The pharmacokinetic and thrombolytic properties were determined of two recombinant single-chain chimeric plasminogen activators (PA) consisting of u-PA-33k, a low-molecular weight derivative of single-chain urokinase-type PA (scu-PA) comprising amino acids Ala32 through Leu49, and of either a single-chain variable region fragment (Fv) derived from the fibrin fragment D-dimer–specific monoclonal antibody MA-15C5 (K12G2S32) or of the deglycosylated single-chain Fv fragment obtained by substitution of Asn86 with Glu (K12G2S32). Following bolus injection in hamsters, clearances of recombinant scu-PA (rscu-PA) and K12G2S32 were similar. In contrast, clearance of K12G2S32 was fourfold slower than that of rscu-PA. The thrombolytic potency (percent lysis per u-PA administered in milligrams per kilogram body weight) and specific thrombolytic activity (percent lysis per microgram per milliliter steady-state plasma u-PA antigen level) of these compounds were studied in hamsters with an experimental pulmonary embolus consisting of a human plasma clot injected via the jugular vein. The doses of K12G2S32 and K12G2S32 required to obtain maximal rate of clot lysis were sixfold and 11-fold lower than that of rscu-PA. The steady-state u-PA–related plasma antigen levels of K12G2S32 and K12G2S32 required to obtain maximal rate of clot lysis were 10-fold and fourfold lower than that of rscu-PA. Thus, targeting of K12G2S32 to the clot surface by means of its glycosylated Fv fragment results in a 10-fold increase of its specific thrombolytic activity and sixfold increase of its thrombolytic potency as compared with those of rscu-PA. Targeting of K12G2S32 to the clot surface by means of its deglycosylated Fv fragment results in only a twofold increase of its thrombolytic activity. However, its fourfold slower clearance, combined with its two-fold higher specific thrombolytic activity, results in an 11-fold increase of its thrombolytic potency over that of rscu-PA. These findings indicate that the thrombolytic potency of chimeric antibody–targeted PA may be increased by increasing the specific thrombolytic activity, reducing the clearance, or both.

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Pharmacokinetic and Thrombolytic Properties of Chimeric Plasminogen Activators Consisting of a Single-Chain Fv Fragment of a Fibrin-Specific Antibody Fused to Single-Chain Urokinase

By Paul Holvoet, Yves Laroche, Jean Marie Stassen, H. Roger Lijnen, Berthe Van Hoef, Frans De Cock, Annemie Van Houtven, Yannick Gansemans, Gaston MatthysSENS, and Désiré Collen

The BLOOD fibrinolytic system comprises an inactive proenzyme, plasminogen, that can be converted to the active enzyme, plasmin, that degrades fibrin into soluble fibrin degradation products. Two immunologically distinct physiological plasminogen activators (PA) have been identified: the tissue-type PA (t-PA) and the urokinase-type PA (u-PA). Inhibition of the fibrinolytic system may occur either at the level of the PA, by specific plasminogen activator inhibitors, or at the level of plasmin, mainly by α2-antiplasmin.1

In contrast to t-PA, single-chain u-PA (scu-PA) does not bind to fibrin, possibly limiting its thrombolytic potential. Targeting of scu-PA to a thrombus by combining the antigen-binding properties of a fibrin-specific antibody with its catalytic domain in a single molecule, has resulted in a significant increase of both its in vitro fibrinolytic and its in vivo thrombolytic potencies.2 Targeting has been achieved by chemical conjugation of scu-PA and a monoclonal antibody directed against the NH2-terminal region of the β-chain of fibrin, 59D8, or a monoclonal antibody specific for fragment D-dimer of human fibrin (MA-15C5).3,8 A recombinant chimeric PA, rscu-PA-32k/59D8, in which intact 59D8 was fused to scu-PA-32k (a low Mr, truncated scu-PA), yielded a 20-fold higher thrombolytic potency than scu-PA in a rabbit jugular vein thrombosis model.9 A recombinant chimeric PA, rscu-PA-32k/MA-15C5, composed of a scu-PA-32k and intact, humanized MA-15C5 (MA-15C5Hu), yielded an 11-fold higher thrombolytic potency than scu-PA in the same model.10

