Multifaceted Role of Plasminogen Activator Inhibitor-1 in Regulating Early Remodeling of Vein Bypass Grafts

Yan Ji, Tammy L. Strawn, Elizabeth A. Grunz, Meredith J. Stevenson, Alexander W. Lohman, Daniel A. Lawrence, William P. Fay

Objective—The role of plasminogen activator inhibitor-1 (PAI-1) in vein graft (VG) remodeling is undefined. We examined the effect of PAI-1 on VG intimal hyperplasia and tested the hypothesis that PAI-1 regulates VG thrombin activity.

Methods and Results—VGs from wild-type (WT), Pai1−/−, and PAI-1-transgenic mice were implanted into WT, Pai1−/−, or PAI-1-transgenic arteries. VG remodeling was assessed 4 weeks later. Intimal hyperplasia was significantly greater in PAI-1-deficient mice than in WT mice. The proliferative effect of PAI-1 deficiency was retained in vitronectin-deficient mice, suggesting that PAI-1’s antiproteolytic function plays a key role in regulating intimal hyperplasia. Thrombin-induced proliferation of PAI-1-deficient venous smooth muscle cells (SMC) was significantly greater than that of WT SMC, and thrombin activity was significantly higher in PAI-1-deficient VGs than in WT VGs. Increased PAI-1 expression, which has been associated with obstructive VG disease, did not increase intimal hyperplasia.

Conclusion—Decreased PAI-1 expression (1) promotes intimal hyperplasia by pathways that do not require vitronectin and (2) increases thrombin activity in VG. PAI-1 overexpression, although it promotes SMC migration in vitro, did not increase intimal hyperplasia. These results challenge the concept that PAI-1 drives nonthrombotic obstructive disease in VG and suggest that PAI-1’s antiproteolytic function, including its antithrombin activity, inhibits intimal hyperplasia.

Key Words: coronary artery disease ■ vascular muscle ■ plasminogen activator inhibitor-1 ■ thrombin ■ vein graft disease

Internal thoracic arteries and saphenous veins are used to perform coronary artery bypass grafting in patients with advanced coronary artery disease. However, the development of obstructive disease is significantly more common in venous than arterial grafts, with approximately 40% of vein grafts (VGs) occluding within 10 years after coronary artery bypass grafting. The initial pathophysiological process in adverse VG remodeling is intimal hyperplasia. Although some degree of intimal hyperplasia in VGs is an adaptive response to arterial blood pressure and flow, excessive intimal hyperplasia is common and constitutes the substrate for the development of VG atherosclerosis. The molecular and cellular processes that regulate intimal hyperplasia within VGs are poorly understood and likely exhibit significant differences from those that regulate intimal hyperplasia in native arteries. Hence, additional studies are needed to define the factors that regulate intimal hyperplasia in VGs.

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type plasminogen activator and urinary-type plasminogen activator. PAI-1 is present in plasma, platelets, endothelial cells, vascular smooth muscle cells (SMC), and extracellular matrix (ECM). PAI-1 binds to and is stabilized by its cofactor, vitronectin (VN), which is present in plasma and ECM. In addition to regulating fibrinolysis, PAI-1 stimulates migration of vascular SMC by binding to low-density lipoprotein receptor–related protein, present on SMC. However, PAI-1 can also inhibit SMC migration by binding to VN in the ECM, thereby blocking VN binding to integrin and nonintegrin receptors present on SMC. In addition, PAI-1 inhibits thrombin. Given that thrombin stimulates SMC proliferation and is hypothesized to stimulate intimal hyperplasia independently of its prothrombotic effects, it is possible that PAI-1 could regulate intimal hyperplasia by inhibiting thrombin. However, little is known about the roles of PAI-1 in regulating VG intimal hyperplasia and thrombin activity in vivo. Elevated plasma PAI-1 concentration is associated with VG occlusion in humans, and PAI-1 expression is upregulated in obstructed human VGs. However, it is unknown whether PAI-1 actively regulates VG intimal hyperplasia or is simply a biomarker associated with VG disease. Given the potential for PAI-1 to produce both stimulatory and inhibitory effects on cell migration in vitro, it is important to determine the net effects of enhanced and reduced PAI-1 expression on VG...
intimal hyperplasia in vivo. Consequently, the main objective of this study was to determine the impact of primary alterations in PAI-1 expression, both localized and systemic, on the development of VG intimal hyperplasia. A secondary objective was to examine the potential role of PAI-1 as a thrombin inhibitor in vivo in the VG wall. To accomplish these objectives, we studied wild-type (WT), PAI-1-deficient (Pai1−/−), and PAI-1-overexpressing mice in a model of vein bypass grafting.

Materials and Methods

A detailed Methods section describing mouse strains, morphometric and immunohistochemical assessments of VGs, measurement of plasma PAI-1, reverse transcription–polymerase chain reaction analysis of PAI-1 gene expression, isolation and functional assessment of venous SMC, and statistical methods is provided in the Supplemental Data, available online at http://atvb.ahajournals.org.

