Effect of Chemical Conjugation of Recombinant Single-Chain Urokinase-Type Plasminogen Activator With Monoclonal Antiplatelet Antibodies on Platelet Aggregation and on Plasma Clot Lysis In Vitro and In Vivo

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The murine monoclonal antiplatelet antibodies MA-TSPI-1 (directed against human thrombospondin) and MA-PMI-2, MA-PMI-1, and MA-LIBS-1 (directed against ligand-induced binding sites [LIBS] on human platelet glycoprotein lib/IIIa) were conjugated with recombinant single-chain urokinase-type plasminogen activator (rscu-PA) using the cross-linking reagent N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). The conjugates (rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, and rscu-PA/MA-LIBS-1), purified by immunoadsorption and gel filtration, were obtained with recoveries of 34% to 45%, with an average stoichiometry of 1.6 to 1.8 IgG molecules per rscu-PA molecule, and with unaltered specific activities and affinities. Preincubation of human platelet-rich plasma with rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, or unconjugated rscu-PA resulted in partial inhibition of ADP-induced aggregation; 25% inhibition was obtained with 63 μg/mL rscu-PA and with 6 μg u-PA/mL rscu-PA/MA-PMI-2 or 1.2 μg u-PA/mL rscu-PA/MA-PMI-1. In an in vitro system composed of a 125I-fibrin-labeled platelet-rich human plasma clot immersed in normal human plasma, the conjugates had threefold to greater than 15-fold less fibrinolytic potency than unconjugated rscu-PA. The thrombolytic potency of rscu-PA/MA-PMI-1 and rscu-PA/MA-LIBS-1 was compared with that of rscu-PA and that of a control conjugate rscu-PA/MA-1CB in a pulmonary embolism model in the hamster, using clots prepared from platelet-poor or platelet-rich human plasma. Lysis was measured 30 minutes after the end of a 60-minute intravenous infusion of the thrombolytic agents. rscu-PA, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, as well as rscu-PA/MA-1CB had comparable thrombolytic potencies (percent lysis per dose administered) towards platelet-poor human plasma clots. In contrast, the thrombolytic potency of rscu-PA/MA-PMI-1 and of rscu-PA/MA-LIBS-1 towards platelet-rich clots was 2.3- to 3-fold higher than that of rscu-PA (P < .005) and fivefold to sevenfold higher than that of the control conjugate (P < .01).

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Materials and Methods

Proteins and Reagents

rscu-PA, prepared by expression of cDNA encoding scu-PA in Escherichia coli, was a gift from Grünenthal AG (Aachen, Germany). Recombinant human t-PA (rt-PA) was Activase, kindly supplied by Genentech Inc (South San Francisco, CA). The murine MoAbs MA-TSPI-1, MA-PMI-1, MA-PMI-2, MA-LIBS-1, and MA-1C8 were prepared and characterized as previously described.6,10 Human plasminogen, plasmin, fibrinogen, and cyanogen bromide (CNBr)-digested fibrinogen were obtained and characterized as previously described.6,13 Bovine thrombin was Topostasin (Hoffmann-La Roche, Basel, Switzerland). rscu-PA, the antibodies MA-TSPI-1, MA-PMI-2, MA-PMI-1, MA-LIBS-1, and MA-1C8, and the conjugates of the antibodies with rscu-PA were radiolabeled using the iodoogen method.14 125I-labeled fibrin-
ogen was purchased from Amersham Research Products (Amersham, UK). Horse-radish peroxidase-labeled goat antihorse IgG or goat antirabbit IgG was obtained from BioRad Laboratories (Richmond, CA). The chromogenic substrates pyroglytanyl-glutamyl-arginine-p-nitroanilide (S-2444) for two-chain u-PA (tcu-PA, urokinase) and D-valyl-leucyl-lysine-p-nitroanilide (S-2251) for plasmin were purchased from KabiVitrum (Brussels, Belgium) and aprotilin (Trasylol) from Bayer (Leverkusen, Germany). The synthetic inhibitor glutamyl-glycinyl-arginyl-chloromethyl ketone (Glu-Gly-Arg-CH,Cl) for tcu-PA was from Union Chimique Belge (Brussels, Belgium) and the thrombin inhibitor D-phenylalanyl-L-proplyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem Corporation (La Jolla, CA). The International Reference Preparation for Urokinase (66146) was obtained from the National Institute for Biological Standards and Control (London, UK). Sephacryl S-200 superfine was obtained from Pharmacia (Uppsala, Sweden). N-succinimidyl 3-(2-pyridyl)dithio)propionate (SPDP) was from Pharmacia; a 50 mmol/L stock solution in dioxane was stored frozen and diluted before use in 0.01 mol/L sodium acetate buffer, pH 4.5, containing 0.1 mol/L NaCl. Platelet-rich human plasma was prepared from fresh blood, collected in 0.1 mol/L citrate (final concentration) unless otherwise indicated, from volunteers who had not taken aspirin for at least 1 week. Normal human plasma was pooled fresh frozen blood bank plasma, obtained from blood collected in acid-citrate-dextrose, from at least five healthy blood donors.

Preparation of the Conjugates

rscu-PA and the antibodies MA-TSPI-1, MA-PMI-2, MA-PMI-1, or MA-LIBS-1 were conjugated with the heterobifunctional cross-linking reagent SPDP using a three-step procedure as described previously, but with minor modifications: (1) introduction of 2-pyridyl disulphide groups in rscu-PA by treating the protein at a concentration of 1 mg/mL (containing approximately 10^10 cpm of ^14C-labeled rscu-PA/mL), with a fourfold molar excess of SPDP (degree of substitution: 1 to 3 pyridyl-disulphide [PDF] groups per molecule rscu-PA); (2) introduction of thiol groups in the antibodies with a 10-fold molar excess of SPDP (degree of substitution: 3 to 5 PDF groups per IgG molecule), followed by specific reduction of the protein-bound PDF disulfide bonds (degree of substitution: 2.9 to 5.5 thiol groups per IgG molecule); and (3) conjugation by overnight incubation of thiolated antibody with the PDF derivative of rscu-PA in a 1:1 molar ratio. Remaining reactive thiol-groups were then alkylated by addition of 1/100 (vol/vol) of 0.1 mol/L sodium iodoacetate. Alternatively, rscu-PA was conjugated to the control antibody MA-1C8 using the same procedure.

Purification of the Conjugates

Each of the reaction mixtures was applied to a 5-mL Sepharose 4B column, coupled to the u-PA-specific MoAb MA-4D1E82 (2 mg antibody/mL Sepharose) in 0.1 mol/L sodium phosphate, 0.1 mol/L NaCl buffer, pH 7.5, containing 10 kallikrein inhibitor units/mL aprotilin (buffer A) at 4°C. About 2 mg total protein was applied per milliliter of gel. Bound rscu-PA (both conjugated and unconjugated) was eluted with 1.6 mol/L KSCN in buffer A and dialyzed extensively against buffer A. The partially purified conjugates were then gel-filtered at 4°C on Sephacryl S-200 superfine in 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 0.1 mol/L NaCl (5 to 15 mg total protein was applied in a volume of 2 mL to a 1.4 x 75 cm column). The pooled purified conjugates were concentrated by centrifugation on Centricon 30 microconcentrators (Amicon, Danvers, MA).

Analytical Techniques

rscu-PA concentration in the conjugate was determined from the specific radioactivity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 7.5% gels without reduction, and on 10% to 15% gradient gels after reduction with diithioerythritol, using the Phast System (Pharmacia). Immunoblotting on nitrocellulose sheets was performed according to Towbin et al. Specific fibrinolytic activities of u-PA moieties were determined on fibrin plates by comparison with the International Reference Preparation for Urokinase. The rscu-PA/antiplalet antibody conjugates were tested after inhibition of trace amounts of two-chain activity with Glu-Gly-Arg-CH,Cl and removal of excess inhibitor by washing on Centricon 30 microconcentrators.

