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

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The pharmacokinetic and thrombolytic properties were determined of two recombinant single-chain chimeric plasminogen activators (PA) consisting of u-PA-32k, a low-molecular weight derivative of single-chain urokinase-type PA (scu-PA) comprising amino acids Ala1 through Leu673, 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 Asn253 with Glu (K12G2S32). Following bolus injection in hamsters, concentrations of recombinant scu-PA (rscu-PA) and of 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 twofold 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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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 as 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 in an affinity similar to that of intact MA-15C5.1 The scFv-K12G2S32 fragment was linked to rscu-PA-32k, yielding a single-chain chimeric PA K12G2S32, with a 13-fold higher in vitro thrombolytic potency than rscu-PA-32k.

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.
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 D. E. 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 plasmid (ie, phage/plasmid hybrid) vectors allowing cloning, site-directed mutagenesis, as well as sequencing to be performed with the same vector without recloning. Oligonucleotides were synthesized by the 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

pVLK12G2S32 encoding K12G2S32 for expression in S9 cells was constructed as described previously. K12G2S32 comprises the scFv-K12G2S32 sequence consisting of the Asp1-Arg168 light chain–variable domain of MA-15C5 linked with the Ala-Gly-Gln-Gly-Ser-Ser-Val synthetic linker to the Gin11-Ser119 heavy chain–variable domain that has been fused to the Ala127-Leu128-Asp129-Phe130 sequence of u-PA-33k. In K12G2S32, the thrombin cleavage site was removed by substitution of Phe130 with Asp130. Site-directed mutagenesis in the pMa/c-12G2S32 plasmid for expression of scFv-K12G2S32, using the 27-mer deoxyoligonucleotide dATGGCTGGTGTCTTGAGCTTG-

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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. rscu-PA was purified by affinity chromatography as described previously.

**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 to the method of Laemmli. Western blot analysis of the proteins separated by SDS-PAGE was performed essentially as described. 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 antisera conjugated to HRP in the second step, and the HRP substrate, 4-chloro-1-naphthol (BioRad).

Amino-terminal amino acid sequence analysis was performed using automated Edman degradation on an Applied Biosystems 470A gas-phase sequenator, and identification of amino acids by high-pressure liquid chromatography.

The concentrations of the different chimeric PA were determined initially by amino acid analysis performed in a Beckman (Palo Alto, CA) 119CL 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 assay (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. D-dimer-binding protein was quantitated 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) = Ac e -at + Be -bt, by graphical curve peeling. 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 these 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 (a and β) describing the disposition of the different activators from plasma, using standard formulas derived by Perier and Ghibaldi: volume of the central compartment, Vc = dose/ (A + B); total volume of distribution, Vd = dose/B; t1/2α = In 2/α, t1/2β = In 2/β, extrapolated area under the curve, AUC = A/α + B/β; plasma clearance, CL = dose/AUC. In addition, the pharmacokinetic properties of KcGfS2 and KcGfS3 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. 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 of u-PA (final concentration, 1 µg/mL) was determined in a similar manner.

**Pharmacokinetics**

The pharmacokinetic properties of rscu-PA, KcGfS2, and KcGfS3 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) = Ac e -at + Be -bt, by graphical curve peeling. 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 these 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 (a and β) describing the disposition of the different activators from plasma, using standard formulas derived by Perier and Ghibaldi: volume of the central compartment, Vc = dose/ (A + B); total volume of distribution, Vd = dose/B; t1/2α = In 2/α, t1/2β = In 2/β, extrapolated area under the curve, AUC = A/α + B/β; plasma clearance, CL = dose/AUC. In addition, the pharmacokinetic properties of KcGfS2 and KcGfS3 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.
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 was constructed starting from pULscu-PA as described in Materials and Methods.

