The Mechanism of Plasminogen Activation and Fibrin Dissolution by Single Chain Urokinase-Type Plasminogen Activator in a Plasma Milieu In Vitro

By H.R. Lijnen, B. Van Hoef, F. De Cock, and D. Collen

The relative contribution of several mechanisms to plasminogen activation and fibrin dissolution by urokinase-type plasminogen activator (u-PA) in vitro was quantitated. The activation of plasminogen by recombinant single chain u-PA (rscu-PA), by its two chain derivative (rtcu-PA) and by a plasmin-resistant mutant, rscu-PA-Glu158, obeys Michaelis-Menten kinetics with catalytic efficiencies of 0.00064, 0.046, and 0.00005 L/mmol-s for native plasminogen (Glu-plasminogen) and of 0.0061, 1.21, and 0.0004 L/mmol-s for partially degraded plasminogen (Lys-plasminogen). In a purified system consisting of a fibrin clot submersed in a plasminogen solution, the equi-effective doses (50% lysis in one hour) for rscu-PA, rtcu-PA, and rscu-PA-Glu158 were 16, 6.5, and 32,000 ng/mL for Glu-plasminogen and two- to fourfold lower for Lys-plasminogen. In a plasma milieu, 50% lysis in two hours was obtained for a plasma clot with 2.1 μg/mL rscu-PA, 0.5 μg/mL rtcu-PA, and >200 μg/mL rscu-PA-Glu158 and for a purified fibrin clot with 1.3 μg/mL rscu-PA and 0.27 μg/mL rtcu-PA. After predigestion of a purified fibrin clot with plasmin, the apparent potency of rscu-PA and rtcu-PA increased by 40% and 20%, respectively. In conclusion, rscu-PA has an intrinsic plasminogen activating potential that is only about 1% of that of rtcu-PA and that is 13 times higher than that of rscu-PA-Glu158. Conformational transition of Glu-plasminogen to Lys-plasminogen enhances its sensitivity to activation by all u-PA moieties ten- to 20-fold. Predigestion of fibrin clots with associated increased binding of plasminogen results in a minor apparent increase of the fibrinolytic potency of rscu-PA and rtcu-PA. The relative fibrinolytic potency of rtcu-PA is two to three orders of magnitude higher than that of rscu-PA-Glu158 but only two- to five-fold higher than that of rscu-PA, both in purified systems and in a plasma milieu. These results indicate that conversion of rscu-PA to rtcu-PA constitutes the primary mechanism of fibrin dissolution.

© 1989 by Grune & Stratton, Inc.

UROKINASE-TYPE plasminogen activator (u-PA) can be obtained as a single-chain molecule (single chain u-PA, scu-PA) or as a two-chain derivative (tcu-PA, urokinase) generated by specific cleavage of the Lys18-Ile19 peptide bond by plasmin (see reference 1). Both in animal models of thrombosis and in patients with acute myocardial infarction, scu-PA was found to be a more fibrin-specific thrombolytic agent than tcu-PA (see reference 2). Initial results of therapeutic thrombolysis with scu-PA in patients with acute myocardial infarction suggest, however, that the fibrin-specificity of scu-PA in humans is not as pronounced as in animals.3

Several hypotheses for the mechanism of plasminogen activation and the fibrin-specificity of clot lysis with scu-PA in a plasma milieu have been proposed. One hypothesis claimed that scu-PA has intrinsic plasminogen activating potential that is counteracted by a competitive inhibitory mechanism in plasma, which is reversed by fibrin.4 Alternatively, scu-PA was claimed to be inactive toward circulating plasminogen but active toward conformationally altered plasminogen bound to partially digested fibrin.5 Third, scu-PA has been proposed to be a genuine proenzyme with negligible activity toward plasminogen.6,7 and fibrinolysis with scu-PA would thus entirely depend on generation of tcu-PA. The original hypothesis that scu-PA would exert its fibrin-specific fibrinolytic action via binding to fibrin4 has been abandoned.5,8

Several methodological difficulties have hampered the quantitative investigation of these alternative hypotheses and the interpretation of results. These include: (a) variability in kinetic properties of scu-PA obtained from different sources,9,10 (b) different sensitivity to activation of alternative molecular or conformational forms of plasminogen (Glu-plasminogen–like or Lys-plasminogen–like),11–14 (c) efficient conversion of scu-PA to tcu-PA by generated plasmin, which is associated with a marked increase in catalytic efficiency toward plasminogen,15,16 and (d) positive feedback of partial degradation of fibrin on the activation of plasminogen.17 In addition, dose-response curves of clot lysis with scu-PA in a plasma milieu are strongly nonlinear,18 which invalidates linear extrapolation of potencies derived from clot lysis v time curves.19

In the present study we have reinvestigated the mechanism of plasminogen activation and fibrin-specificity of scu-PA in in vitro systems, by direct comparison of the relative catalytic efficiency and fibrinolytic potency of rscu-PA, rtcu-PA, and rscu-PA-Glu158 with the use of quantitative dose-effect relations. In addition the effect on the potency of rscu-PA and rtcu-PA of the conformation of plasminogen, of the presence of plasma proteins, or of partial fibrin digestion was determined.

