Vampire Bat Salivary Plasminogen Activator Is Quiescent in Human Plasma in the Absence of Fibrin Unlike Human Tissue Plasminogen Activator

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The vampire bat salivary plasminogen activator (Bat-PA) is a potent PA that exhibits remarkable selectivity toward fibrin-bound plasminogen (Gardell et al, J Biol Chem 256: 3568, 1989). Herein, we describe the activity of recombinant DNA-derived Bat-PA (rBat-PA) in a human plasma milieu. rBat-PA and recombinant human single-chain tissue plasminogen activator (rt-PA) are similarly efficacious at lysing plasma clots. In stark contrast to rt-PA, the addition of 250 nmol/L rBat-PA to plasma in the absence of a clot failed to deplete plasminogen, α2-antiplasmin and fibrinogen. The lytic activities exhibited by finger-domain minus Bat-PA (F-rBat-PA) and finger and epidermal growth factor-like domains minus Bat-PA (FG-rBat-PA) were less than rBat-PA, especially at low concentrations of PA; nevertheless, these truncated forms also possessed a strict requirement for a fibrin cofactor. The loss of PA activity following the addition of rBat-PA to plasma was slower than that observed when either rt-PA or two-chain rt-PA was added. The efficacy, fibrin selectivity, and decreased susceptibility to inactivation exhibited by rBat-PA in vitro in a human plasma milieu suggests that rBat-PA may be superior to rt-PA for the treatment of thrombotic complications.

MATERIALS AND METHODS

Recombinant DNA derived Bat-PA (rBat-PA) and its truncated forms, finger-domain minus Bat-PA (F-rBat-PA) and finger and epidermal growth factor-like domains minus Bat-PA (FG-rBat-PA), were synthesized using expression vectors introduced into the human embryonic kidney cell line 293 (J.-S. Tung et al, manuscript submitted for publication). The Bat-PA species were purified to apparent homogeneity using immobilized erythrina trypsin inhibitor and quantitated by active site titration. These preparations were shown to be free of contaminating t-PA by Western immunoblot analysis using t-PA specific antibodies and fibrin autography. The specific activities of rBat-PA, F-rBat-PA, and FG-rBat-PA are 24,000 ± 1,800, 4,700 ± 1,900, and 2,000 ± 200 IU/nmol, respectively, as determined with the coupled enzymatic PA assay (see below). Recombinant DNA derived t-PA (rt-PA), predominantly single chain, was produced by Genentech, South San Francisco, CA (Activase). The rt-PA concentration was based on the manufacturer’s specifications and its specific activity was determined to be 49,000 ± 2,000 IU/nmol. Two-chain t-PA was generated from rt-PA by treatment with plasma-Sepharose as described elsewhere and quantitated by active site titration. Poled normal human plasma was purchased from George King Bio-Medical Inc (Overland Park, KS). Thrombin was obtained from Sigma (St Louis, MO). Coatest Plasminogen, Coatest Antiplasmin assay kits, as well as S-2251 and S-2288 were purchased from Kabi-Vitrum (Stockholm, Sweden). 125I-radionabeled human fibrinogen was purchased from Amersham (Arlington Heights, IL). HTI080-derived PAI-1, Glu-plasminogen, fibrinogen, hirudin, Spectrozyme P1, and PAI-1-depleted human plasma were obtained from American Diagnostica (Greenwich, CT). The fluorogenic substrates, P3, P4, P5, P6, P7, P8, P9, P10, P11, and P12 were purchased from Peninsula Laboratories (Belmont, CA) and Enzyme Systems Products (Livermore, CA), respectively.

Coupled enzymatic PA assay. Solutions (190 μL) placed in the wells of a microtitration plate (Flow Laboratories, McLean, VA) containing fibrinogen (0.132 mg/mL), Glu-plasminogen (0.66 μmol/
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NaCl, and 0.24 mmol/L Triton X-100, pH 7.5, were incubated at 37°C for 30 minutes. Ten microliters of hirudin (200 U/mL) was added and the clots were incubated an additional 30 minutes at 37°C. Thirty microliters of 3.33 mmol/L Spectrozyme P1 and 20 µL of PA were added and the release of p-nitroaniline from the chromogenic substrate was measured spectrophotometrically using a Thermomax microplate reader (Molecular Devices, Palo Alto, CA). The activities (international units per milliliter) are reported by comparison with a standard curve generated with two-chain t-PA activity standard (American Diagnostica).

