Plasminogen Activator Inhibitor 1 and Vitronectin Protect Against Stenosis in a Murine Carotid Artery Ligation Model

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Objective—We previously reported that plasminogen activator inhibitor 1 (PAI-1), in the presence of vitronectin (VN), inhibits thrombin activity in vitro. Furthermore, we demonstrated in human atherosclerotic plaques the colocalization of thrombin, PAI-1, and VN, as well as activity of thrombin and PAI-1. Here, we show that PAI-1 is a local thrombin inhibitor in vivo.

Methods and Results—We used the murine carotid artery ligation model to assess the role of PAI-1 and VN in stenosis by using PAI-1–deficient (PAI-1−/−) and VN−/− mice. Ligation resulted in a smooth muscle cell (SMC)-rich intima without infiltrating cells. We show that PAI-1−/− and VN−/− mice generate a larger intima than wild-type mice as the result of more extensive SMC proliferation, as evidenced by cell counting and staining for proliferating cell-nuclear antigen.

Conclusions—In PAI-1−/− mice, excessive intima formation is prevented by the thrombin-specific inhibitor hirudin. Finally, immunohistochemical analysis revealed PAI-1, VN, and (pro)thrombin antigen in intimal lesions. Our observations are compatible with inhibition of thrombin-mediated SMC proliferation by PAI-1/VN complexes.

Key Words: restenosis ■ smooth muscle cells ■ plasminogen activator inhibitor 1 ■ vitronectin ■ thrombin

Plasminogen activator inhibitor 1 (PAI-1) is a multifunctional protein that is best known for its regulation of plasminogen activation.1 It rapidly forms inactive complexes with the serine proteases tissue-type and urokinase-type (uPA) plasminogen activator, thereby preventing the conversion of plasminogen into plasmin. Furthermore, PAI-1 binds to vitronectin (VN), an adhesive, extracellular matrix protein. It has been shown that the binding of PAI-1 to VN affects the migration of smooth muscle cells (SMCs) by preventing the binding of VN to the αvβ3 integrin.2

Delineating the role of PAI-1 in atherogenesis and restenosis is particularly cumbersome, partially as a result of the different design of experimental models that often accentuate a single feature of the human pathology. For instance, comparison of mechanical and electrical injury-induced intima formation in PAI-1–deficient (−/−) and wild-type mice revealed that PAI-1 blocks intimal thickening by inhibiting the migration of SMCs.3 Moreover, PAI-1–overexpressing SMCs, seeded on a denuded carotid artery, inhibited intima formation as the result of decreased SMC migration.4 In contrast, by using a copper- or ferrichloride-induced oxidative vascular injury model, PAI-1 deficiency attenuated lesion formation.5–7 Contradictory observations have been noted in high-fat, macrophage-rich/SMC-poor models (apoE−/− and/or LDLR−/− mice), revealing that PAI-1 deficiency may reduce, enhance, or not influence lesion formation, possibly depending on strain differences.8–10 In addition, in a balloon-injured rat carotid artery model, PAI-1 overexpression resulted in increased SMC proliferation that was associated with fibrin accumulation.11 In the murine carotid artery ligation model, formation of a fibrin matrix apparently accounted for increased intima formation in uPA−/− mice, whereas PAI-1−/− mice responded like wild-type mice.12 However, in similar study that used the carotid artery ligation model, decreased intima formation was observed in VN−/− and PAI-1−/− mice.7 A satisfactory explanation for these discrepancies is presently not available, but it is possible that the different models and/or methodologies accentuate the various, distinct functions of PAI-1.

Here, we studied the role of PAI-1 as a local thrombin inhibitor in SMC proliferation by using the carotid artery ligation model, which mimics restenosis after angioplasty. In this model, the endothelium remains intact, and lesions virtually lack infiltrating cells.13 It is assumed that reduced shear stress, hypoxia, and arterial wall pressure are determinants for intima formation as a result of SMC proliferation.14 A possible function for PAI-1 in controlling SMC proliferation is suggested by the following observations and explored in this study. First, PAI-1 inhibits thrombin in vitro, provided either VN or heparin is present as a cofactor.15–17 Second, thrombin is a potent mitogen for cultured SMCs.18,19 Accordingly, the thrombin-specific inhibitor hirudin significantly reduces intima formation in various animal models.20–22 Third, the presence of active thrombin, as well as active
PAI-1, was reported in human atherosclerotic lesions.²⁻²⁴ Moreover, the latter studies revealed colocalization of thrombin, PAI-1, and VN, supporting the potential significance of PAI-1/VN complexes in regulating the mitogenic activity of thrombin.²³ Importantly, the (atherosclerotic) vessel wall is virtually devoid of the traditional plasma thrombin inhibitors,²⁵ whereas PAI-1 is synthesized by vascular cells and abundantly present in lesions.²⁶²⁷ Similarly, large amounts of prothrombin and VN have been encountered in the human vessel wall.²⁵²⁸ In addition, components of the intrinsic and extrinsic coagulation pathway are encountered in diseased vessels, suggesting that prothrombin can be activated.²⁵ Hence, the requirements are met for controlling thrombin activity by PAI-1/VN complexes in the vessel wall.