Vein Grafting Surgery

Surgery was performed as described. In brief, the right common carotid artery of a male mouse was ligated proximally and distally and transected at its midportion. The transected ends were each passed through polyethylene cuffs, everted back over them, and transected at its midportion. The transected ends were each carotid artery of a male mouse was ligated proximally and distally surgical procedures and histological analyses were performed with intact vitronectin (VN) expression (left 2 bars of each graph) and mice with complete VN deficiency (right 2 bars of each graph). DKO indicates double knockout (ie, mice with complete deficiency of PAI-1 and VN). Differences in mean values between all groups were statistically significant (P<0.001). *P≤0.05 vs all other groups (pairwise comparisons).

Results

PAI-1 and VN Deficiency Exert Distinct Effects on VG Remodeling

To examine the role of PAI-1 in VG remodeling, we compared VG intimal hyperplasia in WT and PAI-1-deficient mice. For these experiments, the genotype of the recipient mouse was the same as that of the VG donor. At 4 weeks after VG implantation, mean intimal thickness and intimal area indexed to lumen area were significantly greater in Pai1−/− mice than in WT mice (Figure 1), suggesting that PAI-1 deficiency promotes VG intimal hyperplasia. Enhanced VG neointima formation in PAI-1-deficient mice could result not only from loss of direct effects of PAI-1 on VG remodeling but also from changes in VN function induced by PAI-1 deficiency, given that PAI-1 and VN regulate each other’s functions. To address this issue, we studied VG neointima formation in Vn−/− mice and mice with combined PAI-1 and VN deficiency (double knockout mice), with the genotypes of all VGs being identical to those of recipient mice. Double knockout mice exhibited significantly greater intimal hyperplasia than Vn−/− mice (Figure 1). These results, in conjunction with the data derived from WT and Pai1−/− mice, suggested that PAI-1 deficiency promotes intimal hyperplasia whether or not VN was present—ie, that the effect of PAI-1 deficiency on intimal hyperplasia is not solely mediated indirectly via VN. They also suggested that net effects of deficiency of PAI-1 or VN on VG intimal hyperplasia differ significantly and supported the hypothesis that PAI-1 deficiency promotes the capacity of VN to support intimal hyperplasia, as the proliferative effect of PAI-1 deficiency was significantly less in mice also lacking VN.
Local and Systemic Expression of PAI-1 Regulate Its Concentration in VGs

PAI-1 is present in the VG wall, although the origin of this pool is poorly defined. To examine this issue, we conducted experiments involving various combinations of VG donors and recipients. VG PAI-1 content was assessed by quantitative immunohistochemistry 4 weeks after surgery. Undetectable or extremely low levels of immunostaining, consistent with background signal, were detected in VGs of Pai1/Tg/WT recipients (n=7, Figure 2A), and plasma PAI-1 antigen and activity were undetectable in this group (Table). PAI-1 immunostaining was significantly less in VGs of Pai1/Tg/WT recipients (n=6) than in those of WTgraft/WT recipients (Figure 2B), whereas plasma PAI-1 antigen and activity levels did not differ significantly between these groups. PAI-1 immunostaining was significantly higher in VGs of Pai1/Tg IVC than WT IVC, and real-time reverse transcription–polymerase chain reaction analysis revealed an approximately 4-fold increase of PAI-1 gene expression in Pai1/Tg IVC compared with WT IVC (Supplemental Figure II). These results confirmed that PAI-1 expression was increased in Pai1/Tg veins at the time of initial graft implantation. To assess the impact of increased systemic PAI-1 expression on VG PAI-1 concentration, we compared WTgraft/Pai1/Tg recipients (n=7) with WTgraft/WT recipients controls. The former group exhibited significantly higher VG PAI-1 concentration than the latter (Figure 2B). Plasma PAI-1 antigen and activity concentrations were significantly higher in WTgraft/Pai1/Tg recipients than in WTgraft/WT recipients (Table). Pai1/Tggraft/Pai1/Tg recipients exhibited the highest concentration of PAI-1 in VG, although results did not differ significantly from those of WTgraft/Pai1/Tg recipients. Plasma PAI-1 levels did not differ significantly between Pai1/Tggraft/Pai1/Tg recipients and WTgraft/Pai1/Tg recipients. As a whole, these results suggested that local and systemic PAI-1 expression are both important determinants of VG PAI-1 concentration, whereas plasma PAI-1 concentration, as expected, is not significantly influenced by VG PAI-1 expression.

