Role of Plasminogen Activator Inhibitor-1 in Promoting Fibrin Deposition in Rabbits Infused With Ancrod or Thrombin

By Chitra Krishnamurti, Charles Bolan, Curtis A. Colleton, Thomas M. Reilly, and Barbara M. Alving

The role of defective fibrinolysis caused by elevated activity of plasminogen activator inhibitor-1 (PAI-1) in promoting fibrin deposition in vivo has not been well established. The present study compared the efficacy of thrombin or ancrod, a venom-derived enzyme that clots fibrinogen, to induce fibrin formation in rabbits with elevated PAI-1 levels. One set of male New Zealand rabbits received intravenous endotoxin to increase endogenous PAI-1 activity followed by a 1-hour infusion of ancrod or thrombin; another set of normal rabbits received intravenous human recombinant PAI-1 (rPAI-1) during an infusion of ancrod or thrombin. Thirty minutes after the end of the infusion, renal fibrin deposition was assessed by histopathology. Animals receiving endotoxin, rPAI-1, ancrod, or thrombin alone did not develop renal thrombi. All endotoxin-treated rabbits developed fibrin deposition when infused with ancrod (n = 4) or thrombin (n = 6). Fibrin deposition occurred in 7 of 7 rabbits receiving both rPAI-1 and ancrod and in only 1 of 6 receiving rPAI-1 and thrombin (P < .01). In vitro, thrombin but not ancrod was inactivated by normal rabbit plasma and by purified antithrombin III or thrombomodulin. The data indicate that elevated levels of PAI-1 promote fibrin deposition in rabbits infused with ancrod but not with thrombin. In endotoxin-treated rabbits, fibrin deposition that occurs with thrombin infusion may be caused by decreased inhibition of procoagulant activity and not increased PAI-1 activity. This is a US government work. There are no restrictions on its use.

Intravascular fibrinolysis is regulated by plasminogen activator inhibitor-1 (PAI-1), a 52-kD inhibitor of tissue-type plasminogen activator (t-PA). In rabbits, PAI-1 can increase from basal values of less than 0.1 nmol/L to levels 40-fold or higher within 4 hours of endotoxin infusion. Endotoxin induces other procoagulant responses that include stimulation of tissue factor expression on monocytes and endothelial cells as well as downregulation of thrombomodulin (TM), an endothelial receptor that inhibits the procoagulant activity of thrombin.

We have previously shown that thrombi do not occur in animals infused with endotoxin alone. However, fibrin deposition can be induced in endotoxin-treated rabbits by the infusion of ancrod, an enzyme from the Malayan pit viper that specifically clots fibrinogen without demonstrating any other procoagulant activity. Because ancrod does not induce thrombi in normal rabbits, we have postulated that ancrod-induced fibrin deposition in endotoxin-treated rabbits is caused by increased PAI-1 activity. This hypothesis assumes that ancrod does not undergo significant interaction with endogenous inhibitors such as TM or antithrombin III (AT-III) and is therefore not affected by downregulation of TM in endotoxin-treated animals. However, the fibrinolytic system may not be the major regulator of thrombin-induced fibrin deposition, because thrombin is rapidly inactivated on the endothelium by AT-III and TM.

These hypotheses were further tested in the present study by determining whether ancrod or thrombin induced fibrin deposition in normal rabbits that were infused with human recombinant PAI-1 (rPAI-1) to achieve high plasma levels of PAI-1. The relative thrombogenicity of thrombin and ancrod were also compared in endotoxin-treated rabbits that had increased endogenous PAI-1 activity.

Materials and Methods

Materials Human rPAI-1 (specific activity, 250,000 AU/mg) was obtained from the Du Pont Merck Pharmaceutical Comp (Wilmington, DE). The rPAI-1 was produced by Escherichia coli pE1200 that carried the PAI-1 cDNA and was purified from lysates.

Blood was collected into polypropylene tubes containing EDTA (0.9 mL blood and 0.1 mL 2%
Rabbits were killed 30 minutes after the end of the thrombogenic stimulus. Ancrod (5 U/kg) or thrombin (1 30 U/kg) was infused from 4 to 5 hours. The dose of thrombin infused was equivalent to 590 U ancrod/kg.