Here, we used the carotid artery ligation model in PAI-1⁻/⁻, VN⁻⁻, and wild-type mice to explore the effect of PAI-1 and VN on intima formation.³ We report that increased intima formation is observed in PAI-1⁻/⁻ and VN⁻⁻ mice as compared with wild-type mice. Excessive intima formation in PAI-1⁻/⁻ mice is inhibited by the thrombin-specific inhibitor hirudin. These results provide support for a function of PAI-1/VN complexes in thrombin-mediated SMC proliferation.

Methods

Mice and Experimental Protocol

Breeding of PAI-1⁻/⁻ mice and backcrossing with wild-type C57Bl6/J has been described.²⁹ VN⁻⁻ mice were kindly provided by Randal Westrick and Dr. David Ginsburg (University of Michigan, Ann Arbor,).³⁰ VN⁻⁻ mice had been backcrossed for at least 8 generations with C57Bl6/J (Charles River Laboratories Inc., Iffa-Credo, l’Arbresle, France). All mice were maintained on a standard chow diet. The mice used were 2 to 4 months old and weighed 20 to 30 g. Ligation of the left carotid artery, near the distal bifurcation, was performed as described previously.³¹ The mice were sacrificed either at 2.5 or 4 weeks after ligation of the carotid artery. Four to five mice were used for each time point. In a subsequent carotid artery ligation experiment, only wild-type and PAI-1⁻/⁻ mice were used, which were then subjected to daily subcutaneous injections with placebo (50 mg/mL D-mannitol; Merck) or polyethylene glycol (PEG)-hirudin (1 mg/kg body weight; kindly provided by Dr. Klaus Rübsamen and Dr. Wilfried Hornberger, Knoll AG, Ludwigshafen, Germany) in 50 mg/mL D-mannitol. Seven to eight mice were used in this experiment. All mice were operated and sacrificed under ketamine/xylazine anesthesia and subsequently fixed by perfusion with 4% (v/v) paraformaldehyde in PBS. The carotid arteries were excised, immersion fixed in 70% (v/v) ethanol, and embedded in paraffin. The nonligated, right carotid artery served as internal control and was embedded together with the ligated vessel. Serial sections of 5 μm were cut for morphometric analysis. After sectioning and mounting on Superfrost Plus slides (Menzel-Gläser), the specimens were subjected to hematoxylin-eosin staining. Animal care and experimental procedures were approved by the local Ethical Committee on Animal Experiments.

Morphometry

The ligated carotid artery was sectioned from the ligature toward the aortic arch. A standardized reference point was set at which location the ligature did not distort the vessel and where elastic laminae were intact. The reference point is situated between 0.05 mm and 0.13 mm from the ligation. Cross sections at 0.2, 0.3, and 0.4 mm from the reference point were morphometrically analyzed by using the QWin software (Leica Microsystems) on digital images of the vessel, obtained with a Sony DMC-950 3CCD color videocamera. The circumferences of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL) were measured by tracing along the luminal surface, IEL, and EEL. The medial area (M) was calculated by subtracting the area within the IEL from the area defined by the EEL. Similarly, the intimal area (I) was calculated by subtracting the luminal area from the IEL area. The circumference of the control nonligated carotid artery is independent of the ligation time and identical between deficient and wild-type mice. To establish vascular remodeling, the total vessel area of the ligated carotid arteries (EEL of the left carotid) is expressed as percentage of the total vessel area of the nonligated artery (EEL of the right carotid).

