Studies of Recombinant Plasminogen Activator Inhibitor-1 in Rabbits
Pharmacokinetics and Evidence for Reactivation of Latent Plasminogen Activator Inhibitor-1 In Vivo

Douglas E. Vaughan, Paul J. Declerck, Elisabeth Van Houtte, Maria De Mol, and Désiré Collen

The pharmacokinetics of human recombinant plasminogen activator inhibitor-1 (rPAI-1) was studied in rabbits. Latent rPAI-1 (0–2 units of tissue-type plasminogen activator neutralizing activity per microgram protein); reactivated rPAI-1 (approximately 150 units/µg); and chloramine T-oxidized, nonreactivable rPAI-1 (approximately 0.7 units/µg) were studied. The pharmacokinetic parameters for the disposition of rPAI-1 antigen after an intravenous bolus injection of 1.0 or 2.5 mg/kg rPAI-1 were very similar for all three forms: the initial volume of distribution was approximately 60 ml/kg, the initial half-life in plasma was 6 minutes, and the plasma clearance was approximately 4 ml/kg/min. The disposition of PAI activity after injection of reactivated rPAI-1 was similar to that of rPAI-1 antigen. Injection of latent rPAI-1 was associated with a nearly threefold increase in the specific activity of circulating PAI-1 from 2 units/µg to 5.0±1.1 units/µg (p<0.01) within 1 minute, followed by a cumulative 25-fold increase in specific activity over 1 hour (p=0.01). In contrast, the specific activity of oxidized or reactivated preparations of rPAI-1 did not increase in the first several minutes after injection. These findings support the existence of a fast-acting but low-capacity mechanism for the reactivation of rPAI-1 in vivo. (Circulation Research 1990;67:1281–1286)

Plasminogen activator inhibitor-1 (PAI-1) is one of several related proteins belonging to the superfamily of serine protease inhibitors (serpins).1–5 PAI-1 rapidly neutralizes tissue-type plasminogen activator (t-PA) and urokinase, with second-order rate constants of approximately 107 M–1 sec–1.6,7 PAI-1 differs from other serpins in that it exists in both active and inactive, “latent” forms.8 In plasma, most of the PAI-1 circulates in the active configuration;9–11 whereas latent PAI-1 is the predominant species isolated from culture media in vitro.12 Active PAI-1 inactivates spontaneously with a half-life of 2–4 hours at 37°C.13,14 PAI-1 in plasma may originate from the vascular endothelium,15 the liver,16 or blood platelets,17 although PAI-1 released from platelets is mainly recovered in the inactive form.11,18 Latent PAI-1 can be reactivated in vitro by exposure to protein denaturants, such as urea or guanidine hydrochloride,8 or to negatively charged phospholipids.19 This latter observation has led to the suggestion that activation or reactivation of latent PAI-1 may take place on membrane surfaces in vivo.19

The present study was designed to examine the pharmacokinetics of recombinant human PAI-1 (rPAI-1) in rabbits. rPAI-1 was found to have a short circulatory half-life, and our findings suggest that some reactivation of latent rPAI-1 may occur in vivo.

Materials and Methods

Recombinant, predominantly single-chain t-PA (r-PA, Actilyse) was provided by Boehringer Ingelheim, Ingelheim, FRG. The Second International Reference Preparation of t-PA (86/670) was obtained from the National Institute for Biological Standards and Control, London, UK. CNBr-activated Sepharose 4B was purchased from Pharmacia, Uppsala, Sweden. The chromogenic substrate S-2423 (Ac-Ile-Glu-Gly-Arg-pNA) for endotoxin assay was purchased from Kabi Diagnostica, Stockholm, Sweden. Chloramine T was purchased from Fluka AG, Buchs, Switzerland.
Recombinant Plasminogen Activator Inhibitor-1 Preparation