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 × 10^10 mol/L^-1 and 1.6 ± 10^10 mol/L^-1, respectively, as compared to 1.2 × 10^10 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 k2 values obtained by linear regression analysis, are summarized in Table 1.

\[ \text{Fibrin clot lysis in plasma.} \quad \text{K12G0S32}, \quad \text{K12G2S32, and rscu-PA caused a time- and concentration-dependent lysis of a [125I]fibrin-labeled human plasma clot submerged in normal human plasma. The concentrations of the single-chain chimeric PA that were required to obtain 50% clot lysis in 2 hours (C50) were twofold lower than that of rscu-PA (Table 2). The C50 values for the two-chain derivatives obtained by cleavage of the single-chain forms with plasmin (at a 7.5% molar ratio), were twofold lower than those for their single-chain counterparts (Table 2).

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
concentration increased to 1.1 ± 0.06 μg/mL (mean ± SEM) for rscu-PA, 1.2 ± 0.12 μg/mL for K12G2S32, and 2.2 ± 0.2 μg/mL for K12G2S52. For both chimeric PA, more than 95% of the u-PA-related antigen that was detected in the ELISA for u-PA was also detected in the ELISA based on an IgG-specific catch antibody and a u-PA-specific tag antibody. This indicates that study proteins circulate primarily as intact chimeric molecules. The disappearance rate of all activators could be described by a sum of two exponential terms by graphical curve peeling. This yielded coefficients and exponents and calculated pharmacokinetic parameters as summarized in Table 3. The plasma clearances were 2.5 ± 0.06 mL·min⁻¹ for rscu-PA, 3.1 ± 0.05 mL·min⁻¹ for K12G2S32, and 0.67 ± 0.14 mL·min⁻¹ for K12G2S52.

When the chimeric PA were injected as a bolus (20 μg/kg) in two rabbits each, the plasma concentration increased to 0.02 pg/mL (mean ± SEM) as determined by Bradford assay. Data represent the mean ± SEM of three to six experiments.

Abbreviation: C0, concentration required to obtain 50% lysis within 2 hours of a human plasma clot submerged in human plasma.

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>C0 (μg/mL)</th>
<th>A (μg/mL)</th>
<th>B (μg/mL)</th>
<th>α (min⁻¹)</th>
<th>β (min⁻¹)</th>
<th>Vc (mL)</th>
<th>Vo (mL)</th>
<th>t1/2a (min)</th>
<th>t1/2b (min)</th>
<th>AUC (μg·min·mL⁻¹)</th>
<th>Clp (mL·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rscu-PA</td>
<td>1.1 ± 0.06*</td>
<td>1.0 ± 0.07</td>
<td>0.10 ± 0.01</td>
<td>0.43</td>
<td>0.06</td>
<td>9.1 ± 0.5</td>
<td>100 ± 9.3</td>
<td>1.6 ± 12</td>
<td>4.1 ± 0.1</td>
<td>2.5 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>K12G2S32</td>
<td>1.2 ± 0.12</td>
<td>1.1 ± 0.11</td>
<td>0.073 ± 0.02</td>
<td>0.58</td>
<td>0.06</td>
<td>8.4 ± 0.8</td>
<td>150 ± 4.4</td>
<td>1.2 ± 13</td>
<td>3.3 ± 0.5</td>
<td>3.1 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>K12G2S52</td>
<td>2.2 ± 0.2</td>
<td>2.0 ± 0.2</td>
<td>0.19 ± 0.03</td>
<td>0.25</td>
<td>0.03</td>
<td>4.6 ± 0.5</td>
<td>53 ± 6.2</td>
<td>2.8 ± 24</td>
<td>15 ± 1.7</td>
<td>0.67 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

In hamsters

In rabbits

Abbreviation: Clp, plasma clearance.

These data represent the mean ± SEM of three to six experiments.

These data represent the mean ± SD of two experiments.

