Targeting of plasminogen activators to the fibrin component of a thrombus with the use of monoclonal antibodies (MA) directed against human fibrin may enhance their thrombolytic potency and fibrin-specificity. The thrombolytic and pharmacokinetic properties of rscu-PA/MA-FU1-74, an immunoconjugate of recombinant single-chain urokinase-type plasminogen activator (rscu-PA) and a bispecific MA directed against u-PA and against the β-chain of human fibrin (MA-FU1-74), were investigated in baboons with a [125I]fibrin-labeled autologous blood clot in the femoral vein. Continuous intravenous infusion of rscu-PA/MA-FU1-74 (1:1.2 molar ratio) over 2 hours showed a fivefold increased thrombolytic potency (lysis per unit dose) over that of unconjugated rscu-PA, as evidenced both by a higher maximal rate of lysis (300% ± 68% vs 78% ± 25% lysis per mg u-PA equivalent of compound administered per kg body weight; P < .001), and by a lower dose at which the maximal rate of lysis occurs (0.19 ± 0.03 vs 0.82 ± 0.10 mg compound per kg body weight, P < .001). The specific thrombolytic activity (percent lysis per unit steady-state plasma u-PA antigen level) was lower for rscu-PA/MA-FU1-74 than for rscu-PA, as shown by both a lower maximal rate of lysis (60% ± 13% vs 220% ± 22% lysis per μg/mL u-PA antigen level in plasma, P < .001) and a higher plasma antigen level at which maximal lysis is achieved (1.2 ± 0.17 vs 0.20 ± 0.01 μg/mL, P < .001). The thrombolytic potency of rscu-PA/MA-UK1-3, an immunoconjugate of rscu-PA with the parental anti-u-PA antibody was similar to that of unconjugated rscu-PA. Clot lysis was achieved without systemic fibrinogen or α2-antiplasmin consumption, and with a minor transient prolongation of the bleeding time. After the end of the infusions, u-PA-related antigen disappeared from plasma in a biphasic manner, with an initial half-life of 3.3 ± 0.4 minutes for rscu-PA, 13 ± 1 minutes for rscu-PA/MA-FU1-74, and 13 ± 1 minutes for rscu-PA/MA-UK1-3, with corresponding plasma clearances of 340 ± 28, 10 ± 1, and 37 ± 4 mL/min, respectively (mean ± SEM). rscu-PA/MA-FU1-74 has a fivefold higher thrombolytic potency than unconjugated rscu-PA, as a result both of fibrin targeting by the specific idiotype of the antibody and of a slower plasma clearance.

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Supported by grants from the Geconcerteerde Onderzoeksacties and from Takeda Chemical Industries Ltd.

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search Products (Buckinghamshire, UK). Normal human plasma was pooled, fresh frozen, citrated blood bank plasma from at least five healthy blood donors. Normal baboon plasma and rat plasma were fresh frozen citrated pools.

**Plasma clot lysis in vitro.** The effect of MA-FU1-74 on the fibrinolytic potency of rscu-PA was determined in a clot lysis assay in human, baboon and rat plasma. One milliliter of plasma, containing different concentrations of rscu-PA without or with preincubation for 30 minutes at room temperature with a 1.2-fold molar excess of MA-FU1-74, was clotted by addition of thrombin (final concentration 2 NIH U/mL). The mixture was incubated at 37°C and the lysis time was determined by measurement of light transmission. This is performed automatically with a "lysometer," which records the time between clotting of the plasma sample and its lysis. The start of the assay corresponds to a change in light transmission on introduction of the clotted plasma sample and the end-point is detected by a change in light transmission caused by a small marble that drops in the liquified plasma.

