Comparative Thrombolytic Properties of Single-Chain Forms of Urokinase-Type Plasminogen Activator

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The specific thrombolytic properties of urokinase and three molecular forms of single-chain urokinase-type plasminogen activator (scu-PA) were compared in a human plasma milieu in vitro and in an experimental thrombosis model in rabbits. These scu-PA molecules included M₄, 54,000 scu-PA from human urine (urinary scu-PA), scu-PA from conditioned media of a human lung adenocarcinoma cell line (CALU-3, ATCC, HTB-55) (cellular scu-PA) and an M₃, 32,000 proteolytic derivative of cellular scu-PA (scu-PA-32k). All four molecular forms induced significant lysis of a ¹²⁵I-labeled human plasma clot immersed in citrated human plasma at concentrations between 50 and 200 IU/mL. None of the four showed absolute fibrin-specificity, but at equivalent lytic dose the three single-chain forms appeared to cause less fibrinogen degradation and α₂-antiplasmin consumption than two-chain urokinase. In addition, the fibrinolytic potential of the three single-chain forms was largely maintained during pre-incubation in plasma for up to 48 hours whereas that of urokinase was completely inhibited. Intravenous (IV) infusion of cellular scu-PA or scu-PA-32k into rabbits with a ¹²⁵I-labeled thrombus in the jugular vein caused significant dose-dependent lysis at concentrations ranging from 8,700 to 35,000 and from 9,000 to 36,000 IU/kg respectively. Clot lysis was accompanied by minor α₂-antiplasmin consumption or fibrinogen breakdown. In contrast, urokinase induced lysis at doses between 20,000 and 200,000 IU/kg, but at higher doses was associated with significant systemic activation of the fibrinolytic system. It is concluded that scu-PA obtained from CALU-3 cell cultures has identical thrombolytic properties to that obtained from urine. In addition, the scu-PA-32k proteolytic derivative has the same fibrin-specific thrombolytic properties as the intact molecule. Cellular scu-PA and scu-PA-32k may therefore constitute more readily available alternatives for clot-selective thrombolytic therapy in man.

CONVERSION of plasminogen to plasmin is the key event occurring during physiologic fibrinolysis. Most efficiently it occurs at or near the fibrin surface where rapid inhibition of the generated plasmin does not take place.¹ Urokinase, obtained from human urine,² activates both circulating and fibrin-bound plasminogen similarly. As a result, it induces fibrinolysis but only in association with the so-called “lytic state,” characterized by consumption of α₂-antiplasmin and fibrinogen breakdown, which may predispose to bleeding.³ In contrast, fibrin-specific thrombolysis is now possible with tissue-type plasminogen activator (t-PA), which efficiently activates plasminogen only in the vicinity of a blood clot⁴ as the result of its specific affinity for fibrin.⁵ Its promise as a fibrin-specific thrombolytic-in-man agent has now been confirmed.⁶

More recently, a single-chain molecular form of urokinase has also been recognized as a potential fibrin-specific thrombolytic agent.⁷⁻¹² These molecular forms of urokinase have been designated pro-urokinase or preferably single-chain urokinase-type plasminogen activator (scu-PA).¹³ According to some studies, scu-PA activates plasminogen with high affinity (Kₘ < 1 μmol/L), even in the absence of fibrin,¹⁴⁻¹⁵ but this activity is inhibited by plasma. However, with the presence of fibrin in plasma, scu-PA regains its plasminogen-activating potential, though not via direct binding to fibrin. To date scu-PA has not become widely available for use in biochemical and clinical studies. The most readily available biological source of scu-PA, human urine, indeed contains such small amounts (<15 μg/L)¹⁶⁻¹⁷ that it is impractical for use in large-scale purification. Moreover, to date, urinary scu-PA, though fully characterized physicochemically and kinetically,¹⁷ has not been shown to be stable in plasma or to have clot-selective fibrinolytic properties in plasma.

