Recombinant Plasminogen Activator Inhibitor-1 Inhibits Intimal Hyperplasia

Jianbo Wu, Lin Peng, Grainne A. McMahon, Daniel A. Lawrence, William P. Fay

Objective—Plasminogen activator inhibitor-1 (PAI-1) overexpression is implicated in vascular disease. However, the effects of a primary increase in PAI-1 expression on arterial remodeling are poorly defined. We tested the hypothesis that recombinant PAI-1 inhibits intimal hyperplasia after vascular injury.

Methods and Results—Rats underwent carotid artery injury and received intraperitoneal injections of saline or mutant forms of PAI-1 for 14 days, including an active stable mutant (PAI-1-14-1b), a mutant lacking anti-PA activity (PAI-1-R), or a mutant defective in vitronectin (VN) binding (PAI-1-K). All forms of PAI-1 significantly inhibited neointima formation, whereas elastase-cleaved PAI-1, which lacks both anti-PA and VN-binding functions, did not. Similar effects were observed in a murine model. However, the antiproliferative effect of PAI-1-R was lost in Vn−/− mice, suggesting that PAI-1 can inhibit intimal hyperplasia in vivo by a VN-dependent pathway not involving direct inhibition of proteases. In vitro, recombinant PAI-1 inhibited wild-type vascular smooth muscle cell (VSMC) proliferation, promoted apoptosis, and inhibited migration. These effects were lost in VN-deficient VSMCs.

Conclusion—Recombinant PAI-1 inhibits intimal hyperplasia by inhibiting proteases and binding VN. VN is a key determinant of the antiproliferative effect of PAI-1 overexpression. PAI-1-R has therapeutic potential to inhibit vascular restenosis without promoting thrombosis.

Key Words: plasminogen activator inhibitor-1 ■ vitronectin ■ neointima ■ vascular smooth muscle cell

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of urinary-type and tissue-type plasminogen activators and a key regulator of fibrinolysis.1,2 PAI-1 also regulates the function of vascular cells, including vascular smooth muscle cells (VSMCs). PAI-1 inhibits VSMC migration by inhibiting plasmin formation and preventing degradation of extracellular matrix (ECM) and elastic laminae.3 PAI-1 binds vitronectin (VN), an ECM protein whose PAI-1 binding site overlaps with those on VN for integrin αvβ3 and u-PA receptor (uPAR), cell surface proteins that control VSMC migration.4 Therefore, PAI-1 can competitively block VSMC–VN interactions and inhibit migration. However, PAI-1 can also promote VSMC migration by binding to uPAR-bound u-PA, leading to conformational changes in PAI-1 and exposure of its high-affinity binding site for LDL receptor-related protein (LRP).5,6 Binding of PAI-1 to LRP triggers internalization of PAI-1, along with associated u-PA, uPAR, and integrin.7 This internalization process, which appears to occur predominantly at the trailing edge of cells, allows VSMCs to detach from the ECM, a process necessary for migration.7–9 In addition to modulating VSMC migration, PAI-1 controls VSMC proliferation and apoptosis.10–12 Several diseases, including diabetes mellitus and atherosclerosis, are characterized by increased PAI-1 expression, both within blood and the vascular wall.13,14 PAI-1 overexpression may promote intimal hyperplasia and adverse vascular remodeling.15 However, published animal studies have predominantly used knock-out mice to compare the effect of normal versus absent PAI-1 expression on arterial remodeling.16–20 Relatively little is known about the impact of enhanced PAI-1 expression on arterial remodeling.21 In addition, the role of the cofactor of PAI-1, VN, in controlling the vascular response to PAI-1 overexpression is poorly defined. To examine the impact of a primary increase in PAI-1 expression on vascular remodeling, we administered recombinant PAI-1 to rodents after mechanical arterial injury, using not only active PAI-1, but also PAI-1 mutants lacking inhibitory or vitronectin binding functions. To examine the role of VN in determining the vascular response to PAI-1 overexpression, we studied vascular remodeling after arterial injury in VN-deficient (Vn−/−) mice and performed in vitro experiments with wild-type and VN-deficient VSMCs.