Localized and Systemic PAI-1 Deficiency Increases VG Intimal Hyperplasia

We examined the effects of localized and systemic decreases in PAI-1 expression on venous remodeling by examining VGs at 4 weeks after surgery. Mean intimal thickness and intimal area indexed to lumen area were significantly greater in Pai1/Tggraft/WT recipients than in WTgraft/WT recipients (Figure 3), suggesting that a decrease in PAI-1 expression localized to the VG increases intimal hyperplasia. Mean intimal thickness and indexed intimal area were signif-

Table. Plasma PAI-1 Concentrations

<table>
<thead>
<tr>
<th>Group</th>
<th>Plasma PAI-1 Antigen, pg/mL</th>
<th>Plasma PAI-1 Activity, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTgraft/WT recipients (n=6)</td>
<td>59±7</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Pai1/Tggraft/WT recipients (n=6)</td>
<td>55±5</td>
<td>Undetectable</td>
</tr>
<tr>
<td>Pai1/Tggraft/Pai1/Tg recipients (n=4)</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>WTgraft/Pai1/Tg recipients (n=6)</td>
<td>69.5±9</td>
<td>Undetectable</td>
</tr>
<tr>
<td>WTgraft/Pai1/Tg recipients (n=6)</td>
<td>630±50*</td>
<td>245±39*</td>
</tr>
<tr>
<td>Pai1/Tggraft/Pai1/Tg recipients (n=6)</td>
<td>542±41†</td>
<td>228±46†</td>
</tr>
</tbody>
</table>

*P<0.005 vs other groups, except Pai1/Tggraft/Pai1/Tg recipients mice.  †P<0.005 vs other groups, except WTgraft/Pai1/Tg recipients mice.
significantly greater in \( Pai^{1-/-}\text{-graft/}Pai^{1-/-}\text{-recipient} \) mice than in \( Pai^{1-/-}\text{-graft/}WT\text{-recipient} \) mice, suggesting that systemic PAI-1 deficiency promotes VG intimal hyperplasia beyond that observed with localized VG PAI-1 deficiency.

Localized and Systemic PAI-1 Overexpression Does Not Increase VG Intimal Hyperplasia

We compared intimal hyperplasia in \( WT^{\text{graft}}/WT^{\text{recipient}} \) mice and \( WT^{\text{graft}}/WT^{\text{recipient}} \) mice to determine the effects of a primary increase in VG PAI-1 expression on early venous remodeling. We also compared \( WT^{\text{graft}}/\text{PAI-1-Tg}^{\text{recipient}} \) mice and \( WT^{\text{graft}}/\text{PAI-1-Tg}^{\text{recipient}} \) mice to assess the impact of increased systemic PAI-1 expression on VG intimal hyperplasia. However, neither local nor systemic PAI-1 overexpression had any significant effect on mean intimal thickness or mean intimal area indexed to lumen area (Figure 3), suggesting that PAI-1 overexpression does not promote VG intimal hyperplasia. Consistent with these data, intimal hyperplasia in mice with combined overexpression of PAI-1 both locally and systemically (ie, \( PAI-1^{-/}\text{-graft/PAI-1^{-/}}\text{-recipient} \) mice) did not differ significantly from that of \( WT^{\text{graft}}/WT^{\text{recipient}} \) mice (Figure 3). We did not analyze intimal hyperplasia of WT or PAI-1-Tg VGs transplanted into PAI-1-deficient mice because of the anticipated immune reaction to VG PAI-1 in PAI-1-deficient mice.

PAI-1 Regulates Cellular Composition of VG Neointima

We analyzed the composition of VG neointima. Quantitative smooth muscle \( \alpha\)-actin immunostaining revealed a significant increase in the percentage of smooth muscle \( \alpha\)-actin–positive area in \( Pai^{1-/-}\text{-graft/Pai^{1-/-}}\text{-recipient} \) VGs compared with \( WT^{\text{graft}}/WT^{\text{recipient}} \) VGs and all other experimental groups (Supplemental Figure III), suggesting that PAI-1 deficiency increased not only neointima area but also the density of SMC in neointima, which is consistent with a report that PAI-1 increases ECM accumulation within developing neointima.\(^\text{13}\) Differences between other groups and \( WT^{\text{graft}}/WT^{\text{recipient}} \) mice did not achieve statistical significance. There was no significant difference in neointima macrophage concentration between experimental groups (data not shown).

PAI-1 Regulates Venous SMC Proliferation and Migration

SMC express PAI-1 and play a central role in intimal hyperplasia.\(^\text{14}\) To identify potential mechanisms underlying our in vivo findings, we isolated WT, PAI-1-deficient, and PAI-1-Tg SMC from mouse vena cava, established cultured lines, and compared their proliferation and migration in vitro. To study proliferation, we cultured SMC with thrombin (1 U/mL) and measured cell proliferation by the 5-bromo-2-deoxyuridine incorporation method. Thrombin-induced proliferation of PAI-1-deficient SMC was significantly greater than that of WT SMC, whereas no significant differences were observed between these groups in the presence of hirudin, a specific thrombin inhibitor (Figure 4A). These results suggested that basal rates of venous SMC proliferation are not affected by PAI-1 deficiency but that deficient expression of PAI-1 by venous SMC enhances thrombin-induced proliferation. However, we found no significant difference between WT and PAI-1-Tg venous SMC in thrombin-induced proliferation (Figure 4A), including in
experiments (not shown) involving thrombin concentrations as low as 0.125 U/mL. Consistent with these in vitro data, we found that the percentage of actively proliferating (ie, proliferating cell nuclear antigen–positive) cells in VG neointima was significantly higher in Pai1<sup>−/−</sup>/graft/Pai1<sup>−/−</sup>/recipient mice than in WT<sup>graft</sup>/WT<sup>recipient</sup> mice but did not differ significantly between Pai1<sup>−/−</sup>/graft/Pai1<sup>−/−</sup>/recipient mice and WT<sup>graft</sup>/WT<sup>recipient</sup> mice (Supplemental Figure IV). To study the effect of SMC PAI-1 expression on migration, we added SMC to the surface of 3-dimensional collagen gels and triggered migration of cells through gels with platelet-derived growth factor-BB. Pai1<sup>−/−</sup> and Pai1<sup>−/−</sup>deficient venous SMC both migrated significantly faster than WT SMC (Figure 4B), results consistent with a previous study of ours involving arterial SMC.15

