The Pharmacokinetics of Plasminogen Activator Inhibitor-1 in the Rabbit

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The pharmacokinetics of the activated and latent forms of plasminogen activator inhibitor-1 (PAI-1) isolated from HT1080 fibrosarcoma cells (HT1080 PAI-1) and a nonglycosylated form of human PAI-1 isolated from a yeast expression system (rPAI-1) were followed in the rabbit. As assessed by an immunologic assay specific for human PAI-1, guanidine HCI activated HT1080 PAI-1 and rPAI-1 entered the total plasma volume following intravenous bolus administration and exhibited a biphasic clearance pattern. The $t_{1/2}$ of HT1080 PAI-1 for the initial and $\beta$ phases equaled 6.0 and 24.8 minutes, respectively. The $t_{1/2}$ of rPAI-1 for the initial and $\beta$ phases equaled 8.8 and 34.0 minutes, respectively. Similar results were obtained by measuring PAI-1 activity in plasma and with trace amounts of $^{125}$I-rPAI-1, suggesting that the above pharmacokinetic behavior could also apply to endogenous PAI-1.

PLASMINOGEN activator inhibitor-1 (PAI-1) is a member of the SERPIN class of proteinase inhibitors and is a fast-acting inhibitor of the plasminogen activators, tissue plasminogen activator (t-PA), and urokinase (UK). PAI-1 is synthesized by a variety of cell types in culture, including endothelial cells, and is found in human blood plasma at low levels. Platelets contain large amounts of PAI-1, which is released on activation, suggesting that PAI-1 could be present in significant concentrations at localized sites of vascular injury and in forming thrombi. The conversion of plasminogen to the fibrin degrading enzyme, plasmin, by t-PA and UK is a key step in fibrinolysis. PAI-1 could play a critical role in governing the fibrinolytic pathway by regulating the activity of endogenous plasminogen activators. Clinical studies show a correlation between elevated levels of PAI activity in plasma with venous thrombosis, coronary artery disease, and myocardial infarction. Thus, increased levels of PAI-1 could result in a decreased fibrinolytic potential with a concomitant tendency to thrombose.

Unlike other members of the SERPIN superfamily, PAI-1 can exist in a latent form which can be activated by exposure to strong denaturants such as sodium dodecyl sulfate (SDS) and guanidine HCl. Once activated, the PAI-1 is inherently unstable at 37°C and reverts to the latent state with a $t_{1/2} = 2$ hours. Mechanically, PAI-1 behaves similarly to the other SERPINS, functioning as a pseudo-substrate and reacting rapidly with its target proteinases to form SDS-stable complexes. Studies comparing PAI-1 antigen levels and PAI-1 activity levels suggest that human plasma and serum contain both active and latent forms of PAI-1, although in different ratios. It is not known whether latent PAI-1 possesses other functional roles or can be activated in vivo.

Previous studies of the fate of PAI-1 in the circulation are limited to a single report involving plasma transfusion from endotoxin-treated rabbits which possess elevated PAI activity. Herein, we describe the pharmacokinetics of glycosylated and nonglycosylated forms of PAI-1 as well as PAI-1 in the activated and latent states. The data show that PAI-1 is cleared rapidly from the circulation with glycosylation playing a small role in its clearance behavior. The latent and activated forms of PAI-1 clear far differently from one another and also from other members of the SERPIN superfamily.

MATERIALS AND METHODS

Materials. Two-chain t-PA (920 IU/µg), glu-plasminogen, des-AA-ibrinogen, and PAI-1-depleted human plasma were obtained from American Diagnostica, New York, NY. D-Val-Leu-Lys-pNA (S2251) and cyanogen bromide fibrinogen fragments were obtained from Helena Laboratories, Beaumont, TX. Sephacryl S-300, NAP 10 gel filtration columns, and molecular weight standards were obtained from Pharmacia, Uppsala, Sweden. Guanidine HCI was obtained from Schwarz/Mann, Cleveland, OH. HT1080 PAI-1, purified from dexamethasone treated human fibrosarcoma cells (HT1080) by the procedure of Chmielewska et al., was provided by American Diagnostica. A recombinant, nonglycosylated form of human PAI-1 (rPAI-1) was expressed intracellularly in Saccharomyces cerevisiae and purified to homogeneity as described by Gardell et al., rPAI-1 was radiolabeled with $^{125}$I-jodine using the Bolton-Hunter reagent (New England Nuclear, Boston, MA) to a specific activity of 34,400 to 68,500 cpm/µg. $^{125}$I-t-PA forms SDS stable complexes with t-PA and retains inhibitory activity against t-PA in the coupled amidolytic assay described below.