A more widely available and enriched secondary source (CALU-3 cells) of scu-PA has been identified and a straightforward method for its purification developed.¹⁸ This has also led to the identification of a novel proteolytically modified M₃, 32,000 form of scu-PA,¹⁹ which has similar kinetic properties as intact scu-PA. The purpose of the present study was to compare the thrombolytic properties of urinary scu-PA, cellular scu-PA, and scu-PA-32k with those of urokinase.

MATERIALS AND METHODS

Human urinary scu-PA was purified as described¹⁷ with a specific activity of 51,000 IU/mg as determined by calibration against the International Reference Preparation for Urokinase (66/96) obtained from Dr P. J. Gaffney, National Institute for Biological Standards and Control, London, UK. Cellular scu-PA was purified from conditioned media of cultured human lung adenocarcinoma cells (CALU-3, ATCC, HTB-55)¹⁸ with a specific activity of 35,000 IU/mg. M₃, 32,000 scu-PA (scu-PA-32k) with a specific activity of 71,000 IU/mg was obtained from this same cell line as described elsewhere.¹⁸ Prior to use all three forms of scu-PA were freed of contaminating urokinase by chromatography on benzamidine-sepharose,²⁰ which reduced the urokinase content to less than 1%. Urinary urokinase (Winkovks) was a gift from Dr. E. Murano, Bureau of Biologics, Bethesda, MD.

Human fibrinogen¹¹ and thrombin²² were prepared by the methods described. Fibrinogen labeling with ¹²⁵I (Amersham Research

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were less than 5% to 10% of total releasable radioactivity over the fibrinogenolytic properties of the different forms of scu-PA and of urokinase were determined in an in vitro system consisting of a 125I-labeled human plasma clot immersed in citrated human plasma, as previously described. 23 The 125I-labeled plasma clots were added to 2.5 mL aliquots of pooled plasma, counted, and warmed to 37 °C in a water bath. Fifty microliters of plasminogen activator were then added. At one-hour intervals, 250 mL of plasma were removed, the 125I content measured, and the percent lysis determined from the solubilized radioisotope. Controls without added plasminogen activator were determined at each hourly sampling point and always were less than 5% to 10% of total releasable radioactivity over the four-hour experimental period. Plasma fibrinogen levels were determined immediately on each hourly sample,24 and a2-antiplasmin levels were later measured on frozen samples.25 Reported results are means of at least three separate experiments.

The stability of the plasminogen activators in plasma was measured in the following way. Mixtures were incubated at room temperature for varying periods of time with concentrations of plasminogen activator chosen to give at least 50% lysis within four hours. After pre-incubation, freshly prepared 125I-labeled plasma clots were added to the mixtures that were placed at 37 °C. Lysis rates were measured and calculated as above on duplicate samples.

**Thrombolysis in a rabbit jugular vein thrombosis model.** The in vivo thrombolytic properties of cellular scu-PA and scu-PA-32k were compared to those of urokinase in the rabbit jugular vein thrombosis model previously described.26 In the present study 125I-labeled jugular vein thrombi were aged for 30 minutes, then the plasminogen activator was administered as a 10% bolus injection, followed by continuous infusion of the remaining 90% dose over four hours via a contralateral marginal ear vein. Thrombolysis was quantitated 30 minutes after the end of the infusion by counting the residual radioactivity in the jugular vein segment. Blood samples of 1 mL were drawn into 0.01 mol/L citrate and used for determinations of residual fibrinogen and a2-antiplasmin levels.