Functional Characterization of the Conjugates

Treatment with plasmin. rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA (final concentration 2 μmol/L u-PA equivalent) were treated at 37°C with plasmin (0% to 5% molar ratio) in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80. At timed intervals (0 to 20 minutes) the urokinase-like amidolytic activity was measured with the chromogenic substrate S-2444 (final concentration, 0.3 mmol/L) after 160-fold dilution of the samples. Urokinase activity was expressed in IU by comparison with the International Reference Preparation for Urokinase.

Activation of plasminogen. Activation of plasminogen (final concentration, 0.25 to 1.5 μmol/L) was measured with rscu-PA, rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, or rscu-PA (final concentration, 5 to 20 μmol/L) at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, 0.01% Tween 80, and 1 mmol/L S-2251. Generation of plasmin was monitored continuously for 3 to 4 minutes by measuring the absorbance at 405 nm. Before use, traces of two-chain derivatives in the conjugates were inhibited by addition of the urokinase inhibitor Glu-Gly-Arg-CH,Cl as described above.

The effect of platelets on the rate of plasminogen activation was determined by incubating plasminogen (final concentration, 1.5 μmol/L) in the presence or absence of freshly prepared, washed human platelets (final platelet count 3 x 10^4/μL) with rscu-PA, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 (final u-PA concentration, 5 mmol/L) at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, 0.01% Tween 80, and 0.1 U/mL thrombin. At different time intervals (0 to 10 minutes) samples were removed, centrifuged for 30 seconds, and the amount of plasmin generated was measured with the substrate S-2251 after 20-fold dilution of the samples. Initial activation rates were obtained from plots of the concentration of generated plasmin versus time. Activation of plasminogen (final concentration, 1 μmol/L) was measured in the same way in the presence or absence of platelets with rt-PA (final concentration, 10 mmol/L).

The effect of fibrin on the rate of plasminogen activation was determined by incubating plasminogen (final concentration, 1.5 μmol/L) in the presence or absence of CNBr-digested fibrinogen (final concentration, 1 μmol/L) with rscu-PA, rscu-PA/MA-PMI-2, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 (final u-PA concentration, 1 mmol/L) at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80. CNBr-digested fibrinogen has been shown to be a valid alternative for solid phase fibrin in kinetic experiments. At different time intervals (0 to 15 minutes) samples were removed for quantification of the amount of plasmogen generated. Therefore, the sample was diluted eightfold in 0.05 mol/L Tris-HCl buffer, pH 7.4,
containing 0.038 mol/L NaCl, 0.01% Tween 80, and 1 mmol/L S-2251. After incubation for 3 minutes at 37°C the hydrolysis of the substrate was arrested by addition of acetic acid to a final concentration of 15% and the absorbance was measured at 405 nm. Absorbance at 405 nm was converted to plasmin concentration using a calibration curve constructed with purified plasmin. Initial activation rates were obtained as described above. Activation of plasminogen (final concentration, 1 μmol/L) was measured in the same way in the presence or absence of CNBr-digested fibrinogen with rscu-PA (final concentration, 25 nmoVL in the absence or 0.25 mmol/L in the presence of 1 μmol/L CNBr-digested fibrinogen).

**Binding of the conjugates to their antigen.** Binding experiments with MA-PMI-1, MA-PMI-2, MA-LIBS-1, or their conjugates with rscu-PA were performed in microtiter plates coated with thrombin-stimulated, formaldehyde-fixed human platelets. The assay plates were prepared by addition of 100-μL aliquots of a modified Tyrode’s buffer (134 mmol/L NaCl, 12 mmol/L NaHCO₃, 2.9 mmol/L KCl, 0.34 mmol/L Na₂HPO₄, 5 mmol/L HEPES, pH 7.4) containing 5 × 10⁸ washed platelets to the wells of a 96-well microtiter plate precoated with poly-L-lysine (1 μg per well). The plates were centrifuged at 1,000 rpm for 15 minutes at 10°C, and the platelets were activated by addition of 5 μL per well of an α-thrombin solution of 100 U/mL. After incubation for 15 minutes at room temperature, the platelets were fixed by addition of formaldehyde (50 μL of a 0.5% solution per well) and further incubated for 15 minutes at room temperature. The plates were then washed with phosphate-buffered saline (PBS) containing 0.002% Tween 80, and saturated with 200 μL of 3% gelatin per well. After incubation for 1 hour at room temperature and 30 minutes at 37°C, the plates were washed and used in the binding assay.

For binding experiments with the anti-thrombospondin antibody MA-TSPI-1 or its conjugate with rscu-PA, platelet releasate was used instead of whole platelets. Therefore, washed human platelets, suspended in PBS at a platelet count of 5 × 10⁹/μL, were stimulated with thrombin (final concentration, 5 U/mL) and after incubation for 10 minutes at room temperature, the suspension was centrifuged for 10 minutes at 3,000 rpm. The platelet-free supernatant was diluted 25-fold with PBS, and 100-μL aliquots of the diluted releasate were added to the wells of a 96-well microtiter plate. After overnight incubation at 4°C, the plate was washed four times with 200 μL/well of 0.01 mol/L sodium phosphate buffer, pH 7.3, containing 0.02% azide, 0.05% Tween 20, and 0.1% bovine serum albumin (BSA), and saturated with 3% gelatin as described above.

The binding of the antibody moieties to their antigen was evaluated in competition experiments. Therefore, ¹²⁵I-labeled unconjugated MoAb (12.5 ng) was mixed in PBS containing 0.002% Tween 80 and 0.1% BSA in a total volume of 25 μL, with either the corresponding unlabeled antibody (0 to 3.75 μg) or with the corresponding rscu-PA conjugate (0 to 3.75 μg antibody equivalent). The mixtures were then incubated for 1 hour at room temperature in wells of microtiter plates coated with thrombin-activated platelets as described above (for experiments with MA-PMI-2, MA-PMI-1, or MA-LIBS-1) or with platelet releasate (for experiments with MA-TSPI-1). After extensive washing, bound MoAb was quantitated by measurement of the radioactivity associated with the wells.

**Binding of the conjugates to human platelets.** Binding to platelets was evaluated with the antibodies MA-PMI-1, MA-PMI-2, MA-LIBS-1, and MA-1C8 and with the conjugates rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, and rscu-PA/MA-1C8. Plasma clots were produced by mixing either platelet-poor human plasma (fresh-frozen citrated blood bank plasma) or freshly prepared platelet-rich human plasma (diluted with platelet-poor plasma to a platelet count of 3 × 10⁹/mL) with CaCl₂ (final concentration, 38 mmol/L) and thrombin (final concentration, 2 U/mL). The solution was immediately drawn into Silastic tubing (inner diameter, 4 mm) and incubated for 1 hour at 37°C. The silastic tubing containing clotted platelet-poor or platelet-rich plasma was then cut into 0.5-cm sections, yielding clots of about 0.1 mL. After removal from the tubing sections, the clots were washed with 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80 and each clot was suspended in 0.5 mL citrated normal human plasma containing 10⁻⁴ mol/L urokinase inhibitor Glu-Gly-Arg-CH₂Cl, 10⁻³ mol/L thrombin inhibitor PPACK, and aprotinin at 20 kallikrein inhibitor units/mL. The assay was initiated by addition of 300 ng ¹²⁵I-labeled unconjugated antibody (200,000 to 500,000 cpm) or 300 ng antibody equivalent of ¹²⁵I-labeled conjugate (30,000 to 150,000 cpm). Following 2 hours of incubation at 37°C, the clots were washed extensively with 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, 0.01% Tween 80, and aprotinin at 20 kallikrein inhibitor units/mL, and counted for radioactivity.