rPAI-1 was isolated from conditioned cell culture media of Chinese hamster ovary cells transfected with the cDNA encoding human PAI-1. This material was predominantly inactive (specific activity, approximately 2 units/µg, with 1 unit being defined as the amount that neutralizes 1 IU of t-PA activity, determined by titration against the reference preparation of t-PA). The rPAI-1 could be reactivated by incubation with urea or guanidine hydrochloride according to the method of Hekman and Loskutoff. After reactivation and extensive dialysis against cold phosphate buffered saline (0.04 M phosphate, 0.14 M NaCl, pH 7.4), the rPAI-1 had a specific activity of between 140 and 160 units/µg. This activity corresponds to approximately 20% of the theoretical value for fully active PAI-1 (i.e., 700 units/µg), calculated on the basis of a specific activity for t-PA of 500 IU/µg and molecular weights of t-PA and rPAI-1 of approximately 70,000 and 50,000, respectively. For some experiments, active rPAI-1 present in trace amounts (0.3% of the theoretical activity of fully active rPAI-1 or 1.5% of the actual activity of reactivated rPAI-1) was removed from the latent preparation by titration with rt-PA and incubation at 37°C for 15 minutes. The rt-PA and rt-PA/rPAI-1 complex were removed from this solution by immunoadsorption at 4°C on Sepharose 4B coupled with MA-62E8, a monoclonal antibody directed against t-PA. The residual activity of rPAI-1 treated in this manner was approximately 0.008 units/µg. Nonreactivatable rPAI-1 was prepared by oxidative inactivation with chloramine T according to the method of Lawrence and Loskutoff. This oxidized preparation of rPAI-1 had a specific activity of approximately 0.7 units/µg, and less than 0.3% of the protein could be reactivated with guanidine hydrochloride. The endotoxin concentration in the purified and reactivated preparations of rPAI-1 was measured using the chromogenic substrate S-2423 and was determined to be less than 150 ng/mg rPAI-1.

Pharmacokinetic Analysis

New Zealand White rabbits, 2.3–3.3 kg body weight, were anesthetized by intramuscular injection of 0.4 ml/kg Hypnorm (Duphar, Amsterdam, The Netherlands), containing 10 mg/ml fluanisone and 0.2 mg/ml fentanyl. A catheter was placed in the right femoral vein for blood sampling.

Pharmacokinetic studies were performed in rabbits that received rPAI-1 (1.0 or 2.5 mg/kg intravenous bolus injection over 15 seconds) through a femoral venous catheter. Blood samples (2 ml each) were drawn in trisodium citrate, pH 4.5 (final concentration, 13 mM), before the bolus injection and at frequent intervals thereafter. The blood was immediately placed on ice, and plasma was obtained by centrifugation at 4°C at 2,000g for 10 minutes within 2 hours after collection and stored at −20°C until assayed.

The results of measurements of rPAI-1 concentrations in plasma versus time, [C(t)], after intravenous bolus injection were plotted on semilogarithmic paper and fitted with a sum of two exponential terms, 

\[ C(t) = Ae^{-dt} + Be^{-ft} \]

by graphical curve peeling according to the method of Gibaldi and Perrier. The disposition of rPAI-1 was represented by a two-compartment mammalian model composed of one central and one peripheral compartment with elimination occurring from the central compartment. Pharmacokinetic parameters were calculated from the coefficients and exponents of the equation describing the plasma concentration versus time curve by using the following formulas: volume of the central compartment, \( V_c = \text{dose/(A+B)} \); extrapolated area under the curve, \( AUC = A\alpha + B\beta \); where \( \alpha = -0.693/\text{initial half-life} \) and \( \beta = -0.693/\text{terminal half-life} \); and plasma clearance, \( Cl_p = \text{dose/AUC} \). The specific activity of PAI-1 in plasma after injection was determined by dividing the measured PAI activity (in units) at a given time point by the corresponding PAI-1 antigen value (in micrograms). In all cases, the plasma PAI activity was corrected with respect to the pretreatment plasma PAI activity for a given animal, yielding the following formula: specific activity = [PAI activity (x) − PAI activity (0)]/PAI-1 antigen (x). Pretreatment plasma PAI activities averaged 4.6±1.6 units/ml (mean±SEM, n=16) and ranged from nondetectable to 26 units/ml.

Analytical Methods

PAI-1-related antigen was measured with a specific enzyme-linked immunosorbent assay. PAI activity was measured according to the method of Verheijen et al by or an immunofunctional enzyme-linked immunosorbent assay (specific for PAI-1 activity), as previously described.