MATERIALS AND METHODS

**Proteins and Reagents**

rscu-PA and rscu-PA-Glu158 (obtained by site-directed mutagenesis of Lys158 to Glu) were prepared and characterized as described elsewhere.13 Before use, these proteins were chromatographed on benzamidine-Sepharose in order to remove traces of two chain derivatives. Protein concentrations were determined by amino acid analysis or with an ELISA specific for u-PA.20 rtcu-PA was prepared by treatment of rscu-PA with plasmin and rscu-PA-Glu158 by treatment of rscu-PA-Glu158 with Endoproteinase Glu-C, followed by chromatography on benzamidine-Sepharose, as de-
concentration, MECHANISM OF ACTION OF scu-PA

rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58

Kinetics of the Activation of Plasminogen (Glu-plasminogen or Lys-plasminogen) by rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58

Kinetic analysis of the activation of Glu-plasminogen (final concentration, 10 to 100 nmol/L) or Lys-plasminogen (final concentration, 2 to 12 nmol/L) by rscu-PA-Glu'58 (final concentration, 50 nmol/L) was performed 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. The extent of fibrin digestion was measured at different time intervals (zero to 20 minutes) and quantitated by the release of radioactivity from the clot, corrected for background radioactivity. Fibrinogen was neutralized by incubation of the clot in 1 mL normal human plasma for 30 minutes, at 37°C, which abolished further release of radioactivity. Generation of new COOH-terminal amino acids following plasmin predigestion of fibrin clots was determined as follows. Ten unlabeled fibrin clots (with or without predigestion) were suspended in 1.5 mL 0.2 mol/L N-ethylmorpholine-acetate buffer, pH 8.0, and treated with 5.0 U carboxypeptidase D (specific activity 150 U/mg; Boehringer Mannheim, FRG) for one hour at room temperature. Samples of the supernatant (300 μL) were removed before and 15 to 60 minutes after addition of enzyme, heated to 100°C for five minutes and treated with cold 20% CCl4COOH with addition of norleucine as internal standard to calculate the recovery of amino acids. Samples were dried and used for amino acid analysis (Beckman 119 CL amino acid analyzer).

Fibrinolytic Potency of rscu-PA, rtcu-PA, rscu-PA-Glu'58, and rtcu-PA-Glu'58

In purified systems. 125I-labeled clots of purified human fibrinogen were prepared by addition of CaCl2 (final concentration, 50 mmol/L) and thrombin (final concentration, 3 NIH U/mL) to purified fibrinogen (final concentration, 3 mg/mL) containing approximately 250,000 cpm/mL of 125I-labeled fibrinogen in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 mol/L NaCl. After incubation at 37°C for 60 minutes in silicon tubing (internal diameter 4 mm), pieces of about 1.0 cm length were cut off and the fibrin clots were extensively washed in 0.15 mol/L NaCl. Purified 125I-labeled fibrin clots were then incubated at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80, 0.038 mol/L NaCl, 1 KIU/mL aprotinin and 1.5 μmol/L Glu-plasminogen or Lys-plasminogen, and lysis of fibrin clots by rscu-PA, rtcu-PA, rscu-PA-Glu'58, or rtcu-PA-Glu'58 was monitored for one hour at 15-minute intervals and quantitated by the release of radioactivity from the clot into the surrounding liquid. The concentration of u-PA yielding 50% lysis in one hour was determined from plots of the extent of clot lysis (in percent) against the concentration of the plasminogen activators.

In human plasma. 125I-labeled plasma clots were prepared as described above, following addition to normal human plasma of 500,000 cpm/mL of 125I-labeled fibrinogen, and coagulation with CaCl2 (final concentration, 25 mmol/L), and thrombin (final concentration, 2 NIH U/mL). Lysis of 125I-labeled plasma clots by rscu-PA (final concentration, 0 to 5.0 μg/mL), rtcu-PA (final concentration, 0 to 2.0 μg/mL), rscu-PA-Glu'58 (final concentration, 0 to 200 μg/mL), or rtcu-PA-Glu'58 (final concentration, 0 to 1.0 μg/mL) was measured over four hours as previously described, using normal human plasma or plasminogen-depleted plasma reconstituted with 1.5 μmol/L Glu-plasminogen or Lys-plasminogen. The concentration of the u-PA moiety, required to obtain 50% clot lysis in two hours, was determined from plots of percent lysis vs the concentration of the u-PA moiety.

