Endogenous Vitronectin and Plasminogen Activator Inhibitor-1 Promote Neointima Formation in Murine Carotid Arteries

Lin Peng, Nitin Bhatia, Andrew C. Parker, Yanhong Zhu, William P. Fay

Abstract—We examined the roles of vitronectin and plasminogen activator inhibitor-1 (PAI-1) in neointima development. Neointima formation after carotid artery ligation or chemical injury was significantly greater in wild-type mice than in vitronectin-deficient (Vn−/−) mice. Vascular smooth muscle cell (VSMC) proliferation did not differ between groups, suggesting that vitronectin promoted neointima development by enhancing VSMC migration. Neointima formation was significantly attenuated in PAI-1–deficient (PAI-1−/−) mice compared with control mice. Because intravascular fibrin may function as a provisional matrix for invading VSMCs, we examined potential mechanisms by which vitronectin and PAI-1 regulate fibrin stability and fibrin-VSMC interactions. Inhibition of activated protein C by PAI-1 was markedly attenuated in vitronectin-deficient plasma. The capacity of PAI-1 to inhibit clot lysis was significantly attenuated in vitronectin-deficient plasma, and this effect was not explained simply by the PAI-1–stabilizing properties of vitronectin. The adhesion and spreading of VSMCs were significantly greater on wild-type plasma clots and PAI-1–deficient plasma clots than on vitronectin-deficient plasma clots. We conclude that endogenous levels of vitronectin and PAI-1 enhance neointima formation in response to vascular occlusion or injury. Their effects may be mediated to a significant extent by their capacity to promote intravascular fibrin deposition and by the capacity of vitronectin to enhance VSMC-fibrin interactions. (Arterioscler Thromb Vasc Biol. 2002;22;0000-0000.00000.)

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

Vascular smooth muscle cells (VSMCs) proliferate and migrate in response to various forms of stress, such as mechanical trauma, inflammation, growth factors, and alterations in blood pressure or flow. Clinical disorders that are characterized by VSMC proliferation, migration, and the consequent neointima formation include atherosclerosis, posttransplant vasculopathy, and restenosis after balloon angioplasty. Vitronectin is a 78-kDa glycoprotein that is found in plasma, platelets, and extracellular matrices. Several lines of evidence suggest that vitronectin plays an important role in VSMC migration. Vitronectin binds and stabilizes plasminogen activator inhibitor-1 (PAI-1), the fast-acting inhibitor of tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Therefore, vitronectin may control cell migration by regulating the generation of plasmin, a serine protease that degrades extracellular matrix proteins either directly or indirectly by activating matrix metalloproteinases. Vitronectin also regulates the interactions of PAI-1 with other proteases that may modulate the vascular response to injury, including thrombin and activated protein C (APC). Vitronectin binds to integrin αvβ3, and to the uPA receptor, both of which are expressed on VSMCs. Interactions of these receptors with vitronectin regulate VSMC migration in vitro.

Although the in vitro function of vitronectin has been studied extensively, its role in regulating VSMC migration in vivo is not well defined. Vitronectin-deficient (Vn−/−) mice have been generated by a gene-targeting approach. The main goal of the present study was to test the hypothesis that vitronectin is an important determinant of neointima formation by comparing wild-type mice and Vn−/− mice in 2 murine models of neointima formation. To gain insight into the potential interactions between vitronectin and PAI-1 in regulating neointima formation, we also studied PAI-1–deficient (PAI-1−/−) mice in these models of neointima formation. In addition, we performed in vitro experiments to explore mechanisms by which vitronectin and PAI-1 may affect neointima formation by modulating the formation of fibrin, lysis, and interactions with VSMCs.

Methods

Animals
C57BL/6j mice were purchased from Jackson Laboratories, Bar Harbor, ME. Vn−/− mice (backcrossed >10 generations into the
C57BL/6J (genetic background) were a gift from Dr David Ginsburg, University of Michigan, Ann Arbor. PAI-1+/− mice (backcrossed >10 generations into the C57BL/6J (genetic background)) were a gift from Dr Peter Carmeliet, University of Leuven, Leuven, Belgium. Mice lacking both vitronectin and PAI-1 were generated by standard breeding methods. Genotypes were confirmed by polymerase chain reaction analysis of tail DNA. All experimental groups contained approximately equal mixtures of male and female mice. Animals were fed normal chow (Rodent Diet 5001, LabDiet). All animal care and experimental procedures complied with National Institutes of Health guidelines and were approved by the University of Michigan Committee on Use and Care of Animals. All surgical procedures and morphometric analyses were performed with the investigator blinded to mouse genotype.