PAI-1 activity assay. The inhibitory activity of PAI-1 was determined by adding increasing volumes of PAI-1 to a constant amount of t-PA (46.8 mIU) in the wells of microtiter plates (Nunc Microwell) in 50 mmol/L Tris-HCl, pH 7.4, 0.1 mol/L sodium chloride, 0.01% Tween 80 (TNT buffer). After 1 hour at 25°C, an equal volume of TNT buffer (100 µL) containing 0.5 µmol/L glu-plasminogen, 50 µg/mL des-AA-ibrinogen, and 1 mmol/L S2251 was added. Residual t-PA activity was determined by measuring the increase in optical density at 405 nm after a 1.5-hour
incubation at 37°C. The linear portion of the inhibition curve was extrapolated to obtain the concentration of PAI-1 necessary for complete inhibition of the t-PA. One arbitrary unit (AU) is defined as the amount of PAI-1 required to inhibit one IU of t-PA.

**Activation of PAI-1.** The purified preparations of HT-1080 PAI-1 and rPAI-1 were in a latent, inactive form (<2 AU/μg) but could be activated by exposure to guanidine HCl. Activation was achieved by incubating latent PAI-1 in TNT buffer containing 4.0 mol/L guanidine HCl. After a 5-minute incubation at 25°C, the sample was desalted by gel filtration through an NAP 10 column. The activated forms of PAI-1 were 35% to 40% active and possessed specific activities ranging from 441 to 504 AU/μg for HT1080 PAI-1 and 511 to 584 AU/μg for rPAI-1. Similar results have been reported by other investigators.15,16

**Blood clearance studies.** New Zealand White (NZW) rabbits with a body weight of 1.6 to 2.3 kg were anesthetized with 0.35 mg/kg sodium pentobarbital in the marginal ear vein. Surgery was conducted under aseptic conditions and sterile solutions were used throughout the studies. All solutions, including PAI-1, were endotoxin free. PAI-1, in a volume of 1.5 mL of TNT buffer, was delivered by bolus administration into the femoral vein. Blood samples were drawn from a canulated femoral artery into syringes containing 1/10 vol of 3.8% sodium citrate. The blood was immediately centrifuged at 10,000 rpm for 2 minutes in an Eppendorf microfuge and the plasma removed and stored at −70°C.

**Determination of PAI-1 antigen levels.** The amount of human PAI-1 in rabbit plasma was determined with a solid-phase immunologic assay specific for human PAI-1 (Tintelize PAI-1 ELISA kit [American Diagnostica], "N" wells only). The plasma samples were tested undiluted and after diluting twofold and fivefold into TNT buffer. The absorbance values obtained were compared with the appropriate standard curves constructed by diluting known amounts of activated and latent rPAI-1 and activated HT1080 PAI-1 into PAI-1 depleted human plasma. Representative standard curves from a single experiment are shown in Fig 1. The PAI-1 levels in the plasma samples were determined from the values which fell within the middle range of the standard curves (10 to 70 ng/mL). Similar results were obtained when PAI-1 standards were added to pooled rabbit plasma.

**Determination of PAI activity in plasma.** The PAI activity in plasma was determined using a modification of the method described by Chmielewska and Wiman.17 In brief, 25 μL of plasma, undiluted and diluted 2-, 5-, and 10-fold in 50 mmol/L Tris-HCl, pH 0.01% Tween 80, pH 8.3 (TT buffer) was mixed with 25 μL of t-PA (50 IU/mL). After a 15-minute incubation at 25°C, the samples were diluted with 1 mL of TT Buffer, and 22 μL tested for residual t-PA activity in a coupled amidolytic assay containing 0.5 μmol/L glu-plasminogen, 130 μg/mL CNBr fibrinogen fragments, and 1 mmol/L S2251 in TT buffer. After incubation at 37°C for 4 hours, the absorbance at 405 nm was determined. The residual t-PA activity was determined from standard curves generated by mixing 25 μL of t-PA (0 to 50 IU/mL) with 25 μL of PAI-1 depleted human plasma at the appropriate dilutions. The results are expressed in AU as defined above.