**PAI-1 Regulates Fibrin/Fibrinogen Deposition in VG**

PAI-1 regulates fibrinolysis, and deposition of fibrin within the vascular wall has been hypothesized to regulate intimal hyperplasia.16 We used quantitative immunohistochemistry to assess fibrin/fibrinogen concentration in VGs. There was no significant difference in fibrin/fibrinogen concentration in the VG wall of Pai1<sup>−/−</sup>/graft/WT<sup>recipient</sup>, WT<sup>graft</sup>/Pai1<sup>−/−</sup>/recipient, and WT<sup>graft</sup>/WT<sup>recipient</sup> mice (Supplemental Figure V). However, fibrin/fibrinogen concentration was significantly elevated in Pai1<sup>−/−</sup>/graft/Pai1<sup>−/−</sup>/recipient mice compared with WT<sup>graft</sup>/WT<sup>recipient</sup> mice (P<0.05). Fibrin/fibrinogen concentration in VGs of Pai1<sup>−/−</sup>/graft/WT<sup>recipient</sup>, Pai1<sup>−/−</sup>/graft/Pai1<sup>−/−</sup>/recipient, and WT<sup>graft</sup>/WT<sup>recipient</sup> mice did not differ significantly, although there was a statistically insignificant trend toward lower fibrin/fibrinogen concentration in the former 2 groups. Overall, these data supported the hypothesis that PAI-1 regulates fibrin deposition in the VG wall but did not suggest that enhanced fibrin deposition necessarily promotes intimal hyperplasia.

**PAI-1 Regulates Thrombin Activity in the VG Wall**

It is hypothesized that thrombin signaling promotes intimal hyperplasia.6,9 To test the hypothesis that PAI-1 functions in vivo to inhibit thrombin in VGs, we grafted WT vein segments into WT mice and Pai1<sup>−/−</sup>deficient vein segments into Pai1<sup>−/−</sup>deficient mice. After 5 days, VGs were harvested and homogenized, and soluble extracts were prepared. Microscopic examination of longitudinally incised VGs before homogenization revealed no evidence of thrombus. Thrombin activity was significantly greater in VG extracts of Pai1<sup>−/−</sup>/graft/Pai1<sup>−/−</sup>/recipient mice than in those of WT<sup>graft</sup>/WT<sup>recipient</sup> mice (Figure 5A), suggesting that PAI-1 functions as a physiological thrombin inhibitor in vivo within the VG wall. To examine the role of PAI-1 produced locally in VGs in thrombin inhibition, we performed an experiment in which WT mice received Pai1<sup>−/−</sup>deficient or WT VGs and thrombin activity in VGs was compared 5 days later. Thrombin activity in VG extracts was significantly higher in Pai1<sup>−/−</sup>/graft/WT<sup>recipient</sup> mice than in WT<sup>graft</sup>/WT<sup>recipient</sup> mice (Figure 5B), suggesting that local expression of PAI-1 regulates thrombin activity in VGs.

**Discussion**

Several studies have examined fundamental processes by which PAI-1 regulates intimal hyperplasia, including via effects on cell proliferation, migration, and apoptosis.17 These studies have included analyses of the molecular signaling events underlying the effects of PAI-1 on vascular cell function, including cellular receptors, intracellular signaling pathways, and regulation of ECM proteolysis. Together, they have shown that PAI-1 regulates multiple pathways and exerts effects predicted to both increase and decrease intimal hyperplasia. However, no previous single study has examined the in vivo effects of both primary increases and decreases in vascular wall PAI-1 gene expression on intimal hyperplasia, which is essential to understanding the net effect of abnormal PAI-1 expression on vascular remodeling. Although previous studies examined the role of PAI-1 in regulating intimal hyperplasia in arteries,9,13,18–23 we focused this study on the role of PAI-1 in VG remodeling, as PAI-1 accumulates in diseased VG,11 but it has previously been unknown whether PAI-1 actively regulates VG remodeling or is simply a passive marker of VG disease. The main findings of our study are that (1) PAI-1 regulates VG remodeling, with decreased PAI-1 expression leading to increased intimal hyperplasia; (2) PAI-1’s effects are mediated not only by the plasma pool but also by a local VG pool that is not discernable by measurement of plasma PAI-1; (3) a primary increase in PAI-1 expression locally in VG or systemically does not increase VG intimal hyperplasia; (4) PAI-1 regulates the function of VG SMC; and (5) PAI-1 regulates thrombin activity in VGs.