**Thrombolysis in a baboon femoral vein thrombosis model.** The thrombolytic properties of rscu-PA, rscu-PA/MA-FU1-74, and rscu-PA/MA-UK1-3 were compared in baboons (Papio hamadryas) with femoral vein blood clots, using procedures for anesthesia and surgery as described in detail elsewhere. Briefly, a [125I]fibrin-labeled autologous blood clot with a volume of 0.5 to 1.0 mL (containing ~0.5 μCi of [125I]-labeled fibrinogen) was produced in an isolated femoral vein segment. rscu-PA, either alone or after preincubation for 30 minutes at 37°C with a 1.2-fold molar excess of monoclonal antibodies (MA-FU1-74 or MA-UK1-3), was administered via a brachial vein as a 10% bolus injection, followed by continuous infusion of the remaining 90% dose over 2 hours. Alternatively, the agents were administered as a 5-minute bolus injection. The animals were anticoagulated with heparin (300 U/kg as an intravenous bolus given just before removal of the vessel clamps, immediately followed by a continuous infusion of 60 U/kg/h until the end of the experiment). Thrombolysis was quantitated both by determination of the residual radioactivity in the femoral vein segment 30 minutes after the end of the infusion or 150 minutes after bolus injection, and by external gamma counting. The degree of lysis (in percent) was expressed as the difference between the radioactivity originally calculated to be incorporated in the clot and the residual radioactivity in the vein segment. The time course of clot lysis was monitored continuously by external gamma counting using a 3 × 0.5 inch sodium iodide/thallium crystal (Bicron, Newbury, OH) positioned over the thrombosed femoral vein segment and the data were analyzed as described elsewhere.

Two- or 20-μL blood samples were drawn into sodium citrate (final concentration, 0.01 mol/L) before, at hourly intervals after the start of the infusion, and at the end of the experiment when the test compounds were administered by continuous infusion, or before, 1 minute after the injection, and at the end of the experiment when the test compounds were given as a bolus. These samples were used for measurement of radioactivity, fibrinogen, clotting, antiplasmin, u-PA-related antigen, u-PA/MA complex, and as well for determination of anti-mouse IgG antibody titers (see below). Activated partial thromboplastin time was measured using a routine laboratory assay.

Template bleeding times were measured before and at 120 and 240 minutes after injection of study drugs. The bleeding time incidence was made using an automated spring-loaded device (Simplate-II, General Diagnostics, Morris Plains, NJ) applied to the volar surface of the foreleg. The region of the incision site was washed, shaved, and dried before performance of the first bleeding time.

The relative thrombolytic potency (lysis per unit dose) and the specific thrombolytic activity (lysis per unit steady-state plasma u-PA antigen level) of the compounds were determined as follows. The individual values of percent lysis versus dose of rscu-PA administered, expressed in milligrams u-PA equivalent of compound administered per kilogram body weight, or of percent lysis versus steady-state u-PA-related antigen level in plasma, expressed in micrograms per milliliter, were fitted with an exponentially transformed sigmoidal function y = 100/[1 + e^(ac-μt)], using the statistical program GraFit (Erithacus Software, Middlesex, UK). The mean ± SEM of the parameters c (maximal lysis in percent), b (dose in mg u-PA/kg, or steady-state plasma antigen level in μg/mL, at which the rate of clot lysis is maximal), and z = (ac/4(b^2)) (maximal rate of lysis, expressed as percent lysis per mg u-PA equivalent of compound infused per kg body weight, or percent lysis per μg/mL steady-state u-PA-related antigen level in plasma) were determined. Significance of the differences between these parameters was determined using Student's t-test.

To limit the number of primates used in the present study to an acceptable minimum, we performed 17 experiments in nine baboons (three each with 0.063, 0.125, or 0.250 mg u-PA equivalent per kg of rscu-PA/MA-FU1-74, two with 0.50 mg u-PA equivalent per kg of rscu-PA/MA-UK1-3, one with 0.5 mg/kg rscu-PA, one with 1 mg/kg rscu-PA, two with 2 mg/kg rscu-PA, and two with 0.2 mg/kg rscu-PA/MA-FU1-74 bolus administration). The other experiments in the rscu-PA groups were taken from a previous study performed with an identical experimental protocol.

When two experiments were performed in the same animal, both femoral veins were used once. The two experiments were performed at an interval of 2 to 7 days. No correlation was found between the sequence of the experiments and the clot lysis results. These studies conformed to the guiding principles of the American Physiological Society and the International Committee on Thrombosis and Haemostasis.