Recently, we have produced a single-chain variable region antigen-binding fragment (scFv) derived from MA-15C5, scFv-K12G2S32, that bound to fragment D-dimer of human cross-linked fibrin with an affinity similar to that of intact MA-15C5.11 The scFv-K12G2S32 fragment was linked to rscu-PA-33k, yielding a single-chain chimeric PA K12G2S32, with a 13-fold higher in vitro fibrinolytic potency than rscu-PA-32k.12

In the present study, we determined the comparative in vivo pharmacokinetic and thrombolytic properties of rscu-PA, of K12G2S32, and of K12G2S32, a variant without Asn-based glycosylation of the scFv moiety, in hamsters.

MATERIALS AND METHODS

Proteins and Reagents

Murine monoclonal antibody MA-15C5, which reacts specifically with fragment D-dimer of human cross-linked fibrin, was produced and characterized as described elsewhere.7,4 Human plasminogen, plasmin, fibrinogen, and fibrin fragment D-dimer were obtained and characterized as previously described.13 Saruplase, a preparation of recombinant scu-PA (rscu-PA) obtained by expression of cDNA encoding human scu-PA in Escherichia coli, was a gift from Grünenthal AG (Aachen, Germany). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG or goat anti-rabbit IgG was obtained from BioRad Laboratories (Richmond, CA). The chromogenic substrates S-2444 and S-2251 were purchased from KabiVitrum (Brussels, Belgium) and aprotinin (Trasylo1) from Bayer (Leverkusen, Germany). The International Reference Preparation for Urokinase (batch 66/46) was
obtained from the National Institute for Biological Standards and Control (London, UK). SP-Sephadex and Sephadex-G100 superfine were from LKB (Bromma, Sweden). Normal human plasma was pooled, fresh, frozen citrated blood bank plasma from at least five healthy blood donors.

Klenow fragment and T4 DNA ligase were from Boehringer Mannheim (Mannheim, Germany), polynucleotide kinase from New England Biolabs (Beverly, MA), restriction endonucleases and nucleoside triphosphates from Pharmacia (Brussels, Belgium), and [α-32P]dATP from Amersham (Brussels, Belgium).

Autographa californica nuclear polyhedrosis virus (AcNPV), a baculovirus, was a generous gift of Dr M. Summers, Texas A&M University, College Station, TX. Spodoptera frugiperda (Sf9) cells were obtained from the American Type Culture Collection (Rockville, MD). All cell culture media and solutions were obtained from Gibco-BRL (Ghent, Belgium).

**Oligonucleotide-Directed Mutagenesis and DNA Sequencing**

All DNA manipulations were performed essentially as described by Maniatis et al. Oligonucleotide-directed mutagenesis was performed by the gapped-duplex method of Kramer et al, using the pMa/c vector system of Stanssens et al. This system uses phosphoramidite method on an Applied Biosystems (Foster City, CA) 380A DNA synthesizer and purified by polyacrylamide gel electrophoresis as described. DNA sequencing was performed on single-stranded or double-stranded DNA by the dideoxynucleotide termination method of Sanger et al using the USB (Cleveland, OH) sequence kit, according to the manufacturer's manual.

**Construction of cDNAs**

pVLK2-GoGa encoding K12GoG32 for expression in Sf9 cells and scu-PA. A 1,5 10-bp restriction fragment of was then replaced by the corresponding Bam-HI-XbaI fragments from pMa/c-12G2S32 encoding scFv-K12Go, using the 27-mer deoxyoligonucleotide dATGGCCTGGTCTTGAAGCTTCGAGACTG, exchanged the Asn-linked glycosylation site at the AAT end of the cDNA encoding scu-PA consisting of amino acids 1 to 411, connected to the 3'-untranslated sequence, was then ligated in the XbaI/XmaI site of the poly linker of the transfection vector pVL1393 (British Biotechnology, Oxford, UK), yielding pVLscu-PA.