Saline + ancrod
Saline + thrombin

Endotoxin + ancrod
Endotoxin + thrombin
Endotoxin + saline

The plasma was diluted and mixed with a calcium-thrombin solution for 1 hour; the fibrin clot was then wound on a glass rod, washed in normal saline, and solubilized in alkaline urea as previously described. The concentration was determined spectrophotometrically at 280 nm.

**Inhibition of thrombin and ancrod by rabbit plasma, AT-III, and TM.**

The inhibitory effect of rabbit plasmas, purified human AT-III, and rabbit TM toward thrombin and ancrod was determined in a two-stage assay. The plasmas tested were normal rabbit plasma (PAI-1 <5 U/mL), plasma from an endotoxin-treated rabbit (PAI-1, 115 AU/mL), and plasma containing rPAI-1 (115 AU/mL).

Before incubation with thrombin or ancrod, plasma was first defibrinogenated and the fibrin clot removed. This was accomplished by diluting 1 mL plasma with an equal volume of veronal-saline buffer (1 part veronal consisting of 0.028 mol/L sodium diethylbarbiturate, 0.125 mol/L sodium chloride, and 0.023 mol/L HCl with 9 parts 0.15 mol/L NaCl. NaCl, pH 7.35) and adding 0.06 mL human thrombin (3 U/mL, final concentration). After incubation for 10 minutes at 37°C, the clot was removed by washing on a glass rod.

In the first stage of the inhibition assay, the defibrinogenated plasma (1.6 mL) was incubated with thrombin or ancrod (0.18 mL, 100 U/mL) at 37°C; in the second stage, the residual coagulant activity was determined by admixing a 0.1-mL aliquot with 0.2 mL human fibrinogen (0.4 mg/mL in veronal saline buffer) at 37°C. The clotting time was recorded with a Dataclot 2 fibrometer (Helena Labs, Beaumont, TX).

Thrombin (10 U/mL, final concentration) and ancrod (10 U/mL, final concentration) were also incubated with AT-III (1 U/mL, final concentration) in veronal buffer and the residual coagulant activity determined as described above. The inhibitory activity of TM was determined by mixing thrombin (2 U/mL, final concentration) or ancrod (2 U/mL, final concentration) with rabbit TM (5 U/mL, final concentration) in veronal buffer and determining residual coagulant activity.

**AT-III activity.** The activity was determined in test plasmas in the presence of heparin (3 U/mL). Thrombin (0.1 mL, approximately 20 U/mL) was mixed with 0.4 mL diluted plasma at 37°C for exactly 30 seconds. S-2238 (0.3 mL, 0.48 mmol/L) was then added to the mixture and the reaction was stopped 1 minute later by the addition of 50% acetic acid (0.3 mL). The residual thrombin activity was measured spectrophotometrically at 405 nm, and the concentration of AT-III was calculated using standard curves prepared from pooled normal rabbit plasma.

**Comparison of coagulant activity of thrombin and ancrod in vitro.** Normal rabbit plasma (0.1 mL) was incubated with 0.1 mL veronal saline buffer for 2 minutes at 37°C in a fibrometer cup.

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**Table 1. PAI-1 and t-PA Activities in Rabbits Receiving Endotoxin and Ancrod or Thrombin**

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>0 h</th>
<th>4 h</th>
<th>5 h</th>
<th>0 h</th>
<th>4 h</th>
<th>5 h</th>
<th>0 h</th>
<th>4 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin + saline</td>
<td>&lt;1</td>
<td>188±5</td>
<td>167±16</td>
<td>1.3±0.1</td>
<td>0.2±0.2</td>
<td>0</td>
<td>0</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>Endotoxin + ancrod</td>
<td>&lt;1</td>
<td>143±11</td>
<td>147±8</td>
<td>1.0±0.3</td>
<td>0.5±0.2</td>
<td>0</td>
<td>0</td>
<td>4/4</td>
<td></td>
</tr>
<tr>
<td>Endotoxin + thrombin</td>
<td>&lt;1</td>
<td>126±10</td>
<td>95±11</td>
<td>1.7±0.2</td>
<td>0.6±0.1</td>
<td>0.1±0.1</td>
<td>6/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + ancrod</td>
<td>1.9±1.8</td>
<td>2.3±1.7</td>
<td>3.7±1.6</td>
<td>0.4±0.2</td>
<td>0.7±0.2</td>
<td>1.8±0.6</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline + thrombin</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>0.7±0.3</td>
<td>0.8±0.2</td>
<td>1.0±0.2</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The activities (mean ± SEM) for plasma PAI-1 and t-PA are given for the rabbits presented in Fig. 2. After an infusion of endotoxin (10 μg/kg) at time 0, the thrombogenic stimulus ancrod or thrombin was infused from 4 to 5 hours.