**Carotid Artery Ligation Model**

Adult mice (aged 6 to 8 weeks, weight ~25 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (120 mg/kg). The left common carotid artery was isolated and ligated at the distal bifurcation with a 6-0 silk suture, as described previously. The surgical incision was sutured, and mice were allowed to recover from anesthesia. Five days or 4 weeks later, mice were anesthetized, and the left carotid artery was surgically exposed and perfusion-fixed. Mice were injected intraperitoneally with bromodeoxyuridine (BrdU, 100 mg/kg) 16 hours and 2 hours before carotid artery retrieval. The left common carotid artery, from its origin at the aorta to its distal bifurcation (approximate length 9 mm), was excised and embedded in paraffin. Five evenly spaced hematoxylin-eosin–stained cross sections (thickness 5 μm) were prepared from the central 5 mm of the common carotid artery. The 2 mm of the artery immediately proximal to the ligation site was excluded from analysis, as described previously.

**FeCl₃ Injury Model**

The left carotid arteries of mice (aged 6 to 8 weeks, weight ~25 g) were injured by topical application of FeCl₃ as described. Three weeks later, the injured segment of the carotid artery was surgically exposed, perfusion-fixed, and embedded in paraffin. Five evenly spaced hematoxylin-eosin–stained cross sections (thickness 5 μm) were prepared from the injured segment of the artery.

**Histological and Morphometric Analyses**

Images of carotid artery cross sections were imported into a computer software program (Image-Pro Plus, Media Cybernetics), and the lumen–blood vessel wall interface, the internal elastic lamina, and the external elastic lamina were traced. Intima area, media area, and the intima/media ratio were calculated. Mean values for each artery were calculated from the 5 cross sections. Anti-vitronectin and anti–PAI-1 immunohistochemistry were performed with the use of affinity-purified polyclonal antibodies raised in goats against murine vitronectin or PAI-1, respectively (Santa Cruz Biotechnology), and with the Histostain-SP kit (Zymed Laboratories). Smooth muscle α-actin staining was performed with anti-human smooth muscle α-actin monoclonal antibody (clone 1A4, Dako). Anti-BrdU staining was performed with a BrdU staining kit (Zymed Laboratories). For each artery, 3 cross sections were analyzed, and the percentage of BrdU-positive cells was determined from analysis of >50 cells per cross section. Mean values for each artery were calculated. A positive control (murine intestine) was performed with each assay to confirm its sensitivity for detecting actively proliferating cells.

**VSMC Adhesion and Migration In Vitro**

Rat embryonal aortic VSMCs (cell line A7r5) were from American Type Culture Collection. The cells used in these experiments had been passaged 3 times. Cells were cultured in a humidified atmosphere of 5% CO₂/95% air in DMEM containing 4.5 g/L glucose, 4 mmol/L L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After attaining confluence, the cells were treated with 0.25% trypsin and 1 mmol/L EDTA and were washed once with DMEM containing 10% FBS and then twice with DMEM. Cells were resuspended in DMEM at a density of 10⁵ cells per milliliter. Citrated platelet-poor plasma was prepared from wild-type mice, Vn−/− mice, and PAI-1−/− mice. Plasma clots were prepared by adding plasma (0.5 mL), thrombin (2 U/mL), and CaCl₂ (5 mmol/L) to 12-well (diameter 20 mm) polystyrene cell culture plates (Costar). One milliliter of VSMC suspension was added to individual wells. After 1 hour at 37°C, the wells were washed 5 times with DMEM (1 mL, 3 minutes of gentle agitation per wash), and then 1 mL of DMEM was added to each well. The total number of adherent cells present in 5 randomly selected microscopic fields (magnification ×100) of each well was counted. Adherent cells were incubated 4 hours at 37°C. Five microscopic fields from each well were examined, and the percentage of total cells exhibiting spreading (defined by assumption of a flatter [ie, nonspherical] shape and the development of irregular borders) was determined. The investigator was blinded to the genotype of plasma clots when assessing cell adhesion and spreading. Control experiments revealed no differences in the adhesion of rat VSMCs to purified rat versus mouse vitronectin.