Statistical Analysis

All data are presented as mean±SD unless otherwise indicated. Statistical analyses of the specific activity of PAI-1 in plasma were made using an analysis of variance (ANOVA) corrected for repeated measures of a single factor with a commercial statistical software package (PC ANOVA, Human Systems Dynamics, Northridge, Calif.). Time-dependent, within-group differences in specific activity were compared using Newman-Keuls method. A one-sample t test was used to compare the preinjection specific activity of PAI-1 preparations with the plasma specific activity values obtained at 1 minute for each group. Values of \( p > 0.05 \) were considered not significant.
Results

Pharmacokinetics of Recombinant Plasminogen Activator Inhibitor-1 in Rabbits

The evolution of the plasma concentration versus time curves of the three forms of rPAI-1 after an intravenous bolus injection are shown in Figure 1. When latent rPAI-1 was injected at a dose of 2.5 mg/kg (total dose, 6.8±0.2 mg), the plasma concentration increased to 51±8 μg/ml. The disappearance rate of antigen from plasma could be described adequately by a sum of two exponential terms by graphic curve peeling, yielding the following equation: \[ C(t) = 27e^{-0.11t} + 25e^{-0.050t} \] with an initial half-life of 6.3 minutes. The pharmacokinetic parameters of the disposition of latent rPAI-1 as derived from these values are summarized in Table 1.

When reactivated rPAI-1 was injected at a dose of 1 mg/kg (average dose, 3.0±0.2 mg), the plasma concentration increased to 19±1 μg/ml. This was followed by a rapid disposition of antigen (Figure 1B), which could be described by \[ C(t) = 12e^{-0.12t} + 7e^{-0.034t} \], with an initial half-life of 6 minutes. Pharmacokinetic

Table 1. Pharmacokinetic Parameters of the Disposition of Recombinant Plasminogen Activator Inhibitor-1 After Bolus Intravenous Injections

<table>
<thead>
<tr>
<th>Compound</th>
<th>(n)</th>
<th>Dose (mg)</th>
<th>(C(0)) (μg/ml)</th>
<th>(V_C) (ml)</th>
<th>(V_D) (ml)</th>
<th>(Cl_p) (ml·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latent rPAI-1</td>
<td>4</td>
<td>6.8±0.2</td>
<td>51±8</td>
<td>130±17</td>
<td>360±260</td>
<td>10±3</td>
</tr>
<tr>
<td>Reactivated rPAI-1</td>
<td>3</td>
<td>3.0±0.2</td>
<td>19±1</td>
<td>170±13</td>
<td>450±50</td>
<td>10±1</td>
</tr>
<tr>
<td>Oxidized rPAI-1</td>
<td>3</td>
<td>3.0±0.2</td>
<td>17±3</td>
<td>180±41</td>
<td>470±150</td>
<td>11±2</td>
</tr>
</tbody>
</table>

Values are mean±SD. \(C(0)\), plasma concentration at time 0; \(V_C\), volume of central compartment; \(V_D\), total volume of distribution; \(Cl_p\), clearance of recombinant plasminogen activator inhibitor-1 (rPAI-1) from plasma.
parameters derived from these values are summarized in Table 1.

Injection of a chloramine T–oxidized preparation of rPAI-1 at a dose of 1 mg/kg (average total dose, 3.0±0.2 mg) resulted in a plasma concentration of 17±3 μg/ml. The disposition of antigen was described by C(t)=151e^{-0.18t}+6e^{-0.008t}, yielding the pharmacokinetic parameters reported in Table 1 and illustrated in Figure 1C.

PAI-1 activity (determined using the immunofunctional method) in plasma after bolus injection was markedly different for the three preparations, as illustrated in Figure 1. Whereas the disappearance of PAI activity paralleled the disappearance of antigen in the case of reactivated rPAI-1, the disposition of activity diverged from the disposition of antigen in the case of latent rPAI-1 and oxidized rPAI-1.