**Measurement of baboon anti-mouse IgG antibodies.** Anti-mouse IgG antibody titers in the plasma of baboons given rscu-PA/MA-FU1-74 were determined as follows. Baboon plasma samples were diluted serially with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA). A 100-μL aliquot of each sample was added to microtiter plates, which were coated with MA-FU1-74 (100 μL of a 5-μg/mL solution) and blocked with PBS containing 2% BSA. The plates were incubated for 2 hours at room temperature and after washing with PBS containing 0.05% Tween-20 PBS (PBS-T), a 100-μL portion of rabbit anti-mouse IgG antibody (H- and L-chain-specific) labeled with horseradish peroxidase or rabbit anti-human IgM antibody (μ-chain-specific) labeled with horseradish peroxidase (Organon Teknika, Westchester, PA) was added to the plates. After incubation for 2 hours at room temperature and additional washings with PBS-T, the plates were developed with 6 mmol/L hydrogen peroxide and 40 mmol/L o-phenylenediamine in 0.1 mol/L citrate buffer, pH 5.5, and the absorbance at 492 nm was measured using a Titertek Multiskan (Flow Laboratories, McLean, VA). Antibody titers in this assay represent the maximum dilution factor that gives an absorbance reading of 0.2 with a negative control value of 0.05.

**Pharmacokinetics.** The disappearance of u-PA-related antigen from blood was monitored, in blood samples taken before the start of infusion and 1, 2, 5, 7, 10, 15, 20, and 30 minutes after cessation of infusion for rscu-PA and before and 1, 2, 3, 5, 10, 15, 20, 30, 45, 60, 75, 90, 105, and 120 minutes after the end of the infusion for the conjugates, or in samples taken 1, 2, 3, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240, and 240 minutes after bolus injection of the conjugate. u-PA-related antigen was measured with a specific enzyme-linked immunosorbent assay (ELISA). The ELISA was calibrated with purified rscu-PA or with the
respectively. The immunoreactivity of the conjugates was less than twofold different from that of rscu-PA, and the recoveries in this assay of rscu-PA/MA-FU1-74 and rscu-PA/MA-UK1-3 added to baboon plasma were 140% and 93%, respectively.

u-PA/MA complexes were measured by ELISA as follows. Microtiter plates were coated with 50 μL per well of a 5-μg/mL solution of goat anti-human urokinase antibodies in 10 mmol/L phosphate buffer, pH 8.0, for 4 hours at room temperature. The plates were saturated with 150 μL of 2% BSA in PBS. Aliquots of 50 μL of samples containing rscu-PA/MA complexes were added to the wells, and the plates were incubated at room temperature for 2 hours. After several washings with PBS-T, a 50-μL aliquot of horseradish peroxidase–labeled rabbit antibody raised with anti-mouse IgG was added to the plates, followed by incubation at room temperature for 2 hours. After additional washings with PBS-T, the plates were developed as described above.

The experimental data describing the disappearance of u-PA–related antigen from plasma after the end of the infusion were fitted with a sum of two exponential terms: 

\[ C(t) = R \exp(-\alpha t) + S \exp(-\beta t) \]

This function was obtained from semilogarithmic plots of plasma antigen levels versus time by graphic curve peeling performed as follows. The terminal phase of the curve was fitted with a straight line yielding the ordinate intercept \( R \) 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 \( -\alpha \).

Pharmacokinetic parameters were calculated from these coefficients and exponents using standard formulas derived by Gibaldi and Perrier. The variables \( A \) and \( B \) were first calculated, assuming steady state at the end of infusion, using the formulas 

\[ A = RX_0k_e/k_0 \quad \text{and} \quad B = SX_0\beta/k_0 \]

where \( X_0 \) is the total administered dose, and \( k_e \) 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 \( (\text{AUC}) = A/\alpha + B/\beta \); and (3) plasma clearance \( (Cl_p) = X_0/\text{AUC} \). The clearance (mL/min) during the steady-state phase accompanying continuous intravenous infusion was also calculated from the ratio between the infusion rate (μg/min) and the steady-state plasma antigen concentration (μg/mL).