**Platelet aggregation assay.** Platelet-rich plasma (125 μL, platelet count 3 to 6 × 10⁹/μL) was preincubated for 30 minutes at room temperature with rscu-PA (final concentration, 1 to 300 μg/mL) with the antibodies MA-TSPI-1, MA-PMI-2, MA-PMI-1, or MA-LIBS-1 (final concentration, 3 to 300 μg/mL), or with their conjugates with rscu-PA (final concentration, 0.1 to 300 μg u-PA equivalent/mL), in a final volume of 250 μL. As a control, platelet-rich plasma was preincubated with 0.1 mol/L sodium phosphate buffer, pH 7.5, containing 0.1 mol/L NaCl. ADP was then added at the minimal concentration required for full aggregation, and platelet aggregation was monitored for about 4 minutes at 37°C by measurement of the light transmission using an Elvi 840 dual channel aggregometer (Elvi, Milan, Italy). The extent of aggregation was determined as the change in light transmission measured 3 minutes after addition of ADP, expressed in percent of the difference between platelet-rich and platelet-poor plasma, and calculated as percent of the control value (preincubation with buffer).

**Lysis of ¹²⁵I-fibrin labeled human plasma clots in a plasma milieu in vitro.** ¹²⁵I-fibrin–labeled platelet-rich plasma clots were prepared
Physicochemical Characterization of the Conjugates

SDS-PAGE of the conjugates under nonreducing conditions (Fig 1A) showed a similar migration pattern for each of the conjugates, with a main band with a mobility between that of ferritin and of the unconjugated antibodies (lanes 2, 4, 6, and 8). SDS-PAGE under reducing conditions (Fig 1B) showed the dissociation of the conjugates into their constituents, the heavy and light chains of the antibody with Mr, approximately 50,000 and 25,000, and rscu-PA, migrating below the heavy chain of the antibody. Immunoblotting of nonreduced SDS-PAGE with horseradish peroxidase-labeled goat antimouse IgG (Fig 2A) or with anti-u-PA antiserum (Fig 2B) confirmed the presence of both mouse IgG and u-PA material in the conjugates. Trace amounts of unconjugated rscu-PA were observed in all four conjugates. The stoichiometry of IgG and rscu-PA in the conjugates, as determined from the ratio of the rscu-PA content (measured as 125I=rcsu-PA) and the IgG content (determined as the difference between total protein, calculated from the absorbance at 280 nm, and rscu-PA protein), was 1.8 ± 0.6, 1.6 ± 0.3, 1.6 ± 0.25, and 1.6 ± 0.1 (mean ± SD, n = 3) IgG molecules/rscu-PA molecule for rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, and rscu-PA/MA-LIBS-1, respectively.

Functional Characterization of the Conjugates

Treatment of the conjugates with plasmin. Plasmin caused a time- and concentration-dependent conversion of rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1 (Fig 3), and rscu-PA (not shown) to amido-lytically active two-chain derivatives with an increase in specific activity from less than 2,500 IU/mg to 83,000 ± 15,000 IU/mg u-PA equivalent for rscu-PA/MA-TSPI-1, to 70,000 ± 9,200 IU/mg u-PA for rscu-PA/MA-PMI-2, to 100,000 ± 5,500 IU/mg u-PA for rscu-PA/MA-PMI-1, and to 120,000 ± 6,400 IU/mg u-PA for rscu-PA/MA-LIBS-1, as compared with 100,000 ± 9,800 IU/mg for rscu-PA (mean ± SEM, n = 3) and 94,000 IU/mg for rscu-PA/MA-1C8.8 Maximal activation with plasmin coincided with quantitative conversion of the scu-PA moieties to two-chain

| Table 1. Recoveries of rscu-PA/Antiplatelet Antibody Conjugates During Purification |
|-------------------------------|------------------|------------------|------------------|
| Conjugate Purification Step   | rscu-PA (%)      | Antibody (%)     | Total Protein (%)|
| Immunoabsorption              | 65 ± 1           | 61 ± 10          | 62 ± 8           |
| Gel filtration                | 23 ± 5           | 38 ± 8           | 34 ± 6           |
| Immunoabsorption              | 67 ± 3           | 62 ± 8           | 63 ± 5           |
| Gel filtration                | 30 ± 2           | 46 ± 10          | 42 ± 7           |
| Immunoabsorption              | 63 ± 5           | 62 ± 17          | 62 ± 12          |
| Gel filtration                | 27 ± 5           | 45 ± 15          | 41 ± 12          |
| Immunoabsorption              | 60 ± 4           | 68 ± 6           | 66 ± 5           |
| Gel filtration                | 31 ± 3           | 50 ± 2           | 46 ± 3           |

The data represent mean ± SD of percent recovery obtained with three preparations of each conjugate.
Kinetics of plasminogen activation. Activation of plasminogen by the conjugates obeyed Michaelis-Menten kinetics as evidenced by linear double reciprocal plots of the activation rate versus the plasminogen concentration (not shown). Linear regression analysis of results obtained with three different preparations of each conjugate yielded $K_m$ and $k_2$ values comparable with those of rscu-PA: $K_m = 0.63 \pm 0.17 \mu$mol/L and $k_2 = 0.0003 \pm 0.0002 \text{s}^{-1}$ for rscu-PA/MA-TSPI-1; $K_m = 0.64 \pm 0.2 \mu$mol/L and $k_2 = 0.002 \pm 0.001 \text{s}^{-1}$ for rscu-PA/MA-PMI-2; $K_m = 1.0 \pm 0.3 \mu$mol/L and $k_2 = 0.002 \pm 0.0003 \text{s}^{-1}$ for rscu-PA/MA-PMI-1; and $K_m = 1.3 \pm 0.4 \mu$mol/L and $k_2 = 0.0013 \pm 0.001 \text{s}^{-1}$ for rscu-PA/MA-LIBS-1 (mean $\pm$ SD), as compared with $K_m = 0.54 \mu$mol/L and $k_2 = 0.0065 \text{s}^{-1}$ for rscu-PA/MA-1C8 and $K_m = 0.74 \mu$mol/L and $k_2 = 0.002 \text{s}^{-1}$ for rscu-PA.$^a$

Plasminogen activation by rscu-PA, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 was not altered in the presence of human platelets; initial activation rates (expressed in nmol/L of plasmin generated per minute) in the absence or presence of platelets were 27 and 28, respectively, with rscu-PA, 29 and 27 with rscu-PA/MA-PMI-1, 26 and 28 with rscu-PA/MA-LIBS-1, and 21 and 22, respectively, with rscu-PA/MA-1C8. With rt-PA in the same system, a 4.7-fold enhancement of the initial activation rate was found in the presence of human platelets (6.6 nmol/L plasmin generated per minute in the presence of 1.4 nmol/L per minute in the absence of platelets).

Similarly, plasminogen activation with rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 was not altered in the presence of CNBr-digested fibrinogen; initial activation rates without or with CNBr-digested fibrinogen were 1.3 and 1.4 nmol/L, respectively, of plasmin generated per minute with rscu-PA/MA-PMI-1, 1.0 and 1.1 nmol/L plasmin per minute with rscu-PA/MA-LIBS-1, and 0.56 and 0.58 nmol/L, respectively, of plasmin generated per minute with rscu-PA/MA-1C8. Plasminogen activation with rscu-PA was threefold higher in the presence of CNBr-digested fibrinogen (0.81 nmol/L per minute in the presence of 0.27 nmol/L per minute in the absence) whereas plasminogen activation with rt-PA was enhanced 66-fold in the presence of CNBr-digested fibrinogen (0.33 nmol/L per minute $\times$ 0.005 nmol/L per minute in the absence of CNBr-digested fibrinogen).

Binding of the conjugates to their antigen. Binding of $^{125}$I-labeled MA-TSPI-1 to insolubilized thrombospondin-containing platelet releasate was inhibited in a concentration-dependent manner by addition of unlabeled MA-TSPI-1 or rscu-PA/MA-TSPI-1 (Fig 4). Fifty percent reduction of the binding was obtained at a fourfold molar excess for MA-TSPI-1 and at an eightfold molar antibody excess for rscu-PA/MA-TSPI-1. No competition was observed with the control MoAb MA-15C5 directed against fragment D-dimer of cross-linked human fibrin$^b$ (Fig 4).