The schematic structures of K12GoG32, K12GoG32, scu-PA and u-PA-33k are illustrated in Fig 1. Isolation of rscu-PA and Chimeric PA From Infected Sf9 Insect Cells

The Sf9 cells were grown essentially as described by Summers and Smith. Recombinant baculovirus was produced and purified as described previously and the purity of the isolated recombinant virus was confirmed in filter-hybridization experiments. For the large-scale production of the different proteins, 40 x 10^6 Sf9 cells in 175-cm² culture flasks were infected with 200 x 10^6 plaque forming units of recombinant virus. After incubation for 48 hours at 27°C, the
conditioned medium was removed and centrifuged at 1,000g for removal of cell debris.

Chimeric PA were purified by ion exchange chromatography on SP-Sephadex followed by gel filtration on Sephadex-G100 superfine, as described previously.29 rscu-PA was purified by affinity chromatography as described previously.30

Analytical Techniques

Culture supernatants and purified proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% to 25% gradient gels according.38 Western blot analysis of the proteins separated by SDS-PAGE was performed essentially as described.25 The transferred proteins were analyzed by automated Edman degradation26 on an Applied Biosystems 470A protein sequencer. Western blot analysis of the proteins separated by SDS-PAGE was performed essentially as described.25 The transferred proteins were visualized with rabbit anti-MA-15C5 or anti-rscu-PA antisera in the first step, affinity purified goat anti-rabbit IgG antiserum conjugated to HRP in the second step, and the HRP substrate, 4-chloro-1-naphtol (BioRad).

Aminoterminal amino acid sequence analysis was performed using automated Edman degradation26 on an Applied Biosystems 470A gas-phase sequencer, and identification of amino acids by high-pressure liquid chromatography.27

The concentrations of the different chimeric PA were determined initially by amino acid analysis performed in a Beckman (Palo Alto, CA) 119C1 amino acid analyzer after hydrolysis in 6 mol/L HCl in sealed ampules at 110°C for 20 hours, and subsequently with the Bradford protein assay28 (BioRad), which was calibrated against a secondary albumin standard (conversion factor, 1.0 ± 0.1; mean ± SD; n = 4). The concentration of purified MA-15C5 was determined with the Bradford protein assay calibrated with bovine γ-globulin.

The concentrations of u-PA-related antigen in rscu-PA, recombinant two-chain u-PA (rscu-PA), and chimeric PA preparations were determined by enzyme-linked immunosorbent assay (ELISA) as previously described.29 D-dimer-binding protein was quantitated in international Reference Preparation for Urokinase.

Ratio between bound immunoglobulin (as derived from the absorbance at 280 nm) and total immunoglobulin was determined using specific fibrinolytic activity measured on bovine fibrin plates35 by comparison with the International Reference Preparation for Urokinase.

Purified rscu-PA or chimeric PA (final concentration, 1 μmol/L) were incubated with plasmin (0% to 7.5% molar ratio) in 0.05 mol/L Tris-HCl buffer, pH 7.4, containing 0.038 mol/L NaCl and 0.01% Tween 80 (TNT buffer) at 37°C. At timed intervals (0 to 30 minutes), aliquots were removed and generated urokinase activity was measured with S-2444 (final concentration, 0.3 mmol/L) after 50-fold dilution. The conversion of one-chain to two-chain u-PA was monitored by SDS-PAGE on 10% to 15% gradient gels according with dithioerythritol. Two-chain u-PA molarities were isolated by chromatography on benzamidine-Sepharose as described previously.32

Plasminogen activation was monitored by incubation of plasminogen (final concentration, 5 to 67 μmol/L) at 37°C in 0.1 mol/L phosphate buffer, pH 7.4, with rscu-PA or the respective two-chain chimeric PA (final concentration, 1.8 or 0.8 mmol/L). Generated plasmin was measured at different time intervals (0 to 5 minutes) with S-2251 (final concentration, 1 mmol/L) after 25-fold dilution of samples. Initial activation rates were obtained from plots of the concentration of generated plasmin versus time, and kinetic parameters (K_m and k_cat) were determined from Lineweaver-Burk plots by linear regression analysis.