RESULTS

Plasma clot lysis in vitro. The effect of MA-FU1-74 on plasma clot lysis with rscu-PA in baboon and human plasma in vitro is illustrated in Fig 1. rscu-PA caused concentration-dependent plasma clot lysis both in citrated baboon and human plasma. Addition of MA-FU1-74 at a 1.2-fold molar excess over rscu-PA enhanced the fibrinolytic activity of rscu-PA approximately 20-fold both in baboon plasma and in human plasma. In contrast, MA-UK1-3, the parental anti-u-PA monoclonal antibody, had no effect on the fibrinolytic activity of rscu-PA (data not shown). In rat plasma, rscu-PA had a much lower fibrinolytic activity than in baboon or human plasma, but its activity was not enhanced by addition of MA-FU1-74 (data not shown).

Thrombolytic properties of rscu-PA and its immunocomplexes. The immunocomplexes were less than twofold different from that of rscu-PA, and the recoveries in this assay of rscu-PA/MA-FU1-74 and rscu-PA/MA-UK1-3 added to baboon plasma were 140% and 93%, respectively. The terminal phase of the curve was fitted with a straight line yielding the ordinate intercept \( R \) 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 mammillary model composed of one central and one peripheral compartment, with elimination occurring from the central compartment.

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Table 1. Clot Lysis and Hemostasis Parameters Following Administration of rscu-PA and Its Immunoconjugates to Baboons With Femoral Vein Thrombosis

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose* (mg/kg)</th>
<th>n</th>
<th>Ex Vivo Isotope Recovery (%)</th>
<th>External Gamma Counting (%)</th>
<th>Residual Fibrinogen (%)</th>
<th>α2-Antiplasmin† (%)</th>
<th>u-PA-Related Antigen (ng/mL)</th>
<th>Clr (mL · min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline Infusion</td>
<td></td>
<td>3</td>
<td>8 ± 2</td>
<td>6 ± 1</td>
<td>110 ± 15</td>
<td>100 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rscu-PA</td>
<td>0.50</td>
<td>5</td>
<td>26 ± 9</td>
<td>27 ± 6</td>
<td>110 ± 7</td>
<td>94 ± 1</td>
<td>120 ± 17</td>
<td>440 ± 65</td>
</tr>
<tr>
<td></td>
<td>1.00</td>
<td>5</td>
<td>58 ± 11</td>
<td>57 ± 13</td>
<td>110 ± 6</td>
<td>80 ± 7</td>
<td>290 ± 35</td>
<td>360 ± 42</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>6</td>
<td>90 ± 6</td>
<td>81 ± 4</td>
<td>66 ± 8</td>
<td>68 ± 9</td>
<td>420 ± 110</td>
<td>530 ± 110</td>
</tr>
<tr>
<td>rscu-PA/MA-FU1-74</td>
<td>0.063</td>
<td>3</td>
<td>18 ± 4</td>
<td>14 ± 4</td>
<td>77 ± 11</td>
<td>92 ± 10</td>
<td>460 ± 120</td>
<td>13 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>3</td>
<td>25 ± 5</td>
<td>14 ± 7</td>
<td>100 ± 8</td>
<td>110 ± 12</td>
<td>770 ± 28</td>
<td>13 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.250</td>
<td>3</td>
<td>74 ± 13</td>
<td>67 ± 17</td>
<td>85 ± 12</td>
<td>89 ± 14</td>
<td>2,300 ± 720</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>rscu-PA/MA-UK1-3</td>
<td>0.500</td>
<td>2</td>
<td>42 ± 14</td>
<td>31 ± 10</td>
<td>85 ± 2</td>
<td>130 ± 47</td>
<td>1,100 ± 150</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Bolus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rscu-PA/MA-FU1-74</td>
<td>0.20</td>
<td>2</td>
<td>64 ± 6</td>
<td>57 ± 3</td>
<td>130 ± 6</td>
<td>120 ± 30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rscu-PA</td>
<td>0.25</td>
<td>2</td>
<td>20 ± 6</td>
<td>8 ± 5</td>
<td>100 ± 23</td>
<td>81 ± 8</td>
<td>190 ± 270</td>
<td>70 ± 7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>3</td>
<td>58 ± 28</td>
<td>40 ± 23</td>
<td>92 ± 8</td>
<td>70 ± 7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data are mean ± SEM of the number of experiments indicated by n.
*Expressed as mg u-PA equivalent per kg body weight.
†Measured 150 minutes after the start of the infusion.
‡Measured at the end of the 2-hour infusion.
§Mean values of determinations at 1 and 2 hours.