Methods

Proteins

The following recombinant human proteins were expressed and purified as described.22 (1) PAI-1-14-1b (PAI-1 N150H, K154T, Q319L, M354I), an active stable mutant that binds VN normally23; (2) PAI-1-R (T333R, A335R), a reactive center mutant that binds VN normally, but has no detectable antiproteolytic activity and cannot assume a latent conformation22; (3) PAI-1-K (PAI-1 N150H, K154T, Q319L, M354I, Q123K), which exhibits markedly reduced VN binding, but stable antiprotease activity24,25; and (4) PAI-1-AK

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Inhibition of neointima formation in rats by PAI-1. A, Representative images of injured carotid segments treated with saline, PAI-1-14-1b, PAI-1-K, and PAI-1-R (2 mg/kg/d). An uninjured (Uninj.) segment of a saline-treated rat is shown. Arrow heads denote internal elastic lamina. Magnification: ×400. B, Composite morphometric data, including 2 mg/kg/d and 0.2 mg/kg/d PAI-1 treatment groups. EC-14-1b indicates elastase-cleaved PAI-1-14-1b; PBS, phosphate-buffered-saline. Intima/media ratio of each group was statistically significantly less (P < 0.05) than that of PBS group, except as denoted by asterisk.

Animals
Male Sprague-Dawley rats (300 to 350 g) were from Harlan Laboratories. C57BL/6J mice were from Jackson Labs. C57BL/6J-congenic VN-deficient (Vn−/−) mice were from Dr David Ginsburg (University of Michigan, Ann Arbor).28 Animals received normal chow (Mice: Diet 1-H1006/3) or PAI-1-R treated rats did not differ significantly from that of PAI-1-14-1b (Figure 1B). Additional rats underwent balloon carotid injury and received 10-fold lower doses of PAI-1 (ie, 0.2 mg/kg/d for 14 days). Inhibition of neointima formation was preserved at the lower dose, though the reduced intima/media ratio in PAI-1-R-treated rats did not differ statistically from that of saline-treated controls (Figure 1B). As another control, 5 rats received elastase-cleaved PAI-1-14-1b (0.2 mg/kg/d for 14 days), which completely lacks protease inhibitory activity and has markedly reduced VN binding affinity. Elastase-cleaved PAI-1-14-1b had no apparent effect on neointima formation (Figure 1B). To determine whether the inhibition of neointima formation by recombinant PAI-1 was accompanied by an inhibition of vascular cell proliferation, we administered BrdU to subsets of the rats described above and detected proliferating cells in the intima by anti-BrdU antibody staining. The % BrdU-positive cells at 14 days after injury was significantly higher (P < 0.01) in injured carotid segments of rats treated with saline (38.3 ± 2.8%, n = 3) than in corresponding segments of rats treated with PAI-1-14-1b (9.7 ± 2.8%, n = 3) or PAI-1-R (11.3 ± 3.3%, n = 3). As shown in the Table, peak plasma PAI-1 antigen levels were >1.5 μg/mL in rats treated with recombinant PAI-1 (2 mg/kg/d). Trough plasma PAI-1 antigen levels

Table. Plasma Levels of Recombinant PAI-1 Achieved in Rats by Intraperitoneal Administration (2 mg/kg/day)

<table>
<thead>
<tr>
<th>PAI-1 Mutant</th>
<th>Plasma PAI-1 Antigen (μg/mL)</th>
<th>Plasma PAI-1 Activity (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle control</td>
<td>Peak</td>
<td>Trough</td>
</tr>
<tr>
<td>14-1b</td>
<td>1.61 ± 0.08</td>
<td>0.41 ± 0.04</td>
</tr>
<tr>
<td>R</td>
<td>1.87 ± 0.14</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>K</td>
<td>1.85 ± 0.16</td>
<td>0.42 ± 0.05</td>
</tr>
</tbody>
</table>

Peak and trough levels were measured in 8 rats/group.

stained with hematoxylin-eosin and intima and media areas were measured. Cell proliferation was assessed by anti-BrdU staining.