Our data suggest that PAI-1 functions in vivo to regulate the cell adhesion function of VN in VGs. VN is synthesized by vascular SMC and other cell types and secreted into the ECM of the vascular wall, where it binds collagen and supports cell migration.24–26 PAI-1 competitively blocks binding of integrins (eg, α<sub>v</sub>β<sub>3</sub>) and urinary-type plasminogen activator receptor to VN.5 Therefore, decreased PAI-1 ex-
pression could promote VN’s interactions with its receptors and increase cell migration. In this study, we showed that PAI-1-deficient venous SMC migrate more rapidly through 3-dimensional collagen matrices than WT venous SMC do. We also showed that the proliferative effect of PAI-1 deficiency is blunted in mice also lacking VN, suggesting that this mechanism (ie, PAI-1 deficiency promoting VN’s cell adhesive function) is active in vivo in VGs. In a recent study, we showed that VN-deficient arterial SMC migrate faster through 3-dimensional collagen matrix than WT-SMC, and we provided data to suggest that the effect was mediated by an increase in the pool of free, motogenic PAI-1. On the basis of this finding, one might hypothesize that VN deficiency would enhance intimal hyperplasia, which was observed in a carotid artery ligation model. However, we observed reduced VG intimal hyperplasia in VN-deficient mice, suggesting that during early venous remodeling after bypass grafting, VN supports intimal hyperplasia, most likely by increasing integrin-dependent cell migration.

Overexpression of PAI-1 by vascular SMC, as occurs in diabetes mellitus and other diseases, promotes cell proliferation and migration and inhibits apoptosis in vitro. We found that overexpression of PAI-1 by venous SMC promoted their migration through 3-dimensional collagen but did not increase SMC proliferation. However, we did not find that a primary increase of PAI-1 expression, either in VG or systemically, increased VG intimal hyperplasia. These results suggest that although PAI-1 can promote cell migration and proliferation in vitro, the net effect of PAI-1 overexpression on intimal hyperplasia in vivo during the early phase after vein grafting is neutral, perhaps because the proliferative and promigratory effects of PAI-1 are counterbalanced by its antimigratory and antithrombin effects. It is important to realize that our results, observed at a single time point in a murine system, do not disprove the idea that PAI-1 could promote VG intimal hyperplasia at other time points, in other preclinical models, or in human VGs.

An important aspect of our study was the in vivo analysis of the role of PAI-1 as a thrombin inhibitor. Although PAI-1 is known to bind and inhibit thrombin in purified, in vitro systems, little is known about the significance of this molecular interaction in vivo. Active thrombin can be recovered from the VG wall. We have shown that VG thrombin activity is significantly greater in PAI-1-deficient mice, arguing that PAI-1 functions in vivo to regulate vascular wall thrombin activity in VGs. We also showed that PAI-1 produced locally within VGs regulates thrombin and that PAI-1 produced by SMC regulates their proliferative response to thrombin stimulation. Based on our data, we hypothesize that PAI-1 deficiency promotes VG intimal hyperplasia by effects on thrombin. This hypothesis is supported by reports that thrombin stimulates SMC proliferation and by studies suggesting that sustained thrombin signaling after vascular injury promotes intimal hyperplasia independently of effects on thrombosis. Although we found that PAI-1-deficient SMC exhibited hyperresponsiveness to thrombin in vitro, we did not observe lessened thrombin responsiveness in PAI-1-Tg SMC, as might be expected. Although our data do not explain the mechanism underlying this observation, we hypothesize that the antithrombin effects of PAI-1 are counterbalanced by the proliferative effects of PAI-1 overexpression. In future studies, it will be of interest to examine thrombin activity in VN-deficient VGs to examine the in vivo function of VN as a cofactor for inhibition of vascular wall thrombin by PAI-1. In addition to possible effects on thrombin, it is possible that upregulation of plasmin activity could account for the increased intimal hyperplasia observed in PAI-1-deficient mice, as plasmin has been hypothesized to promote cell migration by degrading ECM. However, plasminogen deficiency does not significantly affect VG neointima formation in mice, arguing against plasmin as the downstream mediator of PAI-1 deficiency on VG intimal hyperplasia.

PAI-1 regulates vascular fibrin accumulation, which has been proposed as a potential mechanism by which PAI-1 regulates intimal hyperplasia. We found increased VG fibrin deposition in PAI-1-Tg graft/PAI-1-Tg recipient mice and a nonsignificant trend toward reduced fibrin deposition in PAI-1-deficient VGs. These results suggested that PAI-1 regulates fibrin deposition in the VG wall. However, we did not observe a correlation between VG fibrin accumulation and intimal hyperplasia in PAI-1-overexpressing mice, suggesting that PAI-1-driven fibrin deposition does not itself promote intimal hyperplasia. We hypothesize that the lack of a proliferative effect of enhanced fibrin deposition may have been due to the potential antimigratory effects of enhanced PAI-1 expression.