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**Fig 1.** Plasma PAI-1 activity in rabbits treated with endotoxin (10 μg/kg) followed by either (a) saline, (c) ancrod, or (b) thrombin.

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**Table 1.** PAI-1 and t-PA Activities in Rabbits Receiving Endotoxin and Ancrod or Thrombin
Different concentrations of thrombin (0.1 mL) or ancrod (0.1 mL) diluted in veronal saline buffer were then added and the clotting time recorded with a fibrometer. When tested with normal rabbit plasma, 1 U of ancrod was equivalent to 0.22 U of thrombin.

**Animal experimental design.** The animal protocol was approved by the small animal committee at the Walter Reed Army Institute of Research. Male New Zealand rabbits (2.2 to 3.2 kg) received intravenous infusions through the marginal ear vein with a 23-gauge needle. Infusions of thrombin and rPAI-1 were administered in the opposite ear. In all experimental groups in which thrombin or ancrod was infused, a single concentration of each agent was used. The doses were chosen based on previous studies that showed an effective dose that could decrease fibrinogen levels in normal rabbits without inducing apparent thrombosis. For ancrod, the dose was 5 U/kg. For thrombin, the dose was 130 U/kg. Based on in vitro studies comparing the relative coagulant effects of thrombin and ancrod, a dose of thrombin of 130 U/kg was equivalent to 590 U/kg of ancrod.

The thrombosis model has been reported previously. Briefly, one set of rabbits received intravenous infusions of endotoxin (10 μg/kg) in 3 mL saline at time 0, followed by an infusion of ancrod (5 U/kg) or thrombin (130 U/kg) in a volume of 10 mL from 4 to 5 hours. Another set received rPAI-1 as an intravenous bolus (40 μg/kg in 3 mL saline) at time 0, followed by a continuous infusion of rPAI-1 (1.5 μg/kg) either alone or in combination with ancrod (5 U/kg) or thrombin (130 U/kg) for 1 hour.

Rabbits were euthanized 30 minutes after the completion of the infusions by first receiving an intramuscular injection of a solution containing 50% ketamine hydrochloride (Ketastet, 10 mg/mL; dose 1 mg/kg) and 50% xylazine hydrochloride (Rompun, 20 mg/mL; dose, 2.2 mg/kg) as a preanesthetic, followed by an intravenous infusion of sodium pentobarbital (V-Pento C; 325 mg in 5 mL; Euthanasia-6).

**Histopathology.** The kidneys were removed after euthanasia and immersion-fixed in neutral buffered 10% formalin. Sections were processed, embedded in paraffin, and stained by a modified phosphotungstic acid hematoxylin (PTAH) procedure.

Two sections from each kidney were scanned by light microscopy on high power and 50 glomeruli were counted per section. Fibrin deposition was considered to be present if at least two glomeruli stained positive for fibrin. The lung, spleen, and liver of each animal were also examined for the presence of fibrin. Histologic studies were performed by a veterinary pathologist who was unaware of the experimental treatment protocols.

**Statistics.** Results are expressed as the mean ± SEM. Statistical analysis was performed using the Student's t-test on paired samples. Data on fibrin deposition in rabbits receiving rPAI-1 and ancrod or thrombin were evaluated with Fisher's exact test.

**RESULTS**

**Effect of ancrod or thrombin in endotoxin-treated rabbits with elevated levels of PAI-1.** Rabbits were first treated with endotoxin and received infusions of either ancrod or thrombin 4 hours later, when the levels of endogenous PAI-1 were at their maximum value (150 to 200 AU/mL, Fig 1). Fibrin deposition did not occur in rabbits that received only endotoxin, or in control rabbits that received ancrod or thrombin alone. However, endotoxin-treated rabbits that received ancrod or thrombin all had fibrin deposition.

