A Monoclonal Antibody Specific for Two-Chain Urokinase-Type Plasminogen Activator. Application to the Study of the Mechanism of Clot Lysis With Single-Chain Urokinase-Type Plasminogen Activator in Plasma

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A murine monoclonal antibody (MA-126E6A8) was raised against human urokinase-type plasminogen activator (u-PA), which, in an enzyme-linked immunosorbent assay (ELISA), reacted 15,000-fold better with recombinant two-chain u-PA (rtcu-PA) than with recombinant single-chain u-PA (rscu-PA). The antibody had no effect on the activity of rtcu-PA or on its inhibition by a chloromethylketone, but reduced the inhibition of rtcu-PA by recombinant plasminogen activator inhibitor-1 (rPAI-1) at least 10-fold. The dissociation constant of the rtcu-PA/MA-12E6A8 complex was 7 nM/L. An ELISA was developed using MA-126E6A8 as capture antibody and a horseradish peroxidase conjugated u-PA specific antibody for tagging. It recognized free and active site blocked rtcu-PA but not rscu-PA in complex with rPAI-1 or with α2-antiplasmin. This ELISA was used to monitor the generation of rtcu-PA during fibrin clot lysis with rscu-PA in human plasma. Addition of 5 μg/mL rscu-PA to 3 mL plasma containing a 0.2 μL 125I-fibrin labeled plasma clot caused 50% clot lysis in 62 ± 13 minutes (mean ± SD, n = 6), at which time 99 ± 28 ng/mL rtcu-PA was detected but no fibrinogen breakdown had occurred. Fifty percent fibrinogen breakdown did occur only when rtcu-PA had reached a level of 1.000 ± 270 ng/mL (at 160 ± 21 minutes). rscu-PA, 2 μg/mL, induced 50% clot lysis in 160 ± 41 minutes (n = 6); no fibrinogen degradation occurred within 4 hours and rtcu-PA levels did not exceed 80 ng/mL. In the absence of a fibrin clot, 5 μg/mL rscu-PA added to human plasma did not result in significant generation of rtcu-PA (<50 ng/mL after 4 hours) and no fibrinogen degradation was observed. These results indicate that clot lysis with rscu-PA in a plasma milieu does not require extensive systemic conversion of rscu-PA to rtcu-PA, and that fibrinogen degradation occurs secondarily to systemic conversion of rscu-PA to rtcu-PA.

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Cell hybridization and screening of hybridomas. MoAbs against u-PA were produced essentially by the method of Galfré and Milstein. BALB/c mice were immunized by subcutaneous injection of 15 µg rtcu-PA in complete Freund's adjuvant, followed 2 weeks later by intraperitoneal injection of 15 µg rtcu-PA in incomplete Freund's adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with 15 µg rtcu-PA in saline on days 4 and 2 before the cell fusion. Spleen cells were isolated and fused with P3X63-Ag8.65.3 myeloma cells (obtained from Dr O. Schönherr, Organon, Oss, the Netherlands) according to Fazekas de St Groth and Scheidegger. After selection in medium with hypoxanthine, aminopterine, and thymidine, the supernatants were screened for specific antibody production with a one-site noncompetitive micro-enzyme-linked immunosorbent assay (ELISA) using microtiter plates coated with rtcu-PA (1 µg/mL, solution incubated overnight at 4°C, washed, and blocked with 10 mg/mL bovine serum albumin). The bound immunoglobulins (Igs) were then detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG. Positive clones were used for the production of ascitic fluid in pristane-primed BALB/c mice. The IgG fraction of the monoclonal antibodies was purified from ascites by affinity chromatography on protein A-Sepharose. Specificity of the positive clones for rtcu-PA was determined with a micro-ELISA using plates coated with rtcu-PA or rscu-PA. MoAbs specific for rtcu-PA were further characterized for their reactivity with reduced urokinase; other two-chain u-PA moieties, including rtcu-PA/T, rtcu-PA/T, P, rtcu-PA-Arg, rtcu-PA-Glu, and rtcu-PA-Gly, and rt-PA, plasmin, trypsin, chymotrypsin, and thrombin, using a similar micro-ELISA.