Table 3. Pharmacokinetic Parameters of rscu-PA, K12G2S32, and K12G2S52 Following Bolus Injections (100 μg/kg) in Hamsters

<table>
<thead>
<tr>
<th>Compound</th>
<th>C0 (μg/mL)</th>
<th>A (μg/mL)</th>
<th>B (μg/mL)</th>
<th>α (min⁻¹)</th>
<th>β (min⁻¹)</th>
<th>Vc (mL)</th>
<th>Vo (mL)</th>
<th>t1/2a (min)</th>
<th>t1/2b (min)</th>
<th>AUC (μg·min·mL⁻¹)</th>
<th>Clp (mL·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rscu-PA</td>
<td>0.48 ± 0.021</td>
<td>0.38 ± 0.04</td>
<td>0.085 ± 0.02</td>
<td>0.45</td>
<td>0.11</td>
<td>98 ± 3</td>
<td>590 ± 70</td>
<td>1.5 ± 5.3</td>
<td>1.9 ± 0.2</td>
<td>28 ± 4</td>
<td></td>
</tr>
<tr>
<td>K12G2S32</td>
<td>0.51 ± 0.04</td>
<td>0.33 ± 0.02</td>
<td>0.18 ± 0.16</td>
<td>0.15</td>
<td>0.012</td>
<td>98 ± 4</td>
<td>280 ± 15</td>
<td>5 ± 60</td>
<td>17 ± 1.8</td>
<td>2.9 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>K12G2S52</td>
<td>0.48 ± 0.021</td>
<td>0.38 ± 0.04</td>
<td>0.085 ± 0.02</td>
<td>0.45</td>
<td>0.11</td>
<td>98 ± 3</td>
<td>590 ± 70</td>
<td>1.5 ± 5.3</td>
<td>1.9 ± 0.2</td>
<td>28 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: Clp, plasma clearance.

These data represent the mean ± SEM of three to six experiments.

These data represent the mean ± SD of two experiments.
Table 4. Clot Lysis and Hemostasis Parameters After Intravenous Infusion of rscu-PA, K12G2S32, and K12G8S32 in Hamsters With Pulmonary Embolism Consisting of a Human Plasma Clot

<table>
<thead>
<tr>
<th>Compound</th>
<th>n</th>
<th>Dose (mg/kg)</th>
<th>Clot Lysis at 90 min (%)</th>
<th>Residual Fibrinogen (% of baseline)</th>
<th>Residual a2-Antiplasmin (% of baseline)</th>
<th>Antigen at End of Infusion (µg/mL)</th>
<th>CI, (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>13</td>
<td>—</td>
<td>19 ± 2</td>
<td>140 ± 11</td>
<td>110 ± 2</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>rscu-PA</td>
<td></td>
<td>0.25</td>
<td>30 ± 4</td>
<td>130 ± 5</td>
<td>120 ± 5</td>
<td>0.15 ± 0.02</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.50</td>
<td>29 ± 4</td>
<td>130 ± 22</td>
<td>82 ± 3</td>
<td>0.26 ± 0.02</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1.00</td>
<td>41 ± 4</td>
<td>110 ± 3</td>
<td>80 ± 8</td>
<td>0.65 ± 0.03</td>
<td>2.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2.00</td>
<td>68 ± 4</td>
<td>81 ± 2</td>
<td>54 ± 7</td>
<td>1.5 ± 0.09</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>K12G2S32</td>
<td>4</td>
<td>0.08</td>
<td>22 ± 1</td>
<td>130 ± 4</td>
<td>120 ± 5</td>
<td>0.037 ± 0.002</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.16</td>
<td>46 ± 6</td>
<td>120 ± 6</td>
<td>120 ± 3</td>
<td>0.083 ± 0.006</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.32</td>
<td>61 ± 4</td>
<td>110 ± 5</td>
<td>100 ± 5</td>
<td>0.15 ± 0.01</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>0.64</td>
<td>71 ± 3</td>
<td>130 ± 18</td>
<td>120 ± 2</td>
<td>0.31 ± 0.06</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>K12G8S32</td>
<td>4</td>
<td>0.025</td>
<td>20 ± 3</td>
<td>82 ± 6</td>
<td>93 ± 6</td>
<td>0.071 ± 0.001</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.050</td>
<td>34 ± 4</td>
<td>96 ± 6</td>
<td>92 ± 7</td>
<td>0.12 ± 0.004</td>
<td>0.67 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>0.10</td>
<td>44 ± 3</td>
<td>75 ± 7</td>
<td>88 ± 9</td>
<td>0.19 ± 0.01</td>
<td>0.84 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.20</td>
<td>64 ± 5</td>
<td>80 ± 5</td>
<td>88 ± 8</td>
<td>0.41 ± 0.04</td>
<td>0.78 ± 0.1</td>
</tr>
</tbody>
</table>