Pharmacokinetics

The pharmacokinetic properties of rscu-PA, K_12GOS32, and K_12GZS32 were determined in anesthetized hamsters by measurements of the residual plasma u-PA levels after bolus injection of 100 μg/kg. Blood samples were taken at times 0.5, 1, 2, 3, 5, 7, 10, 15, 20, 30, 60, and 90 minutes after injection. The results of plasma levels versus time were plotted on semilogarithmic paper and fitted with a sum of two exponential terms, C(t) = A*exp(-t/B) + C*exp(-t/D), by graphical curve peeling.34 Therefore, the linear terminal portion of the antigen versus time curves were extrapolated to yield the ordinate intercept B. This line had a slope = β. The extrapolated values were subtracted from the initial values and the corrected values were fitted with a line which had a slope = -α and an ordinate intercept A. The following protein clearance parameters were calculated from the coefficients (A and B) and exponents (α and β) describing the disposition of the different activators from plasma, using standard formulas derived by Perrier and Gibaldi34: volume of the central compartment, V_c = dose/ (A + B); total volume of distribution, V_d = dose/B; t_1/2α = ln(2)/α, t_1/2β = ln(2)/β; extrapolated area under the curve, AUC = A/α + B/β; plasma clearance, C_l = dose/AUC. In addition, the pharmacokinetic properties of K_12GOS32 and K_12GZS32 were determined in anesthetized New Zealand White rabbits by measurements of the residual plasma u-PA levels after bolus injection of 20 μg/kg of the chimeric PA.

Lysis of [125I]Fibrin-Labeled Human Plasma Clot Lysis

Lysis of [125I]-labeled human plasma clots (volume, 60 μL), immersed in 0.5 mL human citrated plasma, after addition of single-chain or two-chain u-PA-related protein, was monitored over 2 hours as described elsewhere.33 The effect of the addition to plasma of purified fibrin fragment D-dimer (final concentration, 0 to 330 nmol/L) on the fibrinolytic activity of single-chain or two-chain chimeric PA or u-PA (final concentration, 1 μg/mL) was determined in a similar manner.

Analysis of the Thrombolytic Data

The relative thrombolytic potency and specific thrombolytic activity of the compounds in hamsters and rabbits were determined by fitting the individual dose-response data (percent lysis versus dose in milligrams per kilogram, or percent lysis versus steady-state u-PA-related antigen in micrograms per milliliter) with an exponentially transformed sigmoidal function y = 100c/(1 + c*exp(-b)), using the statistical program GraFit (Erithacus, Middlesex, UK). The mean ± SEM of the parameters c (maximal lysis in percent), b (dose in mg/kg or in μg/mL plasma antigen at which the rate of lysis is maximal), and z = (ac4)·c^6 (maximal rate of lysis, expressed as percent lysis per mg/kg compound infused or as percent lysis per μg/mL plasma antigen) were determined.35 The significance of the differences between these parameters was determined using Student's t-test.

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RESULTS

Construction and Expression of rscu-PA, K12G0S32, and K12G2S32

The construction of the transfection vector pVLK12G0S32 for expression of K12G0S32 has been described in detail elsewhere. In pVLK12G0S32, the expression vector of K12G0S32, the Asn-linked glycosylation site in the scFv moiety was removed by substitution of Asn with Glu. In addition, the Phe residue was replaced with Ala to eliminate the thrombin cleavage site in the u-PA moiety. The pVLscu-PA transfection vector for expression of K12G0S32 has been described in detail elsewhere. The structures of pVLK12G0S32, pVLK12G2S32, and pVLscu-PA were confirmed by cDNA sequencing (results not shown). The structure of these molecules is schematically represented in Fig 1.

Biochemical Characterization of Purified rscu-PA, K12G0S32, and K12G2S32

SDS-PAGE of purified rscu-PA, K12G0S32, and K12G2S32 showed homogeneous preparations migrating with an apparent molecular weight of 54,000 for rscu-PA and 57,000 for both K12G0S32 and K12G2S32. All proteins were obtained primarily as single-chain form, as evidenced by SDS-PAGE after reduction with dithioerythritol (Fig 2).