Predigestion of fibrin clots. Purified 125I-labeled fibrin clots were prepared as described above (125 μL volume with 375 μg fibrinogen), and incubated in 1 mL 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.01% Tween 80 and 0.038 mol/L NaCl. The clots were predigested with human plasmin (0.6 μg/mL, final concentration) for 30 minutes at 37°C. The extent of fibrin digestion was calculated from the radioactivity released from the clot, corrected for the background radioactivity. Plasmin was neutralized by incubation of the clot in 1 mL normal human plasma for 30 minutes, at 37°C, which abolished further release of radioactivity. Generation of new COOH-terminal amino acids following plasmin predigestion of fibrin clots was determined as follows. Ten unlabeled fibrin clots (with or without predigestion) were suspended in 1.5 mL 0.2 mol/L N-ethylmorpholine-acetate buffer, pH 8.0, and treated with 5.0 U carboxypeptidase D (specific activity 150 U/mg; Boehringer Mannheim, FRG) for one hour at room temperature. Samples of the supernatant (300 μL) were removed before and 15 to 60 minutes after addition of enzyme, heated to 100°C for five minutes and treated with cold 20% CCl4COOH with addition of norleucine as internal standard to calculate the recovery of amino acids. Samples were dried and used for amino acid analysis (Beckman 119 CL amino acid analyzer).

Lysis of normal or predigested clots following addition of rscu-PA, rtcu-PA, rscu-PA-Glu'58, or rtcu-PA-Glu'58 (range, 0 to 2.0 μg/mL) or t-PA (range, 0 to 1.6 μg/mL), or t-PA (range, 0 to 0.80 μg/mL) was then monitored over four hours as described. The concentration of plasminogen activator required to obtain 50% clot lysis in two hours was then determined from plots of percent lysis at two hours vs the concentration of plasminogen activator. The effect of predigestion on lysis of fibrin clots by these equi-effective concentrations of the enzymes was then investigated using intact and predigested clots.

Binding of plasminogen to normal or predigested purified unlabeled fibrin clots was determined by incubation for one hour at 37°C in normal human plasma containing traces of 125I-labeled Glu-plasminogen. Bound plasminogen was quantitated from the radioactivity associated with the clots after extensive washing in buffer.

Purified 125I-labeled fibrin clots, with or without predigestion,
Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu\textsuperscript{58}, and rtcu-PA-Glu\textsuperscript{58} in the Absence of Fibrin

In purified systems. Activation of Glu-plasminogen or Lys-plasminogen (final concentration, 1.5 \mu mol/L) at 37°C in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl, by rscu-PA (seven concentrations; range, 0 to 0.5 \mu g/mL) for Glu-plasminogen and 0 to 0.1 \mu g/mL for Lys-plasminogen or rtcu-PA (seven concentrations; range, 0 to 0.5 \mu g/mL) for Glu-plasminogen and 0 to 0.5 \mu g/mL for Lys-plasminogen, was monitored during one hour by measuring residual plasminogen, after conversion to plasminogen-streptokinase complex. At 15-minute intervals, 5 \mu L samples were removed from the incubation mixtures, incubated for ten minutes at 37°C in 300 \mu L 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80, and 1,000 lU of streptokinase. Under these conditions, plasminogen was measured as plasminogen-streptokinase complex.

In addition, activation of Glu-plasminogen or Lys-plasminogen (final concentration, 1.5 \mu mol/L) by rscu-PA-Glu\textsuperscript{58} (seven concentrations; range, 0 to 200 \mu g/mL) for both Glu-plasminogen or Lys-plasminogen or rtcu-PA-Glu\textsuperscript{58} (seven concentrations; range, 0 to 1.0 \mu g/mL) for Glu-plasminogen and 0 to 0.13 \mu g/mL for Lys-plasminogen) was monitored as described above. The concentration of u-PA moieties required to obtain 50\% plasminogen activation in one hour was then determined graphically from plots of plasminogen activation \( v \) the concentration of plasminogen activator.

In plasma. Activation of plasminogen at 37°C in normal human plasma or in plasminogen-depleted plasma reconstituted with Glu-plasminogen or Lys-plasminogen, by rscu-PA (seven concentrations; range, 0 to 5.0 \mu g/mL) or rtcu-PA (seven concentrations; range, 0 to 2.0 \mu g/mL) was monitored over four hours at hourly intervals, as described above. Activation of plasminogen by rscu-PA-Glu\textsuperscript{58} in normal human plasma or in plasminogen-depleted plasma reconstituted with Glu-plasminogen or Lys-plasminogen (seven concentrations; range, 0 to 200 \mu g/mL) or by rtcu-PA-Glu\textsuperscript{58} (seven concentrations; range, 0 to 1.0 \mu g/mL) was also evaluated as described above. The concentration of u-PA required to obtain 50\% plasminogen activation in two hours was determined graphically from dose-response curves of plasminogen activation \( v \) u-PA concentration.

Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu\textsuperscript{58}, and rtcu-PA-Glu\textsuperscript{58} in Fibrin Clot Lysis Systems

Plasminogen activation during fibrin clot lysis with these u-PA moieties was monitored both in purified systems and in human plasma by quantitation of residual plasminogen as plasminogen-streptokinase complex. In addition, the extent of plasminogen activation caused by u-PA concentrations that induced 50\% clot lysis in one hour in purified systems or in two hours in plasma was determined both in the presence and in the absence of clots.

Plasminogen Activation With rscu-PA in the Presence of \( \alpha_2\)-Antiplasmin

Activation of Glu-plasminogen by rscu-PA was measured in the presence and the absence of \( \alpha_2\)-antiplasmin. Therefore, Glu-plasminogen (1.5 \mu mol/L) was added to incubation mixtures (0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80 at 37°C), containing \( \alpha_2\)-antiplasmin (0 to 7.5 \mu mol/L). The mixture was activated with rscu-PA (230 ng/mL) and residual plasminogen was measured as plasminogen-streptokinase complex.

Control experiments on the prevention of conversion of scu-PA to tcu-PA in this experimental system were performed by preincubation of rscu-PA (final concentration, 2.5 \mu g/mL) at 4°C for 20 hours with \( \alpha_2\)-antiplasmin (final concentration, 1.6 \mu mol/L) before addition of Glu-plasminogen (final concentration, 1.5 \mu mol/L). Under the latter conditions, added rtcu-PA up to 20\% of the concentration of rscu-PA, is completely inhibited (as measured either with S-2444 or via plasminogen activation in the same system), while rscu-PA incubated in buffer fully retained its plasminogen activating activity.

Effect of Plasma on Clot Lysis by u-PA

Clot lysis in purified systems with Glu-plasminogen (1.5 \mu mol/L) using concentrations of rscu-PA (20 ng/mL) or rtcu-PA (10 ng/mL) yielding about 50\% clot lysis in one hour was monitored in the absence or the presence of increasing amounts of plasma depleted in both plasminogen and \( \alpha_2\)-antiplasmin (0 to 40\%, vol/vol) or of this depleted plasma reconstituted with purified \( \alpha_2\)-antiplasmin to a concentration of 1 \mu mol/L. For each amount of added plasma, the apparent residual concentration of rscu-PA or rtcu-PA was expressed as percent of the total concentration, using a calibration curve constructed in the absence of depleted plasma.

Plasma depleted both in plasminogen and \( \alpha_2\)-antiplasmin was passed three times over a column of insolubilized rscu-PA (0.5 mL plasma/mg rscu-PA). After each chromatography, the column was eluted with 1.6 mol/L KSCN and the pooled eluates were dialyzed against 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80. The effect of the plasma on clot lysis in purified systems was then monitored as described above.

RESULTS

Kinetics of the Activation of Plasminogen (Glu-plasminogen or Lys-plasminogen) by rscu-PA, rtcu-PA, rscu-PA-Glu\textsuperscript{58}, and rtcu-PA-Glu\textsuperscript{58}

Activation of both Glu-plasminogen and Lys-plasminogen by all u-PA moieties studied obeyed Michaelis-Menten kinetics as evidenced by linear double reciprocal plots of the initial activation rate \( \gamma \) the plasminogen concentration (not shown). The kinetic constants, obtained by linear regression analysis (\( r \geq 0.995 \) in all cases), are summarized in Table 1. Activation of both Glu-plasminogen and Lys-plasminogen by...
rscu-PA-Glu$^{158}$ occurs with lower affinity (higher $K_a$) than observed with rscu-PA, resulting in a 13- to 15-fold lower catalytic efficiency ($k_2/K_a$). The catalytic efficiency of rscu-PA and rscu-PA-Glu$^{158}$ for the activation of Lys-plasminogen is about tenfold higher than that of Glu-plasminogen. This is apparently due to an increase in $k_2$ for rscu-PA and to a decrease in $K_a$ for rscu-PA-Glu$^{158}$. Activation of plasminogen by the two chain u-PA moieties occurs with a catalytic efficiency that is two to three orders of magnitude higher than that with their single chain precursors: for Glu-plasminogen the ratio is 70-fold for the wild-type forms and 850-fold for the Glu$^{158}$ mutants; for Lys-plasminogen corresponding values are 200-fold for wild-type forms.

Pretreatment of rscu-PA-Glu$^{158}$ with plasmin to convert potential traces of contaminating rscu-PA to rtcu-PA, followed by addition of excess plasmin and tcu-PA inhibitor, did not significantly affect the activation rate of Glu-plasminogen ($k_2/K_a = 3.6 \times 10^{-5}$ or $3.1 \times 10^{-5}$ L/µmol·s before or after plasmin treatment).