Data represent the mean ± SEM of the number of 4 to 13 experiments.

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

respectively; P < .001). The maximal rate of thrombolysis with K12G2S32 (z' = 140 ± 7) was twofold lower than that of K12G8S32 (P < .001), but still fourfold higher than that of rscu-PA (P < .001). Maximal rates of lysis with rscu-PA, K12G2S32, and K12G8S32 were obtained at steady-state plasma u-PA antigen levels of 0.63 ± 0.12, 0.060 ± 0.011, and 0.17 ± 0.04 µg/mL, respectively. However, when steady-state plasma levels of K12G2S32 and K12G8S32 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 Ala132 through Leu411 in which the thrombin cleavage site is removed by substitution of Phe153 with Asp.15 K12G8S32 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 K12G0S32 counterpart. However, following bolus injection in hamsters, K12G2S32 was cleared from the circulation at a fourfold to fivefold slower rate than rscu-PA and K12G0S32, whereas in rabbits K12G2S32 had a 10-fold slower clearance than rscu-PA and K12G0S32. 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, K12G0S32, and K12G8S32 were determined in hamsters with an experimental pulmonary embolus consisting of a human plasma clot. The maximal rate of lysis with K12G0S32 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 K12G0S32 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 K12G8S32 was obtained at a twofold lower dose, but at a threefold higher steady plasma u-PA antigen level than with K12G0S32, which is in agreement with the fourfold slower clearance and with the twofold lower specific thrombolytic activity of K12G8S32 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 K12G0S32 and K12G8S32 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 K12G0S32, whereas the clearance of rscu-PA-32k/MA-15C5 was fivefold slower than that of K12G0S32. 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 therapeutic 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.

Table 5. Thrombolytic Potencies and Specific Thrombolytic Activities of rscu-PA, K12G2S32, and K12G2S32 Following Intravenous Infusion in Hamsters With Pulmonary Embolism Consisting of a Human Plasma Clot

<table>
<thead>
<tr>
<th>Compound</th>
<th>Thrombolytic Potency</th>
<th>Specific Thrombolytic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Dose (mg/kg)</td>
</tr>
<tr>
<td>rscu-PA</td>
<td>34</td>
<td>27 ± 5</td>
</tr>
<tr>
<td>K12G2S32</td>
<td>40</td>
<td>160 ± 22</td>
</tr>
<tr>
<td>K12G2S32</td>
<td>37</td>
<td>290 ± 41</td>
</tr>
</tbody>
</table>

The individual dose-response data of lysis versus dose or lysis versus steady-state antigen level were fitted with an exponentially transformed sigmoidal function y = (100c)/(1 + e-(z-b)/c). The mean ± SEM of the parameters b, c, and z, respectively, b', c' and z' were determined.

Abbreviations: z, z', maximal rate of lysis expressed as percent lysis per mg/kg dose (z), or percent lysis per µg/mL steady-state plasma antigen (z'). The values of z and z' were calculated as (ac/4) - e^b and (ac/4) - e^b', respectively. b, b', Dose expressed in mg/kg (b), or antigen level expressed in µg/mL plasma (b'), at which the rate of clot lysis is maximal. c, c', maximal rate of lysis achieved, expressed in percent.

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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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

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