The changes in t-PA and PAI-1 in the animals receiving endotoxin are shown in Table 1. Infusion of ancrod into endotoxin-treated rabbits caused no change in PAI-1 activity, whereas thrombin infusion caused a significant decrease (P = .01). Thrombin has been previously reported to decrease endogenous PAI-1 levels through activation of protein C.

The increased PAI-1 activity induced by endotoxin was associated with reduced t-PA activity that persisted throughout the infusion of ancrod or thrombin. Administration of thrombin to normal rabbits was not associated with

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**Table 2. PAI-1 and t-PA Activities in Rabbits Receiving rPAI-1 and Ancrod or Thrombin**

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>PAI-1 (AU/mL)</th>
<th>t-PA (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>5 min</td>
</tr>
<tr>
<td>rPAI-1 + saline</td>
<td>&lt;1</td>
<td>147 ± 18</td>
</tr>
<tr>
<td>rPAI-1 + ancrod</td>
<td>&lt;1</td>
<td>168 ± 6</td>
</tr>
<tr>
<td>rPAI-1 + thrombin</td>
<td>&gt;231 ± 27</td>
<td>281 ± 13</td>
</tr>
</tbody>
</table>

The activities (mean ± SEM) for plasma PAI-1 and t-PA are given for the rabbits presented in Fig 3. After a bolus infusion of rPAI-1 (40 μg/kg) at time 0, ancrod or thrombin was infused with rPAI-1 (1.5 μg/kg) from 5 to 65 minutes.
any alteration of PAI-1 or t-PA activity. Although normal rabbits treated with ancrod beginning at 4 hours showed a trend towards an increase in mean PAI-1 and t-PA activities at the end of ancrod infusion, the changes were not significant, P = .09 for PAI-1 activity and P = .17 for t-PA activity.

Effect of ancrod or thrombin on fibrinogen levels and on AT-III activity. Although examination of glomerular fibrin deposition in experimental animals provided a quantitative estimate of fibrin deposition, other organs were also evaluated to determine if there were differences among the experimental groups with respect to the distribution of fibrin deposition (Table 4). The minimal prothrombotic effect of high levels of rPAI-1 in thrombin-treated rabbits was underscored by the finding of fibrin deposition in only 1 of 6 animals; in this animal, fibrin deposition was confined to the kidney.

For animals receiving both rPAI-1 and ancrod or for animals receiving endotoxin followed by ancrod or thrombin, fibrin deposition was present in other organs as well as the glomeruli (Table 4). In animals receiving endotoxin and ancrod, renal fibrin deposition was more prominent than in those receiving rPAI-1 and ancrod.

In vitro studies: Inhibition of ancrod and thrombin by rabbit plasma, AT-III, and TM. We postulated that, for the ancrod-treated rabbits, the major determinant in preventing fibrin deposition would be a functional fibrinolytic system, because ancrod was unlikely to be inhibited by AT-III or TM. In contrast, in rabbits infused with thrombin, fibrin deposition could be prevented primarily by inactivation of thrombin through the inhibitors AT-III and TM. This could greatly reduce the initial procoagulant activity of thrombin, and the fibrinolytic system could then degrade forming fibrin before deposition could occur.

We further compared the interaction of thrombin and ancrod with plasma inhibitors by measuring the residual coagulant activity after incubation with normal rabbit plasma and plasma with increased concentrations of PAI-1 (Fig 3). The procoagulant activity of ancrod was not inhibited by incubation of ancrod with normal plasma, plasma from endotoxin-treated rabbits (PAI-1, 115 AU/mL), plasma containing rPAI-1 (115 AU/mL), purified AT-III, or TM (Fig 3). In contrast, thrombin was rapidly neutralized within 30 seconds after incubation with TM and within 3 minutes when incubated with AT-III or with rabbit plasma containing high levels of endogenous PAI-1 or rPAI-1.