The equilibrium association constants for K12G0S32 and K12G2S32 for immobilized fragment D-dimer were 0.5 × 10⁻⁹ mol/L⁻¹ and 1.6 ± 10⁻⁹ mol/L⁻¹, respectively, as compared to 1.2 × 10⁻⁹ mol/L⁻¹ for MA-15C5.

The amidolytic activity of all preparations of rscu-PA, K12G0S32, and K12G2S32 was less than 5,000 IU/mg u-PA-related antigen before treatment and less than 1,000 IU/mg u-PA-related antigen after treatment with benzamidine-Sepharose, confirming that these proteins were secreted primarily as single-chain inactive forms. Plasmin caused a time- and concentration-dependent increase of the amidolytic activity of rscu-PA and of the chimeric PA (Table 1).

The specific fibrinolytic activities, measured on fibrin plates, of single-chain K12G0S32, K12G2S32, and rscu-PA were similar (Table 1). The specific fibrinolytic activities of two-chain K12G0S32 and K12G2S32 were twofold lower than that of rscu-PA.

Functional Characterization

Treatment with plasmin. Plasminogen activation by the two-chain derivatives of all chimeras followed Michaelis-Menten kinetics, as evidenced by double reciprocal plots of the initial activation rate versus the plasminogen concentration (not shown). The catalytic efficiencies, derived from kcat and km values obtained by linear regression analysis, are summarized in Table 1.

Pharmacokinetics in Hamsters and Rabbits

When rscu-PA or the chimeric PA were injected as a bolus (100 μg/kg) in groups of three to six hamsters, the plasma

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Table 1. Comparative Biochemical Properties

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific Activity* (IU/mg)</th>
<th>Plasminogen Activation†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fibrin Plate</td>
<td>S-2444</td>
</tr>
<tr>
<td></td>
<td>Single-Chain</td>
<td>Two-Chain</td>
</tr>
<tr>
<td>rscu-PA</td>
<td>72,000 ± 5,000</td>
<td>220,000 ± 7,400</td>
</tr>
<tr>
<td>K12G0S32</td>
<td>67,000 ± 4,000</td>
<td>140,000 ± 10,000</td>
</tr>
<tr>
<td>K12G2S32</td>
<td>72,000 ± 12,000</td>
<td>110,000 ± 10,000</td>
</tr>
</tbody>
</table>

Results represent the mean ± SEM of the number of experiments ranging from three to six.

* Specific activities are expressed in IU/mg by comparison with the International Reference Preparation of u-PA; S-2444 is pyroglu-Gly-Arg-pNA, a chromogenic substrate for u-PA.

† Kinetic parameters were determined by linear regression analysis (r ≥ .99) of Lineweaver-Burk plots obtained with six substrate concentrations (ranging between 1.0 and 15 μmol/L) using 1.25 nmol/L of the enzyme.
model in which the pulmonary clot was prepared from normal human plasma. Dose-response data are summarized in Table 4. Thirteen experiments with saline infusion yielded a value for spontaneous lysis at 90 minutes of 19% ± 2% (mean ± SEM). Fibrinogen levels and α2-antiplasmin levels were not different from the baseline values. With rsu-PA at doses ranging from 0.25 to 2.0 mg/kg, lysis increased from 30% ± 4% to 68% ± 4%. The dose of rsu-PA at which the human plasma clots were lysed for 50% (ED50) was 1 mg/kg. Fibrinogen levels were 81% ± 2% at the highest dose of rsu-PA, whereas the α2-antiplasmin levels at this dose were 54% ± 7% of the baseline values. With K12G3S32 at doses ranging from 0.08 to 0.64 mg/kg, lysis increased from 22% ± 1% to 71% ± 3%. The value of ED50 was 0.23 mg/kg. Fibrinogen and α2-antiplasmin levels remained unchanged.