Cell Culture Experiments
Murine aortic VSMCs were grown in culture. Effects of recombinant PAI-1 on VSMC proliferation, apoptosis, and migration were measured.

Results
Recombinant PAI-1 Inhibits Intimal Hyperplasia After Arterial Injury
Rat carotid arteries were subjected to balloon injury, after which animals received twice-daily injections of PAI-1-14-1b, PAI-1-R, PAI-1-K (2 mg/kg/d), or saline. Fourteen days later the injured and uninjured carotid arteries were retrieved. Injured arteries in all groups were patent with no detectable thrombus. Injured arteries from saline-treated animals exhibited a robust neointima with significantly increased intima/media ratio (Figure 1A). Noninjured carotid arteries from all groups appeared normal. Injured arterial segments of rats treated with PAI-1-14-1b demonstrated a marked reduction in intima/media ratio as compared to saline-treated controls. Treatment with PAI-1-R and PAI-1-K also markedly inhibited neointima formation, producing an effect that did not differ significantly from that of PAI-1-14-1b (Figure 1B).

Statistical Analyses
Data are presented as mean ± SEM. Experimental groups were compared by 2-tailed Student t test, Mann–Whitney rank sum test, or 1-way analysis of variance (ANOVA).

Online Methods Supplement
A detailed description of the methods briefly described below is available as a supplement online at http://atvb.ahajournals.org.

Animal Experiments
Recombinant PAI-1 was administered by daily intraperitoneal injection. Plasma PAI-1 antigen and activity were measured by ELISA and functional assays. Cross-sections of injured arterial segments were

Figure 1. Inhibition of neointima formation in rats by PAI-1. A, Representative images of injured carotid segments treated with saline, PAI-1-14-1b, PAI-1-K, and PAI-1-R (2 mg/kg/d). An uninjured (Uninj.) segment of a saline-treated rat is shown. Arrow heads denote internal elastic lamina. Magnification: ×400. B, Composite morphometric data, including 2 mg/kg/d and 0.2 mg/kg/d PAI-1 treatment groups. EC-14-1b indicates elastase-cleaved PAI-1-14-1b; PBS, phosphate-buffered-saline. Intima/media ratio of each group was statistically significantly less (P < 0.05) than that of PBS group, except as denoted by asterisk.
were approximately 0.5 \mu g/mL. Rats treated with PAI-1-R showed an increase in plasma PAI-1 antigen, but no increase in plasma PAI-1 activity.

**VN Deficiency Inhibits Intimal Hyperplasia and Blocks Suppression of Neointima Formation by PAI-1**

Wild-type mice were subjected to femoral artery injury, after which PAI-1 (2 mg/kg/d) or saline were administered for 21 days. Mean intima-media ratio of mice administered PAI-1-14-1b (0.97 \pm 0.19, n=10) was significantly less than that of saline-treated mice (1.73 \pm 0.18, n=15, *P < 0.01). PAI-1-AK, a mutant with no detectable VN binding, also significantly inhibited neointima formation (mean intima-media ratio 0.72 \pm 0.12, n=10, *P < 0.005 versus saline treated mice). To examine the role of VN in mediating the antiproliferative effect of PAI-1, wild-type- and Vn-/- mice (n=5/group) were given PAI-1-R (2 mg/kg/d) or saline for 21 days after femoral artery injury. Saline-treated Vn-/- mice exhibited less neointima formation than saline-treated wild-type mice did (Figure 2). PAI-1-R markedly suppressed neointima formation in wild-type mice but did not inhibit neointima formation in Vn-/- mice. Peak (Pk) and trough (Tr) plasma PAI-1-R antigen did not differ significantly (*P > 0.5) between wild-type mice (Pk: 1.76 \pm 0.24; Tr: 0.48 \pm 0.11 \mu g/mL) and Vn-/- mice (Pk: 1.66 \pm 0.07; Tr: 0.51 \pm 0.01 \mu g/mL). These results suggested that VN supports intimal hyperplasia after arterial injury and that the inhibition of intimal hyperplasia by recombinant PAI-1-R is VN-dependent.