Table 2. Comparative Thrombolytic Potencies and Specific Thrombolytic Activities of rscu-PA/MA-FU1-74 and rscu-PA in Baboons With Femoral Vein Thrombosis

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>z</th>
<th>b</th>
<th>c</th>
<th>n</th>
<th>z</th>
<th>b</th>
<th>c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ex vivo isotope recovery</td>
<td>19</td>
<td>78 ± 25</td>
<td>0.82 ± 0.10</td>
<td>88 ± 7</td>
<td>18</td>
<td>220 ± 22</td>
<td>0.20 ± 0.01</td>
<td>100 ± 0.03</td>
</tr>
<tr>
<td>rscu-PA/MA-FU1-74</td>
<td>12</td>
<td>380 ± 68*</td>
<td>0.19 ± 0.03*</td>
<td>99 ± 17</td>
<td>12</td>
<td>60 ± 13*</td>
<td>1.20 ± 0.17*</td>
<td>87 ± 11</td>
</tr>
<tr>
<td>External gamma counting</td>
<td>19</td>
<td>73 ± 27</td>
<td>0.76 ± 0.13</td>
<td>79 ± 8</td>
<td>18</td>
<td>220 ± 7</td>
<td>0.20 ± 0.003</td>
<td>100 ± 0.01</td>
</tr>
<tr>
<td>rscu-PA/MA-FU1-74</td>
<td>12</td>
<td>410 ± 76*</td>
<td>0.21 ± 0.02*</td>
<td>99 ± 10</td>
<td>12</td>
<td>60 ± 13*</td>
<td>1.20 ± 0.17*</td>
<td>86 ± 11</td>
</tr>
</tbody>
</table>

The data represent mean ± SEM; n indicates the number of data points used for curve fitting.
*P < .001 v rscu-PA.
thrombolytic agents or heparin were 4.0 ± 0.4 minutes (mean ± SEM, n = 15). In animals infused with rscu-PA/MA-FU1-74, bleeding times at the end of the 2-hour infusion were twofold to threefold prolonged: values of 8.5 ± 1.5, 8.0 ± 1.0, and 11 ± 2.8 minutes were measured at doses of 0.063, 0.125, and 0.25 mg u-PA equivalent administered per kg body weight, respectively. However, 4 hours after the start of the infusion, the bleeding times were shortened to 6.7 ± 3.2, 4.8 ± 2.1, and 6.2 ± 1.9 minutes in animals given 0.063, 0.125, or 0.25 mg u-PA equivalent per kg, respectively. Immediately after bolus injection of rscu-PA/MA-FU1-74 at a dose of 0.2 mg u-PA equivalent per kg, the bleeding time was markedly prolonged to 48 ± 13 minutes (mean ± SEM, n = 2) and then decreased to 12 ± 2.8 minutes and to 8.8 ± 4.3 minutes at 2 and 4 hours after the injection, respectively.

