Thrombolytic Properties of Desmodus rotundus (vampire bat) Salivary Plasminogen Activator in Experimental Pulmonary Embolism in Rats

By Werner Witt, Berthold Baldus, Peter Bringmann, Linda Cashion, Peter Donner, and Wolf-Dieter Schleuning

rDSP\textsubscript{A} (recombinant Desmodus salivary plasminogen activator α1) is a recombinant protein corresponding to a natural plasminogen activator from the vampire bat Desmodus rotundus. The thrombolytic properties of rDSP\textsubscript{A}, and tissue-type plasminogen activator (t-PA) were compared in a rat model of pulmonary embolism. Whole blood clots, produced in vitro and labeled with \textsuperscript{125}I-fibrinogen, were embolized into the lungs of anesthetized rats. Thrombolysis was calculated from the difference between initial clot radioactivity and that remaining in the lungs at 60 minutes.

The initial clot radioactivity was then sampled for gamma counting, measurement of hemostatic factors, and plasminogen activator antigen levels. Thrombolysis at 3, 10, 30, and 100 nmol/kg intravenously (10% bolus, 90% over 60 minutes) amounted to 30% ± 2%, 51% ± 4%, 85% ± 4%, 98% ± 0% for rDSP\textsubscript{A}, and 30% ± 3%, 41% ± 3%, 57% ± 6%, 93% ± 2% for t-PA (controls: 29% ± 2%; mean ± SEM, n = 6). t-PA at 100 nmol/kg significantly decreased fibrinogen, plasminogen, and α-antiplasmin levels by 33% ± 7%, 38% ± 8%, and 61% ± 9%, whereas rDSP\textsubscript{A}, at 100 nmol/kg only lowered α-antiplasmin significantly (by 29% ± 6%). Compared with t-PA, rDSP\textsubscript{A} is more potent and more clot selective (fibrin specific) thrombolytic agent. These results suggest that rDSP\textsubscript{A} may be safer and more efficacious than currently used thrombolytics.

MATERIALS AND METHODS

Animal model. The method is based on that described by Clozel et al. Normotensive male Wistar rats (320 to 400 g) were anesthetized with 90 mg/kg i.p. pentobarbitone sodium. Blood was withdrawn from a carotid artery catheter and anticoagulated with 3.18% sodium citrate solution (9 vol citrated whole blood + 1 vol). One milliliter of citrated whole blood + 40 μL (about 1.5 × 10\textsuperscript{5} Bq) radiolabeled fibrinogen (3.4 MBq/mg \textsuperscript{125}I-fibrinogen; Amersham, Buckinghamshire, England) was then clotted by the addition of 20 μL (500 nmol/L) calcium and 30 μL thromboplastin (Thromborel Behring, Marburg, Germany) in a silicone catheter (0.058 in. ID). The clot was then aged for 2 hours at 37°C including the time for withdrawal from the catheter and careful washing in warm saline to remove \textsuperscript{125}I-fibrinogen not bound to the clot. Thereafter, 12-mm pieces of the clot (about 3 × 10\textsuperscript{5} Bq) were counted in a gamma counter, aspirated into the tip of a polyethylene catheter (PE205), and injected into the left external jugular vein. The clot was flushed into the venous circulation with 0.5 mL warm saline. Embolization into the lungs was successfully achieved in all but three animals, which did not show radioactivity in the lungs nor in blood at any time and were therefore excluded. Immediately after clot injection thrombolytic treatment was started and continued for 1 hour.

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during which blood samples for radioactivity measurement were withdrawn (0, 10, 20, 40, and 60 minutes). Before the treatment period and at the end (60 minutes) blood samples were taken to obtain plasma for determination of hemostasis factors and plasminogen activator antigen levels (see below). The heart and lungs were then removed and residual radioactivity was counted. Thrombolysis was determined as the remaining radioactivity in the lungs divided by that injected with the clot.

Whole blood samples (200 µL) were used for gamma counting of radioactivity. Plasma was obtained from 400 µL of whole blood to which 40 µL of 3.16% sodium citrate and 50 µL of 10 nmol/L PPACK (Calbiochem, La Jolla, CA) were added to stabilize the plasma against proteolytic degradation. All plasma samples were stored frozen at −80°C.

**Hemostasis factors.** Plasma fibrinogen was measured as clottable protein according to the method of Clauss.13 Plasminogen and α2-antiplasmin were determined on microtiter plates using the chromogenic substrate S-2251 (Kabi/Vitrum, Munich, Germany) and expressed as percentage of that in normal pooled rat plasma. For comparisons among groups, the percent changes from initial values were calculated from the original data.

**Plasminogen activator antigen determinations.** rDSP4, antigen levels in rat plasma were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) employing biotinylated rabbit anti-rDSP4 antibodies. This assay had a linear range expanding from 2 ng/mL to 100 ng/mL with a lower detection limit of 1 ng/mL.

r-PA antigen concentrations in rat plasma were also determined by ELISA using a commercially available kit (Biopool, Umeå, Sweden).