**Organ distribution studies.** 125I-rPAI-1 (2.3 × 10⁶ cpm; 91 ng) was injected into the marginal ear vein of anesthetized NZW rabbits. Ten, 20, and 135 minutes following injection of 125I-rPAI-1, 0.5 mL of heparin (1,000 IU/mL) and sufficient sodium pentobarbital to insure deep anesthesia were administered. The organs were exposed, perfused with saline pumped through the left ventricle, and tissue samples excised, weighed, and counted for radioactivity.

**Gel filtration chromatography of activated and latent PAI-1.** Plasma samples (100 μL) containing 125I-rPAI-1 were chromatographed on a Sephacryl S-300 column (16 cm × 1.0 cm) in phosphate-buffered saline, 0.01% Tween 80 at a flow rate of 10 mL/h at 4°C. Fractions were collected every 2 minutes and the cpm determined.

**Analysis of data.** The clearance of PAI-1 was resolved into individual components by analysis of the semi-logarithmic plots using the curve peeling technique.18 The initial volume of distribution (VD@) and the VD of the β phase (VDβ) were calculated by dividing the dose by Cα + Cβ and Cβ alone, respectively. Cα and Cβ equal the initial concentrations of PAI-1 for the α and β phases, respectively, and were determined by extrapolation of the α and β phase curves to 0 time (T₀). Clearance was determined by dividing the dose by the area under the curve.

**RESULTS**

**The clearance of activated human PAI-1 in the rabbit.** The clearance of HT1080 PAI-1, a glycosylated species isolated from human HT1080 fibrosarcoma cells, and rPAI-1, a nonglycosylated recombinant form of human PAI-1, were followed in the rabbit using an immunologic assay which recognizes human PAI-1 (Fig 1). HT1080 PAI-1 and rPAI-1 were activated by exposure to guanidine HCl as described in Materials and Methods immediately before
administration. The clearance curves obtained from a single representative experiment for each type of PAI-1 are shown in Fig 2. Table 1 summarizes the pharmacokinetic parameters obtained from multiple experiments (n = 3) for both HT1080 PAI-1 and rPAI-1. Following intravenous bolus administration of HT1080 PAI-1 (25 μg/kg), the concentration of human PAI-1 in plasma increased from nondetectable levels to 329 ± 51 ng/mL at T₀. This value represents 68% of the theoretical amount of PAI-1 administered, assuming a plasma volume of 50 mL/kg. The clearance of HT1080 PAI-1 was described by a two-compartment model. The initial α (distribution) phase was rapid (t₁/₂α = 6.0 ± 1.6 minutes) while the β (elimination) phase was considerably slower (t₁/₂β = 24.8 ± 2.0 minutes). Similar results were obtained with rPAI-1 (22 μg/kg) (Fig 2B). At T₀, the rPAI-1 concentration in plasma equaled 378 ± 25 ng/mL, which represents 79% of the theoretical amount of PAI-1 administered. The t₁/₂α was 8.8 ± 1.0 minutes and the t₁/₂β equaled 34.0 ± 6.4 minutes. Using the above data, the plasma clearance rates were calculated for HT1080 PAI-1 (3.7 ± 0.5 mL minutes⁻¹ kg⁻¹) and rPAI-1 (1.9 ± 0.1 mL minutes⁻¹ kg⁻¹).