Similarly, binding of $^{125}$I-labeled MA-PMI-2 (125I-MA-PMI-2), 125I-MA-PMI-1, or 125I-MA-LIBS-1 to thrombin-
Fig 2. Immunoblotting of nonreduced SDS-PAGE. (A) Staining with horseradish peroxidase-labeled goat antimouse IgG (1:1,000 dilution in Tris-buffered saline containing 1% gelatin). Lane 1, MA-TSPI-1; lane 2, rscu-PA/MA-TSPI-1; lane 3, MA-PMI-2; lane 4, rscu-PA/MA-PMI-2; lane 5, MA-PMI-1; lane 6, rscu-PA/MA-PMI-1; lane 7, MA-LIBS-1; lane 8, rscu-PA/MA-LIBS-1. (B) Staining with rabbit antisera against u-PA (1:500 dilution) and horseradish peroxidase-labeled goat antirabbit IgG (1:2,000 dilution). Lane 1, rscu-PA/MA-TSPI-1; lane 2, rscu-PA/MA-PMI-2; lane 3, rscu-PA/MA-PMI-1; lane 4, rscu-PA/MA-LIBS-1; lane 5, rscu-PA.

Activated formaldehyde-fixed platelets was reduced in a concentration-dependent manner by addition of the corresponding unlabeled antibody or conjugate (Fig 5). Fifty percent reduction of the binding of 125I-MA-PMI-2 was obtained at a 12-fold molar excess with MA-PMI-2 and at a 45-fold molar excess with rscu-PA/MA-PMI-2 (Fig 5A). Fifty percent reduction of the binding of 125I-MA-PMI-1 was obtained at a 55-fold molar excess with MA-PMI-1 and at a 110-fold molar excess with rscu-PA/MA-PMI-1 (Fig 5B). Fifty percent reduction of the binding of 125I-MA-LIBS-1 was obtained at a sevenfold or at an 11-fold molar excess with MA-LIBS-1 or with rscu-PA/MA-LIBS-1, respectively (Fig 5C).

Binding of the conjugates to human platelets. Binding to unstimulated or ADP-stimulated platelets in plasma was evaluated with the antibodies MA-PMI-1, MA-LIBS-1, and

Fig 3. Treatment of the antiplatelet antibody/ rscu-PA conjugates with plasmin. Generated urokinase-like amidolytic activity is expressed in IU/μg u-PA equivalent. Plasmin was added at a final concentration of 0 nmol/L (•), 20 nmol/L (△), 40 nmol/L (●), or 100 nmol/L (○) to a solution of 2 μmol/L rscu-PA/MA-TSPI-1 (A), rscu-PA/MA-PMI-2 (B), rscu-PA/MA-PMI-1 (C), or rscu-PA/MA-LIBS-1 (D). Data represent mean values of result obtained with three different preparations of each conjugate. Vertical bars represent SEM.
unconjugated or conjugated antibody (containing 30,000 to 400,000 cpm of radiolabeled ligand) at room temperature in a final volume of 150 μL. Results are summarized in Table 2. ADP-stimulated platelets bound threefold to fourfold more molecules of rscu-PA/MA-PMI-1, MA-LIBS-1, or rscu-PA/MA-LIBS-1 than unstimulated platelets (P < .0005). In contrast, binding to stimulated platelets with MA-1C8, rscu-PA/MA-1C8, and, surprisingly, with MA-PMI-1 was not increased over the binding to unstimulated platelets. ADP-stimulated platelets bound a fivefold higher number of MA-LIBS-1 molecules than of the control antibody MA-1C8 (P < .0005), whereas this difference was not observed with unstimulated platelets. Binding to ADP-stimulated platelets was, respectively, 13- and 18-fold higher with rscu-PA/MA-PMI-1 or rscu-PA/MA-LIBS-1 as compared with the control conjugate rscu-PA/MA-1C8 (P < .0005). With unstimulated platelets, binding with rscu-PA/MA-PMI-1 or rscu-PA/MA-LIBS-1 was threefold higher than with rscu-PA/MA-1C8 (P < .05).

Binding of the conjugates to human plasma clots. Binding to platelet-poor or platelet-rich human plasma clots (0.1 mL clots containing, respectively, <3 × 10^6 and 3 × 10^7 platelets) immersed in 0.5 mL citrated normal human plasma was evaluated with the antibodies MA-PMI-1, MA-LIBS-1, and MA-1C8 and with the conjugates of rscu-PA with these antibodies. Binding, expressed as the number of molecules bound per clot, was calculated from the radioactivity associated with the clot following 2 hours of incubation at 37°C with 300 ng ^35S-labeled unconjugated or conjugated antibody. Results are summarized in Table 2. Binding to platelet-rich clots with rscu-PA/MA-PMI-1, MA-LIBS-1, or rscu-PA/MA-LIBS-1 was, respectively, two- (P < .11), 2.6-fold (P < .0005), or 2.7-fold (P = .002) higher than binding to platelet-poor clots. As compared with platelet-poor clots, binding to platelet-rich clots was not increased with MA-1C8 or rscu-PA/MA-1C8 nor with MA-PMI-1. No differences were observed for binding to platelet-poor clots between MA-PMI-1 or MA-LIBS-1 and the control antibody MA-1C8, or between rscu-PA/MA-PMI-1 or rscu-PA/MA-LIBS-1 and the control conjugate rscu-PA/MA-1C8. In contrast, with platelet-rich clot binding of MA-PMI-1 or MA-LIBS-1 was twofold to fivefold higher than that of MA-1C8 (P < .015) and binding of rscu-PA/MA-PMI-1 or rscu-PA/MA-LIBS-1 was 2.3- to 3.5-fold higher than that of rscu-PA/MA-1C8 (P < .1).

Effect of the conjugates on platelet aggregation. rscu-PA, the antiplatelet antibodies, and the rscu-PA/antiplatelet antibody conjugates were tested for their effect on ADP-induced platelet aggregation in platelet-rich plasma. Aggregation was determined as the change in light transmittance measured 3 minutes after the addition of stimulus, expressed in percent of the difference between platelet-rich and platelet-poor plasma. Platelet aggregation decreased to 75% of the control value when the platelets were preincubated for 30 minutes at room temperature with rscu-PA at a concentration of 63 μg/mL (n = 6) (Fig 6A). No significant effect on aggregation was observed with either MA-TSPI-1, MA-PMI-2, or MA-PMI-1 at concentrations up to 300 μg IgG/mL (Fig 6B), nor with the conjugate rscu-PA/MA-
TSPI-1 at concentrations up to 200 µg u-PA/mL (Fig 6A). Inhibition to 75% of control was obtained at concentrations of 1.2 µg u-PA/mL for rscu-PA/MA-PMI-1 and 6 µg u-PA/mL for rscu-PA/MA-PMI-2, which is 50 and 10 times lower than the equipotent concentration of rscu-PA. With the conjugate rscu-PA/MA-LIBS-1, a decrease to 75% of the control value was obtained at a concentration of 7.6 µg u-PA/mL and complete inhibition was obtained at higher concentrations (Fig 6A). However, this effect could be fully ascribed to the antibody-moiety present in the conjugate (about 34 µg IgG/mL at a concentration of 7.6 µg u-PA/mL). Indeed, the antibody MA-LIBS-1 itself was also able to fully inhibit aggregation, reaching 75% of the control value at a concentration of 12 µg/mL (Fig 6B).