**RESULTS**

The dose response of fibrinolysis in human plasma in vitro of the three forms of scu-PA and of urokinase is shown in Fig 1. Urinary scu-PA induced significant clot lysis at a concentration of 200 IU/mL in two to three hours (Fig 1A). At a twofold higher concentration, more rapid lysis occurred, 35% within one hour and 95% within two hours. No significant lysis was observed over the four-hour observation period at concentrations below 100 IU/mL. Similar dose-related fibrinolytic responses were also seen with cellular scu-PA (Fig 1B) and scu-PA-32k (Fig 1C), with significant lysis occurring with 100 IU/mL or more and to lysis with 50 IU/mL or less. Lysis progressed with time with all three molecular forms of scu-PA. When lysis induced by urokinase was quantitated (Fig 1D), initially a more rapid and more potent effect was seen. The addition of only 50 IU/mL induced 50% lysis within one hour. This rapid initial lysis was proportional to the amount of urokinase added, but in contrast to that observed with the scu-PA forms, the subsequent lysis rates decreased with time. With urokinase, lysis thus occurred predominantly within the first two hours.

The relative fibrin-specificities of the urokinase-type plasminogen activators (u-PAs) are shown in Fig 2. At concentrations of 200 IU/mL (urinary), 160 IU/mL (cellular), or 95 IU/mL (scu-PA-32k) 50% or more lysis was observed within four hours, all with less than 25% fibrinogen breakdown. At concentrations of 400 IU/mL of urinary scu-PA (Fig 2A) or of 200 IU/mL of scu-PA-32k (Fig 2B), which gave 100% after three hours, plasma fibrinogen levels decreased to below 25% of the baseline level. With urokinase (Fig 2D), 45% to 55% lysis occurred after one hour with both 48 and 96 IU/mL, concomitant with 30% and 70% fibrinogen degradation respectively. At a concentration of 96 IU/mL urokinase was also associated with a greater than 75% decrease in fibrinogen levels after two hours while not causing 100% lysis even after four hours. Thus, although none of the urokinase-type plasminogen activators was absolutely fibrin-specific in this in vitro model, equivalent clot lysis appeared to be accompanied by less extensive fibrinogen degradation with all three single-chain forms. Measurement of a2-antiplasmin levels confirmed this, with less than 50% consumption associated with 50% lysis by the three single-chain forms, while 50% lysis by urokinase was always accompanied by greater than 50% a2-antiplasmin consumption (not shown).
clots labeled with 2H-fibrin following addition of urinary scu-PA were at one hour: 0.99 ± 0.025; at two hours: 0.92 ± 0.059; at three hours: 0.96 ± 0.034; and at four hours: 0.90 ± 0.045. Each data point represents the mean value of at least three separate experiments. ± SD for one concentration of each giving significant lysis.

The relative stability of scu-PA in plasma was illustrated by pre-incubation studies with results shown in Fig 3. Lysis of freshly prepared plasma clots was measured after pre-incubation of mixtures of plasma with each of the four forms of u-PA at room temperature. Without preincubation all three single-chain forms, at concentrations between 160 and 400 IU/mL, gave 50% lysis within 1.5 to 2.5 hours. The more rapid initial rate for urokinase was evident by 50% lysis with 48 IU/mL at only one hour (panel A). After pre-incubation for 24 hours (panel B) and 48 hours (panel C), the potency of the single-chain forms was largely maintained, with 50% lysis still occurring within 2.5 to 4 hours. In contrast, urokinase was completely inhibited within the first 24 hours.

The thrombolytic properties and the fibrin specificity of the more readily available cellular scu-PA and scu-PA-32k were compared with those of urokinase in a rabbit jugular vein thrombosis model with results summarized in Table 1. Lysis measured 30 minutes after the end of infusion of solvent into control animals was 8.9% ± 0.6%. Dose-dependent lysis (12% to 48%) was observed for urokinase over a range of 20,000 to 200,000 IU (0.2 to 2.0 mg) per kilogram. A similar range of lysis (13% to 40%) was produced by infusion of cellular scu-PA over a concentration range of 8,700 to 35,000 IU (0.25 to 1.0 mg) per kilogram and by scu-PA-32k between 9,000 and 36,000 IU (0.13 to 0.5 mg) per kilogram. Comparable thrombolysis (20% to 25%) was obtained with 100,000 IU/kg urokinase, 17,000 IU/kg scu-PA, and 18,000 IU/kg scu-PA-32k, ie, five- to eightfold lower total doses for both forms of scu-PA than for urokinase. In addition, both forms of scu-PA failed to induce systemic fibrinolytic activation, as evidenced by normal