Immunohistochemistry

Paraffin sections (5 μm) were deparaffinized, rehydrated, and incubated with 0.3% hydrogen peroxide and blocked with 10% (v/v) preimmune goat serum (DAKO). Subsequently, sections were incubated overnight at 4°C with primary antibodies, followed by incubation with biotinylated secondary rat antimurine light-chain anti-serum or goat antirabbit antisera, which were detected with streptavidin-horseradish peroxidase conjugates (DAKO) and, subsequently, with amino-ethylcarbazole and hydrogen peroxide. After counterstaining with hematoxylin, sections were embedded in glycergel (Sigma). Proliferating cell-nuclear antigen (PCNA) staining was performed by using an anti-PCNA monoclonal antibody (DAKO) with 0.1% (v/v) Triton X-100. Antigen retrieval was performed by boiling three times for 5 minutes at 700 Watt in a microwave in citrate buffer (pH 6). A polyclonal anti-PAI-1 antisem was kindly provided by Dr. D.J. Loskutoff (The Scripps Research Institute, La Jolla, Calif) and used as in the PCNA-staining protocol. A polyclonal antiserum against murine thrombin was kindly provided by Dr. J. Timmerman (this institute) and affinity-purified on a thrombin-containing column.

Thrombin Activity and Hirudin Assays

Thrombin-mediated coagulation was determined with the Pathromtin SL kit to measure the activated partial thromboplastin time (APTT) of citrated plasma samples from wild-type and PAI-1⁻/⁻ mice by using the BCS Coagulation System (Dade Behring). Plasma was incubated with phospholipids and calcium to trigger coagulation, and time to clot formation was recorded. The activity of PEG-hirudin was measured by using a hirudin-activity assay in the BCS Coagulation System. Hirudin inactivates a fixed amount of added thrombin: Residual thrombin activity is determined by continuously measuring the increase in absorbance (405 nm) caused by conversion of the chromogenic substrate tosylglycyl-l-prolyl-l-arginyl-5-amino-2-nitrobenzoic acid isopropylamide. On the final experimental day, the mice received a last placebo or PEG-hirudin injection. Blood collection and subsequent sacrifice of the mice started at least 1 hour after the above-mentioned injections.

Statistical Analysis

All statistical analyses were performed with the SPSS version 10.0.5 software. Experimental values are expressed as mean±standard deviation. The significance of differences in intima formation between the PAI-1⁻/⁻, VN⁻⁻, and the corresponding wild-type groups was determined by using a nonparametric test (Mann–Whitney U 2-tailed test with a significance of P<0.05).

Results

Vascular Remodeling in Ligated Carotid Arteries of VN⁻⁻, PAI-1⁻/⁻, and Wild-Type Mice

The total vessel area was measured in ligated carotids at set distances from the reference point (Figure 1A). By comparing the vessel area of nonligated with the ligated carotid arteries of wild-type mice (Figure 1B and 1C), we confirmed that ligation results in narrowing of arteries (constrictive remodeling).³¹ After 1 week of ligation, equal constrictive remodeling to approximately 50% of its original size was observed in the ligated carotids of wild-type, VN⁻⁻, and PAI-1⁻/⁻ mice.
carotid) reveals less constrictive remodeling in the VN
alized. Total vessel area (percentage of unligated control
1D) and PAI-1
constrictive remodeling was observed in both VN
(data not shown). Yet, after 2.5 and 4 weeks of ligation, less
constrictive remodeling was observed in both VN
mice (Figure
IC through 1F). Consequently, the VN
and PAI-1
mice display a substantially larger intima than wild-type mice.

Intima of Ligated Carotid Arteries of VN
, PAI-1
, and Wild-Type Mice
We measured the length of the lesion by counting the number of
sections containing intimal SMCs, and determined the intima/
media (I/M) ratio for the deficient and wild-type mice. Clearly,
the lesions of both VN
(Figure 2A) and PAI-1
mice (Figure 2B) were substantially longer than those of wild-type
mice. At 2.5 and 4 weeks after ligation, the intimal lesion in
mice was approximately 1.5-fold longer than that of the
corresponding wild-type mice. PAI-1
mice displayed a 2-fold
longer lesion than their wild-type counterparts.

The I/M ratios were calculated on morphometric analysis of
sections of ligated carotid arteries that were taken at a fixed
distance (0.3 mm) from the reference point. For VN
mice (C), PAI-1
mice (D), and their wild-type counterparts.

To determine whether larger intimal lesions are caused by SMC
proliferation, we counted the nuclei present in sections that were
eosin–stained sections: bright-field microscopy, original magnifi-
cation ×200. ○ indicates VN
; ●, WT; ⌉, PAI-1
; ▽, WT.