**Inhibition of APC by PAI-1**

Recombinant murine PAI-1 (0.5 μmol/L, Molecular Innovations) or an equal volume of PBS was added to citrated murine plasma. Protein C was activated by adding Protac (American Diagnostica) according to the manufacturer’s instructions. After 1 hour at 37°C, APC activity was determined by adding Spectrozyme APC (American Diagnostica) and measuring the absorbance of the reaction mixture at 405 nm.

**Clot Lysis Assay**

Pooled citrated murine plasma (50 μL, obtained from wild-type mice or Vn−/− mice), containing a trace amount of 125I-human fibrinogen (Amersham Pharmacia) and recombinant murine PAI-1 (200 nmol/L, Molecular Innovations) or an equal volume of Tris-buffered saline, was clotted for 1 hour at 37°C by the addition of thrombin (1 U/mL) and CaCl₂ (5 mmol/L). Clots were formed on polypropylene rods to facilitate their washing and transfer. Clots were washed 5 times with Tris-buffered saline containing hirudin (1 μg/mL) and then suspended in 37°C citrated murine plasma (400 μL, obtained from either wild-type mice or Vn−/− mice) containing recombinant human tPA (50 nmol/L, Genentech) and hirudin (1 μg/mL). At timed intervals (0 to 3 hours), 10 μL of plasma was removed, and radioactivity counts were measured in a γ-counter. Percent clot lysis was calculated by dividing plasma radioactive counts by those present in the washed clot before its placement in plasma.

**Statistical Analyses**

Data are presented as mean ± 1 SEM. The unpaired Student t test was used to compare 2 groups, and the Kruskal-Wallis 1-way ANOVA on ranks (pairwise multiple comparison procedures) was used to compare multiple groups.

**Results**

**Effects of Vitronectin on Neointima Formation**

We studied neointima formation after carotid artery ligation in 13 wild-type mice and 10 Vn−/− mice. Consistent with earlier studies, neointima was present in carotid arteries 4 weeks after ligation. The mean intima cross-sectional area of wild-type mice was significantly greater than that of Vn−/− mice (Figure 1). There was a nonsignificant trend toward greater media cross-sectional area in wild-type mice compared with Vn−/− mice. Mean intima/media area ratios were significantly greater in wild-type mice than in Vn−/− mice. The gross and microscopic appearances of the right (ie, nonligated) carotid arteries of wild-type mice and Vn−/− mice were normal. The mean frequency of BrdU-positive cells 4 weeks after carotid artery ligation was very low (<1% of total
cells) for wild-type mice (n=5) and for Vn$^{-/-}$ mice (n=6). To determine whether cell proliferation differed between groups at earlier time points, wild-type mice (n=4) and Vn$^{-/-}$ mice (n=4) were subjected to carotid artery ligation. Five days later, the ligated arteries were retrieved, and the percentage of BrdU-positive cells was determined. The mean percentage of BrdU-positive cells was 7.9 ± 1.9% for wild-type mice and 9.3 ± 1.7% for Vn$^{-/-}$ mice (P=0.35). No significant neointima formation was observed at day 5, and essentially all BrdU-positive cells were located within the media. Vitronectin and PAI-1 immunostaining revealed similar patterns of distribution, with the strongest staining in the intima and adventitia (Figure 2).

Because pathological neointima formation often occurs after vascular injury, we also studied carotid artery neointima formation after chemical injury with FeCl$_3$. This method induces full-thickness injury, endothelial cell denudation, necrosis of media cells, and a subsequent proliferative response that results in the formation of a smooth-muscle α-actin–positive neointima within 3 weeks. We confirmed this response by performing smooth muscle α-actin immunostaining of carotid arteries 3 weeks after FeCl$_3$ injury (Figure 3D). Neointima formation 3 weeks after injury was assessed in wild-type mice (n=16) and Vn$^{-/-}$ mice (n=14). Mean intima cross-sectional area of wild-type mice was significantly greater than that of Vn$^{-/-}$ mice (Figure 4). There was no difference in media cross-sectional area between wild-type mice and Vn$^{-/-}$ mice. Mean intima/media area ratios were significantly greater in wild-type mice than in Vn$^{-/-}$ mice. Therefore, the effects of vitronectin deficiency on neointima formation were similar in the carotid ligation model and the FeCl$_3$ injury model.