**In Vivo Reactivation of Recombinant Plasminogen Activator Inhibitor-1**

In an effort to elucidate the origin of the discrepancies between the disposition of antigen and activity (measured by immunofunctional assay) after injection of the rPAI-1 preparations, the specific activities of PAI-1 in plasma were determined in four groups of animals during the initial 60 minutes after the bolus injections (Table 2). In four animals given latent rPAI-1 (2.5 mg/kg), the specific activity of PAI-1 in plasma increased over twofold within 1 minute, from 2 units/μg to 5.0±1.1 units/μg (p<0.01) after correction for an endogenous baseline PAI-1 activity in plasma of 2±2 units/ml. The specific activity of PAI-1 in plasma steadily increased over the following hour to 7.3±1.5 units/μg at 10 minutes, 16±2.0 units/μg at 30 minutes, and 53±16 units/μg at 60 minutes (p<0.01). This biphasic pattern of increase in activity was also observed in six animals that received a bolus injection of 1 mg/kg of latent rPAI-1, from which the active rPAI-1 had been removed by titration with t-PA. This titrated, latent rPAI-1 had a specific activity of approximately 0.008 units/μg, which increased in the first minute after injection to 1.6±0.9 units/μg (p<0.05). This initial increase of 200-fold is equivalent to the release or activation of approximately 5 pmol in the rabbits’ circulation. The specific activity of PAI-1 in plasma increased another eightfold over the subsequent hour, to 13±9 units/μg (p=0.01). The third group of animals received oxidized rPAI-1 in a dose of 1 mg/kg as a bolus injection. There was no measurable increase in specific activity in the first 15 minutes after injection, and only a gradual increase was noted over 60 minutes, from 0.7 units/μg before injection to 3.4±1.5 units/μg at 1 hour (p=0.05). The specific activity of reactivated rPAI-1 did not change significantly throughout the observation period.

In control experiments, latent rPAI-1 (final concentration, 1–50 μg/ml) was added to either fresh plasma, phosphate buffered saline, or fresh, whole, rabbit blood. The mixtures were incubated at 37°C and sequential determinations of PAI activity were performed. No change in the specific activity of latent rPAI-1 was observed over a period of 30 minutes in any of these control experiments performed in vitro.

**Discussion**

Both active and latent forms of PAI-1 have been identified in plasma,9–11 in platelets,12 and in culture media.12 In vitro, inactivation occurs spontaneously with a 50% inhibition of active PAI-1 at 37°C within 2–4 hours.13,14 Inactivation of PAI-1 can be reversed by exposing the latent protein to denaturants or to negatively charged phospholipids.19 It is not currently known if, in vivo, biological activity can be restored to latent PAI-1, nor have specific endogenous mechanisms of reactivation been identified.

The issue of reactivation of latent rPAI-1 was addressed in the pharmacokinetic studies described herein. After the administration of latent rPAI-1 (2.5 mg/kg), we observed a divergent clearance of antigen and activity from plasma. Potential explanations for this observation include the following: 1) latent rPAI-1 may be reactivated in vivo; 2) active rPAI-1 may be cleared more slowly than inactive material, possibly because of binding to and stabilization by vitronectin14; or 3) the bolus of rPAI-1 might displace endogenous, active PAI-1 from the extracellular matrix. Our findings suggest that the first explanation is most likely correct. First, the increase in specific activity of rPAI-1 in plasma was most pronounced in the first minute after

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### Table 2. Specific Activity of Circulating Recombinant Plasminogen Activator Inhibitor-1 After Bolus Injection