Table 1. Thrombolysis After Infusion of Different Molecular Forms of scu-PA or Two-Chain Urokinase in Rabbits With Jugular Vein Thrombosis

<table>
<thead>
<tr>
<th>Agent</th>
<th>Dose (IU/kg)</th>
<th>n</th>
<th>Lysis (percent)</th>
<th>Recovery (percent)</th>
<th>Fibrinogen (percent)</th>
<th>α2-Antiplasmin (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>5</td>
<td>8.9 ± 0.6</td>
<td>99 ± 1.8</td>
<td>101 ± 3</td>
<td>94 ± 4</td>
</tr>
<tr>
<td>Urokinase</td>
<td>20,000</td>
<td>3</td>
<td>12 ± 0.8</td>
<td>99 ± 1.5</td>
<td>101 ± 5</td>
<td>90 ± 6</td>
</tr>
<tr>
<td></td>
<td>50,000</td>
<td>3</td>
<td>16 ± 2.3</td>
<td>94 ± 2.2</td>
<td>95 ± 5</td>
<td>87 ± 2</td>
</tr>
<tr>
<td></td>
<td>100,000</td>
<td>3</td>
<td>22 ± 0.8</td>
<td>95 ± 0.9</td>
<td>91 ± 7</td>
<td>74 ± 6</td>
</tr>
<tr>
<td></td>
<td>200,000</td>
<td>4</td>
<td>48 ± 4.3</td>
<td>93 ± 5.3</td>
<td>39 ± 24</td>
<td>42 ± 14</td>
</tr>
<tr>
<td>Cellular scu-PA</td>
<td>8,700</td>
<td>3</td>
<td>13 ± 1.3</td>
<td>94 ± 1.0</td>
<td>92 ± 12</td>
<td>92 ± 7</td>
</tr>
<tr>
<td></td>
<td>17,000</td>
<td>6</td>
<td>21 ± 1.4</td>
<td>94 ± 1.8</td>
<td>99 ± 3</td>
<td>85 ± 3</td>
</tr>
<tr>
<td></td>
<td>35,000</td>
<td>4</td>
<td>34 ± 3.8</td>
<td>88 ± 1.8</td>
<td>92 ± 2</td>
<td>97 ± 4</td>
</tr>
<tr>
<td></td>
<td>9,000</td>
<td>3</td>
<td>15 ± 1.1</td>
<td>96 ± 1.0</td>
<td>98 ± 2</td>
<td>90 ± 8</td>
</tr>
<tr>
<td>scu-PA-32K</td>
<td>18,000</td>
<td>3</td>
<td>24 ± 1.5</td>
<td>94 ± 3.3</td>
<td>99 ± 4</td>
<td>95 ± 2</td>
</tr>
<tr>
<td></td>
<td>36,000</td>
<td>3</td>
<td>40 ± 4.1</td>
<td>93 ± 3.2</td>
<td>90 ± 5</td>
<td>100 ± 2</td>
</tr>
</tbody>
</table>

Each figure represents the mean ± standard error of the mean of the number of experiments indicated by n.
fibrinogen and $a_2$-antiplasmin levels. Lysis with urokinase occurred with less fibrin-specificity as reflected by the dose-dependent consumption of $a_2$-antiplasmin. Fibrinogen degradation with urokinase was very pronounced at the highest dose (200,000 IU/kg) with plasma levels falling to 39% of baseline.