The clearance of activated PAI-1 was also followed by measuring PAI-1 activity in plasma. Following administration of HT1080 PAI-1 (25 μg/kg), the PAI-1 activity in plasma increased from 8.6 AU/mL to 208 AU/mL at T₀, while the levels of PAI-1 antigen increased from nondetectable levels to 385 ng/mL. The increase in PAI-1 activity is close to the value predicted from this level of PAI-1 antigen (194 AU/mL). Figure 3 indicates that PAI-1 activity cleared rapidly from the circulation. Importantly, analysis of the same plasma samples for the presence of human PAI-1
antigen generated a nearly superimposable clearance curve. Similar results were obtained in a second experiment with HT1080 PAI-1 and in experiments performed with rPAI-1 (data not shown). Thus, PAI-1 activated in vitro retains inhibitory activity in vivo. Moreover, the data suggest that the pharmacokinetic profile of PAI-1 presented in Table 1 is indicative of biologically active PAI-1.

The clearance of physiologic levels of PAI-1. The clearance behavior of PAI-1 presented in Figs 1 and 2 and Table 1 were determined from initial PAI-1 concentrations, which are considerably above the levels of PAI-1 encountered in the normal human circulation. To more closely mimic physiologic conditions, the clearance of a small amount of activated $^{125}$I-rPAI-1 was followed (Fig 4). Following bolus administration (30 ng/kg), the level of $^{125}$I-rPAI-1 in the plasma equalled 0.53 ng/mL ($T_s$) or 88% of the predicted dose. Measurements of radioactivity in whole blood and plasma indicated that the PAI-1 was almost exclusively present (>90%) in the plasma fraction at each time point examined (data not shown). Thus, PAI-1 does not bind to a significant extent to the cellular components of blood. The clearance behavior of $^{125}$I-rPAI-1 was biexponential and, in general, resembled that obtained using high levels of unlabeled PAI-1 (Fig 2 and Table 1). $^{125}$I-rPAI-1 had a $t_{1/2a} = 1.9$ minutes and a $t_{1/2b} = 37.2$ minutes. Figure 3 also indicates that the administration of a large excess of unlabeled rPAI-1 in conjunction with $^{125}$I-rPAI-1 had little effect on the clearance of $^{125}$I-rPAI-1 ($t_{1/2a} = 2.8$ minutes, $t_{1/2b} = 30$ minutes).

Organ distribution. The organ distribution of PAI-1 in rabbits killed 10, 20, and 135 minutes after injection of activated $^{125}$I-rPAI-1 is presented in Table 2. Excluding blood, the bulk of the $^{125}$I-rPAI-1 was detected in the liver at 10 and 20 minutes and the kidney at 135 minutes. Little radioactivity (<1%) was present in the spleen, lungs, heart, intestine, or striated muscle at any time.

Clearance of latent PAI-1. The pharmacokinetic data presented above was generated with activated PAI-1. To determine whether latent rPAI-1 behaves in a similar manner, its distribution and clearance were followed by antigen analysis following bolus administration of 22 µg/kg. A representative experiment indicates that approximately 80% of the latent rPAI-1 cleared from the circulation within 10 minutes (Fig 5). The clearance behavior of latent rPAI-1 was represented by a two-compartment model. The $t_{1/2a}$ equaled 1.70 ± 1.0 minutes while the $t_{1/2b}$ equaled 7.12 ± 0.61 minutes (average ± range from two experiments). The clearance rate of latent rPAI-1 equaled 9.08 ± 1.74 mL minutes⁻¹ kg⁻¹. Thus, relative to activated rPAI-1 (Table 1), latent rPAI-1 has a 4.8-fold faster rate of clearance.

The interaction of activated PAI-1 with plasma binding components. To determine whether the different pharmacokinetic profiles of activated and latent PAI-1 could be related to a differential association of PAI-1 with plasma binding components, activated and latent $^{125}$I-rPAI-1 were mixed with rabbit plasma in vitro for 2 minutes and 30 minutes, respectively, and immediately analyzed by gel filtration chromatography. Activated $^{125}$I-rPAI-1 eluted as a doublet (Fig 6A); 75% emerging at a position corresponding to a molecular weight (mol wt) of approximately 400,000 and the remainder eluting in the void volume. In contrast, latent $^{125}$I-rPAI emerged as a single peak with an apparent mol wt equal to 30,500, despite the prolonged preincubation time. In the absence of plasma, activated and latent $^{125}$I-rPAI-1 eluted with apparent mol wt less than 40,000 (data not shown). Thus, the differences in elution profiles between activated and latent PAI-1 do not appear to be due to self-association of the activated form.
Fig 5. The clearance of latent PAI-1. Latent rPAI-1 (22 μg/kg) was delivered by bolus administration and its clearance determined immunologically. For comparison, the initial portion of the clearance curve of activated rPAI-1 from Fig 2B is included. Latent rPAI-1: (■), activated rPAI-1.