**Fibrinolytic Potency of rscu-PA, rtcu-PA, rscu-PA-Glu$^{158}$, and rtcu-PA-Glu$^{158}$**

In purified systems. Addition of u-PA moieties to labeled fibrin clots caused a time- and dose-dependent lysis as quantitated by the release of $^{125}$I-labeled degradation products. Sigmoidal curves similar to those described in detail elsewhere were obtained. Fifty percent lysis in one hour of a purified labeled fibrin clot immersed in a Glu-plasminogen solution was obtained with 6.5 to 7.0 ng/mL of rtcu-PA and rscu-PA-Glu$^{158}$, while this required 16 ng/mL rscu-PA and 32,000 ng/mL rscu-PA-Glu$^{158}$ (Table 2). Two- to fourfold lower concentrations were required for equivalent clot lysis using a Lys-plasminogen solution.

In human plasma. Fifty percent lysis in two hours of a labeled plasma clot immersed in normal plasma was obtained with approximately 0.5 µg/mL of rtcu-PA and rscu-PA-Glu$^{158}$, with 2.1 µg/mL rscu-PA but not with up to 200 µg/mL rscu-PA-Glu$^{158}$. Similar concentrations were required for lysis in plasminogen depleted plasma reconstituted with Glu-plasminogen whereas two- to four-fold lower concentrations were required for depleted plasma reconstituted with Lys-plasminogen (Table 2).

**Predigestion of fibrin clots**. Incubation of purified fibrin clots with plasmin as described under Materials and Methods resulted in 6.9 ± 1.6% (n = 48) predigestion of the clots, as determined from the release of radioactivity.

<table>
<thead>
<tr>
<th>Glu-plasminogen</th>
<th>Lys-plasminogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_a$ (µmol/L)</td>
<td>$k_2$ (s$^{-1}$)</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>rscu-PA</td>
<td>7.1</td>
</tr>
<tr>
<td>rtcu-PA</td>
<td>78</td>
</tr>
<tr>
<td>rscu-PA-Glu$^{158}$</td>
<td>91</td>
</tr>
<tr>
<td>rtcu-PA-Glu$^{158}$</td>
<td>129</td>
</tr>
</tbody>
</table>

**Table 2. Concentration (ng/mL) of u-PA Required to Obtain 50% Clot Lysis**

<table>
<thead>
<tr>
<th>Glu-plasminogen</th>
<th>Lys-plasminogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Reconstituted</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Purified system*</td>
<td></td>
</tr>
<tr>
<td>rscu-PA</td>
<td>16</td>
</tr>
<tr>
<td>rtcu-PA</td>
<td>6.5</td>
</tr>
<tr>
<td>rscu-PA-Glu$^{158}$</td>
<td>32,000</td>
</tr>
<tr>
<td>rtcu-PA-Glu$^{158}$</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasma system†</th>
<th>Reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-plasminogen</td>
<td>Lys-plasminogen</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>rscu-PA</td>
<td>2.100</td>
</tr>
<tr>
<td>rtcu-PA</td>
<td>500</td>
</tr>
<tr>
<td>rscu-PA-Glu$^{158}$</td>
<td>&gt;200,000</td>
</tr>
<tr>
<td>rtcu-PA-Glu$^{158}$</td>
<td>500</td>
</tr>
</tbody>
</table>

* Purified labeled fibrin clots in purified systems, with 1.5 µmol/L Glu-plasminogen or Lys-plasminogen, within one hour.
† Plasma clots labeled with $^{125}$I-fibrin in normal human plasma or in plasminogen-depleted plasma reconstituted with either Glu-plasminogen or Lys-plasminogen, within two hours.
rt-PA, respectively (not shown). On predigestion of fibrin clots before incubation for one hour in normal human plasma, subsequent lysis in buffer induced after one hour by rscu-PA (130 ng/mL), rtcu-PA (70 ng/mL), or rt-PA (120 ng/mL) increased from 46 ± 16% to 80 ± 12% for rscu-PA (n = 10; P < .001), from 39 ± 15% to 73 ± 13% for rtcu-PA (n = 10; P < .001), or from 43 ± 3% to 75 ± 7% for rt-PA (n = 10; P < .001). At lower concentrations of rscu-PA, rtcu-PA, or rt-PA, yielding 5% to 10% lysis of intact fibrin clots in one hour, predigestion of the clots also resulted in comparable relative increases of lysis with all three enzymes (not shown).

**Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu158, and rtcu-PA-Glu158 in the Absence of Fibrin**

*In purified systems.* In the absence of fibrin, Glu-plasminogen or Lys-plasminogen (1.5 μmol/L) in buffer were activated in a concentration-dependent manner by rscu-PA, rtcu-PA, rscu-PA-Glu158, or rtcu-PA-Glu158 (not shown). The concentrations of rtcu-PA required to obtain 50% plasminogen activation in one hour, were about twofold lower than those of rscu-PA (Table 3). Concentrations of rtcu-PA-Glu158 yielding 50% plasminogen activation in one hour are comparable with those of rscu-PA and rtcu-PA (Table 3). With rscu-PA-Glu158, 50% activation was obtained with 65 μg/mL in the presence of Lys-plasminogen, whereas with Glu-plasminogen, 50% activation was not reached with 200 μg/mL. Fifty percent activation of Lys-plasminogen was obtained with 10- to 15-fold lower concentrations for all u-PA moieties, as compared with Glu-plasminogen.