Influence of MA-12E6A8 on the reactivity of rtcu-PA with inhibitors. The effect of MA-12E6A8 on the inhibition of rtcu-PA by Glu-Gly-Arg-CH₂Cl or rPAI-1 was studied in the following way. rtcu-PA (100 nmol/L) was preincubated with a 20-fold molar excess of MA-12E6A8 for 1 hour 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 mixture was then incubated with Glu-Gly-Arg-CH₂Cl or rPAI-1 (final concentration 1 to 5 µmol/L), and residual rtcu-PA activity was measured at different time intervals (0 to 10 minutes) with S-2444 (final concentration 0.3 mmol/L) after 20-fold dilution of the samples. The pseudo-first-order rate constant was determined from semilogarithmic plots of residual enzymatic activity versus time. The efficiency index of the inhibition was calculated as the ratio of the rate constant and the inhibitor concentration.

Influence of MA-12E6A8 on the reactivity of rtcu-PA with substrates. Activation of plasminogen (final concentration 50 µmol/L) by rtcu-PA (final concentration 2 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 generated plasmin was measured at different time intervals (0 to 10 minutes) with S-2251 (final concentration 1 mmol/L) after 50-fold dilution of the samples. The influence of MA-12E6A8 on plasma activity by rtcu-PA was studied after preincubation of rtcu-PA (final concentration 50 nmol/L) with MA-12E6A8 (final concentration 16 nmol/L to 4 µmol/L) for 1 hour at 37°C. The catalytic efficiency was calculated as the ratio of the catalytic rate constant and the Michaelis constant, and expressed in L/µmol · s. In control experiments, rtcu-PA (50 nmol/L) was preincubated with MA-7C7 (0.05 to 2 µmol/L) under the same conditions.

The kinetic parameters of the hydrolysis of S-2444 (final concentration 0.05 to 0.75 mmol/L) by rtcu-PA (final concentration 5 mol/L) were determined 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, before and after incubation of rtcu-PA with a 20-fold molar excess of MA-12E6A8. Vₚₜ was expressed in mmol/L · s of released p-nitroaniline, using a molar extinction coefficient of 10,500 L/mol · cm at 410 nm.

Determination of dissociation constant (kd) of rtcu-PA/MA-12E6A8. The kd for the interaction between MA-12E6A8 and rtcu-PA was determined under equilibrium conditions as follows. rtcu-PA (final concentration 1.3 nmol/L) was incubated with various concentrations of MA-12E6A8 (0 to 52 nmol/L) for 1 hour 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. u-PA activity was then measured with S-2444 (final concentration 0.3 mmol/L) before and after the addition of an excess of rPAI-1 (final concentration 11 nmol/L of active rPAI-1). Under these conditions rtcu-PA (in the absence of MA-12E6A8) was instantaneously blocked. The dissociation constant was then determined as the concentration of MA-12E6A8 needed to reduce the extent of inhibition of rtcu-PA by rPAI-1 to 50%.

Two-site ELISA specific for rtcu-PA. The IgG fraction of MA-12E6A8 was diluted in 0.04 mol/L phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl (PBS). Two hundred-microliter samples of this solution were incubated for 48 hours at 4°C in the wells of polystyrene microtiter plates (Costar, Cambridge, MA). The plates were emptied, and the wells were treated for 2 hours at room temperature with 200 µL PBS containing 10 g/L bovine serum albumin. Then the wells were washed with 200 µL PBS and finally with a solution containing 10 g of mannitol and 20 g saccharose/L. The plates were stored at −20°C. Immediately before use, the plates were washed 5 times with PBS containing 0.05% Tween 80. The samples were diluted in PBS containing Tween 80 (0.002%), EDTA (5 mmol/L), bovine serum albumin (1 g/L), and aprotinin (1 KIU/mL) (dilution buffer), and 180 µL samples were added to the wells. After incubation for 18 hours at 4°C in a moist chamber, the wells were emptied and washed with PBS containing 0.002% Tween 80 (PBS-Tween). Tagging was performed using an HRP-conjugated monoclonal antibody (MA-7C7), which reacts equally well with scu-PA and rtcu-PA. The MA-7C7-HRP conjugate was prepared as described by Nakane and Kawai.37 MA-7C7-HRP, 180 pL, diluted to 0.3 µg/mL in PBS-Tween, was applied to the wells and incubated for 2 hours at room temperature. After washing of the plates, 150 µL aliquots of a 0.1 mol/L citrate, 0.2 mol/L sodium phosphate buffer pH 5.0, containing 300 µg/mL o-phenylene-diamine and 0.003% hydrogen peroxide were added. After 30 minutes at room temperature the peroxidase reaction was stopped with 50 µL of 4 mol/L H₂SO₄. The absorbance was measured at 492 nm with a multiscan spectrophotometer EAR 400 AT (SLT-Lab Instruments, Salzburg, Austria).