Our study has some limitations. Although we used a published method to study intimal hyperplasia in VG, we observed less intimal hyperplasia at 4 weeks after VG implantation than reported previously. The variation between our study and that of Zou et al may be due to minor differences in surgical technique, differences in the location of cross-sections used to assess intimal hyperplasia, and environmental and dietary differences between studies. Our studies involving implantation of genotype-mismatched VGs into recipient mice provided insights into the function of PAI-1 produced locally within VGs during early venous remodeling. However, some of the PAI-1 in VGs observed in our study could be of systemic origin (ie, from plasma or cells that invade VGs from blood or adjacent artery), which complicates our functional assessment of VG PAI-1. Nevertheless, our analyses of VG and plasma PAI-1 suggest that cells present in VGs at the time of graft insertion (or subsequently derived from them) are the major source of PAI-1 within VG neointima. These results are consistent with published studies that found that the majority of cells present in VGs are derived from the transplanted graft.

In summary, we have shown that PAI-1 regulates intimal hyperplasia in a clinically relevant model. Our experiments involving PAI-1-deficient mice and SMC suggest mechanisms by which downregulation of PAI-1 could promote intimal hyperplasia (ie, by enhancing VN-dependent SMC migration and thrombin-induced SMC proliferation). These findings are relevant to ongoing development of PAI-1 inhibitors to treat vascular disease—ie, pharmacological PAI-1 inhibition could potentially upregulate cell migration and VG wall thrombin activity. Our experiments involving...
PAI-1-Tg mice suggest that PAI-1 overexpression does not necessarily promote intimal hyperplasia. This finding is significant, given that previous clinical studies, based on associations of PAI-1 concentration and VG disease, had hypothesized that increased PAI-1 expression promotes intimal hyperplasia.10,11 Additional studies involving large animal models of bypass grafting and chemical inhibitors of PAI-1 are warranted to determine the effects of pharmacological targeting of PAI-1 on venous remodeling under clinically relevant conditions.

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Disclosures

None.

References

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Supplement Material

Supplemental Methods, Figures, and Figure Legends

Detailed Methods

**Mice.** C57BL/6J mice were purchased from Jackson Laboratories. *Pai1*−/− mice and *Vn*−/− mice (backcrossed >20 generations into the C57BL/6J genetic background) were described previously.1,2 C57BL/6J-congenic PAI-1-transgenic (Tg) mice that over-express murine PAI-1 under the control of the CMV promoter were from Dr. David Ginsburg, University of Michigan.3 To determine transgene copy number, we performed real-time, quantitative PCR analyses of genomic DNA isolated from PAI-1-Tg and wild-type mice, using oligonucleotide primers (5'-GTTCTGGTCTCTGGAAAGG-3' and 5'-GCTGAAACTTTTTACTCCGAAG-3') located within exon 2 of the murine PAI-1 gene, CYBR Green I dye (BIO-RAD), and the comparative cycle threshold (2−[delta][delta]Ct) method.4 Our data (not shown) suggested that the PAI-1 transgene copy number of the line of PAI-1-Tg mice used in our experiments was two. To assess for the possibility that integration of the transgene disrupted function of an endogenous gene that regulates intimal hyperplasia, we compared VG intimal hyperplasia in mice homozygous (n=5) vs. heterozygous (n=7) for the PAI-1 transgene (transgene zygosity was determined by quantitative real-time PCR).5 We observed no significant differences between transgene-homozygous and transgene-heterozygous mice (data not shown), suggesting that transgene integration did not disrupt function of an endogenous gene.
that regulates VG intimal hyperplasia. All animal care and experimental procedures were approved by the University of Missouri Animal Care and Use Committee. Mice received normal chow (5008/LabDiet) before and after surgery. All surgical procedures and histological analyses were performed with the investigator blinded to mouse genotype.