PA-mediated lysis of plasma clots. Human thrombin (0.1 U) was added to human plasma (0.1 mL) dispensed in the wells of a 96-well microtitration plate. The fibrin clots were aged for 1 hour at 37°C. Aliquots of the PAS (0.1 mL) were added to the fibrin clots. The progressive decreases in turbidity due to reductions in the fibrin clot mass were measured every 30 seconds for 1 hour at 37°C with a Thermomax microplate reader equipped with a 650-nm filter. Data was analyzed with the Softmax kinetic software (Molecular Devices). All PAS exhibited clot lysis profiles characterized by a nonlinear lag phase followed by a linear phase. The velocity of clot lysis was defined as the maximum negative slope that occurred during a 1-hour incubation period.

Activation of plasminogen in plasma. Varying concentrations of rBat-PA, F-rBat-PA, FG-rBat-PA, or rt-PA were added to human plasma (0.5 mL), incubated at 37°C for 30 minutes, and quick-frozen on dry ice. Plasminogen levels were determined by the sequential addition of streptokinase and S-2251 according to the Coatest plasminogen method. a2-Antiplasmin levels were determined by the sequential addition of plasmin and S-2251 according to the Coatest antiplasmin method.

PA-mediated degradation of fibrinogen. rBat-PA or rt-PA was added to 0.5 mL of human plasma containing 125I-labeled fibrinogen (35,000 cpm/mL) and samples were incubated at 37°C. Aliquots were added to 0.5 mL of human plasma containing '2SI-labeled fibrinogen (35,000 cpm/mL). The samples were incubated at 37°C and 0.04-mL aliquots were added to sodium dodecyl sulfate (SDS) and dithiothreitol-containing loading buffer, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

Inactivation of PAS in plasma. Human platelet-poor plasma, 0.5 mL, containing 21 IU/mL of PAI-1 activity (corresponding to approximately 460 pmol/L PAI-1) was added to 0.5 mL of 10 mmol/L Na phosphate, 150 mmol/L NaCl, pH 7.4, 0.01% Tween 80 containing 130 mmol/L of rt-PA, two-chain rt-PA, or rBat-PA. The samples were incubated at 37°C and 0.04-mL aliquots were withdrawn at the indicated times and mixed with 0.05 mL of 1 mol/L Na acetate, pH 3.9. Following the acidification step, all samples were incubated at 37°C for 10 minutes before assay of residual PA activity. A similar experimental protocol using PAI-1-depleted human plasma was also performed. PA activity was determined using the coupled enzymatic assay described above.

Determination of the association rate constants for the interactions between PAS and PAI-1. Purified PAI-1 was activated by treatment with guanidine hydrochloride (4 mol/L) at 37°C for 1 hour. The PAI-1 was reneutralized by gel filtration on a NAP-10 column (Pharmacia, Piscataway, NJ). The concentration of activated PAI-1 was determined by quantitative neutralization of two-chain rt-PA in a direct amidolytic assay using S-2288. The kinetics of inhibition were monitored as follows: PAS (1 nmol/L) were added to cuvettes containing activated PAI-1 (2 to 10 nmol/L), a fluorogenic substrate (either MIGAM or PGAM) and, when included, human plasminogen-free fibrinogen (0.3 µmol/L) in HEPES (10 mmol/L), NaCl (150 mmol/L), Triton X-100 (0.24 mmol/L), and dimethyl sulfoxide (1.0%, vol/vol), pH 7.5. The concentration of fluorogenic substrate was in all cases twice the experimentally determined Kᵅ. The respective Michaelis-Menten constants of two-chain rt-PA for MIGAM and of rt-PA and rBat-PA for PGAM were 0.54, 1.16, and 0.83 mmol/L in the absence of fibrinogen, and 0.46, 0.25, and 0.61 mmol/L in the presence of fibrinogen; these values were determined by nonlinear regression analysis. The fluorescence was monitored with a Perkin-Elmer LS-3 Fluorescence Spectrophotometer at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The time-dependent inhibition of PAS at varying concentrations of PAI-1 was recorded as a family of progress curves, each of which reflected slow, tight-binding inhibition as described by Williams and Morrison.6 The data from each curve were fit to the integrated first-order rate equation: F = x(t) + (x₀ - x(t))(1 - e⁻kt)/k by nonlinear regression, which allowed for the calculation of the apparent constant, k, where k = k₀ + (k₀/(1 + s/Kᵅ)). A replot of k as a function of PAI-1 concentration yielded a line whose slope was equal to k₀/(1 + s/Kᵅ), from which the second-order rate constant was calculated.