The dose-response data (% lysis vs mg/kg u-PA-related antigen administered) were fitted with the exponentially transformed sigmoidal function as described in the Methods (Fig 3A), yielding values for c (maximal lysis achieved, expressed in %), z (maximal rate of lysis, expressed in % lysis per mg/kg administered), and b (dose in mg/kg at which maximal rate of lysis occurs) as summarized in Table 5. Maximal rates of lysis with rsu-PA, K12G3S32, and K12G2S32 were 27% ± 5% (mean ± SEM, n = 34), 150% ± 22% (n = 40, P < .001 v rsu-PA), and 290% ± 41% per mg/kg (n = 37, P < .001 v rsu-PA and P = .003 v K12G2S32), respectively. Maximal rates were obtained at dose of 0.77 ± 0.10, 0.13 ± 0.02, and 0.066 mg/kg, respectively.

The specific thrombolytic activities of rsu-PA, K12G3S32, and K12G2S32 were determined by plotting percent lysis versus level of steady-state plasma u-PA-related antigen (µg/mL). The dose-response data, summarized in Table 4, were fitted with the exponentially transformed sigmoidal function (Fig 3B), yielding corresponding values for c, z and b as illustrated in Table 5. With K12G2S32, the maximal rate of lysis was ninefold higher than with rsu-PA (z’ = 310% ± 49%/ µg/mL and 36% ± 5%/µg/mL, respectively; P < .001) and occurred at an 11-fold lower plasma antigen level than with rsu-PA (b’ = 0.060 ± 0.001 µg/mL and 0.63 ± 0.12 µg/mL, 

Table 2. Comparative Fibrinolytic Properties in a Human Plasma Milieu In Vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>C80 (nmol/L)</th>
<th>C50 (nmol/L)</th>
<th>C20 (nmol/L)</th>
<th>C10 (nmol/L)</th>
<th>C5 (nmol/L)</th>
<th>C2 (nmol/L)</th>
<th>C1 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rsu-PA</td>
<td>24 ± 0.4 (6)</td>
<td>11 ± 0.9 (6)</td>
<td>4.4 ± 0.06 (3)</td>
<td>5.9 ± 0.06 (3)</td>
<td>700 ± 400 (5)</td>
<td>200 ± 100 (4)</td>
<td>50 ± 5 (3)</td>
</tr>
<tr>
<td>K12G3S32</td>
<td>12 ± 0.1 (6)</td>
<td>4.4 ± 0.06 (3)</td>
<td>11 ± 0.2 (4)</td>
<td>5.9 ± 0.06 (3)</td>
<td>700 ± 400 (5)</td>
<td>200 ± 100 (4)</td>
<td>50 ± 5 (3)</td>
</tr>
<tr>
<td>K12G2S32</td>
<td>11 ± 0.2 (4)</td>
<td>5.9 ± 0.06 (3)</td>
<td>11 ± 0.2 (4)</td>
<td>5.9 ± 0.06 (3)</td>
<td>700 ± 400 (5)</td>
<td>200 ± 100 (4)</td>
<td>50 ± 5 (3)</td>
</tr>
</tbody>
</table>

The concentration expressed in nmol/L is the total protein concentration as determined by Bradford assay. Data represent the mean ± SD of the number of experiments indicated in parentheses.

Abbreviation: C80, concentration required to obtain 50% lysis within 2 hours of a human plasma clot submerged in human plasma.

Thrombolytic Properties in a Hamster Pulmonary Embolism Model

The thrombolytic potencies of rsu-PA, K12G3S32, and K12G2S32 were determined in a hamster pulmonary embolism model in which the pulmonary clot was prepared from normal human plasma. Dose-response data are summarized in Table 4. Thirteen experiments with saline infusion yielded a value for spontaneous lysis at 90 minutes of 19% ± 2% (mean ± SEM). Fibrinogen levels and α2-antiplasmin levels were not different from the baseline values. With rsu-PA at doses ranging from 0.25 to 2.0 mg/kg, lysis increased from 30% ± 4% to 68% ± 4%. The dose of rsu-PA at which the human plasma clots were lysed for 50% (ED50) was 1 mg/kg. Fibrinogen levels were 81% ± 2% at the highest dose of rsu-PA, whereas the α2-antiplasmin levels at this dose were 54% ± 7% of the baseline values. With K12G3S32 at doses ranging from 0.08 to 0.64 mg/kg, lysis increased from 22% ± 1% to 71% ± 3%. The value of ED50 was 0.23 mg/kg. Fibrinogen and α2-antiplasmin levels remained unchanged.