Morphometric analyses. Four weeks after surgery the VG was surgically exposed. The vasculature was perfused with phosphate-buffered saline (PBS) and 4% phosphate-buffered paraformaldehyde for 2 and 5 minutes, respectively, which were injected into the left ventricle and exited the circulation through a laceration created in the liver. The VG was embedded in paraffin. Three evenly spaced cross-sections (5 µm thick, 70 µm between sections) from its mid-portion were prepared, mounted, and stained with hematoxylin and eosin. Microscopic images were imported into Image-Pro Plus (Media Cybernetics) and lumen-vascular wall and tunica media-adventitia interfaces were traced. Intima area was defined as the difference between the two traced cross-sectional areas.\(^6\) Lumen area (A) was calculated from the length of the lumen-vascular wall interface (p), using the formula \(A = \frac{p^2}{4\pi}\). Mean intimal area/lumen area was calculated for each VG. To measure intima thickness, the thinnest portion of the intima was designated the 12 o’clock position. Intima thickness was measured at 1:30, 3, 4:30, 6, 7:30, 9, 10:30, and 12 o’clock. Mean intima thickness was calculated for each cross-section and VG.
**Immunohistochemistry.** Cross-sections obtained from the mid-portions of VGs were mounted on slides, de-paraffinized, hydrated, incubated 10 minutes in 3% H$_2$O$_2$ in methanol, and rinsed with PBS. Antigens of interest were detected using the Histomouse-MAX Kit (Invitrogen) and appropriate primary and secondary antibodies. Negative control reactions lacking primary antibody confirmed the specificity of each secondary antibody. Identical, simultaneously performed immunostaining techniques (i.e. antibody dilutions, incubation and wash times) were used for all samples. PAI-1 immunostaining was performed using rabbit anti-mouse PAI-1 polyclonal antibody (Santa Cruz Biotechnology) and peroxidase-conjugated anti-rabbit-IgG. Control Western blotting experiments performed with anti-PAI-1 antibody confirmed its capacity to detect purified murine PAI-1 in concentrations as low as 10 ng/gel lane, very low non-specific immunoreactivity with antigens present in detergent extracts prepared from murine inferior vena cava (IVC), and the lack of any apparent differences in non-specific immunoreactivity between wild-type, PAI-1-deficient, and PAI-1-trangsenic IVC (data not shown). To quantify PAI-1 immunostaining, images of cross-sections were captured under identical imaging conditions and imported into Image-Pro Plus software (MediaCybernetics). Four regions of interest (ROI) were drawn at the 12, 3, 6, and 9 o’clock positions within the neointima of each VG. The amount of positive immunostaining within each ROI (expressed as positive pixels/mm$^2$) was determined by false color segmentation analysis, using PAI-1-positive threshold settings determined from a WT$^{\text{graft/WT-recipient}}$ sample. Mean values of the ROIs were calculated for each VG. Mean immunostaining intensity observed in PAI-1-deficient samples (i.e. $Pai1^{\text{-/graft}}/Pai1^{\text{-/recipient}}$ mice, n=3) was defined as background immunostaining, and this value was
subtracted from mean observed immunostaining intensity in each VG to calculate PAI-1 immunostaining. Specificity of anti-PAI-1 antibody was demonstrated by the observed lack of significant staining in PAI-1-deficient VG, and the marked inhibition of immunostaining of PAI-1-expressing VG after preincubation of antibody with recombinant murine PAI-1 (Molecular Innovations, Supplemental Fig. IA).

Fibrinogen/fibrin immunostaining was performed using goat anti-mouse fibrinogen polyclonal primary antibody (Nordic Immunology) and Texas-Red-conjugated rabbit-anti-goat-IgG antibody (Vector). Specificity of anti-fibrinogen/fibrin antibody was demonstrated by the observed lack of immunostaining in liver tissue of fibrinogen-deficient mice (provided by J. Degen, PhD, Children’s Hospital Research Foundation, University of Cincinnati, Supplemental Fig. IB). Immunostaining of macrophages was performed with rat anti-Mac-3 IgG (BD Pharmingen) and Texas-Red-conjugated goat-anti-rat IgG antibody (Vector). Smooth muscle alpha-actin (SMAA) and proliferating cell nuclear antigen (PCNA) were detected with mouse-anti-human-SMAA and mouse-anti-rat-PCNA monoclonal antibodies (Santa Cruz Biotechnology) and peroxidase-conjugated anti-mouse-IgG (Invitrogen). Quantification of immunostaining of specific cell types and fibrinogen was performed as described.8

**Measurement of plasma PAI-1.** Blood was collected into citrate anticoagulant by cardiac puncture. Platelet-poor plasma was prepared by centrifugation. Plasma PAI-1 concentration was determined by a conventional sandwich assay with a Luminex 100 System (Luminex Austin, TX). Murine anti-rodent PAI-1 monoclonal antibody H34G6 (Molecular Innovations, Novi, MI) linked to carboxylated microspheres was used to
capture total PAI- antigen. Human urokinase (Molecular Innovations) linked to carboxylated microspheres was used to capture active PAI-1. To detect microsphere-bound active and total PAI-1 antigen, biotinylated rabbit anti-mouse PAI-1 (Molecular Innovations) was used followed by streptavidin-R-phycoerythrin (S-866, Molecular Probes). The concentrations of active and total PAI-1 in each sample were calculated against standard curves for active and total murine PAI-1 generated from purified active murine PAI-1 (MPAI, Molecular Innovations). Lower limits of PAI-1 detection were 3.2 pg/mL and 16 pg/mL for the antigen and activity assays, respectively. Values less than these are reported as undetectable.

Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis of PAI-1 gene expression. Veins were retrieved from adult mice immediately after euthanasia and rinsed extensively. Total cellular RNA was extracted using the Recover All Total Nucleic Acid Isolation Kit (Ambion). Reverse-transcriptase real-time polymerase chain reaction (RT-PCR) was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), oligonucleotide primers specific for PAI-1 cDNA (5’-GCTGCAGATGACCACAGCGGG-3’ and 5’-CCGCAGTACTGATCTCATC-3’), and a Bio-Rad iQ5 real-time PCR detection system. Beta actin gene expression was simultaneously assessed in all samples as an internal control. Relative PAI-1 gene expression was determined by the \(2^{-\Delta\Delta CT} \) method.\(^9\)