Effects of PAI-1 on Neointima Formation
Because the effects of vitronectin on neointima formation could potentially be mediated via the interactions of vitronectin with PAI-1, we studied PAI-1$^{-/-}$ mice in models of carotid occlusion and chemical injury. Ten PAI-1$^{-/-}$ mice underwent carotid ligation. The mean intima area 4 weeks later was significantly less in PAI-1$^{-/-}$ mice (n=10) than in wild-type mice (n=13, Figure 1). The mean media area was significantly reduced in PAI-1$^{-/-}$ mice, and the mean intima/media ratio was significantly reduced in PAI-1$^{-/-}$ mice compared with wild-type mice. PAI-1$^{-/-}$ mice (n=10) also were studied in the FeCl$_3$ model and were found to have a significant reduction in neointima formation compared with that in wild-type mice (n=16, Figure 4). We also studied Vn$^{-/-}$+PAI-1$^{-/-}$ mice (n=13) in the FeCl$_3$ model to determine whether combined deficiency produced an additive effect. The decrease in neointima formation observed in mice

**Figure 1.** Effects of vitronectin deficiency and PAI-1 deficiency on neointima formation after carotid artery ligation. The distal left carotid arteries of wild-type (Wt) mice, Vn$^{-/-}$ mice, and PAI-1$^{-/-}$ mice were ligated to completely obstruct blood flow. Four weeks later, the arteries were harvested and subjected to morphometric analysis. Mean intimal and medial areas are shown. Mean intima/media ratios are shown in the inset.

**Figure 2.** Immunohistochemical analyses performed 4 weeks after carotid artery ligation. A, Anti-vitronectin staining of a carotid artery from a wild-type mouse. B, Anti–PAI-1 staining of a carotid artery from a wild-type mouse. Insets in each panel are immunostaining controls performed on a Vn$^{-/-}$ artery and a PAI-1$^{-/-}$ artery in a fashion identical to that for wild-type arteries. Bars=25 μm.
lacking both factors did not differ significantly from that observed in mice lacking only vitronectin or only PAI-1 (Figures 3 and 4). However, because deficiency of only PAI-1 or only vitronectin produced nearly complete inhibition of neointima formation (ie, the mean intima areas in arteries after injury of $Vn^{-/-}$ mice and $PAI-1^{-/-}$ mice did not differ significantly from the areas in uninjured wild-type arteries; data not shown), these studies could not exclude additive effects of combined vitronectin/PAI-1 deficiency on neointima formation.

Potential Mechanisms Underlying the Effects of Vitronectin and PAI-1 on Neointima Formation

Both of the neointima models that we used result in thrombus formation. In the carotid ligation model, a thrombus forms in the most distal aspect of the ligated arterial segment (ie, with 2 mm of the ligature), and this thrombus is subsequently organized by invading cells. In the $FeCl_3$ injury model, a thrombus forms at the site of injury and then undergoes lysis within several days. The thrombi formed in these models could contribute to neointima formation by serving as a provisional fibrin matrix that is invaded by VSMCs, as suggested by recent studies involving the carotid ligation model. Although the capacity of PAI-1 to stabilize fibrin is well recognized, less is known about the impact of vitronectin on fibrin formation and fibrinolysis. PAI-1 inactivates APC, an endogenous anticoagulant with profibrinolytic properties. We compared the capacity of PAI-1 to inhibit APC in wild-type plasma and $Vn^{-/-}$ plasma. Although PAI-1 significantly inhibited APC in normal plasma, it had no effect on APC activity in vitronectin-deficient plasma (Figure 5). We also compared the capacity of PAI-1 to inhibit tPA-
mediated clot lysis in vitro in wild-type versus vitronectin-deficient plasma. In the absence of added PAI-1, there was a minor trend toward enhanced clot lysis in vitronectin-deficient plasma compared with wild-type plasma, but this did not achieve statistical significance (Figure 6). Because the concentration of PAI-1 in normal mouse plasma is very low,21 we performed additional experiments in which clots were formed in the presence of recombinant wild-type murine PAI-1 (200 nmol/L). The capacity of exogenous wild-type PAI-1 to inhibit clot lysis was attenuated in vitronectin-deficient plasma compared with wild-type plasma (Figure 6A). This effect could have been mediated by the capacity of vitronectin to stabilize PAI-1 in its active conformation3,4,22 or by its capacity to physically link PAI-1 to fibrin, thereby localizing PAI-1 to the clot surface.18 To address this issue, we performed experiments in which fibrin clots were formed in the presence of PAI-1-14-1b23 (obtained from Molecular Innovations, in catalogue as CPAI), a mutant with a markedly prolonged half-life (>145 hours) compared with that of wild-type PAI-1 (1 to 2 hours), ie, under conditions in which the PAI-1–stabilizing effect of vitronectin would be insignificant. The inhibition of clot lysis by PAI-1-14-1b was significantly attenuated in Vn-/- plasma compared with wild-type plasma (Figure 6B), suggesting that the antifibrinolytic effect of vitronectin was mediated to a significant extent by its capacity to bind PAI-1 to fibrin.

