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

rDSPα4 (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 rDSPα4 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 125I-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 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 rDSPα4, 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 α2-antiplasmin levels by 33% ± 7%, 38% ± 8%, and 61% ± 9%, whereas rDSPα4 at 100 nmol/kg only lowered α2-antiplasmin significantly (by 29% ± 6%). Compared with t-PA, rDSPα4 is the more potent and more clot selective (fibrin specific) thrombolytic agent. These results suggest that rDSPα4 may be safer and more efficacious than currently used thrombolytics. © 1992 by The American Society of Hematology.

THE INITIAL anticipation that the safety of thrombolytic therapy would improve with the introduction of human tissue-type plasminogen activator (t-PA) has not been fulfilled in clinical trials.1 The expectation that bleeding episodes might be reduced with t-PA was based on early in vitro and experimental animal work which suggested that t-PA, unlike other clinically available thrombolytics, would not deplete coagulation factors in effective thrombolytic doses.2,4 These experimental findings were interpreted as the result of the enhancement of t-PA-mediated plasminogen activation in the presence of fibrin.1 Unfortunately, a strict fibrin-specific (clot-specific) plasminogen activation is limited to low doses (low plasma levels) of t-PA in vivo. However, at higher plasma levels that are necessary to achieve satisfactory clinical results in patients with myocardial infarction, the limitation of plasmin formation to the presence of fibrin on the clot surface is lost and systemic plasminogen activation occurs that leads to plasminemia and subsequent degradation of coagulation factors like fibrinogen.1 The lack of a strict fibrin specificity under clinically relevant conditions may explain the failure of t-PA to reduce bleeding. Furthermore, it has been suggested that all fibrinolytic agents, which reduce systemic plasminogen levels, may also show a reduced thrombolytic efficacy.6

The intensive search for new and safer plasminogen activators with increased clot specificity has shown no major breakthrough until recently, when a series of reports described the isolation, cloning, and characterization of a new class of plasminogen activators derived from saliva of the vampire bat Desmodus rotundus.7,8 A family of four Desmodus plasminogen activators encoded by four distinct genes has been discovered.11 Although there is striking structural homology of these bat plasminogen activators to human t-PA, their fibrin specificity is greatly increased in comparison with t-PA. Most interesting, the increased fibrin dependence of bat plasminogen activators does not correlate with fibrin binding because the two smaller forms of the family actually have no fibrin affinity.7

In this study we demonstrate that rDSPα4, the recombinant protein corresponding to one of the two larger natural plasminogen activator forms (α1, α2) from Desmodus, is an effective thrombolytic agent in vivo in experimental pulmonary embolism in rats. rDSPα4 is more potent and more fibrin-specific in vivo than t-PA at doses that achieve full thrombolytic efficacy. There is also evidence that rDSPα4 may lyse blood clots faster and more completely in vivo than t-PA. The in vivo results reported here confirm the in vitro data previously obtained by other investigators (see Discussion) and exhibit some additional interesting features that may further improve the profile of this new thrombolytic agent.

MATERIALS AND METHODS

Animal model. The method is based on that described by Clozel et al.12 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 1 vol). One milliliter of citrated whole blood + 40 μL (about 1.5 × 105 Bq) radiolabeled fibrinogen (3.4 MBq/mg 125I-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 125I-fibrinogen not bound to the clot. Thereafter, 12-mm pieces of the clot (about 3 × 106 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 pmol/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 α,-antiplasmin were determined on microtiter plates using the chromogenic substrate S-2251 (KabiVitrum, Munich, Germany) and expressed as percentage of that in normal pooled rat plasma.

Compared 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.