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The specific thrombolytic activities of rsu-PA, K12G3S32, and K12G2S32 were determined by plotting percent lysis versus level of steady-state plasma u-PA-related antigen (µg/mL). The dose-response data, summarized in Table 4, were fitted with the exponentially transformed sigmoidal function (Fig 3B), yielding corresponding values for c, z and b as illustrated in Table 5. With K12G2S32, the maximal rate of lysis was ninefold higher than with rsu-PA (z’ = 310% ± 49%/ µg/mL and 36% ± 5%/µg/mL, respectively; P < .001) and occurred at an 11-fold lower plasma antigen level than with rsu-PA (b’ = 0.060 ± 0.001 µg/mL and 0.63 ± 0.12 µg/mL,
the PA to the plasma clot surface. Derived from the fibrin fragment D-dimer-specific antibody comparable to those of their suggesting that the single-chain Fv fragment efficiently targets which the thrombin cleavage site is removed by substitution of MA-15C5 fused to a 33,000 molecular weight derivative of meric PA consisting of a 25,000 molecular weight Fv fragment with a 13-fold higher fibrinolytic potency in an in vitro plasma clot lysis system of rscu-PA, K12G2S32, and K12G2S32 were determined in hamsters with an experimental pulmonary embolus consisting of a human plasma clot. The maximal rate of lysis with K12G2S32 was obtained at a sixfold lower dose and at a 10-fold lower steady-state plasma level than with rscu-PA, indicating that the increased thrombolytic potency of K12G2S32 is the result of fibrin-targeting of the chimera to the surface of the plasma clot via the Fv fragment. The maximal rate of lysis with K12G2S32 was obtained at a twofold lower dose, but at a threefold higher steady plasma u-PA antigen level in plasma) of rscu-PA, K12G2S32, and K12G2S32 was removed by substitution of AsnS8 with Asp.I2 (P < 0.001). The maximal rate of thrombolysis with K12G2S32 (z' = 140 ± 7) was twofold lower than that of K12G2S32 (P < 0.001), but still fourfold higher than that of rscu-PA (P < 0.001). Maximal rates of lysis with rscu-PA, K12G2S32, and K12G2S32 were obtained at steady-state plasma u-PA antigen levels of 0.63 ± 0.12, 0.06 ± 0.01, and 0.17 ± 0.04 µg/mL, respectively. However, when steady-state plasma levels of K12G2S32 and K12G2S32 were expressed in µg/mL fibrin D-dimer–binding protein, the values for b' were 0.09 ± 0.01 and 0.12 ± 0.01 µg/mL, respectively.

**DISCUSSION**

K12G2S32 is a 57,000 molecular weight single-chain chimeric PA consisting of a 25,000 molecular weight Fv fragment derived from the fibrin fragment D-dimer–specific antibody MA-15C5 fused to a 33,000 molecular weight derivative of rscu-PA, comprising amino acids Ala91–through Leu411 in which the thrombin cleavage site is removed by substitution of Phe57 with Asp.15 K12G2S32 was previously found to have a 13-fold higher fibrinolytic potency in an in vitro plasma clot lysis system than the unconjugated rscu-PA moiety, suggesting that the single-chain Fv fragment efficiently targets the PA to the plasma clot surface. K12G2S32 is a derivative of K12G0S32 in which the Asn-linked glycosylation site in the Fv moiety of K12G0S32 was removed by substitution of Asn88 with Glu. The specific fibrinolytic activity and the specific plasma clot lysis activities in an in vitro clot lysis system of both single-chain and two-chain K12G2S32 were found to be comparable to those of their K12G2S32 counterpart. However, following bolus injection in hamsters, K12G2S32 was cleared from the circulation at a fourfold to fivefold slower rate than rscu-PA and K12G2S32, whereas in rabbits K12G2S32 had a 10-fold slower clearance than rscu-PA and K12G2S32. Still K12G2S32 was cleared threefold to fivefold faster than rscu-PA-32k/MA-15C5, a recombinant chimera consisting of scu-PA-32k fused to intact humanized MA-15C5.10