Lysis of fibrin-labeled human plasma clots. In vitro clot lysis experiments were performed as described previously, with a 0.2 mL fibrin-labeled human plasma clot submersed in 3 mL citrated human plasma, using rtcu-PA at a final concentration of 2 or 5 µg/mL. At regular time intervals (0 to 4 hours) samples were collected on Glu-Gly-Arg-CH₂Cl (500 µmol/L final concentration), and aprotinin (1,000 KIU/mL final concentration) for antigen determinations. rtcu-PA antigen was determined with the ELISA specific for two-chain forms of urokinase, as described above. rtcu-PA/α₂-antiplasmin complexes were quantitated by a two-site ELISA, as described above, using an MoAb against u-PA (MA-4D1E8) as capture antibody and an MoAb against α₂-antiplasmin (MA-39A1) conjugated to HRP for tagging. In this ELISA a
linear dose response curve was obtained between 0.3 and 3 ng/mL complex-associated u-PA antigen in buffer. The lower limit of sensitivity in plasma (10-fold diluted) was 3 ng/mL. Plasmin-α1-antiplasmin complexes were quantitated as described previously.46 Lys-forms of plasmin(ogen) were determined with a two-site ELISA using an MoAb (MA-Lpm1) specific for Lys-plasmin(ogen)45 as capture antibody, and an MoAb against plasminogen (MA-Tpm1) conjugated to HRP for tagging. Fibrinogen, plasminogen, and α1-antiplasmin were measured as described elsewhere.15

RESULTS
Characterization of MoAbs. Out of five fusions, 160 hybridomas were obtained producing MoAbs directed against u-PA. Eight MoAbs reacted better with rtcu-PA than with rscu-PA. The ratio of reactivity (two-chain v single-chain), as measured with the micro-ELISA using plates coated with rtcu-PA or rscu-PA, varied between 2 and 15,000. For further experiments the most discriminating antibody (MA-12E6A8) was selected.

The specificity of MA-12E6A8 was investigated by measuring its reactivity toward the following antigens: rscu-PA, rtcu-PA, reduced and alkylated rtcu-PA, rtcu-PA/T, rtcu-PA/T,P, rtcu-PA-Arg158, rtcu-PA-Glu158, and rtcu-PA-Gly158. Of these moieties, rtcu-PA, rtcu-PA/T,P, rtcu-PA-Arg158, and rtcu-PA-Glu158 had a comparable enzymatic activity toward S-2444 and plasminogen, whereas rtcu-PA/T and rtcu-PA-Gly158 were virtually inactive (data not shown). MA-12E6A8 reacted with rtcu-PA, rtcu-PA/T,P, rtcu-PA-Arg158, and rtcu-PA-Glu158, but not with rscu-PA, rtcu-PA/T, or rtcu-PA-Gly158 (Fig 1A). MA-7C7 recognized all u-PA moieties to the same extent (Fig 1B). MA-12E6A8 did not recognize reduced rtcu-PA, plasmin, thrombin, trypsin, chymotrypsin, or two-chain rt-PA (data not shown).