*In human plasma.* In the absence of fibrin, 50% activation of plasminogen was obtained with 3.7 μg/mL of rscu-PA and with 0.4 μg/mL of rtcu-PA both in normal plasma and in plasminogen-depleted plasma reconstituted with Glu-plasminogen and with seven- to tenfold lower concentration of rscu-PA or rtcu-PA in plasminogen-depleted plasma reconstituted with Lys-plasminogen (Table 3). Equi-effective plasminogen activation (50% within two hours) was obtained with rtcu-PA-Glu158 at comparable concentrations as with rtcu-PA. A concentration of 200 μg/mL rscu-PA-Glu158 caused 46% plasminogen activation in two hours in plasma reconstituted with Lys-plasminogen but only 13% or 11% in normal plasma or plasma reconstituted with Glu-plasminogen (not shown).

**Plasminogen Activation by rscu-PA, rtcu-PA, rscu-PA-Glu158, and rtcu-PA-Glu158 in Fibrin Clot Lysis Systems**

*In purified systems.* In the presence of fibrin, 50% plasminogen activation in one hour was obtained with 85 ng of rscu-PA in a Glu-plasminogen solution and with 20 ng/mL in a Lys-plasminogen solution (Table 3). Equi-effective concentrations (50% plasminogen activation) of rtcu-PA and rtcu-PA-Glu158 are comparable for both Glu-plasminogen (50 and 68 ng/mL) and Lys-plasminogen (14 and 35 ng/mL), while rscu-PA-Glu158 is up to 2,000-fold less efficient. Concentrations of rscu-PA or rtcu-PA required for 50% lysis in one hour of fibrin clots immersed in Glu-plasminogen or Lys-plasminogen solutions caused <5% plasminogen activation.

*In human plasma.* In the presence of a fibrin clot, 50% plasminogen activation in two hours in normal plasma or in plasminogen-depleted plasma reconstituted with Glu-plasminogen is obtained with 3.3 μg/mL of rscu-PA whereas 50% activation in depleted plasma reconstituted with Lys-plasminogen requires sevenfold less rtcu-PA. Plasminogen activation with rtcu-PA and rtcu-PA-Glu158 occurs at three- to tenfold lower concentrations (Table 3). Only 10% to 15% plasminogen activation is obtained with 200 μg/mL rscu-PA-Glu158 in normal plasma or plasma reconstituted with Glu-plasminogen as compared with 45% in plasma reconstituted with Lys-plasminogen.

The concentrations of rscu-PA required for 50% clot lysis in normal plasma in two hours, cause about 25% plasminogen activation both in the presence or the absence of fibrin (not shown).

### Table 3. Activation of Glu-plasminogen and Lys-plasminogen by Different u-PA Moieties in a Purified System and in Plasma

<table>
<thead>
<tr>
<th></th>
<th>Glu-plg</th>
<th>Lys-plg</th>
<th>Normal</th>
<th>Glu-plg</th>
<th>Lys-plg</th>
<th>Reconstituted</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Without fibrin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rscu-PA</td>
<td>210</td>
<td>15</td>
<td>3,700</td>
<td>3,700</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>rtcu-PA</td>
<td>110</td>
<td>9</td>
<td>400</td>
<td>360</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>rscu-PA-Glu158</td>
<td>&gt;200,000</td>
<td>65,000</td>
<td>&gt;200,000</td>
<td>&gt;200,000</td>
<td>220,000</td>
<td></td>
</tr>
<tr>
<td>rtcu-PA-Glu158</td>
<td>240</td>
<td>25</td>
<td>500</td>
<td>550</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td><strong>With fibrin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rscu-PA</td>
<td>85</td>
<td>20</td>
<td>3,300</td>
<td>3,300</td>
<td>440</td>
<td></td>
</tr>
<tr>
<td>rtcu-PA</td>
<td>50</td>
<td>14</td>
<td>900</td>
<td>700</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>rscu-PA-Glu158</td>
<td>180,000</td>
<td>60,000</td>
<td>&gt;200,000</td>
<td>&gt;200,000</td>
<td>210,000</td>
<td></td>
</tr>
<tr>
<td>rtcu-PA-Glu158</td>
<td>68</td>
<td>35</td>
<td>600</td>
<td>750</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: Glu-plg, Glu-plasminogen; Lys-plg, Lys-plasminogen.