The thrombolytic potencies (% lysis per mg u-PA equivalent administered per kg body weight) and the specific thrombolytic activities (% lysis per µg/mL steady-state u-PA–related antigen level in plasma) of rscu-PA, K12G2S32, and K12G2S32 were determined in hamsters with an experimental pulmonary embolus consisting of a human plasma clot. The maximal rate of lysis with K12G2S32 was obtained at a sixfold lower dose and at a 10-fold lower steady-state plasma level than with rscu-PA, indicating that the increased thrombolytic potency of K12G2S32 is the result of fibrin-targeting of the chimera to the surface of the plasma clot via the Fv fragment. The maximal rate of lysis with K12G2S32 was obtained at a twofold lower dose, but at a threefold higher steady plasma u-PA antigen level than with K12G2S32, which is in agreement with the fourfold slower clearance and with the twofold lower specific thrombolytic activity of K12G2S32 than those of K12G0S32. However, when the specific thrombolytic activities were expressed on the basis of the steady-state plasma levels of D-dimer–binding protein instead of on the basis of steady-state u-PA plasma levels, the values for K12G2S32 and K12G2S32 were similar. These findings can be explained by a twofold to threefold reduced fibrin-targeting capacity of the deglycosylated Fv fragment. With rscu-PA-32k/MA-15C5, the maximal rate of lysis in the hamster pulmonary embolism model was obtained with a fivefold lower dose than with that of K12G2S32, whereas the clearance of rscu-PA-32k/MA-15C5 was fivefold slower than that of K12G2S32. In aggregate, these findings indicate that the in vivo fibrin-targeting capacity of the Fv fragment derived from MA-15C5 fused to scu-PA is similar to that of the intact antibody fused to scu-PA.

Chimeric molecules such as K12G2S32 are designed for potential use as thrombolytic agents. A 10-fold increase of the thrombolytic potency of K12G2S32 as compared with that of rscu-PA, might result in the reduction of the therapeutical dose from 100 to 10 mg, a significant but possibly submaximal improvement. Enhanced targeting of the PA to the thrombus
might further increase its thrombolytic potency. Alternative fibrin-specific monoclonal antibodies, including 59D8, which is directed against an epitope in the aminoterminal region in the β-chain of fibrin,4 and 12B3, which is directed against an epitope in a 27-amino acid region spanning the carboxyterminal end of fragment E and the aminoterminal end of fragment D in the α-chain of fibrin,36 were found to have targeting capacities comparable to that of MA-15C5.36 (unpublished data) both in a hamster pulmonary embolism thrombosis model and in a rabbit jugular vein thrombosis model. Alternatively, reduction of the clearance rate might further increase the thrombolytic potency of the chimera. However, elucidation of the mechanisms of clearance of such single-chain constructs will require detailed structural investigation of the surfaces of both the Fv moiety and of the scu-PA moiety.

Thus, although the thrombolytic potency of K12G2S32 might have to be further improved, K12G2S32 might have significant advantages over scu-PA-32k/MA-15C5 for clinical use. The molecular weight of K12G2S32 is threefold lower than that of scu-PA-32k/MA-15C5. The lower molecular weight of K12G2S32 might allow a better diffusion of the molecule into a thrombus, and the lack of an Fc domain might reduce specific binding to platelets and monocytes which contain Fc receptors at their surface. Moreover, the absence of constant domains in the single-chain constructs might reduce their immunogenicity.

Although the present study does not provide avenues to further improve the thrombolytic potency of single-chain chimeric PA, the demonstration that a single-chain antibody fragment can efficiently target PA to the thrombus provides a rational basis for the further investigation of this approach.

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