Influence of MA-12E6A8 on the reactivity of rtcu-PA with inhibitors and substrates. Preincubation of rtcu-PA with MA-12E6A8 resulted in a concentration-dependent reduction of the second-order rate constant (k) of the inhibition of rtcu-PA by rPAI-1. At an antibody:enzyme molar ratio of 0.25, the apparent rate constant was reduced to 50%, at an equimolar ratio to 23% and at a 20-fold molar excess to 8% (Table 1). MA-7C7 at a 20-fold molar excess had no effect on the inhibition rate.

MA-12E6A8 caused a concentration-dependent decrease of the extent of inhibition of rtcu-PA by rPAI-1. At a 40-fold molar excess of antibody, the residual extent of inhibition was less than 20 percent, allowing titration of the rtcu-PA/MA-12E6A8 complex. When the residual rtcu-PA activity was plotted versus the logarithm of the antibody concentration, a sigmoidal curve was obtained (not shown), compatible with a 1:1 stoichiometric interaction between enzyme and antigen binding site. Fifty percent inhibition of the reaction between rtcu-PA (1.3 nmol/L) and rPAI-1 (11 nmol/L) was obtained at an antibody concentration of 7 nmol/L, which represents the apparent dissociation constant.

The efficiency index of the inhibition of rtcu-PA by Glu-Gly-Arg-CH2Cl was only slightly affected by preincubation of the enzyme with a 20-fold molar excess of MA-12E6A8; it was 0.007 ± 0.001 L/μmol·s in the absence and 0.01 ± 0.002 L/μmol·s in the presence of MA-12E6A8 (Table 1).

Preincubation of rtcu-PA with MA-12E6A8 in increasing molar ratios (antibody:enzyme 0.3 to 80) resulted in a
Table 1. Effect of MA-12E6A8 on the Reactivity of rtcu-PA With Substrates and Inhibitors

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MA-12E6A8 No Antibody</th>
<th>MA-12E6A8 Equimolar</th>
<th>MA-12E6A8 20-Fold Excess</th>
<th>MA-7C7 20-Fold Excess</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrates</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>S-2444</td>
<td>0.16</td>
<td>—</td>
<td>0.18</td>
<td>—</td>
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<tr>
<td>Plasminogen</td>
<td>0.07</td>
<td>0.05</td>
<td>0.02</td>
<td>0.08</td>
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<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glu-Gly-Arg-CH₂Cl</td>
<td>0.007</td>
<td>—</td>
<td>0.01</td>
<td>—</td>
</tr>
<tr>
<td>rPAI-1</td>
<td>14</td>
<td>3.1</td>
<td>1.1</td>
<td>10</td>
</tr>
</tbody>
</table>

Data represent the catalytic efficiency or the efficiency index of the reaction (see Methods), and are expressed as L/pmol s.

gradually decreasing activation rate of plasminogen. The residual activation rate was 90% at a molar ratio of antibody: enzyme of 0.25, 70% at an equimolar ratio, and 30% at a 20-fold molar excess of antibody. MA-7C7 had no effect on the activation rate of plasminogen by rtcu-PA (Table 1). A 20-fold molar excess of MA-12E6A8 had no effect on the hydrolysis of the chromogenic substrate S-2444 by rtcu-PA (Table 1).

Development of an ELISA specific for rtcu-PA. Coating of microtiter plates with MA-12E6A8 at a concentration of 4 µg/mL and development with MA-7C7-HRP at a final concentration of 0.3 µg/mL allowed the accurate measurement of rtcu-PA levels between 0.1 and 2.5 ng/mL in dilution buffer. The apparent cross-reactivity with rscu-PA after chromatography on benzamidine-sepharose was approximately 0.3%, resulting in a background reactivity corresponding to 15 ng/mL rtcu-PA in the experiments using 5 µg/ml rscu-PA. Inhibition of 150 nmol/L rtcu-PA with α₂-antiplasmin or with rPAI-1 resulted in loss of cross-reactivity in the ELISA. Reaction of rtcu-PA with the synthetic inhibitor Glu-Gly-Arg-CH₂Cl produced an inactive molecule with a fourfold decreased antigenic reactivity.