<table>
<thead>
<tr>
<th>rPAI-1 preparation</th>
<th>Dose (mg/kg)</th>
<th>n</th>
<th>0 min</th>
<th>1 min</th>
<th>5 min</th>
<th>10 min</th>
<th>45 min</th>
<th>60 min</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latent</td>
<td>2.5</td>
<td>4</td>
<td>2</td>
<td>5.0±1.1*</td>
<td>5.3±0.7</td>
<td>7.3±1.5</td>
<td>28±8*</td>
<td>53±16*</td>
<td>0.0000</td>
</tr>
<tr>
<td>Latent (active PAI-1 removed)</td>
<td>1</td>
<td>6</td>
<td>0.008</td>
<td>1.6±0.9t</td>
<td>1.6±0.9</td>
<td>2.2±1.6</td>
<td>4.3±2.4</td>
<td>13±9.1*</td>
<td>0.0002</td>
</tr>
<tr>
<td>Oxidized</td>
<td>1</td>
<td>3</td>
<td>0.7</td>
<td>0.6±0.05</td>
<td>0.5±0.1</td>
<td>0.7±0.1</td>
<td>2.9±0.4t</td>
<td>3.4±1.5t</td>
<td>0.0069</td>
</tr>
<tr>
<td>Reactivated</td>
<td>1</td>
<td>3</td>
<td>160</td>
<td>170±20</td>
<td>170±32</td>
<td>150±25</td>
<td>210±50</td>
<td>210±23</td>
<td>0.1274</td>
</tr>
</tbody>
</table>

Values are mean±SD of specific activity of recombinant plasminogen activator inhibitor-1 (rPAI-1) in plasma. Values for specific activity at time 0 refer to measurements made on PAI-1 preparations before injection. Specific activity determinations between samples taken at 1 minute after bolus injection and the preinjection values for each group were made using a one-sample t test. p values for time-dependent changes in specific activity were determined using analysis of variance. Newman-Keuls method was used to compare specific activity values within groups for the time points between 1 and 60 minutes, inclusive.

*p<0.01 (see text).

†p<0.05 (see text).
injection, and this increase occurred before significant clearance of the protein from the circulation had taken place. Accordingly, the role of differential clearance of latent versus active material would appear to be minimal. Second, none of the known stimulants of endogenous PAI-1,26 including endotoxin,27 provokes a very rapid release. Although the rPAI-1 preparations used in the present studies contained a small amount of contaminating endotoxin, the rabbits received, at most, a total endotoxin dose of 0.6 µg/kg. While this dose of endotoxin may have stimulated the synthesis of endogenous PAI-1, increased plasma levels would have occurred only after a significant delay.28 Administration of 0.6 or 3 µg/kg endotoxin did not result in a rapid increase of PAI-1 activity (data not shown), thereby excluding an endotoxin-related effect in the initial phase of the observed reactivation. Additionally, a similar pattern of rapid increase in specific activity was observed in the group of rabbits that received a lower dose of latent rPAI-1 (1 µg/kg) and, consequently, a lower dose of contaminating endotoxin.

The experiments in which oxidized or reactivated rPAI-1 was administered provide additional evidence to support the reactivation hypothesis. After the administration of reactivated rPAI-1, the specific activity of PAI-1 in plasma did not change significantly over the 1-hour observation period. Furthermore, after the injection of oxidized material, which resists reactivation,21 the specific activity of PAI-1 increased slowly over the ensuing hour and, importantly, did not increase in the first 15 minutes after injection. Simultaneously, we observed that oxidized PAI-1 was cleared from the circulation as rapidly as the latent and reactivated preparations. These data, taken together, suggest that the increases in specific activity observed after the administration of latent reactivatable material was not due to the mass-action effect and the displacement of active PAI-1 from the extracellular matrix.

Importantly, we were unable to detect any reactivation of latent PAI-1 in vitro in experiments in which latent PAI-1 was added to citrated whole blood or plasma. This suggests that reactivation is not merely dependent on the addition of latent PAI-1 to the plasma milieu or on the exposure of latent material to formed elements in blood. At present, the localization of an endogenous reactivating mechanism remains obscure, but our findings raise the suspicion that it may be endothelium related.

Although our findings provide evidence that latent PAI-1 can be reactivated in vivo, our study is limited by the fact that the monoclonal antibodies used in the enzyme-linked immunosorbent assays for PAI-1 do not discriminate between reactivated human rPAI-1 and active, native rabbit PAI-1. The availability of a species-specific assay would conceivably permit the precise determination of the relative roles of reactivation of latent, exogenous material versus the release of active, endogenous PAI-1 in producing an increase in specific activity of PAI-1 in plasma.

In conclusion, reactivation of latent rPAI-1 in the circulation appears to occur rapidly but only to a limited extent. The mechanism of reactivation and its role in the regulation of endogenous fibrinolysis remain to be determined.

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References


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