Lysis of 125I-fibrin-labeled human plasma clots in a plasma milieu in vitro. Clot lysis with rscu-PA, rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 was assayed in an in vitro system composed of 125I-fibrin-labeled plasma clots immersed in normal human plasma. Figure 7A (panel I) represents dose-response curves of clot lysis, expressed as lysis after 2 hours versus the concentration of plasminogen activator, using clots prepared from normal human plasma (platelet-poor clots). Fifty percent clot lysis in 2 hours was obtained with a concentration (expressed in u-PA equivalents) of 1.25 µg/mL (n = 7) for rscu-PA, as compared with 5 µg/mL for rscu-PA/MA-TSPI-1 (n = 1), 2 µg/mL for rscu-PA/MA-PMI-2 (n = 1), 0.70 µg/mL for rscu-PA/MA-PMI-1 (n = 3), 1.0 µg/mL for rscu-PA/MA-LIBS-1 (n = 3), or 1.9 µg/mL for rscu-PA/MA-1C8 (n = 2) (not shown). Figure 7B (panel I) represents residual fibrinogen levels at 2 hours versus the concentration of plasminogen activator, during lysis of platelet-poor clots. At concentrations yielding 50% clot lysis in 2 hours, residual fibrinogen levels were 75% for rscu-PA, as compared with 40% for rscu-PA/MA-TSPI-1, less than 20% for rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, or rscu-PA/MA-LIBS-1 (Fig 7B, panel I), and 40% for rscu-PA/MA-1C8 (not shown).

Table 2. Binding of the Conjugates to Human Platelets or to Human Plasma Clots in a Plasma Milieu

<table>
<thead>
<tr>
<th>Binding to Human Platelets*</th>
<th>Binding to Human Plasma Clots†</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Unstimulated Platelets (molecules/platelet)</td>
</tr>
<tr>
<td>MA-PMI-1</td>
<td>6,700 ± 1,600</td>
</tr>
<tr>
<td>rscu-PA/MA-PMI-1</td>
<td>9,900 ± 3,400</td>
</tr>
<tr>
<td>MA-LIBS-1</td>
<td>9,000 ± 1,800</td>
</tr>
<tr>
<td>rscu-PA/MA-LIBS-1</td>
<td>11,000 ± 2,500</td>
</tr>
<tr>
<td>MA-1C8</td>
<td>10,400 ± 1,600</td>
</tr>
<tr>
<td>rscu-PA/MA-1C8</td>
<td>3,500 ± 560</td>
</tr>
</tbody>
</table>

*The data represent mean ± SD obtained from three experiments.
†Binding of 5 µg unconjugated antibody or 5 µg antibody equivalent of the conjugates to 3 × 10⁷ platelets was measured following 30 minutes of incubation at room temperature in plasma (final volume, 150 µL) in the absence or in the presence of ADP (10 µm/L final concentration).

With rscu-PA, 50% lysis in 2 hours was reached at a concentration of 1.2 µg/mL (n = 10). With rscu-PA/MA-LIBS-1, 50% lysis in 2 hours required a concentration of 4 µg u-PA/mL (n = 3). Maximal lysis with rscu-PA/MA-PMI-1 at concentrations up to 16 µg u-PA/mL was only about 45%, and lysis with rscu-PA/MA-TSPI-1 or rscu-PA/MA-PMI-2 at concentrations up to 8 µg u-PA/mL did not exceed 30%. Lysis in 2 hours with rscu-PA/MA-1C8 at concentrations up to 24 µg u-PA/mL did not exceed 25% (not shown). With rscu-PA at the concentration yielding 50% lysis of a platelet-rich clot in 2 hours, a residual fibrinogen level of 83% of the baseline value was measured (Fig 7B, panel II). In contrast, extensive fibrinogen breakdown occurred during lysis with rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, and, somewhat less pronounced, with rscu-PA/MA-TSPI-1 (Fig 7B, panel II). Lysis with rscu-PA/MA-1C8 was also associated with extensive fibrinogen breakdown (not shown). Lysis of platelet-rich clots with rscu-PA was not influenced by addition of unconjugated MA-TSPI-1, MA-PMI-2, MA-PMI-1, or MA-LIBS-1 at a twofold to fourfold molar excess of antibody over rscu-PA (not shown), indicating that interaction of the antibodies with a platelet-rich clot did not alter the susceptibility of the clot to lysis by rscu-PA.

Figure 7A (panel III) represents dose-response curves of lysis using clots prepared from platelet-enriched plasma with a platelet count of 6 to 15 × 10⁷/µL (platelet-enriched clots). With rscu-PA at a concentration of 2 µg/mL, lysis in 2 hours reached a maximum of only about 44% (n = 5) and decreased again at higher rscu-PA concentrations. No significant lysis (<20% in 2 hours) was obtained with the rscu-PA/antiplatelet antibody conjugates at concentrations up to 32 µg u-PA/mL (Fig 7A, panel III) nor with rscu-PA/MA-1C8 at concentrations up to 24 µg u-PA/mL (not shown). At the concentration of rscu-PA yielding maximal lysis in 2 hours, the level of residual fibrinogen was decreased to 45% of the baseline value (Fig 7B, panel III). Significant fibrinogen breakdown was observed during lysis with each of the four rscu-PA/antiplatelet antibody conjugates, which was most pronounced with rscu-PA/MA-PMI-1 and with rscu-PA/MA-LIBS-1 (Fig 7B, panel III). Significant fibrinogen breakdown also occurred during lysis with rscu-PA/MA-1C8 (not shown).
U-PNANTIPLATELET ANTIBODY CONJUGATES

was prepared from platelet-rich (platelet count, 3
value for spontaneous lysis at 90 minutes of 18%  

W
man plasma clots in a hamster pulmonary embolism model.  

TSPI-l(O), rscu-PNMA-PMI-2 (MI, rscu-PA/MA-PMI-1  
addition of ADP, expressed in percent of the difference between 

ries (B) on ADP-induced platelet aggregation. Aggregation was deter-

platelet-rich and platelet-poor plasma and calculated as percent of the  

MA-LIBS-1 (A). Concentrations are expressed in µg u-PA-equivalent/  

MA-LIBS-1 (A). Concentrations are expressed in µg IgG/mL. The data are mean 

values of results obtained from two to six experiments. Vertical bars  

represent SEM.

Fig 6. Effect of a 30-minute preincubation of human platelets with  

rscu-PA/antiplatelet antibody conjugates (A) or antiplatelet antibod-

ies (B) on ADP-induced platelet aggregation. Aggregation was deter-

mined as the change in light transmittance measured 3 minutes after  

addition of ADP, expressed in percent of the difference between  

platelet-rich and platelet-poor plasma and calculated as percent of the  

control value (preincubation with buffer). (A) rscu-PA (•), rscu-PA/MA-

TSPI-1 (□), rscu-PA/MA-PMI-2 (■), rscu-PA/MA-PMI-1 (□), or rscu-PA/  

MA-LIBS-1 (△). Concentrations are expressed in µg u-PA-equivalent/  

mL. (B) MA-TSPI-1 (□), MA-PMI-2 (■), MA-PMI-1 (□), or MA-LIBS-1  

(△). Concentrations are expressed in µg IgG/mL. The data are mean 

values of results obtained from two to six experiments. Vertical bars  

represent SEM.

Lysis of 125I-fibrin–labeled platelet-rich or platelet-poor hu-

man plasma clots in a hamster pulmonary embolism model. The in vivo thrombolytic potency of rscu-PA/MA-PMI-1 and rscu-PA/MA-LIBS-1 was compared with that of rscu-PA or of the control conjugate rscu-PA/MA-1C8 in a hamster pulmonary embolism model, in which the pulmonary clot was prepared from platelet-rich (platelet count, 3 × 10⁵/μL) or from platelet-poor human plasma. The results obtained with platelet-rich clots are summarized in Table 3. Twenty-five experiments with saline infusion yielded a value for spontaneous lysis at 90 minutes of 18% ± 2% (mean ± SEM). Fibrinogen levels at the end of the experiment were 160% ± 13% (n = 13) of the baseline value and α2-antiplasmin levels were 95% ± 5% (n = 14). With  

rscu-PA, lysis at 90 minutes after the start of the infusion  

increased from 25% ± 8% at 0.25 mg/kg to 89% ± 1% at 4  

mg/kg. rscu-PA/MA-PMI-1 at a dose of 0.25, 0.5, or 1 mg  
u-PA equivalent/kg resulted in 19% ± 5%, 36% ± 7%, or  
85% ± 1% lysis at 90 minutes, respectively. rscu-PA/MA-

LIBS-1 at a dose of 0.25, 0.5, or 1 mg u-PA equivalent/kg yielded 35% ± 5%, 41% ± 7% or 58% ± 7% lysis at 90  

minutes, respectively. With the control conjugate rscu-PA/  

MA-1C8, lysis at 90 minutes ranged from 20% ± 4% to  

34% ± 8% at doses from 0.5 to 2 mg u-PA equivalent/kg.  