SMC Proliferation
To determine whether larger intimal lesions are caused by SMC
proliferation, we counted the nuclei present in sections that were
also used for morphometric measurements. For VN
mice, the
cell number after 2.5 weeks of ligation was 4-fold higher than for
the wild-type mice (Figure 3A). After 4 weeks of ligation, a
2.5-fold increase was seen. Wild-type mice had a comparable number of cells present in the intima after 2.5 and 4 weeks of ligation. In PAI-1−/− mice, an increased cell number was seen after both 2.5 and 4 weeks of ligation (Figure 3B). After 2.5 weeks, the cell number was 9-fold higher than that of the corresponding wild-type mice. Although the lesion had increased after 4 weeks of ligation in the PAI-1−/− mice, the increase in cell number was only 6-fold compared with the wild-type mice. This is explained by the 2-fold increase in cell number of the wild-type mice. To further substantiate the increase in cell number observed and to establish whether these cells are actively engaged in proliferation, PCNA staining was performed. After 2.5 weeks of ligation, both VN−/− and PAI-1−/− mice (Figure 3D) had an increased number of PCNA-stained cells, whereas wild-type mice only showed an occasional PCNA-stained cell in their minute lesions. After 4 weeks of ligation, the PAI-1−/− mice still had an increased number of PCNA-stained cells. However, at this time point, the number of PCNA-staining cells was diminished in the VN−/− mice, being the same as for the wild-type counterparts. In conclusion, the data on cell numbers measured by two different methods agree with those recorded for I/M ratios.

**Hirudin Affects Intima Formation**

We measured thrombin-mediated coagulation activity in plasma as a surrogate value for prothrombin levels in the vessel wall. The clotting values for VN−/−, PAI-1−/−, and wild-type genotypes are not significantly different (see online Figure I, which can be accessed at http://atvb.ahajournals.org). Therefore, prothrombin availability is considered similar in all genotypes studied.

To provide evidence for a role of thrombin in intima formation in PAI-1−/− and wild-type mice, we assessed the effect of the thrombin-specific inhibitor hirudin. To that end, we injected PEG-hirudin and measured its bioactivity in plasma of placebo- and PEG-hirudin–treated mice. In PEG-hirudin–treated mice, 3% to 5% (0.3 to 0.5 µg/mL plasma) of the injected PEG-hirudin (10 µg/mL plasma) was recovered (Figure 4A). No significant difference was observed between wild-type and PAI-1−/− mice. Yet, the amount of PEG-hirudin encountered is responsible for a 2-fold longer APTT (Figure 4B).

Carotid artery ligations were performed and I/M ratios were calculated. The I/M ratio of placebo-treated PAI-1−/− mice was 2-fold higher than that of placebo-treated wild-type mice (Figure 4C). In wild-type mice, PEG-hirudin did not cause an alteration of the I/M ratio as compared with placebo-treated wild-type mice. However, in PAI-1−/− mice, PEG-hirudin significantly reduced the I/M ratio, reaching the level of placebo- or hirudin–treated wild-type mice.

**Presence of VN, PAI-1, and Prothrombin**

To verify the presence of the components studied, immunohistochemical detection of VN, PAI-1, and prothrombin was performed in ligated carotids. VN antigen was predominantly present in the adventitia and in the intima of wild-type and PAI-1−/− mice (Figure 5A). PAI-1 antigen was found almost exclusively in the intima of wild-type and VN−/− mice after 2.5 and 4 weeks of ligation (Figure 5B). Furthermore, PAI-1 was encountered both cell associated and in the extracellular matrix. Prothrombin, a plasma-derived protein, was unevenly distributed in the intima and media (Figure 5C). Consequently, all three proteins were present in the intima of ligated arteries, thus potentially positioned to interact.

**Discussion**

We used the murine carotid artery ligation model to study the role of PAI-1 and VN in (re)stenosis. In this model, the size of the intima relies greatly on SMC proliferation because the
adhesion, and detachment by virtue of its overlapping VN-pili-

tion, presumably caused by the PAI-1

proliferation that correlated with persistent fibrin(ogen) accumula-

tion. In contrast to the rat vascular injury model, carotid

artery ligation is not based on injury because the endothelial-cell

layer remains intact. Consequently, after 1 week of ligation, a

thrombus is rarely observed in any of our studied genotypes,

confirming previous observations. At this time point, virtually

no intima has been generated. Thus, in our hands, there are no

indications that thrombus formation plays a prominent role in the
carotid artery ligation model. By contrast, Peng and collaborators

recently used the carotid artery ligation model and observed de-

creased intima formation in PAI-1−/− mice as compared with

wild-type mice. These authors assumed that thrombi had been

generated and attributed a cell migration-promoting role to fibrin,

which would be diminished in the absence of PAI-1, because of

increased fibrinolysis. Apart from fibrin formation, an essential

methodological discrepancy is that their measurements of intima

size were performed at a considerably larger distance from the

ligature (2 to 7 mm) than in our experiments (0.05 to 1.5 mm),
apparently spanning an area that does not harbor proliferating SMC.