Plasminogen activators. Each dose of plasminogen activator was administered in 2 mL of isotonic saline. Ten percent of the total volume was infused 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 pSVLR1.11 Transfection of dhfr-CHO cells was performed according to Chen and Okayama.14 Transfectants were screened for producers by fibrin plate assay and immediately subcloned in α MEM (minimum essential medium) containing increasing amounts of methotrexate. Methotrexate treatment led to substantial amplification of expression levels and one clone was grown in a small bioreactor for fermentation. Purification of the recombinant protein 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 log-dose-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, and t-PA at 3 to 100 nmol/kg IV diminished thrombus radioactivity in a dose-dependent manner achieving significance over controls at total doses ≥ 10 nmol/kg (Fig 1, Table 1). Spontaneous thrombolysis in controls amounted to 28.5% ± 1.5% (n = 13). At 10 and 30 nmol/kg rDSP4, was significantly more effective than equimolar doses of 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, estimated from peak blood counts at 100 nmol/kg and assuming a total blood volume of 50 ml/kg in rats, about 21% of the initial clot radioactivity was recovered in the blood with rDSP4, 43% with t-PA.

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).
THROMBOLYTIC PROPERTIES OF rDSPA,

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) (%)</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>rDSPAₐ</td>
<td>3</td>
<td>0.3 ± 0.1</td>
<td>50.7 ± 3.7*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.8 ± 0.1</td>
<td>85.0 ± 3.8*</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.7 ± 0.2</td>
<td>180 ± 18*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>9.7 ± 0.6</td>
<td>245 ± 34*</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 ± 0.1</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 (a = .05 per comparison, multiple t-test).

Plasminogen activator antigen levels. The plasma levels of both rDSPAₐ 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 rDSPAₐ 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 3). At equimolar doses mean plasma levels were 1.5, 2.7, 3.1, and 1.3 times higher for rDSPAₐ 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 rDSPAₐ or t-PA (Table 2). In contrast, neither plasma fibrinogen nor plasminogen concentrations changed significantly with rDSPAₐ 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 rDSPAₐ has previously been shown to be a potent plasminogen activator in vitro with a markedly higher fibrin specificity than t-PA. We now report that rDSPAₐ is a very effective thrombolytic agent in vivo. rDSPAₐ 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, ie, their specific activity in vivo (thrombolytic activity/plasma level) is about equal. The higher plasma levels for rDSPAₐ 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 rDSPAₐ has a longer β-elimination half-life and a slower clearance than t-PA (unpublished results). Hence, infusion of rDSPAₐ could lead to higher steady-state plasma levels, which would then account for the observed differences in effectiveness of equimolar doses of rDSPAₐ 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 rDSPAₐ 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. A specific advantage of this model is that full effectiveness of t-PA is only achieved at doses where clot specificity is lost,
very similar to the clinical usage of t-PA in patients with plasminogen nor induces fibrinogenolysis in the dose range plasminogen only because it neither depletes circulating contrast to t-PA, rDSPA, seems to activate clot-bound appears to occur at lower plasma levels than in rats. In myocardial infarction, although fibrinogenolysis in humans these results confirm in vivo the higher fibrin specificity of min liberated from the clot during thrombolysis. Therefore, this 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 rDSPA, 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.16

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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REFERENCES


### Table 2. Decrease in Hemostasis Parameters

<table>
<thead>
<tr>
<th>Dose</th>
<th>Fibrinogen (%)</th>
<th>Plasminogen (%)</th>
<th>α2-antiplasmin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 ± 3</td>
<td>2 ± 5</td>
<td>-5 ± 3</td>
</tr>
<tr>
<td>rDSP4</td>
<td>30</td>
<td>8 ± 3</td>
<td>3 ± 4</td>
</tr>
<tr>
<td>100</td>
<td>15 ± 2*</td>
<td>9 ± 6*</td>
<td>29 ± 6*</td>
</tr>
<tr>
<td>t-PA</td>
<td>30</td>
<td>9 ± 4</td>
<td>9 ± 5</td>
</tr>
<tr>
<td>100</td>
<td>33 ± 7†</td>
<td>38 ± 8†</td>
<td>61 ± 9†</td>
</tr>
</tbody>
</table>

Values represent percentage decrease (at 60 minutes) from initial values of hemostasis parameters; mean ± SEM of n = 6 to 8 (control n = 13) animals per group.

*Significant versus equimolar dose of t-PA
†Significant versus control.


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