Role of conversion of rscu-PA to rtcu-PA during clot lysis with scu-PA. The rtcu-PA-specific ELISA was used to monitor the generation of rtcu-PA during clot lysis with rscu-PA in an in vitro plasma clot lysis system. In vitro formation of rtcu-PA/α₂-antiplasmin complex, which is not detected in the ELISA, was prevented by collecting samples on Glu-Gly-Arg-CH₂Cl (final concentration 500 µmol/L). Under these conditions, recoveries of rtcu-PA added to plasma at a final concentration of 1,000, 500, 200, and 100 ng/mL were 84% ± 13% (mean ± SD), 86% ± 18%; 80% ± 5% and 81% ± 10%, respectively, as measured with the ELISA using a calibration curve constructed with rtcu-PA pretreated with Glu-Gly-Arg-CH₂Cl. The half-life of rtcu-PA in plasma at 37°C, measured under these conditions, was 25 ± 6 minutes (mean ± SD, n = 4).

Addition of 5 µg/mL rscu-PA to the plasma clot lysis system caused 50% lysis in 62 ± 13 minutes (mean ± SD, n = 6), at which time only 99 ± 28 ng/mL rtcu-PA (not corrected for the background activity of 15 ng/mL after addition of 5 µg/mL rscu-PA) could be detected (Fig 2A).

![Fig 2. Generation of rtcu-PA, clot lysis, and fibrinogen breakdown after addition of rscu-PA to the in vitro plasma clot lysis system.](https://www.bloodjournal.org)
and 30 ± 16 ng/mL rtcu-PA in complex with α₂-antiplasmin. Fibrinogen breakdown occurred much later, 50% at 150 ± 21 minutes, and was associated with the appearance of rtcu-PA (1,000 ± 270 ng/mL at 150 minutes) and 200 ng/mL rtcu-PA in complex with α₂-antiplasmin. Depletion in α₂-antiplasmin (50% at 120 ± 15 minutes, n = 3) preceded fibrinogen breakdown and correlated with the formation of plasmin-α₂-antiplasmin complexes (Fig 3A). Formation of Lys-plasmin(ogen) was observed when α₂-antiplasmin levels were below 40% (Fig 3A). Addition of 5 μg/mL rscu-PA induced 50% lysis in 160 ± 41 minutes (n = 6). No fibrinogen degradation was observed within 4 hours, whereas rtcu-PA levels never exceeded 80 ng/mL (Fig 2B). α₂-antiplasmin depletion did not occur, and the generation of plasmin-α₂-antiplasmin and Lys-plasminogen derivatives was negligible (Fig 3B). The absence of significant amounts of complexes between generated rtcu-PA and plasma inhibitors, which would not be recognized in the ELISA that is specific for rtcu-PA, was further supported as follows. Clot lysis experiments were performed using ³²P-labeled scu-PA and detection of u-PA related antigen by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (before and after reduction) and autoradiography (data not shown). No complexes were detectable within 4 hours in the experiments using 2 μg/mL rscu-PA. With 5 μg/mL rscu-PA, no significant amounts of rtcu-PA were formed within 90 minutes (at which time complete lysis had occurred but no fibrinogen breakdown was observed). Addition of 5 μg/mL rscu-PA to citrated plasma in the absence of a fibrin clot did not induce fibrinogen breakdown within 4 hours, and did not cause α₂-antiplasmin or plasminogen consumption or formation of plasmin-α₂-antiplasmin complex or Lys-plasmin(ogen) derivatives, whereas rtcu-PA levels remained below 50 ng/mL (data not shown).

DISCUSSION

scu-PA, in contrast to rtcu-PA, induces relatively fibrin-specific thrombolysis in human plasma in vitro and in vivo.¹⁰,¹¹,¹² The mechanism of its fibrin specificity and the role of conversion of scu-PA to rtcu-PA in clot lysis and fibrinogen breakdown is not well-understood. MoAbs specific for either