RESULTS

rt-PA, rBat-PA, F-rBat-PA, and FG rBat-PA catalyzed the dissolution of clots formed from human plasma (Fig 1). Clot lysis was assayed by monitoring the progressive decreases in turbidity that resulted from the addition of these PAS to plasma clots formed in the wells of a microtitration plate. The relative potencies of these PAS were a function of their concentrations. For example, the rate of clot lysis catalyzed by 30 nmol/L rBat-PA slightly exceeded that by 30 nmol/L rt-PA. However, the rate of clot lysis catalyzed by 5 nmol/L rBat-PA was 80% of that exhibited by 5 nmol/L rt-PA and, whereas 0.2 nmol/L rt-PA promoted appreciable clot lysis, 0.2 nmol/L rBat-PA appeared to be ineffective. The rates of clot lysis catalyzed by F-rBat-PA and FG-rBat-PA were generally slower than those promoted by rt-PA and rBat-PA. The decreased potencies of the truncated forms of rBat-PA were especially evident at low concentrations of PA but more closely rivaled those of

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full-length rBat-PA and rt-PA when 30 nmol/L of each were assayed (Fig 1). Incubation of 15 to 250 nmol/L rt-PA in human plasma for 30 minutes at 37°C resulted in prominent decreases in the levels of α2-antiplasmin and plasminogen (Fig 2). In contrast, the addition of equimolar amounts of rBat-PA, F- rBat-PA, or FG- rBat-PA failed to result in significant consumption of α2-antiplasmin or plasminogen. Incubation of comparable amounts of rt-PA and rBat-PA in freshly drawn citrated whole human blood yielded similar results to those observed in plasma (data not shown). The extraordinary fibrin selectivity of rBat-PA was emphatically demonstrated by the relatively modest 35% decrease in the plasminogen level when 3 μmol/L of rBat-PA was incubated in plasma at 37°C for 30 minutes.

The activity of rt-PA and rBat-PA in plasma was also monitored by the formation of fibrinogen degradation products (Fig 3). Degradation of fibrinogen was assessed by SDS-PAGE and autoradiography of human plasma samples containing radiolabeled fibrinogen. Only intact subunits of fibrinogen were observed in samples that were incubated at 37°C for 10 or 40 minutes (lanes 1 and 2, respectively). However, the addition of 250 nmol/L t-PA to plasma resulted in degraded fibrinogen that was apparent at 10 minutes (lane 3) and very prominent after 40 minutes (lane 4). The presence of 250 nmol/L Bat-PA in plasma failed to generate discernible fibrinogen degradation products after incubation at 37°C for 10 or 40 minutes (lanes 6 and 7, respectively). The latency of Bat-PA activity in plasma was corroborated by the appearance of fibrinogen degradation products following the addition of rBat-PA (25 nmol/L) and thrombin (lane 8). The corresponding thrombin-induced degradation of fibrinogen by 25 nmol/L rt-PA is shown in lane 4.

The addition of rBat-PA to plasma and incubation at 37°C resulted in a time-dependent loss of PA activity (Fig 4). However, rBat-PA appeared less susceptible to inactivation than either one- or two-chain rt-PA. The activities of these three PAs were relatively stable in plasma that had been depleted of PAI-1, thus implicating PAI-1 as playing the major role in mediating inactivation. The apparent second-order rate constant for the interaction between rBat-PA and PAI-1 was determined to be 0.48 × 10⁻² M⁻¹ s⁻¹ (Table 1). The corresponding $k_{\text{assoc}}$ values for rt-PA and two-chain rt-PA are 0.30 × 10⁻⁷ M⁻¹ s⁻¹ and 1.4 × 10⁻⁷ M⁻¹ s⁻¹, respectively, which agreed with published values. The similarity in $k_{\text{assoc}}$ values between rt-PA and rBat-PA for PAI-1 failed to explain the disparate inactivation profiles that these two PAs exhibited in plasma. However, the $k_{\text{assoc}}$ value for interaction of rt-PA with PAI-1 was increased approximately 3.2-fold in the presence of fibrinogen (Table 1); in contrast, the corresponding rate constants for rBat-PA and two-chain rt-PA were essentially unaffected by the presence of fibrinogen. Perhaps the dissimilar inhibition profiles exhibited by these PAs in plasma can be explained by differences in their apparent association rates with PAI-1 in the presence of fibrinogen.