### Table 4. Distribution of Fibrin Deposition in Rabbits Receiving Ancrod or Thrombin

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Kidney</th>
<th>Liver</th>
<th>Lung</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin + thrombin</td>
<td>100</td>
<td>50</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>rPAI-1 + thrombin</td>
<td>17 (2)*</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Endotoxin + ancrod</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>rPAI-1 + ancrod</td>
<td>100</td>
<td>43</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate the mean percent ± SEM of glomeruli affected. Values are the percentage of glomeruli affected for the single rabbit with fibrin deposition.
PLASMINOGEN ACTIVATOR INHIBITOR-1 AND THROMBOSIS

3635

Fig 3. In vitro inhibition of thrombin and ancrod. (A) Thrombin or ancrod was incubated with normal rabbit plasma (——) or with plasma from an endotoxin-treated rabbit (PAI-1, 115 AU/mL: ———) or with plasma containing rPAI-1 (115 AU/mL: ·······) as described in Materials and Methods. Thrombin and ancrod were also incubated (B) with purified human AT-III (1 U/mL) and (C) with rabbit TM (5 U/mL) and the residual activities determined (see Materials and Methods). Final concentrations of thrombin and ancrod were 10 U/mL in (A) and (B) and 2 U/mL in (C). Results are expressed as mean ± SEM of two to four separate experiments performed in duplicate.

ously hypothesized that ancrod induces fibrin deposition in endotoxin-treated rabbits because the fibrinolytic activity is inhibited by high levels of PAI-1. The present study validates this hypothesis by showing that ancrod induces fibrin deposition in rabbits whose only hemostatic abnormality is an elevated level of PAI-1. In vivo, ancrod has an initial half-life of 3 to 5 hours, indicating lack of significant interaction with endogenous inhibitors. Furthermore, ancrod does not interact in vitro with rabbit plasma or with purified TM or AT-III.

The marked elevation in PAI-1 activity that occurs in endotoxin-treated rabbits is unlikely to be found in patients. Ancrod prepared by Knoll Pharmaceuticals (Whippany, NJ) has been used in human recipients for a variety of clinical conditions and is currently available as an Investigational New Drug for patients with heparin-associated thrombocytopenia. In such patients the goal is to prevent further clot formation by maintaining the fibrinogen levels between 70 and 90 mg/dL. This is achieved by infusing ancrod during a 12-hour period at half the coagulant activity that was used in the present study. Thus, ancrod would not be expected to induce thrombosis in human recipients if used as directed.

The renal fibrin deposition that occurs after thrombin infusion into endotoxin-treated animals may be due, at least in part, to downregulation of TM activity and perhaps an increased turnover of AT-III. Thrombin has a half-life of seconds when infused in vivo, because of rapid reversible binding to the vascular endothelium. The binding is due presumably to the interaction of thrombin with TM; the dissociation constant for this interaction is 0.5 nmol/L. Binding is not influenced by glycosaminoglycans, and the bound thrombin can still interact with AT-III, perhaps in an accelerated fashion. Although the PAI-1-vitronectin complex can inhibit α-thrombin, it is unlikely, at least as suggested by in vitro experiments, that the inhibition is significant when compared with that of TM.

Thrombin induced a decrease in endogenous PAI-1 in endotoxin-treated rabbits, although the levels remained significantly above baseline values. Thrombin decreases PAI-1 activity by binding to TM and activating protein C, which then neutralizes PAI-1. In this study, infusion of ancrod caused no decrease in endogenous PAI-1 levels, suggesting either that ancrod does not bind to TM or cannot activate protein C. Infusion of thrombin into rabbits with high levels of human rPAI-1 also caused no significant reduction in PAI-1 activity. Because the removal of PAI-1 is mediated through the activation of protein C, it is possible that activated rabbit protein C does not neutralize human PAI-1.

In the clinical setting, stimuli such as endotoxin that increase PAI-1 activity also downregulate TM as part of a generalized coagulant response. Currently, the expression of TM can be monitored indirectly through measurement of activated protein C or the activation peptide of protein C. Tests that measure plasma TM levels are available, but the values may not reflect what is occurring at the endothelial level. The development of methods that can directly measure TM expression on the endothelial cell surface will greatly enhance the understanding of how hemostasis is regulated.

REFERENCES


19. Reggezzi E, Bell W: In vitro behavior of the coagulant enzyme from agistrodon rhodostoma venom: Studies using 111-I-Ar


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