**Plasminogen activators.** Each dose of plasminogen activator was administered in 2 mL of isotonic saline. Ten percent of the total volume was injected as an intravenous (IV) bolus followed by the remaining 1.8 mL infused over 1 hour.

rDSP4 was produced by heterologous expression of its cDNA in CHO cells. The entire cDNA clone was subcloned into the expression plasmid pSVLRI.11 Transfection of dhfr-CHO cells was performed according to Chen and Okayama.14 Transfectants were screened for producers by fibrin plate assay and immediately transfected into CHO cells. The entire cDNA clone was subcloned into the expression plasmid pSVLRI. Transfection of dhfr-CHO cells was achieved by affinity chromatography on immobilized Erythrina trypsin inhibitor followed by ion exchange chromatography on a Mono S resin. The purity of isolated rDSP4 was determined by RP-HPLC, gel electrophoresis, and N-terminal sequence identity. Its apparent molecular weight was assessed to be 52,000 Kd. Bioactivity was measured on fibrin plates and expressed as t-PA units by comparison to the activity of the international standard preparation of t-PA (86/670). Specific activity was estimated as 330,000 U/mg protein using amino acid composition for determination of mass.

A commercial recombinant t-PA preparation registered for clinical use (Actilyse) was purchased from Thomae (Biberach, Germany). Its specific activity on fibrin plates was determined as 1,000,000 U/mg.

**Statistics.** All data were presented as means ± SEM. For comparisons of single doses versus control and equimolar doses of the two plasminogen activators, multiple t-tests (two-tailed, α = .05 per comparison) were used. A comparison of the dose-response relationships (thrombolytic effects, lung radioactivity) was performed by means of linear regression and analysis of covariance. To get linear logdose-response relationships logarithms of percent lysis values (see below) were taken and the highest dose of rDSP4 was excluded. Parallel lines fitted did not show a deviation from the hypothesis of parallelism (α = .05) and their distance differed significantly from zero (α = .05).

## RESULTS

**Thrombolysis (lung radioactivity).** rDSP4 was significantly more effective than t-PA, achieving 50.7% ± 3.7% and 85.0% ± 3.8% lysis for rDSP4, and 40.7% ± 3.0% and 56.7% ± 6.1% for t-PA, respectively (n = 6 to 8). Regression analysis (see above) showed that rDSP4 was significantly more potent than t-PA, 2 times on a molar and 2.5 times on a weight basis.

**Time course of blood radioactivity.** Mean peak levels of whole blood radioactivity increased in a dose-dependent manner for rDSP4 and t-PA (Fig 2, Table 1). Usually in controls, as well as at doses up to 30 nmol/kg, the blood radioactivity increased to a maximum at 60 minutes after the start of (plasminogen activator) infusion. However, with 100 nmol/kg t-PA or rDSP4, the maxima of mean blood radioactivity were already reached at 40 or 20 minutes, respectively (Fig 2). In six of six animals receiving 100 nmol/kg rDSP4, the early maximum in blood radioactivity was followed by a decrease, whereas for the same dose of t-PA this was seen in only three of eight rats, namely the three animals that showed the highest lytic efficacy. Although the extent of thrombolysis measured by lung radioactivity was slightly higher with 100 nmol/kg rDSP4, compared with the equimolar dose of t-PA, peak blood counts were more than 50% lower in the case of rDSP4.

![Graph](https://www.bloodjournal.org/618/28/1214G1.png) **Fig 1.** Thrombolytic effects of 3, 10, 30, and 100 nmol/kg IV (10% bolus, 90%/60 min) rDSP4 and t-PA in pulmonary embolism in rats. Percentage thrombolysis was calculated from the difference between initial radioactivity of the thrombus and that remaining in the lungs at 60 minutes. Asterisks refer to significance versus control (α = .05, multiple t-test; mean ± SEM, n = 6 to 8, control n = 13).
Table 1. Plasma Levels, Thrombolysis, and Peak Blood Radioactivity Levels

<table>
<thead>
<tr>
<th>Dose (nmol/kg)</th>
<th>Plasma Level (μg/mL)</th>
<th>Thrombolysis (lung radioactivity) (% of control)</th>
<th>Peak Levels of Blood Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>28.5 ± 1.5</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>rDSP₄₂₀</td>
<td>3</td>
<td>0.3 ± 0</td>
<td>29.8 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.8 ± 0.1</td>
<td>50.7 ± 3.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.7 ± 0.2</td>
<td>85.0 ± 3.8*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9.7 ± 0.6</td>
<td>98.4 ± 0.2*</td>
</tr>
<tr>
<td>t-PA</td>
<td>3</td>
<td>0.2 ± 0.1</td>
<td>29.9 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.3 ± 0</td>
<td>40.7 ± 3.0*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.2 ± 0.1</td>
<td>56.7 ± 6.1*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.5 ± 2.1</td>
<td>92.5 ± 1.5*</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM of n = 6 to 8 (control n = 13) animals per group; blood radioactivity reflects mean of individual peaks (Fig 2 shows means at a given time).

*Significant versus control.
†Significant versus equimolar dose of t-PA (α = .05 per comparison, multiple t-test).