**Inhibition of VSMC Proliferation by PAI-1 is VN-Dependent**

We conducted in vitro experiments to examine potential mechanisms underlying the effects of recombinant PAI-1 and VN-deficiency observed in vivo. Recombinant PAI-1 (1 \mu g/mL) inhibited wild-type VSMC proliferation, with similar effects observed with PAI-1-14-1b, PAI-1-R, and PAI-1-AK, though lower concentrations of PAI-1 did not produce a significant effect (Figure 3A), nor did latent PAI-1 (1 \mu g/mL) to growth media promote apoptosis of VSMCs isolated from Vn-/- mice grew more slowly in culture than wild-type VSMCs did. However, recombinant PAI-1 did not inhibit proliferation of VN-deficient VSMCs (Figure 3B).

**Effect of PAI-1 and VN on VSMC Apoptosis**

VN-deficient VSMCs exhibited a higher incidence of apoptosis than wild-type VSMCs did (Figure 3C). Addition of PAI-1 mutants (1 \mu g/mL) to growth media promoted apoptosis of wild-type VSMCs, but did not promote apoptosis of VN-deficient VSMCs (Figure 3C). The proapoptotic effects of PAI-1-14-1b and PAI-1-R were accompanied by increases in the incidence of necrotic cells (Figure 3D).

**PAI-1 and VN Coregulate VSMC Migration**

PAI-1-14-1b and PAI-1-R (1 \mu g/mL) inhibited migration (assessed in all experiments 24 hours after initial cell seeding) of wild-type VSMCs on murine VN (10 \mu g/mL), whereas PAI-1 AK had no effect (Figure 4A). Lower concentrations of PAI-1-14-1b and PAI-1-R (ie, 0.01 to 0.1 \mu g/mL) inhibited wild-type VSMC migration to a proportionately lesser extent.
However, none of the forms of PAI-1 inhibited migration of VN-deficient VSMCs on VN (Figure 4D), indicating that even when a VN ECM is provided, VN expression by VSMCs is required for PAI-1 to inhibit migration. ECM VN promoted migration of wild-type VSMCs in a concentration-dependent manner (Figure 5A). However, in the presence of recombinant PAI-1-14-1b (1 μg/mL), the shape of the dose–response curve of ECM VN concentration versus migration was distinctly altered, with increasing concentrations of VN initially inhibiting and subsequently increasing VSMC migration (Figure 5B).

**Discussion**

We administered recombinant PAI-1 to rodents to examine the impact of a primary increase in PAI-1 expression on arterial remodeling. We focused our investigations on: (1) the functional roles of antiproteolytic and VN-binding domains of PAI-1 in arterial remodeling, and (2) the impact of the cofactor of PAI-1, VN, on the effects of PAI-1 in vivo and on cultured VSMCs. Our in vivo experiments demonstrate that systemically administered PAI-1 inhibits intimal hyperplasia after arterial injury. Inhibition of neointima formation by PAI-1-R indicates that the capacity of recombinant PAI-1 to directly inhibit proteases is not required to regulate vascular remodeling. The capacity of PAI-1-K and PAI-1-AK to inhibit intimal hyperplasia suggests that direct binding of PAI-1 to VN is also not required to inhibit intimal hyperplasia. However, elastase-cleaved PAI-1, which lacks both antiprotease and VN-binding functions, did not inhibit neointima formation. Therefore, while the capacity of recombinant PAI-1 to either bind VN or directly inhibit proteases each appears sufficient to inhibit neointima formation, neither function is absolutely required, suggesting that PAI-1 can suppress intimal hyperplasia by multiple mechanisms.