**Pharmacokinetics.** Steady-state plasma concentrations of u-PA–related antigen obtained during infusion of the compounds increased proportionally with the infusion rate, ranging from 120 ± 17 to 420 ± 110 ng/mL for doses of rscu-PA between 0.5 and 2.0 mg/kg, and from 460 ± 120 to 2,300 ± 720 ng/mL for doses of rscu-PA/MA-FU1-74 between 0.063 and 0.25 mg u-PA equivalent per kg (Table 1). From these steady-state plasma levels, plasma clearances of 360 ± 42 to 530 ± 110 mL/min were calculated for rscu-PA and of 11 ± 3 to 13 ± 2 mL/min for rscu-PA/MA-FU1-74. Steady-state plasma levels of u-PA–related antigen after infusion of 0.50 mg u-PA equivalent per kg of rscu-PA/MA-UK1-3, were 1,100 ± 150 ng/mL, corresponding to a plasma clearance of 47 ± 4 mL/min.

The disappearance rates from plasma of rscu-PA, rscu-PA/MA-FU1-74, and rscu-PA/MA-UK1-3 after the end of the infusion, as determined from u-PA–related antigen measurements, were biphasic (Fig 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. The pharmacokinetic parameters of the disposition of rscu-PA, rscu-PA/MA-FU1-74, and rscu-PA/MA-UK1-3 from plasma after the end of a 2-hour continuous infusion, derived from the exponents α and β and the variables R and S, are summarized in Table 3. Results were (1) for rscu-PA: initial half-life (t1/2α) = 2.5 to 4.0 minutes; plasma clearance (Clp) = 320 to 470 mL/min; (2) for rscu-PA/MA-FU1-74: t1/2α = 12 to 16 minutes and Clp = 9 to 11 mL/min; (3) for rscu-PA/MA-UK1-3: t1/2α = 13 minutes and Clp = 37 mL/min. The pharmacokinetic parameters of the disposition of rscu-PA/MA-FU1-74 complex (determined as mouse IgG-related antigen) from plasma after the end of a 2-hour continuous infusion, derived from the exponents α and β and the variables R and S, are summarized in Table 3. Results were (1) for rscu-PA/MA-FU1-74: t1/2α = 9 to 12 minutes and Clp = 13 to 14 mL/min; (2) for rscu-PA/MA-UK1-3: t1/2α = 12 minutes and Clp = 42 mL/min (data not shown). The pharmacokinetic parameters of the disposition of rscu-PA and rscu-PA/MA-FU1-74 from blood following bolus injections were similar to those obtained after infusion, as determined from the disappearance of both u-PA–related antigen (Table 3) and of mouse IgG-related antigen (data not shown).

**Fig 3.** Dose-response curves of femoral vein clot lysis. Lysis (in percent) measured by ex vivo isotope recovery 30 minutes after the end of the infusion is plotted (A) against the dose (in mg u-PA equivalent of compound administered per kg body weight) or (B) against the steady-state plasma antigen level (in μg u-PA–related antigen per mL). (□) rscu-PA, (●) rscu-PA/MA-FU1-74. The data represent mean ± SEM of the data reported in Table 1.

(60% ± 13% v 220% ± 22% lysis per μg/mL steady-state u-PA–related antigen level in plasma [P < .001]) and a higher β value (1.2 ± 0.17 v 0.20 ± 0.01 μg/mL steady-state u-PA–related antigen level [P < .001]). Bolus injection of rscu-PA/MA-FU1-74 at a dose of 0.2 mg u-PA equivalent per kg body weight yielded a similar extent of clot lysis as bolus injection of 1 mg/kg rscu-PA (Table 1).

Template bleeding times before the administration of
Fig 4. Plasma disappearance rate of u-PA-related antigen after the end of the infusion of rscu-PA, rscu-PA/MA-FU1-74, or rscu-PA/MA-UK1-3. Data are mean ± SEM, expressed as a fraction of the first sample, to allow pooling of data obtained with different doses, as summarized in Table 3. (■) rscu-PA, (○) rscu-PA/MA-FU1-74, (▲) rscu-PA/MA-UK1-3.

