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. This study aimed to compare the efficacy of thrombin or antithrombin III (AT III), a vitamin K-dependent inhibitor that degrades PAI-1, to reduce fibrin formation in rabbits with elevated PAI-1 levels. One set of male New Zealand rabbits received intravenous injections of either 100 mg/kg of AT III or 1,000 mg/kg of PAI-1 in order to increase endogenous PAI-1 activity. A thrombin inhibition study was conducted on the remaining rabbits in order to determine the efficacy of AT III in reducing fibrin deposition. Thirty minutes after the injection of AT III, all rabbits received an intravenous infusion of a fibrinogen solution and were then sacrificed. Fibrin deposition was assessed by histology and immunohistochemistry. Animals receiving AT III alone had significantly lower fibrin deposition than those receiving PAI-1 alone. The data indicate that elevated PAI-1 levels promote fibrin deposition in rabbits infused with either anticoagulant, and not with thrombin. In anticoagulant-treated rabbits, fibrin deposition was significantly lower in those receiving AT III alone than those receiving PAI-1 alone.
Role of Plasminogen Activator Inhibitor-I in Promoting Fibrin Deposition in Rabbits Infused With Ancrod or Thrombin

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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.

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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 PAI-1 (specific activity, 250,000 AU/mg) was obtained from the Du Pont Merck Pharmaceutical Co. Wilmington, DE. The rPAI-1 was produced by Escherichia coli pE1200 that carried the PAI-1 cDNA and was purified from lysates by sequential anion exchange and cation exchange chromatography on Q Sepharose and S Sepharose columns. This preparation of rPAI-1 contained no detectable endotoxin units (<0.03 endotoxin units/mL) as determined by the limulus assay.

Single-chain human recombinant t-PA (Activase; specific activity, 580,000 IU/mg) was obtained from Genentech (Berkeley, CA). Lys-plasminogen (specific activity, 20 CTA U/mg) was purified from Cohn fraction III paste (Cutler Laboratories, Berkeley, CA) by affinity chromatography on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Human thrombin (specific activity, 4,360 U/mg) and calcium chloride were purchased from Sigma Chemical Co. (St Louis, MO). Purified human AT-III (ATan) as well as the substrates D-Val-Leu-Lys-pNA (S-2238) and H-D-Phe-Arg-pNA (S-2238) were obtained from Kabi Diagnostica (Stockholm, Sweden). Ancrod, an enzyme derived from the Malayan pit viper, was a gift from Abbott Laboratories (North Chicago, IL). Endotoxin (E. coli 0111:B4) was purchased from Difco Laboratories (Detroit, MI). Rabbit TM (specific activity, 1,000 U/mg) was obtained from American Diagnostica (Greenwich, CT).

Sample collection and handling. Blood was collected into polypepylene tubes containing EDTA (0.9 mL blood and 0.1 mL 2%
After acidification and dilution, residual t-PA activity was determined as previously described. In this assay, one unit (arbitrary unit [AU]) of inhibitor is defined as the amount that inhibits 1 IU of t-PA in 10 minutes at 37°C.

**Fibrinogen assay.** 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-I < 5 U/mL), plasma from an endotoxin-treated rabbit (PAI-I, 115 AU/mL), and plasma containing PAI-I (115 AU/mL). Before incubation with thrombin or ancrod, plasma was first debrinogenated 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, 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 winding on a glass rod.

In the first stage of the inhibition assay, the debrinogenated 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 adding 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 20 minutes. 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. 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>PAI-1 (AU/mL)</th>
<th>t-PA (IU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotoxin + saline</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Endotoxin + ancrod</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Endotoxin + thrombin</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Saline + ancrod</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Saline + thrombin</td>
<td>1</td>
<td>0</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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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 at time 0 followed by a continuous infusion of rPAI-1 (150 U/kg) with either saline, ancrod (5 U/kg), or thrombin (130 U/kg) from 5 to 65 minutes. Rabbits were killed 30 minutes after the end of the infusions. The number of animals with renal fibrin deposition is as follows: ancrod (7 of 7), thrombin (1 of 6), and saline (0 of 6).

The thrombosis model has been reported previously. 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></td>
<td>0 min</td>
<td>5 min</td>
</tr>
<tr>
<td>rPAI-1 + saline</td>
<td>&lt;1</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>rPAI-1 + ancrod</td>
<td>&lt;1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>rPAI-1 + thrombin</td>
<td>&lt;1</td>
<td>1.4 ± 0.2</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 in rabbits with elevated levels of rPAI-1.** We administered to normal rabbits infusions of rPAI-1 to obtain plasma PAI-1 values that were similar to those in endotoxin-treated rabbits. Rabbits received rPAI-1 as a bolus (40 µg/kg) at time 0 followed by a 1-hour infusion of 1.5 µg/kg; this dose resulted in PAI-1 levels of 150 to 200 AU/mL (Fig 2). There was no evidence of renal fibrin deposition in the animals that received only rPAI-1. When ancrod was infused with rPAI-1, renal fibrin deposition developed in all animals (\( n = 7 \)). However, when thrombin was infused with rPAI-1 (\( n = 6 \)), renal deposition was noted in only one rabbit, a significant difference from that of ancrod-treated rabbits (\( P = .0047 \)).

Table 2 shows the values of PAI-1 and of t-PA in rabbits receiving rPAI-1. Infusion of rPAI-1 was associated with a decrease in endogenous t-PA activity, as was noted for the endotoxin-treated rabbits. During the infusion of rPAI-1, the plasma PAI-1 activity increased to a similar extent in the groups receiving ancrod or thrombin, suggesting that human rPAI-1 activity was not affected by thrombin infusion.

**Effect of thrombin and ancrod on fibrinogen levels and on AT-III activity.** The importance of inhibitors in preventing the procoagulant activity of thrombin was suggested by the finding that thrombin, although infused at a dose that was 100 times more potent than ancrod, caused only a mild decrease in fibrinogen levels compared with those measured in ancrod-treated rabbits (Table 3).

Infusion of thrombin into endotoxin-treated rabbits caused a significant reduction in plasma AT-III levels (Table 3). This was not observed when thrombin was infused into saline-treated rabbits or rabbits that received rPAI-1. Infusion of ancrod caused no significant change in levels of AT-III in all experimental groups.

**Histopathology.** Although examination of glomerular fibrin deposition in experimental animals provided a qualitative 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 in 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 or with plasma containing rPAI-1 (115 AU/mL), purified 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.

**DISCUSSION**

This is the first study using rPAI-1 to explore the role of increased PAI-1 activity in promoting fibrin deposition in vivo after infusion of procoagulant enzymes. We had previ-
increased turnover of TH at least in part, to downregulation of TM activity and perhaps an enhanced fibrinolytic activity. 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 presumptively 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 reduction 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.

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