Our murine experiments suggest that VN is a key determinant of the antiproliferative effects of PAI-1, as inhibition of neointima formation by PAI-1-R required VN expression. Multiple mechanisms could account for this effect. Migration of VSMCs from the tunica media into the intima is a critical step in neointima formation. By binding VN, PAI-1 blocks VSMC–VN interactions, thereby inhibiting VSMC migration. However, a proteolysis-independent, antimigratory effect of PAI-1 has only been shown in vitro on purified VN matrices. We show that PAI-1-R inhibits intimal hyperplasia in wild-type mice, but not in VN-deficient mice, suggesting that blockade of VN–VSMC interactions—indeed, independent of protease inhibition—constitutes a mechanism by which PAI-1 functions in vivo to control vascular remodeling. Nevertheless, other mechanisms could account for the antiproliferative effect of PAI-1-R. By binding VN, PAI-1-R could displace and destabilize endogenous PAI-1, thereby decreasing PAI-1 activity in the pericellular environment, which is proposed to play a key role in cell migration.  

In addition, PAI-1 is a downstream mediator of the proliferative effects of TGF-beta, and PAI-1 suppresses TGF-beta expression. Therefore, PAI-1-R could block TGF-beta signaling by a dominant-negative effect on endogenous PAI-1 or by feedback-inhibition of TGF-beta expression. However, the antiproliferative effects of PAI-1-K and PAI-1-AK suggest that PAI-1 can also inhibit intimal hyperplasia by inhibiting cell-associated PAs or other proteases, such as thrombin, which exhibits proliferative and promigratory vascular effects. We showed that VN−/− mice have less neointima formation after arterial injury than wild-type mice do, supporting a key role for VN and its receptors in arterial remodeling after vascular injury.

Our cell culture experiments offer important insights into mechanisms by which PAI-1 controls VSMC function. Recombinant PAI-1-14-1b, PAI-1-R, and PAI-1-AK, at concentrations achieved in plasma in our in vivo experiments, inhibited proliferation and promoted apoptosis of wild-type...
VSMCs. However, these effects were lost in VN-deficient VSMCs, consistent with our in vivo data and suggesting that PAI-1 regulates vascular remodeling by controlling VSMC proliferation and apoptosis. The VN-binding function of PAI-1 would be expected to inhibit proliferation and promote apoptosis by inhibiting cell adhesion to VN.\(^{13}\) Inhibition of plasmin formation could potentially account for the capacity of PAI-1-AK to promote apoptosis, as plasmin exerts antiapoptotic effects.\(^{35}\) However, PAI-1 exerted antiapoptotic effects in some studies.\(^{11,36}\) Further studies are necessary to determine the mechanisms by which PAI-1 mutants lacking VN-binding function promote VSMC apoptosis. In addition, further experiments are needed to determine whether active forms of PAI-1 inhibit intimal hyperplasia in VN-deficient mice. Loss of the antiproliferative effect of wild-type PAI-1 in VN-deficient mice would support the hypotheses that the antiprotease function of PAI-1 requires VN to inhibit intimal hyperplasia (eg, effective inhibition thrombin, a mitogen, by PAI-1 in vivo could require VN\(^{37}\)) and that control of vascular wall cell function by PAI-1 requires engagement of cells with ECM VN (eg, PAI-1 can dissociate cells from VN without directly binding to VN\(^{9}\)). We found that only forms of PAI-1 that bind VN significantly inhibited migration of wild-type VSMCs on VN, consistent with previous studies.\(^{4}\) However, recombinant PAI-1 did not inhibit migration of VN-deficient VSMCs, even when they were seeded on VN. Although VSMCs express VN in vivo, a functional significance of VSMC–VN expression has not previously been reported. Our results suggest that VN production by VSMCs themselves is a critical determinant of the effect of PAI-1 on VSMC proliferation, apoptosis, and migration.

Our studies of the effect of ECM VN concentration on VSMC migration (Figure 5) offer important insights into the interconnected functions of VN and PAI-1. At low PAI-1 concentrations (ie, none added), VN promoted VSMC migration in a linear concentration-dependent fashion, consistent with a previous study.\(^{39}\) However, at high PAI-1 concentrations, the effect of ECM VN was complex, with increasing VN concentration initially inhibiting VSMC migration and subsequently promoting it. The “J-shaped” effect of VN under conditions of PAI-1 overexpression may be explained by the fact that free PAI-1 can promote VSMC migration, whereas VN-bound PAI-1 does not, as VN-bound PAI-1 does not bind LRP.\(^{40}\) Therefore, at high-levels of PAI-1 expression, VN could inhibit VSMC migration by binding PAI-1 and preventing its LRP-dependent motogenic effect, whereas higher VN concentrations (ie, in excess of those needed to saturate PAI-1) could promote VSMC migration by providing “unblocked” integrin attachment sites. These results have important implications regarding the impact of PAI-1 overexpression on vascular remodeling (ie, the concentration of PAI-1 in the ECM may play a key role in determining whether VN promotes or inhibits intimal hyperplasia).