DISCUSSION

The remarkable dependence of Bat-PA activity on the presence of a fibrin cofactor was shown previously in a purified system and has now been demonstrated in a human plasma milieu. The quiescent state of rBat-PA activity in plasma, at concentrations as high as 250 nmol/L, contrasts sharply with the extensive plasminogen activation that is catalyzed by comparable amounts of rt-PA. However, rBat-PA when added to human plasma in the presence of a clot is activated to yield robust lytic activity. These data suggest that there is no component of human plasma that drastically undermines the fibrin selectivity or potency of rBat-PA.

Relatively slow lysis of plasma clots catalyzed by F- rBat-PA and FG- rBat-PA compared with rBat-PA at low PA concentrations was also noted in a purified system using plasminogen-containing fibrin clots (Tung et al, submitted for publication). The F and, perhaps, the G domains of rBat-PA appear to make an important functional contribution to plasminogen activation as with rt-PA. The apparent role of the F domain of Bat-PA in facilitating fibrinolysis was provided by our earlier study that demonstrated the F domain mediated tight binding of Bat-PA to fibrin.

A PA that selectively activates fibrin-bound plasminogen may be a safer thrombolytic agent than one that also activates circulating plasminogen. Several recent studies using rt-PA have shown a statistically significant, although somewhat weak, correlation between the extent of fibrinogen breakdown and the incidence of serious bleeding complications. Furthermore, patients who have suffered
an intracerebral hemorrhage following rt-PA administration invariably exhibited abnormally low fibrinogen levels and elevated fibrinogen degradation products.\textsuperscript{20} The increased serum levels of fibrinogen degradation products, although related to bleeding complications, are not related to coronary patency.\textsuperscript{19} By virtue of the strict fibrin requirement of its activity, rBat-PA should serve to test the hypothesis that avoidance of the activation of circulating plasminogen will decrease the frequency or severity of the bleeding complications which can accompany fibrinolytic therapy.

The fibrin selectivity of the rBat-PA might also have a beneficial effect on efficacy. In addition to the bleeding complications, an unresolved problem with fibrinolytic therapy is that reperfusion of the infarct-related artery is not uniformly successful, approximately 25% of patients exhibit persistent coronary occlusion. It has recently been reported that the patency rate of infarct-related coronary arteries can be improved by using an accelerated dosage regimen of rt-PA.\textsuperscript{31,32} However, a consequence of this modified regimen may be that the higher plasma concentration of rt-PA will lead to an even greater incidence of life-threatening bleeding complications. Indeed, it has been demonstrated that reduction of the total rt-PA dose from 150 to 100 mg over the course of 3 hours decreased the frequency of cerebral bleeds.\textsuperscript{17}

A potential advantage of the remarkable fibrin selectivity of rBat-PA is that large amounts of this agent could be administered by a “front-loading” dosage schedule without concomitantly compromising the patient’s hemostatic system.

An additional characteristic of rBat-PA that may afford an advantage over t-PA is its apparent decreased susceptibility to inactivation when present in plasma. It was previously shown that PAI-1 was primarily responsible for the rapid

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<th>Time (min)</th>
<th>Two-chain tPA</th>
<th>One-chain tPA</th>
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The second-order association rate constants ($k_{assoc}$) were determined as described under Materials and Methods. Where indicated, the reactions included 100 μg/mL fibrinogen (FBG). Each value is presented as the mean ± standard deviation.
inhibition of t-PA in plasma.23 The results of our study agree with this conclusion and, furthermore, show that inhibition of rBat-PA in plasma is also mediated by PAI-1. The potential benefit of a fibrinolytic agent that is relatively resistant to PAI-1 was suggested by a recent clinical study that showed an inverse correlation between elevated initial levels of PAI-1 activity in patients and decreased incidence of recanalization of the infarct-related artery following rt-PA administration.24

Studies are currently underway using animal models of thrombotic complications to determine whether the in vitro fibrin selectivity and potency of rBat-PA will also be exhibited in vivo. If so, then these attributes, along with the relative resistance to PAI-1, may make rBat-PA a safer and more efficacious fibrinolytic agent than those currently available.

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REFERENCES

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