Linear regression analysis of the individual dose-

response data, expressed as percent lysis over the back-

ground value versus dose administered, yielded values for the  

relative thrombolytic potencies (percent lysis per u-PA  
equivalent administered) towards platelet-rich human  

plasma clots as summarized in Table 3 and illustrated in Fig  

8A. With rscu-PA/MA-PMI-1, a threelfold higher throm-

bolytic potency was found than with rscu-PA (58% ± 14%  

v 19% ± 2.4% lysis per mg u-PA/kg, P < .0005). The throm-

bolytic potency of rscu-PA/MA-LIBS-1 was 2.3-fold higher  

than that of rscu-PA (43% ± 11% v 19% ± 2.4% lysis per  

mg u-PA/kg, P = .004). In contrast, conjugation of rscu-PA  

with the control antibody MA-1C8 resulted in a 2.3-fold  

decrease of its thrombolytic potency (8.3% ± 4.1% v  

19% ± 2.4% lysis per mg u-PA/kg, P = .023).  

Linear regression analysis of the individual dose-

response data, expressed as percent lysis over the back-

ground value versus steady state plasma u-PA-related  

antigen level, yielded values for the specific thrombolytic  

activity (percent lysis per microgram per millilitre steady  

state plasma u-PA antigen level) towards platelet-rich  

human plasma clots as summarized in Table 3 and  

illustrated in Fig 8B. As compared with rscu-PA, specific  
thrombolytic activities were reduced 2.6-fold with rscu-PA/  

MA-PMI-1 (P = .028), sixfold with rscu-PA/MA-LIBS-1  
(P = .003), and 30-fold with the control conjugate rscu-PA/  

MA-1C8 (P < .0005). No significant fibrinogen breakdown  
ocurred during lysis with rscu-PA, but α2-antiplasmin  

levels at the end of the experiment were somewhat de-

creased (66% ± 10% residual α2-antiplasmin at a dose of 4  

mg/kg). However, lysis with the conjugates was associated  

with fibrinogen breakdown and extensive α2-antiplasmin  

consumption at doses in excess of 0.5 mg/kg u-PA equiva-

lent. Plasma clearance rates, determined from the infusion  
rate and the steady state u-PA-related antigen levels in  

plasma, were approximately 0.64 mL/min for rscu-PA/MA-

PMI-1, 0.48 mL/min for rscu-PA/MA-LIBS-1, and 0.54  

mL/min for rscu-PA/MA-1C8, as compared with 4.9 mL/ 

min for rscu-PA (Table 3).

Results of the in vivo thrombolytic potency of rscu-PA,  
rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, and rscu-PA/  
MA-1C8 towards platelet-poor human plasma clots are  
summarized in Table 4. Spontaneous lysis at 90 minutes was  
22% ± 3% (mean ± SEM, n = 6). With rscu-PA, lysis at 90  

minutes increased from 30% ± 4% at 0.25 mg/kg to 87% ±  
7% at 2 mg/kg. rscu-PA/MA-PMI-1 at a dose of 0.5, 1, or 2  

mg u-PA/kg yielded 24% ± 7%, 54% ± 7%, and 61% ± 5%  

lysis, respectively, and with rscu-PA/MA-LIBS-1 at a dose  
of 0.25, 0.5, 1, and 2 mg u-PA/kg lysis at 90 minutes was
plasma (panel I), from platelet-rich plasma with a platelet count of 1 to 3 × 10^10/mL, or from platelet-enriched plasma with a platelet count of 6 to 16 × 10^10/mL (panel III). (A) Percent clot lysis after 2 hours versus the concentration of plasminogen activator. (B) Residual fibrinogen levels after 2 hours, expressed in percent of the baseline value, versus the concentration of plasminogen activator. Data represent mean values of results obtained from two to 10 experiments. Vertical bars represent SEM.

The individual dose-response data, expressed as percent lysis over background versus steady state plasma u-PA-related antigen level, yielded values for specific thrombolytic activities towards platelet-poor human plasma clots as summarized in Table 4 and illustrated in Fig 8C. No significant differences were observed between rscu-PA and the conjugates (21% to 37% lysis per mg u-PA/kg, P > .075).

Linear regression analysis of the individual dose-response data, expressed as percent lysis versus steady state plasma u-PA–related antigen level, yielded values for specific thrombolytic activities towards platelet-poor human plasma clots as summarized in Table 4

### Table 3. Clot Lysis and Hemostasis Parameters After Intravenous Infusion of rscu-PA, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 in Hamsters With Pulmonary Embolism Consisting of a Platelet-Rich Human Plasma Clot

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (mg u-PA/kg)</th>
<th>Clot Lysis at 90 min (%)</th>
<th>Residual Fibrinogen (% of baseline)</th>
<th>Residual α1-Antiplasmin (% of baseline)</th>
<th>Antigen at End of Infusion (ng/mL)</th>
<th>Clp* (mL/min)</th>
<th>% Lysis per mg/kg Compound</th>
<th>% Lysis per μg/mL Plasma Level</th>
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<tr>
<td>Saline</td>
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<td>—</td>
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<td>rscu-PA</td>
<td>0.25</td>
<td>25 ± 8 (4)</td>
<td>120 ± 18 (3)</td>
<td>83 ± 5 (2)</td>
<td>110 ± 30 (4)</td>
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<td>0.50</td>
<td>32 ± 5 (4)</td>
<td>140 ± 8 (4)</td>
<td>100 ± 2 (4)</td>
<td>170 ± 34 (4)</td>
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<tr>
<td></td>
<td>1.0</td>
<td>47 ± 9 (7)</td>
<td>140 ± 5 (6)</td>
<td>86 ± 4 (5)</td>
<td>300 ± 39 (5)</td>
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<td>2</td>
<td>52 ± 5 (6)</td>
<td>120 ± 19 (4)</td>
<td>59 ± 11 (6)</td>
<td>860 ± 140 (4)</td>
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<tr>
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<td>4</td>
<td>89 ± 1 (4)</td>
<td>120 ± 11 (3)</td>
<td>66 ± 10 (3)</td>
<td>1,100 ± 73 (4)</td>
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<td>19 ± 5 (3)</td>
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<td>78 ± 8 (3)</td>
<td>650 ± 10 (2)</td>
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<tr>
<td></td>
<td>0.50</td>
<td>36 ± 7 (3)</td>
<td>110 (1)</td>
<td>&lt; 25 (3)</td>
<td>1,400 ± 140 (3)</td>
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<td>85 ± 1 (3)</td>
<td>&lt; 29 (2)</td>
<td>&lt; 25 (3)</td>
<td>2,500 ± 32 (3)</td>
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<tr>
<td>rscu-PA/MA-LIBS-1</td>
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<td>35 ± 5 (3)</td>
<td>120 ± 16 (3)</td>
<td>73 ± 3 (3)</td>
<td>690 ± 25 (3)</td>
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<td>120 ± 12 (3)</td>
<td>51 ± 6 (3)</td>
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<td>4,300 ± 570 (3)</td>
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<td>rscu-PA/MA-1C8</td>
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<td>34 ± 8 (3)</td>
<td>—</td>
<td>&lt; 25 (2)</td>
<td>5,200 ± 740 (3)</td>
<td>0.64</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

*The data represent mean ± SEM of the number of experiments indicated between parentheses.