The data represent concentration (ng/mL) of u-PA required to obtain 50% plasminogen activation, in purified systems within one hour with 1.5 μmol/L Glu-plasminogen or Lys-plasminogen, or within two hours in normal plasma or plasminogen-depleted plasma reconstituted with either Glu-plasminogen or Lys-plasminogen.
Plasminogen Activation With rscu-PA in the Presence of α2-Antiplasmin

rscu-PA caused a time- and concentration-dependent activation of Glu-plasminogen both in the absence and the presence of α2-antiplasmin (Fig 1). In the presence of α2-antiplasmin (both at 1.5 and 7.5 μmol/L), plasminogen activation was significantly reduced, probably as a consequence of plasmin inhibition and prevention of the conversion of rscu-PA to rtcu-PA. Overnight preincubation at 4°C of rscu-PA with α2-antiplasmin, before addition of Glu-plasminogen, did not influence subsequent plasminogen activation, whereas such preincubation with rtcu-PA completely abolished its fibrinolytic activity (not shown). Direct measurement of plasmin activity with S-2251 revealed that the plasmin concentration in the incubation mixtures with α2-antiplasmin remained below the detection limit corresponding to conversion of 0.1% of plasminogen to plasmin.

Effect of Plasma on Clot Lysis by u-PA

Figure 2A shows that lysis by rscu-PA and rtcu-PA of purified fibrin clots immersed in Glu-plasminogen was inhibited in a concentration-dependent way by addition of plasma depleted in both plasminogen and α2-antiplasmin. When the
apparent residual enzyme activity was plotted against the volume fraction of added plasma (Fig 2B), it appeared that rscu-PA is inhibited to 50% in the presence of about 2.5% (vol/vol) of depleted plasma. For rtcu-PA, 50% inhibition required about 40% depleted plasma. When the depleted plasma was reconstituted with α2-antiplasmin, fibrinolytic activity toward rscu-PA, in in vitro systems and with the use of quantitative dose-effect curves, in order to delineate the relative contribution of several pathways to the fibrinolytic potency and fibrin-specificity of rscu-PA. Our results confirm and extend previous observations from several laboratories that rscu-PA has some intrinsic plasminogen activating potential, that conversion of rscu-PA to rtcu-PA results in a markedly increased catalytic efficiency, that conformationally altered plasminogen (Lys-plasminogen) is more susceptible to activation than native plasminogen (Glu-plasminogen), that plasma exerts an inhibitory action on the fibrinolytic potency of rscu-PA, and that predigestion of fibrin renders it more susceptible to lysis.

Recombinant scu-PA, expressed in Chinese hamster ovary cells, appears to have an intrinsic plasminogen activation potential with a catalytic efficiency that is approximating 1% of that of its two chain derivative, rtcu-PA. This is confirmed by the intrinsic enzymatic activity of the cleavage-resistant rscu-PA-GluL158 although this mutant has a catalytic efficiency that is about 15-fold lower than that of wild-type rscu-PA. This intrinsic plasminogen activating activity of rscu-PA-GluL158 is maintained after removal of potential trace contamination of rscu-PA and rtcu-PA. In test systems with plasmin-mediated fibrin clot lysis as an end-point, rscu-PA has a 2,000-fold higher fibrinolytic potency than rscu-PA-GluL158, but only a 2.5-fold lower potency than rtcu-PA. This indicates that conversion of rscu-PA to rtcu-PA during the fibrinolytic process constitutes a major positive feedback mechanism for fibrinolysis. Partially degraded plasminogen (Lys-plasminogen) is ten- to 20-fold more susceptible to activation by both rscu-PA and rtcu-PA in purified systems and in plasma. This higher sensitivity of Lys-plasminogen to activation does not appear to be specific for scu-PA and rtcu-PA, since a similar change in sensitivity has previously been observed for streptokinase and t-PA. Furthermore, in a plasma milieu as well as in purified systems, the fibrinolytic potency (in terms of clot lysis) of rscu-PA is only about fourfold higher in the presence of 1.5 μmol/L Lys-plasminogen as compared with Glu-plasminogen. These findings would suggest that the marked threshold phenomenon in the dose-response curves of clot lysis with rscu-PA is primarily due to conversion of rscu-PA to rtcu-PA during the fibrinolytic process. The binding of plasminogen to fibrin, resulting in a conformational transition to a more susceptible Lys-plasminogen-like form, may thus provide an additional but quantitatively much less important feedback mechanism than the conversion of rscu-PA to rtcu-PA.