Induction of anti-MA-FU1-74 antibodies. The titers of anti-MA-FU1-74 IgG and IgM were determined at baseline and 1, 2, and 3 weeks after the administration of MA-FU1-74 at doses of 0.24, 0.47, and 0.94 mg/kg in five baboons, as the highest dilution which produced an absorbance of 0.2 at 492 nm, over a baseline value 0.05 or less. Results expressed by antibody titer (4th dil, mean ± SEM) were 1.0 ± 0.2, 2.1 ± 0.7, 7.6 ± 0.4, and 7.5 ± 0.4 for IgG, and 1.0 ± 0.2, 2.0 ± 0.3, 6.1 ± 0.2, and 5.4 ± 0.2 for IgM. The anti-MA-FU1-74 titer apparently did not correlate with the dose of MA-FU1-74 that was administered.

DISCUSSION

The efficacy and fibrin-specificity of plasminogen activators may be improved by targeting of the agents to a fibrin clot by conjugation with monoclonal antibodies which are fibrin-specific and which cross-react poorly with fibrinogen. Monoclonal antibodies that have been used for this purpose include antibodies against the β-chain of fibrin and against cross-linked fibrin fragment D-dimer. These antibodies have been conjugated with t-PA and/or with scu-PA or tcu-PA, using either chemical coupling procedures or recombinant DNA technology. The resulting conjugates have been shown to have a significantly higher fibrinolytic potency than the unconjugated plasminogen activators, both in human plasma in vitro and in animal models of venous thrombosis. Antibody targeting thus appears to have the potential to enhance the potency of thrombolytic agents. Alternatively, bispecific monoclonal antibodies containing one site that recognizes the thrombus and one site that binds the plasminogen activator may be used to concentrate the thrombolytic agent at the surface of thrombus.
a thrombus. Bispecific monoclonal antibodies directed both against t-PA and against the β-chain of human fibrin have been produced using the hybrid hybridoma technique. Immunoconjugates of t-PA with such a bispecific monoclonal antibody were shown to have a marginally enhanced thrombolytic potency in a rabbit jugular vein thrombosis model.

In the present study, we have evaluated the thrombolytic and pharmacokinetic properties of immunoconjugates of rsu-PA with MA-FU1-74, a bispecific monoclonal antibody directed against both scu-PA and the β-chain of human fibrin, in baboons with femoral vein thrombosis. In an in vitro plasma clot lysis assay, MA-FU1-74 enhanced the fibrinolytic activity of rsu-PA toward both clotted baboon plasma and human plasma by approximately 20-fold. In contrast, MA-UK1-3, the parental anti-u-PA monoclonal antibody, had no effect on the fibrinolytic activity of rsu-PA (data not shown). MA-FU1-74 had no effect on the fibrinolytic activity of t-PA in the same system (data not shown). Furthermore, MA-FU1-74, which does not cross-react with rat fibrin, did not enhance the fibrinolytic activity of rsu-PA toward clotted rat plasma. These findings suggest that the enhancement of the fibrinolytic activity of rsu-PA with MA-FU1-74 is mediated by specific interaction of the antibody with both fibrin and rsu-PA.

The close similarity of the results obtained with human and baboon plasma in vitro suggests that the baboon is a suitable species to evaluate the targeting effect of rsu-PA with MA-FU1-74. The thrombolytic potency of immunoconjugates of rsu-PA with MA-FU1-74 was therefore investigated in baboons with a [125I]fibrin-labeled autologous blood clot produced in the femoral vein. This model allows continuous monitoring of clot lysis by external isotope scanning and determination of the extent of thrombolysis both by external isotope scanning and by ex vivo recovery of the residual clot.