†The plasma clearance rate (Clp) was calculated as the ratio of the infusion rate (μg/min) and the steady state plasma concentration of antigen (μg/mL) at the end of the infusion, assuming an average body weight of 100 g.

The regression line slope was calculated from the individual dose-response data after correction for background lysis (saline), by linear regression forced through the origin. The data represent mean ± SEM of the regression line slope and, in parentheses, the correlation coefficient of the regression line.
Fig 8. Dose-response curves of thrombolysis in hamsters with pulmonary embolism consisting of a platelet-rich (A and B) or platelet-poor (C and D) human plasma clot. (A and C) Thrombolytic potency expressed as percent lysis versus dose administered in mg u-PA/kg. (B and D) Specific thrombolytic activity expressed as percent lysis versus steady state plasma u-PA-related antigen level in µg/mL. (8) rscu-PA, (9) rscu-PA/MA-PMI-1, (7) rscu-PA/MA-LIBS-1, and (7') rscu-PA/MA-1C8. Symbols shown represent mean values of lysis obtained from two to 10 experiments as summarized in Tables 2 and 3, after correction for background lysis. Vertical bars represent SEM. Regression lines shown were obtained by fitting the individual dose-response data, corrected for background lysis, with a linear regression line forced through the origin. Regression line slopes are summarized in Tables 3 and 4.

DISCUSSION

One approach to improve the clot-selectivity of plasminogen activators consists of targeting the agent to the clot by conjugation with fibrin-specific MoAbs.25,26 Alternatively, plasminogen activators could be targeted to a platelet-rich clot using antiplatelet antibodies. In the present study, chemical conjugates were made of rscu-PA with four different well-characterized murine monoclonal antplatelet antibodies:9-12, MA-TSPI-1, directed against human thrombospordin; MA-PMI-2, directed against the platelet surface glycoprotein IIa; MA-PMI-1, directed against GPIIbα; and MA-LIBS-1, directed against GPIIIa. These antibodies only recognize epitopes on activated platelets, not, or much less, on unactivated platelets.9,11,12 The purified conjugates rscu-PA/MA-TSPI-1, rscu-PA/MA-PMI-2, rscu-PA/MA-PMI-1, and rscu-PA/MA-LIBS-1 contained 34% to 45% of the total protein present in the coupling mixtures, with an average stoichiometry between

Table 4. Clot Lysis and Hemostasis Parameters After Intravenous Infusion of rscu-PA, rscu-PA/MA-PMI-1, rscu-PA/MA-LIBS-1, or rscu-PA/MA-1C8 in Hamsters With Pulmonary Embolism Consisting of a Platelet-Poor Human Plasma Clot

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose at 90 min (%)</th>
<th>Residual Fibrinogen (%)</th>
<th>Residual α2-Antiplasmin (%)</th>
<th>Antigen at End of Infusion (mg/mL)</th>
<th>Clp* (ml/min)</th>
<th>% Lysis per mg/kg Compound</th>
<th>% Lysis per µg/mL Plasma Level</th>
<th>Regression Line Slope†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>22 ± 3 (6)</td>
<td>150 ± 12 (6)</td>
<td>110 ± 6 (4)</td>
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</tr>
<tr>
<td>rscu-PA</td>
<td>0.25 30 ± 4 (9)</td>
<td>140 ± 9 (9)</td>
<td>110 ± 7 (9)</td>
<td>42 ± 4 (10)</td>
<td>10</td>
<td>35 ± 3.8</td>
<td>61 ± 9.8</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.5 40 ± 4 (10)</td>
<td>140 ± 10 (10)</td>
<td>100 ± 8 (10)</td>
<td>150 ± 29 (10)</td>
<td>5.5</td>
<td>(r = .848)</td>
<td>(r = .507)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1.0 64 ± 4 (8)</td>
<td>150 ± 9 (6)</td>
<td>98 ± 8 (7)</td>
<td>410 ± 110 (6)</td>
<td>4.1</td>
<td>(r = .848)</td>
<td>(r = .507)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.0 87 ± 7 (7)</td>
<td>96 ± 12 (6)</td>
<td>64 ± 12 (9)</td>
<td>910 ± 230 (6)</td>
<td>3.7</td>
<td>(r = .848)</td>
<td>(r = .507)</td>
<td>—</td>
</tr>
<tr>
<td>rscu-PA/MA-PMI-1</td>
<td>0.5 24 ± 7 (3)</td>
<td>120 ± 9 (3)</td>
<td>36 ± 9 (3)</td>
<td>1,300 ± 36 (3)</td>
<td>0.6</td>
<td>(r = .848)</td>
<td>(r = .507)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 54 ± 7 (3)</td>
<td>&lt;20 (3)</td>
<td>&lt;25 (3)</td>
<td>2,500 ± 150 (3)</td>
<td>0.7</td>
<td>21 ± 6.2</td>
<td>7.7 ± 2.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2 61 ± 5 (3)</td>
<td>&lt;20 (3)</td>
<td>&lt;25 (3)</td>
<td>5,700 ± 210 (3)</td>
<td>0.8</td>
<td>(r = .887)</td>
<td>(r = .770)</td>
<td>—</td>
</tr>
<tr>
<td>rscu-PA/MA-LIBS-1</td>
<td>0.25 23 ± 9 (3)</td>
<td>96 (1)</td>
<td>63 (1)</td>
<td>660 ± 92 (3)</td>
<td>0.6</td>
<td>(r = .848)</td>
<td>(r = .770)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.5 52 ± 4 (3)</td>
<td>110 ± 9 (3)</td>
<td>37 ± 10 (3)</td>
<td>1,200 ± 3 (3)</td>
<td>0.7</td>
<td>37 ± 6.9</td>
<td>10 ± 2.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1 67 ± 11 (3)</td>
<td>&lt;20 (3)</td>
<td>&lt;26 (3)</td>
<td>3,300 ± 92 (3)</td>
<td>0.5</td>
<td>(r = .828)</td>
<td>(r = .768)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2 86 ± 11 (2)</td>
<td>&lt;20 (2)</td>
<td>&lt;25 (2)</td>
<td>7,300 ± 25 (2)</td>
<td>0.5</td>
<td>(r = .828)</td>
<td>(r = .768)</td>
<td>—</td>
</tr>
<tr>
<td>rscu-PA/MA-1C8</td>
<td>0.25 25 ± 3 (4)</td>
<td>150 ± 14 (4)</td>
<td>78 ± 9 (4)</td>
<td>700 ± 120 (4)</td>
<td>0.6</td>
<td>32 ± 8.8</td>
<td>5.9 ± 2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.5 50 ± 8 (5)</td>
<td>120 ± 7 (5)</td>
<td>62 ± 14 (5)</td>
<td>1,100 ± 100 (5)</td>
<td>0.7</td>
<td>(r = .615)</td>
<td>(&lt; .1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>1.0 57 ± 12 (5)</td>
<td>77 ± 11 (5)</td>
<td>30 ± 4 (5)</td>
<td>2,700 ± 550 (5)</td>
<td>0.6</td>
<td>(r = .615)</td>
<td>(&lt; .1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.0 75 ± 1 (2)</td>
<td>&lt;30 (2)</td>
<td>&lt;30 (2)</td>
<td>11,000 ± 1,000 (2)</td>
<td>0.3</td>
<td>(r = .615)</td>
<td>(&lt; .1)</td>
<td>—</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM of the number of experiments indicated in parentheses.
*The plasma clearance rate (Clp) was calculated as the ratio of the infusion rate (µg/min) and the steady state plasma concentration of antigen (µg/mL) at the end of the infusion, assuming an average body weight of 100 g.
†The regression line slope was calculated from the individual dose-response data after correction for background lysis (saline), by linear regression forced through the origin. The data represent mean ± SEM of the regression line slope with the correlation coefficient given in parentheses.
binding MA-LIBS-1 in these binding assays were compared with the corresponding radiolabeled unconjugated antibody by the SPDP treatment or the conjugation. However, with unconjugated MA-PMI-1, binding with rscu-PA. rscu-PA/MA-PMI-1, or rscu-PA/MA-PMI-2 was obtained at 50- or 10-fold lower u-PA concentrations, respectively. This enhanced inhibitory effect of rscu-PA/MA-PMI-1 or rscu-PA/MA-PMI-2 cannot be explained merely by interaction of MA-PMI-1 or MA-PMI-2 with their epitopes on GPIIb/IIIa, because the antibodies alone did not inhibit platelet aggregation. Targeting of rscu-PA to the platelet surface may, however, result in localized plasminogen activation and breakdown of receptor-bound fibrinogen. ADP-induced platelet aggregation was also inhibited after preincubation of the platelets with the antibody MA-LIBS-1. However, the inhibition by MA-LIBS-1 presumably was not related to specific antigen-antibody interaction, because Fab fragments of this antibody did not inhibit platelet aggregation (not shown). Conjugation of MA-LIBS-1 with rscu-PA did not increase the inhibitory action further.