minutes later. The samples were immediately centrifuged and the plasma examined by gel filtration chromatography. Activated 125I-rPAI-1 eluted as a doublet with the bulk of the material (78%) emerging at a mol wt of approximately 400,000 (Fig 6B). Latent 125I-rPAI-1 eluted as a single peak with a mol wt corresponding to 28,000.

**DISCUSSION**

The pharmacokinetics of the activated and latent forms of human PAI-1 have been determined in the rabbit. The activated form of PAI-1 clears from the circulation in a biphasic manner. The t½ of less than 9 minutes indicates that PAI-1 distributes soon after entering the circulation. The distribution process seems unlikely to be due to PAI-1 interaction with rabbit plasminogen activators since the levels of PAI-1 used for the experiments described in Table 1 (>300 ng/mL) greatly exceed the levels of plasminogen activators believed present in the circulation. PAI-1 does not appear to bind to blood cells, endothelium, or extravasate into the tissues because low amounts of radioactivity were detected in the formed elements of blood, the lung, and tissues other than the liver, following administration of 125I-PAI-1. Rather, the data presented in Fig 6 suggests that activated PAI-1 distributes within the plasma compartment to form high mol wt complexes with plasma binding components. The t½ of the β or clearance phase of activated PAI-1 ranged from 25 minutes (HT-1080 PAI-1) to 34 minutes (rPAI-1). Although the t½ of the β phase is considerably longer than that observed for latent PAI-1 (see below), the clearance of activated PAI-1 is considerably faster than that observed for other members of the SERPIN family of plasma proteinase inhibitors. The plasma half-lives of antithrombin III and α1-antichymotrypsin are greater than 3 hours. Because the SERPINs probably have similar three-dimensional structures, the information governing clearance may reside on relatively few amino acids.

The presence of carbohydrate modestly affected the clearance behavior of PAI-1. HT1080 PAI-1, which is glycosylated, had a rate of clearance of 3.7 mL minutes⁻¹ kg⁻¹. rPAI-1, which contains no detectable carbohydrate, had a rate of clearance of 1.9 mL minutes⁻¹ kg⁻¹. In sharp contrast, the clearance behavior of the SERPIN, α1-antitrypsin, is strongly influenced by its carbohydrate con-
tent and in an opposite manner\textsuperscript{22}; a recombinant form of α1-antitrypsin which is not glycosylated clears within minutes from plasma. The α1-antitrypsin which is isolated from human plasma has a $t_{1/2}$ of over 8 hours.

The bulk of the clearance studies were monitored by a PAI-1–specific immunologic assay. Activated HT1080 PAI-1 was much more immunogenic than either the activated or latent forms of rPAI-1, and latent rPAI-1 was only slightly less (10\% to 20\%) immunogenic than activated rPAI-1. These results suggest that the enzyme-linked immunosorbent assay (ELISA) depends, in part, on the carbohydrate structure of PAI-1 and is relatively impervious to the ability of activated PAI-1 to complex with factors in plasma (see below). The immunologic assay seems to specifically recognize human PAI-1 and not rabbit PAI-1; plasma from non–PAI-1 treated control rabbits never exhibited significant cross-reaction in the ELISA (data not shown).