From the kinetic constants in Table 1, one can calculate that 50% activation in one hour of 1.5 μmol/L Glu-plasminogen or Lys-plasminogen would require rtcu-PA concentrations of 150 ng/mL and 6.0 ng/mL, respectively, rscu-PA-GluL158 concentrations of 150 μg/mL and 18 μg/mL, respectively, and rscu-PA concentrations of, respectively, 12 and 1.2 μg/mL. The values found experimentally for rtcu-PA agree well with these estimates, for rscu-PA-GluL158 they are two- to fourfold higher, but for rscu-PA the measured concentrations are 60- to 80-fold lower, namely 210 ng/mL for Glu-plasminogen and 15 ng/mL for Lys-plasminogen. This further indicates that conversion of rscu-PA to rtcu-PA contributes significantly to the fibrinolytic potency of rscu-PA, via an increased catalytic efficiency for plasminogen activation of the two chain derivative. Following predigestion of a fibrin clot by plasmin, subsequent lysis by rscu-PA, but also by rtcu-PA and rt-PA is enhanced. This may be explained by increased binding of plasminogen to newly exposed COOH-terminal lysine residues on the partially digested fibrin matrix. Enhanced plasmin generation at the fibrin surface may further accelerate conversion of scu-PA to rtcu-PA, resulting in an even more pronounced feedback effect of this conversion. This enhanced lysis of predigested clots thus is observed for all three enzymes evaluated, although the extent appears to be marginally higher for rscu-PA than for rtcu-PA and rt-PA. On preincubation of fibrin clots in human plasma for one hour at 37°C, sufficient plasminogen is bound to allow subsequent clot lysis by rscu-PA, rtcu-PA, and rt-PA in buffer. In this setup, a comparably enhanced lysis of predigested (about 5%) fibrin clots as compared with normal clots is also observed for rscu-PA, rtcu-PA, or rt-PA. These results indicate that partial predigestion of a fibrin clot by plasmin results in...
MECHANISM OF ACTION OF scu-PA

additional plasminogen binding and subsequently enhanced lysis by rscu-PA, rtcu-PA, and t-PA. This effect appears not to be specific for rscu-PA.

The role of conversion of scu-PA to rtcu-PA is further substantiated by activation experiments of plasminogen in the presence of α₂-antiplasmin. Indeed, a significant reduction of plasminogen activation by rscu-PA is observed when generated plasmin is efficiently neutralized. In addition, the results of clot lysis experiments (Table 2) show that equimolar concentrations of rscu-PA-Glu¹⁵⁸ are two- to three-orders of magnitude higher as compared with rscu-PA, whereas their catalytic efficiencies differ only by a factor of 15. This again indicates that the plasminogen activating potency of rscu-PA is lower when conversion to rtcu-PA is prevented.

Plasma depleted both in plasminogen and α₂-antiplasmin has a higher inhibitory potential on fibrinolysis with rscu-PA than with rtcu-PA. This confirms that plasma contains one or more components that interfere with plasminogen activation by rscu-PA. Attempts to remove this activity from plasma by repeated chromatography on insolubilized rscu-PA or on an insolubilized plasmin-resistant mutant²⁵ (rscu-PA-Pro³⁵⁹, results not shown), have, however, failed. This indicates that the inhibitory mechanism for rscu-PA in plasma is complex and suggests that more than one protein or interaction may be involved. The nature of this inhibitory effect in plasma thus remains to be further defined.

In conclusion, our results demonstrate that rscu-PA has some intrinsic plasminogen activating potential, which is, however, only 1% of that of rtcu-PA. Conversion of rscu-PA to rtcu-PA during the fibrinolytic process constitutes the primary positive feedback mechanism, whereas binding of plasminogen to fibrin or predigestion of fibrin may result in relatively minor additional acceleration of the fibrinolytic process.

The mechanism of plasminogen activation and fibrinolysis by rscu-PA in a plasma milieu in vitro, however, may not be identical to its physiological mechanism of action. Indeed, dose-response curves of rscu-PA in plasma clot lysis systems in vitro show a marked threshold phenomenon, whereas the dose-response curves for thrombolysis in vivo, both in rabbit and dog models are linear.¹⁸ The relative contribution of conversion of rscu-PA to rtcu-PA to thrombolysis in vivo remains to be further explored.

REFERENCES

2. Collen D: Tissue-type plasminogen activator (t-PA) and single chain urokinase-type plasminogen activator (scu-PA). Potential for fibrin-specific thrombolytic therapy. Progr Haemost Thromb 8:1, 1986
18. Collen D, De Cock F, Demarsin E, Lijnen HR, Stump DC: Absence of synergism between tissue type plasminogen activator (t-PA), single chain urokinase-type plasminogen activator (scu-PA) and urokinase on clot lysis in a plasma milieu in vitro. Thromb Haemost 56:35, 1986
22. Fraker PJ, Speck JC Jr: Protein and cell membrane iodina-
tions with a sparingly soluble chloroamide 1,3,4,6-tetrachloro-3a,6a-


The mechanism of plasminogen activation and fibrin dissolution by single chain urokinase-type plasminogen activator in a plasma milieu in vitro

HR Lijnen, B Van Hoef, F De Cock and D Collen