Plasminogen activator antigen levels. The plasma levels of both rDSP₄₂₀ and t-PA measured at 60 minutes increased in a dose-dependent manner up to means of 9.7 μg/mL and 7.5 μg/mL for 100 nmol/kg rDSP₄₂₀ and t-PA, respectively (Table 1). The dose-response curves of thrombolysis (lung radioactivity) versus mean plasma levels (instead of total dose as in Fig 1) at 60 minutes show both plasminogen activators to be approximately equipotent (Fig 2). At equimolar doses mean plasma levels were 1.5, 2.7, 3.1, and 1.3 times higher for rDSP₄₂₀ at 3, 10, 30, and 100 nmol/kg, respectively.

Hemostasis factors. α₂-Antiplasmin levels measured at the end of the treatment period (60 minutes) decreased significantly at 100 nmol/kg of either rDSP₄₂₀ or t-PA (Table 2). In contrast, neither plasma fibrinogen nor plasminogen concentrations changed significantly with rDSP₄₂₀ up to 100 nmol/kg, while the same dose of t-PA depleted fibrinogen and plasminogen levels significantly by 33% ± 7% and 38% ± 8%, respectively (n ≥ 6).

DISCUSSION

Desmodus rotundus plasminogen activator rDSP₄₂₀ has previously been shown to be a potent plasminogen activator in vitro with a markedly higher fibrin specificity than t-PA.³⁰ We now report that rDSP₄₂₀ is a very effective thrombolytic agent in vivo. rDSP₄₂₀ is 2.5 times more potent than t-PA (by mass). However, at equal antigen plasma levels both plasminogen activators have a comparable thrombolytic potency, i.e., their specific activity in vivo (thrombolytic activity/plasma level) is about equal. The higher plasma levels for rDSP₄₂₀ compared with t-PA at 60 minutes indicate different pharmacokinetic profiles of the two plasminogen activators. Indeed, preliminary pharmacokinetic studies in rats and dogs show that rDSP₄₂₀ has a longer β-elimination half-life and a slower clearance than t-PA (unpublished results). Hence, infusion of rDSP₄₂₀ could lead to higher steady-state plasma levels, which would then account for the observed differences in effectiveness of equimolar doses of rDSP₄₂₀ and t-PA.

The pulmonary lung embolism model in rats described here is highly reproducible. Therefore, even the small difference in potency between t-PA and rDSP₄₂₀ becomes significant. The rate of spontaneous thrombolysis in controls, which is stable and acceptably low, has been shown to be due to release of endogenous t-PA in the lungs.†² A specific advantage of this model is that full effectiveness of t-PA is only achieved at doses where clot specificity is lost.
and systemic plasminogen activation and consumption of coagulation factors (fibrinogenolysis) are induced. This is very similar to the clinical usage of t-PA in patients with myocardial infarction, although fibrinogenolysis in humans appears to occur at lower plasma levels than in rats. In contrast to t-PA, rDSP4 seems to activate clot-bound plasminogen only because it neither depletes circulating plasminogen nor induces fibrinogenolysis in the dose range tested. The observed decrease in α2-antiplasmin levels probably reflects inhibition of clot-bound plasmin or plasmin liberated from the clot during thrombolysis. Therefore, these results confirm in vivo the higher fibrin specificity of rDSP4 observed in vitro.

The elevation of blood radioactivity during thrombolysis indicates release of fibrin degradation products (FbDP) from the radiolabeled clot. Up to doses of 30 nmol/kg, ie, in the suboptimal dose range, peak radioactivity levels in the blood are consistently attained at the end of the treatment period (60 minutes) for both rDSP4 and t-PA. Using 100 nmol/kg of both agents, nearly complete thrombolysis was achieved in most animals. When clot lysis is complete, clearance of FbDP from the blood should eventually exceed liberation, leading to a decrease of blood radioactivity. Indeed, this was observed in all animals treated with 100 nmol/kg rDSP4, but only in three of eight animals receiving an equimolar dose of t-PA. These differences clearly indicate a more rapid thrombolysis with rDSP4.

Furthermore, although 100 nmol/kg of rDSP4 is slightly more effective than the equimolar dose of t-PA, blood radioactivity levels in the rDSP4 group were always lower. This indicates that FbDP released from the clot by rDSP4, induced lysis are more rapidly cleared from the blood than those liberated with t-PA. Because plasmin is the ultimate effector of clot degradation for both plasminogen activators, this puzzling observation seems to suggest a qualitatively different (acting) plasmin to be generated by rDSP4. Another possible explanation could be that t-PA complexes with FbDP are formed that may have a reduced liver clearance. Most significantly, higher levels of circulating FbDP could be one explanation for the lower clot specificity of t-PA, because FbDP have been shown to stimulate (systemic) plasminogen activation by t-PA.

rDSP4 is an effective thrombolytic agent in experimental pulmonary embolism in rats. Compared with t-PA, rDSP4 is the more potent thrombolytic agent, it may also achieve lysis more rapidly and more completely and, because of its improved clot (fibrin) specificity, rDSP4 may also be the safer agent, reducing the risk of bleeding, which is still the predominant side effect of all thrombolytics.

ACKNOWLEDGMENT

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