Hirudin-mediated reduction in intima formation is observed in

PAI-1−/− mice; however, no effect of hirudin was seen on the

small lesions of wild-type mice. It is conceivable that, simulta-

eous with thrombin, other determinants function to ultimately

induce SMC proliferation (eg, stasis, hypoxia, arterial wall

pressure). Such factors are insensitive to hirudin. Yet, the

discrepancy might also be explained by previous observations

with cultured SMCs, showing that thrombin induces PAI-1

synthesis in these cells. In addition, we and others demonstrated

previously that thrombin is a target serine proteinase

for PAI-1/VN complexes in vitro. Hence, in this environment,
thrombin provides for control of its own activity. Obviously, this

feedback loop is not existent in PAI-1−/− mice, resulting in

abundant thrombin activity and, consequently, excessive SMC

proliferation. Collectively, apart from other functions of PAI-1

and VN, the interaction between thrombin and PAI-1/VN

complexes is conceivably relevant for the generation of SMC-

rich intima.

The interaction between thrombin and PAI-1/VN complexes

is characterized by a so-called suicide-substrate mechanism. This

mechanism consists of two separate pathways for the fate of

PAI-1, namely the generation of covalent thrombin/PAI-1 com-

plexes (ie, suicide reaction) and the cleavage of PAI-1 and

recovery of active thrombin (ie, substrate reaction). In the latter

case, thrombin removes active PAI-1 from the vascular com-

partment and thereby eliminates not only its own inhibitor but

also that of other target proteases, notably uPA. Because uPA

is primarily responsible for the migration of SMCs, the ultimate

effect of the substrate reaction is to remove the uPA inhibitor

and, consequently, an indirect promotion of migration by throm-

bin. Alternatively, if the suicide reaction prevails, then PAI-1

predominantly forms covalent complexes with thrombin, result-

ing in inhibition of thrombin-mediated SMC proliferation. We

believe that the data reported here favor the latter explanation

because we observed an increased cell number and PCNA-

expressing cells in the intima of PAI-1−/− and VN−/− mice.

Finally, it should be noted that the kinetics of intima forma-

tion in PAI-1−/− and VN−/− mice are not identical. This variation

is not caused by differences in apoptosis because nuclear

morphology and the number of active caspase 3-positive SMCs
did not differ between the various genotypes (data not shown).

In summary, observations with the murine carotid artery ligation

model lead us to conclude that PAI-1 and VN protect against

excessive SMC proliferation. Our data provide support for a

mechanism by which PAI-1, presumably in conjunction with the

Figure 5. Presence of VN, PAI-1, and prothrombin. Immunohisto-

chemical staining of VN (A), PAI-1 (B), and prothrombin antigen (C)
in intima, media, or adventitia of lesions obtained after carotid

tissue. Staining of endothelial cells is an artifact because these
cells do not synthesize prothrombin. Photomicrographs: bright-field microscopy, original magnification ×200 (A through C).
essential cofactor VN, controls the mitogenic activity of thrombin in the vessel wall.

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Figure I. Thrombin-mediated coagulation of murine plasma

Thrombin-induced fibrin formation is measured in re-calci fied EDTA plasma samples, from wild-type, VN$^{-/-}$ and PAI-1$^{-/-}$ mice, using Thrombotest reagent (Axis-Shield, Oslo, Norway). Thrombotest reagent contains bovine brain thromboplastin and adsorbed bovine plasma as a source for coagulation factor V and fibrinogen. Thrombotest is used to measure differences in fibrin-formation times due to reduced coagulation factors X, VII and prothrombin. The murine EDTA plasma samples were diluted 40% with 0.9% (w/v) sodium chloride and 20 µl diluted plasma sample was added to 500 µl Thrombotest reagent to measure coagulation. As a control, the clotting time for pooled EDTA plasma derived from five control unligated mice was measured. Clotting times of re-calci fied EDTA plasma samples, derived from VN$^{-/-}$ mice, PAI-1$^{-/-}$ mice and their wild-type counterparts, were determined.

In control pooled plasma, derived from five unligated control mice, fibrin formation occurred after 36 sec. For the VN$^{-/-}$ and wild-type mice, the clotting times were 37 +/- 2 sec (n=2) and 42 +/- 4 sec (n=3), respectively. In addition, we have measured the clotting times for PAI-1$^{-/-}$ and wild-type mice, being 38 +/- 1 sec (n=6) and 37 +/- 2 sec (n=5), respectively. Clearly, the clotting values for any of these genotypes are not significantly different. This Thrombotest assay demonstrates that thrombin, factor VII and factor X are present and can be activated in plasma of all studied genotypes after carotid artery ligation. WT indicates wild-type; -/-, deficient; ns, not significant.