels) of rscu-PA/MA-15C5 is lower than that of unconjugated rscu-PA. Indeed, when the results of clot lysis in Table 1 are plotted against the steady-state u-PA concentration, 50% clot lysis is obtained at a plasma level of 0.15–0.2 µg/ml rscu-PA, whereas the equipotent level of rscu-PA/MA-15C5 is 1.5–1.6 µg/ml. Furthermore, the results obtained with rscu-PA/MA-1C8 in vitro also indicate that coupling of rscu-PA to a control antibody reduces its fibrinolytic potency and fibrin specificity. However, the presence of the fibrin-specific antibody combining site markedly enhances the fibrinolytic potency and fibrin specificity as revealed by comparison of the results obtained with rscu-PA/MA-15C5 and rscu-PA/MA-1C8. Similar results have been observed in in vitro systems using a chemical conjugate between a low molecular weight form of rscu-PA (comprising amino acids Leu14 through Leu411 of native scu-PA) and F(ab')2 fragments of MA-15C5.31 In the in vitro plasma clot lysis assay, 50% lysis in 2 hours was observed with 6.4 µg/ml conjugate and with 1.2 µg/ml low molecular weight rscu-PA.

In conclusion, targeting of rscu-PA to fibrin with the fibrin fragment D-dimer specific antibody increases the thrombolytic potency approximately threefold and reduces its clearance approximately 15-fold. This effect is the result of, on the one hand, a marked reduction of the potency due to coupling of rscu-PA to the antibody molecule and, on the other hand, an enhancement of the thrombolytic potency due to fibrin targeting by the specific idiotype of the antibody. Antibody targeting of thrombolytic agents with constructs optimizing the effect of the specific thrombolytic activity of rscu-PA might provide a clinically useful approach to the treatment of thromboembolic disease.

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D Collen, M Dewerchin, H J Rapold, H R Lijnen and J M Stassen

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