Isolation and functional assessment of venous SMC. Lines of cultured venous SMC, each originating from the IVC of a single mouse, were established by digesting veins
with collagenase (Worthington) after carefully removing loose adventitia, as described.\textsuperscript{10} At least 3 independent lines of cells of each genotype were studied in each set of experiments. Venous SMC were grown in culture under standard conditions in DMEM/F12 medium supplemented with 20% fetal bovine serum (FBS). Cells were passaged a maximum of 7 times before being used in experiments. To study thrombin-induced proliferation, venous SMC ($1.0 \times 10^4$) were seeded in 96-well plates in DMEM/F12 containing 20% FBS, glucose, Fungizone (Sigma), and gentamycin. After 3 days of standard cell culture conditions cells were washed and incubated 3 days in serum-free DMEM/F12 containing insulin-transferrin-selenium-A supplement (GibcoBRL). Alpha-thrombin (Enzyme Research Laboratories, 1 U/mL) or vehicle control was added to cells. Two days later BrdU (10 µM) was added. Eighteen hours later rates of cell proliferation were measured using the Cell Proliferation ELISA, BrdU (colorimetric) Kit (Roche), according to manufacturer’s instructions. Results are reported as % of vehicle-control-treated cells. To study SMC migration, 3-dimensional rat type 1 collagen gels were prepared as described previously.\textsuperscript{11} Polymerizing collagen mixture (30 µL) was pipetted into the upper chambers of 24-well Transwell inserts (Corning) whose bottoms consisted of porous (8 µm pore diameter) membranes. After gels polymerized, DMEM medium (200 µL) containing 0.2% FBS and SMC ($1.0 \times 10^5$) were added to the upper chamber. Inserts were placed into lower chamber wells filled with DMEM (600 µL) containing 2.5% FBS and PDGF-BB (Upstate, 20 ng/mL), which stimulates SMC migration through the collagen gel. After 72 hr of standard cell culture conditions, inserts were removed, collagen gels were scraped away, and membranes were stained with Diff-Quick (Siemens Healthcare Diagnostics). The lower-chamber
side of the membrane, to which cells that migrated through the collagen gel and pores adhere, was visualized en-face with a microscope and cells were counted.

**Statistical Analyses.** Results are expressed as mean ± one standard error of the mean. One way analysis of variance with multiple comparison procedures (Holm-Sidak method) and the Students t test were used, as appropriate, to compare differences between groups. A probability (P) value of <0.05 was considered statistically significant.
Supplemental Figure I. Characterization of specificity of antibodies. (A) Anti-PAI-1 immunostaining. Consecutive cross-sections (5 µm thickness) of a PAI-1-transgenic vein graft were immunostained with anti-PAI-1 antibody as described in Methods. For
one sample (left panel) the anti-PAI-1 antibody was pre-incubated with bovine serum albumin (10 µg/mL) prior to immunostaining. For the other sample (right panel) the anti-PAI-1 antibody was pre-incubated with recombinant murine PAI-1 (10 µg/mL, Molecular Innovations) prior to immunostaining. Note depletion of immunostaining by exposure of anti-PAI-1 antibody to soluble PAI-1. Distance bar denotes 50 µm. (B) Anti-fibrin/fibrinogen immunostaining. Liver tissue from mice exposed to carbon tetrachloride (which induces centrilobular necrosis and fibrin deposition) was generously provided by J. Degen, PhD, Children’s Hospital Research Foundation, University of Cincinnati.\textsuperscript{12,13} Samples were immunostained with anti-fibrin antibody (upper row) or 4',6-diamidino-2-phenylindole (DAPI, a nuclear stain; lower row). Left column is liver from a fibrinogen-expressing (Aα\textsuperscript{+/-}) mouse. Right column is liver from a fibrinogen-null (Aα\textsuperscript{-/-}) mouse. Distance bar denotes 200 µm.
Supplemental Figure II. Analysis of PAI-1 expression in inferior vena cava (IVC) of wild-type (WT) and transgenic (PAI-1-Tg) adult mice. (A) Representative images and quantitative analysis of PAI-1 immunostaining (n=3 mice/group). *P<0.05. Distance bar represents 50 µm. (B) Real-time RT-PCR analysis of total cellular RNA isolated from IVC. AU, arbitrary units. *P<0.001. Control reactions performed with veins from Pai1−/− mice revealed no detectable PAI-1 gene expression (data not shown).
Supplemental Fig. III. Smooth muscle cell density in vein graft neointima, assessed by quantitative analysis of smooth-muscle-alpha-actin-positive pixels/mm². All groups consisted of 5 vein grafts. *P<0.05 vs. all other groups.
Supplemental Fig. IV. Rates of cell proliferation, assessed by anti-PCNA staining.

*P<0.05 vs. other groups.
Supplemental Fig. V. Fibrin/fibrinogen immunostaining in vein graft neointima is increased in PAI-1-Tg\textsuperscript{graft}/PAI-1-Tg\textsuperscript{recipient} mice. *P<0.05 vs. Pai1\textsuperscript{−/−-graft}/Pai1\textsuperscript{−/−-recipient}, Pai1\textsuperscript{−/−-graft}/WT\textsuperscript{recipient}, and WT\textsuperscript{graft}/WT\textsuperscript{recipient} mice.
References for Supplemental Methods and Figure Legends