![Fig 3. α₂-Antiplasmin and plasminogen levels after the addition of racu-PA to the in vitro plasma clot lysis system. (A) 5 μg/mL rscu-PA; (B) 2 μg/mL rscu-PA. (●), residual α₂-antiplasmin; (▲), residual plasminogen; (■), percentage of plasmin (ogen) occurring as Lys-form; (●), percentage of α₂-antiplasmin complexed with plasmin. Data represent mean ± SEM of three separate experiments.](image-url)
gests that the epitope recognized by MA-12E6A8 is only
PLASMA CLOT LYSIS WITH XU-PA 1799
PA/T, rtu-PA-Gly"~, or with reduced rtcu-PA. This sug-
constitution of the catalytic site. Reaction of rtcu-PA with
reacts with enzymatically active two-chain forms of u-PA
but not with rscu-PA, with inactive two-chain forms (rscu-
PA/T,T, rtcu-PA-Gly"~, or with reduced rtcu-PA. This sug-
suggests that the epitope recognized by MA-12E6A8 is only
exposed after cleavage of the Lys"Ile" peptide bond and
constitution of the catalytic site. Reaction of rtcu-PA by
MA-12E6A8 does not block the active site of the enzyme but
prevents its inhibition by rPAI-1.
MA-12E6A8 was used as a capture antibody in a two-site
ELISA specific for rtcu-PA. This ELISA was then used to
study the role of conversion of rscu-PA to rtcu-PA during
fibrin clot lysis in a human plasma milieu. Under conditions
where 50% clot lysis by rscu-PA was obtained within
approximately 1 hour (5 pg/mL of rscu-PA), negligible
levels of rtcu-PA were generated (99 ± 28 ng/mL). In this
system, 50% lysis with rtcu-PA within 2 hours requires 500
ng/mL, which indicates that the levels of rtcu-PA gener-
ated in plasma do not contribute significantly to clot lysis
with rscu-PA, and that clot lysis with rscu-PA does not
require extensive systemic conversion of rscu-PA to rtcu-PA.
Previously reported results suggested that conversion of
rscu-PA to rtcu-PA constitutes a primary positive feedback
mechanism for fibrinolysis with rscu-PA in human plasma in
vitro. These findings, together with the data of this study,
suggest that conversion of rscu-PA to rtcu-PA occurs prima-
arily at the fibrin surface.

To prevent in vitro complex formation between rtcu-PA
and plasma inhibitors associated with loss of reactivity in the
ELISA, plasma samples were collected on excess rtcu-PA
inhibitor (Glu-Gly-Arg-CH2Cl). Thus, the measured rtcu-
PA levels probably closely represent the actual plasma
concentrations. Furthermore, because the half-life of rtcu-
PA in plasma measured under these conditions was found to
be 25 minutes, the measured rtcu-PA levels in plasma
probably are a good indicator of the total amounts generated.
The absence of significant amounts of generated rtcu-PA
complexed to proteinase inhibitors (which are not recognized
in the ELISA) is further substantiated by our finding of only
low levels of rtcu-PA/α2-antiplasmin complex under condi-
tions of extensive conversion of rscu-PA to rtcu-PA. Thus,
using 5 μg/mL rscu-PA, less than 20% of formed rtcu-PA is
recovered as rtcu-PA/α2-antiplasmin complex within 180
minutes.

Our results also indicate that systemic fibrinogen degrada-
tion occurs secondarily to extensive systemic conversion of
rscu-PA to rtcu-PA, resulting in marked plasminogen activa-
tion and α2-antiplasmin depletion. In the absence of fibrin,
rscu-PA did not cause fibrinogen, plasminogen, or
α2-antiplasmin consumption in plasma, whereas rtcu-PA levels
remained below 50 ng/mL. These data add evidence to the
hypothesis that the clot selectivity of rscu-PA may be
mediated by conversion of rscu-PA to rtcu-PA at the fibrin
surface, which in plasma may be prevented by a mecha-
nism of competitive inhibition that is reversed by fibrin. At
present, the nature of this competitive inhibition has not been
elucidated. The ELISA specific for rtcu-PA developed in this
study may be a useful tool to study this mechanism.

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A monoclonal antibody specific for two-chain urokinase-type plasminogen activator. Application to the study of the mechanism of clot lysis with single-chain urokinase-type plasminogen activator in plasma

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