Given that the inhibitory mechanism of PAI-1 involves formation of 1:1 molar complexes with t-PA,\textsuperscript{11} the activated PAI-1 used by us and by others\textsuperscript{15,16} probably consists of two distinct PAI-1 populations: an inhibitory form, which comprises 35\% to 40\% of the total, and a noninhibitory form, which makes up the remainder. This latter form appears to be distinct from latent PAI-1, which has not been exposed to denaturants. Measurements of PAI-1 activity and antigen following activated PAI-1 administration provided almost superimposable clearance patterns. Thus, the pharmacokinetic profile of activated PAI-1 presented in Table 1 most likely applies to the form which is capable of inhibiting t-PA. Moreover, although activated PAI-1 consists of both inhibitory and noninhibitory forms, essentially complete formation of high mol wt, activated PAI-1 plasma complexes was observed (Fig 6). Thus, guanidine HCl treatment of latent PAI-1 may expose different functional sites in the PAI-1 molecule: an active site conferring t-PA inhibition, and a separate site which mediates interactions with plasma-binding factors. The pharmacokinetic behavior of the exogenously added, activated PAI-1 may describe the clearance of endogeneous, active PAI-1 from the circulation. Physiologic doses of $^{125}$I-rPAI-1 cleared with a $t_{1/2}$ of 37 minutes, close to the value obtained when either rPAI-1 or HT1080 PAI-1 was administered at levels greater than 300 ng/mL and the clearance measured immunologically (see below). Organ distribution studies indicate that the liver mediates the initial clearance of activated rPAI-1 from the circulation. The liver is seemingly able to clear large amounts of rPAI-1 from the circulation because the clearance profile of $^{125}$I-rPAI-1 was not affected by the simultaneous administration of excess unlabeled rPAI-1 (Fig 4). At later times following rPAI-1 administration, radioactivity appeared in the kidney. Thus, renal uptake of PAI-1 degradation peptides released into the circulation from the liver may occur. More experiments are needed to determine whether latent PAI-1 and HT1080 PAI-1 also clear by hepatic and renal mechanisms. Latent PAI-1 is cleared more rapidly from the circulation than did activated PAI-1. The rapid $α$ phase ($t_{1/2} = 1.7$ minutes) indicates that latent PAI-1 distributes immediately after entering the circulation. Latent PAI-1 possesses a $t_{1/2}$ of 7.1 minutes and rate of clearance (9.08 mL minutes$^{-1}$ kg$^{-1}$) which were approximately 4.8 times faster than the respective values for activated PAI-1. Some studies\textsuperscript{3,4} but not all,\textsuperscript{23} have suggested that latent PAI-1 accounts for one third to two thirds of the total PAI-1 present in human plasma. If latent PAI-1 is present in plasma, it could arise from active PAI-1, which has been shown to revert to the latent state in vitro.\textsuperscript{10} This scenario appears unlikely, given the more rapid clearance of latent PAI-1 relative to activated PAI-1. It has been suggested that latent PAI-1 could serve as a reservoir for the generation of active PAI-1.\textsuperscript{3,4} However, the short half-life of latent PAI-1 seemingly precludes this possibility, at least in the circulation. The rapid clearance of latent PAI-1 may simply reflect the elimination of a nonfunctional protein.

The results suggest that activated, but not latent PAI-1, binds quickly to components of plasma to form high-mol-wt complexes in vitro and in vivo. The formation of these complexes may be a key determinant affecting clearance. Latent PAI-1, which was unable to form high-mol-wt complexes, distributed and cleared far more rapidly than did activated PAI-1, which did form high-mol-wt complexes. The component(s) in rabbit plasma that associates with activated PAI-1 was not further characterized. However, recent studies suggest that the plasma protein, vitronectin, may be involved in complex formation.\textsuperscript{25,26}

PAI-1 levels are capable of changing rapidly in the circulation. Kluft et al\textsuperscript{27} have established that human plasma PAI activity increases 77\% on the first day following surgery, which suggests that PAI-1 is one of the fastest known acute phase reactants. PAI-1 levels also rise quickly after endotoxin treatment in humans.\textsuperscript{28} Additionally, because platelets store PAI-1,\textsuperscript{29} increased levels of this SERPIN could accompany platelet degradation occurring at sites of vascular injury and thrombosis. The results contained herein indicate that PAI-1 released into the circulation would act systemically for only a short period of time before being cleared. The target proteinases for PAI-1, t-PA and UK, also clear rapidly from the circulation.\textsuperscript{29,30} Thus, rapid elimination, together with molecular targeting,\textsuperscript{31,32} may represent important general mechanisms which regulate the fibrinolytic system.

ACKNOWLEDGMENT

We thank Dr Edgar Ulm for advice on data analysis, and Dr Susan Socher for a critical reading of the manuscript.

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