In an in vitro system composed of 125I-fibrin-labeled platelet-rich human plasma clots immersed in citrated human plasma, none of the rscu-PA/antiplatelet antibody conjugates appeared to have a higher fibrinolytic potency than unconjugated rscu-PA. The poor fibrinolytic potency of the rscu-PA/antiplatelet antibody conjugates towards platelet-rich clots in vitro appeared not to be due to an impairment of the enzymatic activity of conjugated rscu-PA, because the biochemical properties of the u-PA moieties of each of the conjugates were comparable with those of rscu-PA. Furthermore, all four antiplatelet antibody/rscu-PA conjugates were able to lyse platelet-poor clots prepared from normal human plasma, with a fibrinolytic potency similar or even slightly higher than that of rscu-PA; 50% lysis in 2 hours was obtained at a concentration of 0.7 to 3 μg u-PA/mL for the various conjugates, as compared with 1.25 μg/mL for rscu-PA. Recently, Bode et al obtained a 1.3- to 11-fold enhancement of the fibrinolytic potency of urokinase towards platelet-rich human plasma clots in vitro by conjugation of urokinase with the murine MoAb 7E3 directed against platelet membrane GPIIb/IIIa.27

The in vivo thrombolytic potency of the different plasminogen activators towards platelet-rich human plasma clots was evaluated in a hamster pulmonary embolism model. Lysis of platelet-rich clots increased 2.3- to 3-fold by conjugation of rscu-PA with the anti-GPIIb/IIIa antibodies MA-PMI-1 or MA-LIBS-1. Moreover, thrombolysis with these rscu-PA/antiplatelet antibody conjugates was fivefold to sevenfold higher than with the conjugate of rscu-PA with the control antibody MA-1C8. The thrombolytic potency of an anti-thrombospondin antibody/rscu-PA conjugate, rscu-PA/MA-TSP5, with similar biochemical properties as rscu-PA/MA-TSPI-1 described above (data not shown), was not significantly different from that of rscu-PA or of rscu-PA/MA-1C8: 17% ± 7% lysis per mg u-PA/kg v 19% ± 2.4% lysis per mg/kg for rscu-PA, P = .7, or v 8.3% ± 4.1% lysis per mg u-PA/kg for rscu-PA/MA-1C8, P = .3. Lysis of platelet-poor or platelet-rich clots in vivo with the different conjugates was associated, at higher concentrations, with fibrinogen breakdown and with extensive α2-antiplasmin consumption. Chemical conjugation of rscu-PA with antibodies thus seems to increase the potential for systemic activation of the fibrinolytic system, as was also previously observed for rscu-PA/MA-1C8.5 This increased fibrinogenolytic effect of the conjugates was also indicated by the significant fibrinogen breakdown observed during in vitro clot lysis. The reason for the increased systemic activation with conjugated rscu-PA is not clear. With the antibody/rscu-PA conjugates, activation to two-chain moieties by plasmin occurred with initial activation rates that were comparable with the value found with rscu-PA. Thus, the difference in systemic activation between conjugated and unconjugated rscu-PA appears not to be related to differences in the rate of activation to two-chain moieties.

Conjugation of rscu-PA with MA-PMI-1 and MA-LIBS-1 as well as with MA-1C8 decreased its plasma clearance rate approximately 10-fold, yielding significantly higher steady state plasma u-PA antigen levels with the conjugates than with rscu-PA at comparable doses (Tables 3 and 4). The specific thrombolytic activities towards platelet-rich human plasma clots, determined as percent lysis per microgram per milliliter steady state plasma antigen level, were 2.6- and 6-fold lower for rscu-PA/MA-PMI-1 and rscu-PA/MA-LIBS-1, respectively, and 30-fold lower for rscu-PA/MA-1C8, as compared with rscu-PA. Specific thrombolytic activities towards platelet-poor human plasma clots were eightfold to 10-fold lower than for rscu-PA, both for...
rs cu-PA/MA-PMI-1 or rs cu-PA/MA-LIBS-1 and for rs cu-PA/MA-1C8, resulting in similar thrombolytic potencies (potency at comparable dose). Thus, conjugation of rs cu-PA per se reduces its in vivo thrombolytic potency, a finding that has been observed previously with similar chemical conjugates of rs cu-PA with MoAbs. With platelet-poor clots, the prolonged half-life of the conjugates apparently partially compensates for the loss of specific thrombolytic activity, resulting in similar thrombolytic potencies for conjugated and unconjugated rs cu-PA. With platelet-rich clots, the additional targeting effect of the antiplatelet antibody moiety resulted in 2.3- to 3-fold enhanced thrombolytic potencies for rs cu-PA/MA-PMI-1 or rs cu-PA/MA-LIBS-1 as compared with rs cu-PA, and fivefold or sevenfold enhanced thrombolytic potencies as compared with the control conjugate rs cu-PA/MA-1C8.

With rs cu-PA, lysis of platelet-rich clots in vivo occurred with a 1.8-fold lower efficiency than lysis of platelet-poor clot (19% ± 2.4% v 35% ± 3.8% lysis per mg/kg, P = .001). Similarly, with rs cu-PA/MA-1C8 lysis of platelet-rich clots in vivo was 3.8-fold less efficient than lysis of platelet-poor clots (8.3% ± 4.1% v 32% ± 8.8% per mg u-PA/kg, P = .044). No significant difference was found between platelet-rich and platelet-poor clots for lysis with rs cu-PA/MA-LIBS-1 (43% ± 11% v 37% ± 6.8% lysis per mg u-PA/kg, P = .636). In contrast, rs cu-PA/MA-PMI-1 was 2.8-fold more efficient for lysing platelet-rich clots than platelet-poor clots (58% ± 14% v 21% ± 6.2% lysis per mg u-PA/kg, P = .028).

In conclusion, chemical conjugates of rs cu-PA with antiplatelet antibodies were functionally intact, both with respect to the in vitro enzymatic properties of rs cu-PA and the antigen-binding capacity of the antibody. The thrombolytic potency of the conjugates towards platelet-rich human plasma clots in a hamster pulmonary embolism model was 2.3- to 3-fold increased as compared with unconjugated rs cu-PA and was fivefold to sevenfold higher than the value obtained with a control conjugate. The relatively marginal improvement over rs cu-PA appears to be due in part to a marked reduction of the specific thrombolytic activity of rs cu-PA due to the conjugation procedure. Consequently, alternatively prepared conjugates, such as chimeric molecules obtained by recombinant DNA technology, might have maintained specific thrombolytic activities of rs cu-PA and consequently have higher thrombolytic potencies.

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Effect of chemical conjugation of recombinant single-chain urokinase-type plasminogen activator with monoclonal antiplatelet antibodies on platelet aggregation and on plasma clot lysis in vitro and in vivo

M Dewerchin, HR Lijnen, JM Stassen, F De Cock, T Quertermous, MH Ginsberg, EF Plow and D Collen

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