**DISCUSSION**

Evidence is accumulating that the single-chain form of urokinase, presently designated scu-PA,16 shows potential as a fibrin-specific thrombolytic agent. Although scu-PA isolated from its natural source, human urine, has been characterized to some extent,16,17 all information on its fibrin specificity in plasma in vitro6,9 or in vivo7,8,10-12 has been obtained with material isolated from secondary sources, eg, cell culture or recombinant DNA technology. In the present study we compared the specific thrombolytic properties of scu-PA derived from human urine, its natural biological source, with that of scu-PA isolated from CALU-3-conditioned cell culture media. Both forms of scu-PA do induce clot lysis with relatively higher fibrin specificity than urokinase in a human plasma milieu. In addition, both scu-PA forms demonstrate relatively higher stability in plasma than urokinase, with an approximately 50% preservation of fibrinolytic activity after 48 hours incubation of scu-PA with plasma but total inhibition of urokinase within 24 hours. This relative stability, as previously recognized for scu-PA from other secondary natural6 or recombinant sources,8 has been ascribed to the presence of specific plasma inhibitor(s) of urokinase. Such an inhibitor has in fact now been identified in human plasma with inhibitory activity toward urokinase but not scu-PA.27 This inhibition would not account for the partial reduction in fibrinolytic activity of the single-chain forms seen during pre-incubation, though some loss of activity after prolonged room temperature exposure is perhaps not surprising.

From these observations two extrapolations can be made. First, since scu-PA circulating in blood28 is most likely identical to that isolated from urine, it may as such contribute to physiologic fibrin-specific thrombolysis via its relatively unique fibrinolytic properties. Second, scu-PA from cellular origin, specifically isolated from CALU-3-conditioned cell culture media, having biological properties identical to those of urinary scu-PA, may constitute a valid and more practical alternative for the study of the biochemical and biological properties of scu-PA.

In addition, the in vitro and in vivo thrombolytic properties of scu-PA–32k, a previously unrecognized molecular form of scu-PA, were compared with that of its well-characterized $M_r$, 54,000 counterpart. Though structurally different, both forms induced very similar clot lysis, relatively distinct from that of urokinase. Their molar-specific fibrinolytic activities, as measured on fibrin plates, were identical, although their thrombolytic potency in vivo was higher than that of urokinase, as contrasting with the higher in vitro potency of urokinase. This discrepancy may well be related to species differences between the plasma systems utilized, especially with the known lack of sensitivity of the rabbit to urokinase.29

While our results indicate that the moiety(ies) responsible for the fibrin specificity of scu-PA are not dependent upon the NH$_2$-terminal and kringle-containing portions of the $M_r$, 54,000 protein, which are indeed absent in scu-PA–32k,18 the exact structures responsible for its unique action remain, however, to be more precisely defined. Since scu-PA appears to act more via a high affinity for its natural substrate plasminogen than via a specific affinity for fibrin, it is perhaps not surprising that the domains responsible for lysine (fibrin) binding could be deleted without any impact on the fibrinolytic capacity of the enzyme. In fact, scu-PA–32k does display the same high affinity for plasminogen ($K_m$ = 0.9 $\mu$mol/L) as $M_r$, 54,000 scu-PA, a property not present in either molecule following conversion to two-chain u-PA by cleavage of the Lys 158-Ile 159 peptide bond. In any case, scu-PA–32k, a low $M_r$ variant of scu-PA with identical thrombolytic properties, may constitute a more practical alternative to the native high $M_r$, form, particularly for the large-scale production of this thrombolytic agent by recombinant DNA technology. As such it may offer significant potential for future development.

In summary, it is concluded that scu-PA derived from its natural biological source, human urine, has distinctive fibrinolytic properties from urokinase. Furthermore, the more readily available scu-PA, obtained from CALU-3 cell structures, has identical thrombolytic properties to the material of urinary origin. Thus, scu-PA and scu-PA–32k, a proteolytic derivative of scu-PA, may constitute valid alternatives for future physiologic and therapeutic studies in man.

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