Several molecular counterparts of PAI-1 and VN are likely to mediate their effects in our experiments. Integrin \(\alpha_\beta_3\) binds VN in the ECM to promote VSMC migration and neointima formation.\(^{34}\) This interaction also inhibits apoptosis.\(^{12}\) Blockade of this interaction by binding of PAI-1 to VN would be expected to inhibit intimal hyperplasia. Inhibition of cell-surface–bound uPA and other promigratory/mitogenic proteases, such as thrombin, would also be expected to inhibit intimal hyperplasia. However, binding of PAI-1 to LRP can promote VSMC migration in vitro.\(^{5}\) Our in vivo experiments suggest that high concentrations of PAI-1 do not necessarily promote intimal hyperplasia by a LRP-dependent pathway, perhaps because inhibition of integrin-VN interactions or proliferative/promigratory proteases by PAI-1 produce a dominant effect. Alternatively, as PAI-1–LRP interactions mediate cell detachment from ECM via cross-talk with integrins,\(^{9}\) excessive LRP-signaling, produced in our in vivo experiments and in diseases characterized by PAI-1 overexpression, could inhibit intimal hyperplasia by overstimulating cell detachment. Additional experiments are necessary to precisely define the molecular targets that mediate the effects of PAI-1 and VN on intimal hyperplasia.

Plasma PAI-1 concentrations in our experiments were higher than those normally achieved by endogenous mechanisms. We do not know the vascular wall concentration of PAI-1 attained in our experiments, nor are the vascular wall concentrations of PAI-1 attained in vivo by endogenous mechanisms known. However, PAI-1 expression in the arterial wall is markedly increased under pathological conditions.\(^{13,14}\) Our data suggest that enhanced PAI-1 expression can inhibit neointima formation and that the high vascular wall expression of PAI-1 found in diseased blood vessels is not necessarily a cause of the associated intimal hyperplasia. Our data also have therapeutic implications. We hypothesize that recombinant PAI-1-R could be used to inhibit neointima formation without inhibiting fibrinolysis. In fact, PAI-1-R could potentially promote fibrinolysis by competing with endogenous PAI-1 for binding to VN, fibrin, and cells. Further studies are needed to determine whether transient delivery of PAI-1-R produces long-term suppression of neointima formation; DeYoung et al demonstrated that elevated vascular PAI-1 expression, induced by infusion adenovirus into rat carotid arteries, increased neointima formation after balloon injury.\(^{21}\) The differences between these results and ours may be explained by the fact that recombinant PAI-1-R would be expected to remain in the extracellular compartment, whereas in DeYoung’s study PAI-1 was overexpressed within vascular cells. Intracellular PAI-1 expression inhibits apoptosis and promotes proliferation of VSMCs.\(^{10,11}\) In addition, adenovirus may induce inflammatory mediators of intimal hyperplasia and fibrin formation.\(^{18}\)

In summary, we demonstrate that recombinant PAI-1 inhibits intimal hyperplasia after vascular injury. This effect depends both on the antiproteolytic and VN-binding properties of PAI-1. VN expression is a critical determinant of intimal hyperplasia and the antiproliferative properties of PAI-1. The capacity of recombinant PAI-1 to inhibit intimal hyperplasia has important pathological implications, as increased PAI-1 expression in diseased blood vessels may not necessarily be a cause of intimal hyperplasia observed in vascular disease, as has been proposed.\(^{15}\) Our study also has therapeutic implications and supports examination of the antiproliferative effect of PAI-1-R in other preclinical models, as new strategies are needed to inhibit restenosis without increasing thrombotic risk.
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Disclosures
None.