Preincubation of rsu-PA with MA-FU1-74 at a 1.2-fold molar excess resulted in an increase of the thrombolytic potency (percent lysis per unit dose) of rsu-PA, as shown by both an increased maximal rate of lysis (38% ± 6%) lysis per mg u-PA equivalent administered per kg for the complex v 78% ± 25% per mg u-PA equivalent per kg for rsu-PA, as determined by ex vivo isotope recovery) and by a reduction of the dose at which the maximal rate of clot lysis occurs (0.19 ± 0.03 mg u-PA equivalent per kg for complex v 0.82 ± 0.10 mg u-PA equivalent per kg for rsu-PA). Thus, the thrombolytic potency of the complex of rsu-PA with MA-FU1-74 is enhanced approximately fivefold as compared with that of unconjugated rsu-PA. However, the specific thrombolytic activity (percent lysis per unit steady-state plasma level of u-PA-related antigen) was approximately fourfold lower for rsu-PA/MA-FU1-74 than for rsu-PA, as shown by both a lower maximal rate of lysis and a higher plasma antigen level at which maximal lysis is achieved. On bolus injection of rsu-PA/MA-FU1-74 at a dose of 0.2 mg u-PA equivalent per kg body weight, 64% ± 6% clot lysis was obtained, as determined by ex vivo isotope recovery, as compared with 58% ± 28% clot lysis with rsu-PA at a dose of 1 mg u-PA equivalent per kg.

Pharmacokinetic analysis indicated that the plasma clearance of the immunoconjugate of rsu-PA with both MA-FU1-74 and MA-UK1-3 is 10-30 fold decreased. The plasma half-life and the plasma clearance calculated from the disappearance rate of u-PA-related antigen and of IgG-related antigen from plasma were comparable, indicating that u-PA antigen occurs as a complex with the bispecific monoclonal antibodies and that no significant dissociation of the immunoconjugates occurs in vivo.

MA-UK1-3, the parental anti-u-PA monoclonal antibody, did not affect the thrombolytic potency of rsu-PA, although the plasma clearance of this immunoconjugate was also significantly reduced. This is probably due to a decreased specific thrombolytic activity of the immunoconjugate (as also shown for rsu-PA/MA-FU1-74), which is compensated for by the prolonged half-life, but no additional effect of fibrin-targeting is observed. Taken together, these results suggest that the thrombolytic potency of the immunoconjugate of rsu-PA with MA-FU1-74 is the result of a targeting effect of the activator to fibrin and of a reduced plasma clearance of the immunoconjugate. The fivefold enhanced in vivo thrombolytic potency of the immunoconjugate is less pronounced than the 20-fold enhanced fibrinolytic activity in human plasma in vitro (Fig 1). In these in vitro experiments, plasma clots were formed in the presence of the immunoconjugate. Surprisingly, experiments with preformed plasma clots reported elsewhere did not show an enhanced fibrinolytic activity of the rsu-PA/MA-FU1-74 immunoconjugate over rsu-PA, indicating that the static system with preformed plasma clots is not adequate to demonstrate fibrin-targeting by bispecific monoclonal antibodies.

Bleeding times in the groups treated with rsu-PA/MA-FU1-74 were moderately prolonged, but marked fibrinogen or α2-antiplasmin depletion at the end of the infusions or 2 hours after the bolus injections was not observed. Furthermore, bleeding times normalized within 4 hours after drug administration. Two weeks after the administration of the murine antibody, anti-mouse IgG baboon antibody titers were significantly increased. Although these antibody titers are relative values obtained as dilutions producing an increase of absorbance at 492 nm of 0.2 over a background of 0.05 or less, these findings are indicative of consistent immunogenicity following a single intravenous administration.

In conclusion, a bispecific monoclonal antibody directed against both rsu-PA and against the β-chain of human fibrin enhanced the thrombolytic potency of rsu-PA fivefold in baboons with autologous clots in the femoral vein. The use of such immunoconjugates for thrombolytic therapy by bolus administration in patients with thromboembolic disease is promising, although it will require reduction of the immunogenicity, possibly by "humanization" of the murine antibody.

ACKNOWLEDGMENTS

Y. Imura, T. Kurokawa, and S. Iwasa thank Dr Atsushi Kakinuma and Dr Masao Nishikawa for their support and encouragement throughout this work, and Akiko Watanabe for skillful technical assistance.
REFERENCES

Thrombolytic and pharmacokinetic properties of an immunoconjugate of single-chain urokinase-type plasminogen activator (u-PA) and a bispecific monoclonal antibody against fibrin and against u-PA in baboons

Y Imura, JM Stassen, T Kurokawa, S Iwasa, HR Lijnen and D Collen