References
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Methods Supplement

Administration and measurement of PAI-1. PAI-1 (0.2-2.0 mg/kg/day) or an equal volume of saline was administered by twice daily intraperitoneal injection for 14-21 days. First injection was 1 hour prior to vascular injury. For rats, blood was collected into citrate anticoagulant from tail vein and plasma was prepared. “Peak” plasma levels were measured on day 2 and 6 by drawing blood four hours after the first daily PAI-1 injection, while “trough” levels were collected immediately before the first injection on days 4 and 8. For mice, blood was collected 21 days after injury by inferior vena cava venipuncture at the time of euthanasia. Peak and trough plasma levels were measured by euthanizing animals 4 and 16 hours after the last PAI-1 injection, respectively. Human PAI-1 antigen was measured by ELISA (American Diagnostica). PAI-1 activity was measured with the HPAIKT assay (Molecular Innovations).

Histologic and morphometric analyses. Injured arterial segments were perfusion-fixed, excised, and prepared for histologic analysis.\(^1\) Arterial cross-sections were stained with hematoxylin-eosin. Lumen-blood vessel wall interface, internal elastic lamina, and external elastic lamina were traced using a computer software program (Image-Pro Plus, Media Cybernetics). Intima area, media area, and intima-media ratio were calculated. Mean values for each injured vascular segment were calculated from 5 evenly spaced cross-sections. Cell proliferation in arterial segments were studied by anti-BrdU immunostaining.\(^1\)
**Cell culture.** VSMC were isolated from mouse aortas. Detailed methods of assays used to measure proliferation, apoptosis, and migration of VSMC are described in the on-line supplement.

**Cell proliferation assay.** Murine aortic vascular smooth muscle cells (VSMC, $3 \times 10^5$, passage number 3-6) were placed in 96-well plates containing DMEM/F12 with 10% fetal bovine serum (FBS). After overnight incubation medium was removed and replaced with DMEM/F12 containing 0.5% FBS supplemented with recombinant PAI-1 or vehicle control. After 48 hr culture medium was removed and cell number was measured by using the CyQUANT®NF cell Proliferation Assay Kit (Molecular Probes) according to manufacturer’s instructions.

**VSMC apoptosis assay.** Murine aortic VSMC ($2 \times 10^5$, passage number 3-6) were placed in 6-well plates containing DMEM/F12 medium supplemented with 10% FBS. After overnight culture medium was removed and replaced with serum-free DMEM/F12 supplemented with recombinant PAI-1 or vehicle control. After 24-48 hrs trypsin was added and cells were harvested, washed, and resuspended in annexin V-binding buffer (10 mM Hepes/NaOH, 140mM NaCl, 2.5 mM CaCl$_2$, pH 7.4) at a concentration of $10^5$ cells/mL. Apoptosis and necrosis were assessed with annexin V-FITC apoptosis kit (BD Biosciences) and flow cytometry according to manufacturer’s instructions. Annexin V-positive/propidium iodide-negative cells were considered early apoptotic cells and annexin V-positive/propidium-iodide cells were considered necrotic cells.
**VSMC migration assay.** Cell-migration assays were performed using 24-well transwell chemotaxis chambers with 8 µm-size porous membranes (Costar). For some experiments membranes were pre-coated murine multimeric VN by placing a solution of VN (10 µg/mL) in wells for 2 hours at room temperature. Cells (0.5 x 10^6 per ml, passage number 3-6) were seeded in the upper chamber in serum-free DMEM/F12 supplemented with recombinant PAI-1 or vehicle control. DMEM/F12 supplemented with 1% FBS was placed in the lower chamber. After 24 hr incubation at 37°C in a humidified chamber with 5% CO₂ porous membranes were rinsed and cells remaining in the upper chamber were removed with a cotton swab. Membranes were fixed, stained with Giemsa solution, excised from wells, and mounted on glass slides. Migration of cells to the lower-chamber-side of the membrane was quantified by counting the number of cells in five random 200× fields/filter and expressed as the mean number of cells per high-powered field (HPF).

**References**