Because the migration of VSMCs through fibrin matrices may contribute to neointima formation,17 we examined the effects of endogenous vitronectin and PAI-1 on the capacity of cultured VSMCs to adhere to and spread on fibrin. Aortic smooth muscle cells (n=1.0×10^5) were incubated for 1 to 2 hours on plasma clots prepared from wild-type plasma, Vn-/-
Adhesion and Spreading of Cultured Vascular Smooth Muscle Cells on Plasma Clots

<table>
<thead>
<tr>
<th>Mean Number of Adherent Cells per Microscopic Field</th>
<th>Mean % of Adherent Cells Exhibiting Spreading Phenotype 4 Hours Later</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>Vn−/−</td>
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<tr>
<td>89.6±5.9</td>
<td>53.4±4.0*</td>
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Data are mean±SEM of 3 to 6 experiments. *P<0.05 vs other groups by ANOVA.

plasma, or PAI−/− plasma. After the cells were extensively washed, the number of adherent cells was counted. Four hours later, the percentage of adherent cells that exhibited spreading was determined. Smooth muscle cell adhesion and spreading were significantly greater on wild-type plasma clots than on vitronectin-deficient plasma clots (Table). However, there was no significant difference in cell adhesion/spreading between wild-type plasma clots and PAI−/− deficient plasma clots.

Discussion

The main objective of the present study was to examine the impact of endogenous vitronectin and PAI−1 on neointima development in murine carotid arteries. Neointima formation was blunted in Vn−/− mice, suggesting that vitronectin plays an important role in supporting neointima formation. Vascular cell proliferation, assessed by anti-BrdU staining, did not differ between wild-type mice and Vn−/− mice in the carotid artery ligation model, suggesting that altered vascular cell migration, as opposed to proliferation, contributed to the observed effects of vitronectin. VSMCs express αβ, and uPA receptors, which bind extracellular matrix vitronectin to enable VSMC migration in vitro. αβ- The present study suggests that vitronectin modulates VSMC migration in vivo as well, and it supports the hypothesis that vitronectin deficiency “uncouples” VSMCs from the extracellular matrix of the blood vessel wall, thereby limiting cell migration and neointima formation. Vitronectin binds PAI−1, and this interaction appears to play a major role in regulating the function of each protein. αβ We found that neointima formation also was reduced in PAI−/− mice compared with wild-type mice in the carotid ligation and chemical injury models, suggesting that endogenous PAI−1 promotes neointima development. Our results appear to disagree with those reported by Carmeliet et al., who found that neointima formation after electrical or mechanical injury was increased in PAI−/− mice compared with wild-type mice. The differences in the models used and in the genetic background of mice studied may account for the differences between studies. At first glance, our results also appear inconsistent with in vitro studies demonstrating that PAI−1 can retard VSMC migration through extracellular matrices by inhibiting plasmin formation or by binding to vitronectin and preventing it from interacting with its receptors present on VSMCs. αβ We hypothesize that endogenous concentrations of PAI−1, which are relatively low, particularly in mice, may not significantly inhibit VSMC migration because low PAI−1 levels may not (1) sufficiently saturate vascular stores of vitronectin, which is an abundant extracellular matrix protein, and/or (2) sufficiently inhibit cell-associated uPA. However, several studies have demonstrated that thrombolysis after arterial injury is accelerated in PAI−/− mice compared with wild-type mice, suggesting that endogenous PAI−1 expression is sufficient to promote the stability of intravascular fibrin by inhibiting tPA. αβ We also studied the impact of vitronectin on the inhibition of clot lysis by PAI−1. We found that vitronectin significantly enhanced the capacity of PAI−1 to inhibit APC, an endogenous anticoagulant that may also stimulate fibrinolysis. αβ It is possible that in these studies the very high levels of PAI−1 that were achieved at sites of vascular injury were sufficient to saturate vitronectin and/or inhibit cell-associated uPA, thereby inhibiting neointima formation. Therefore, thrombus formation and VSMC migration must both be taken into account when the role of PAI−1 in modulating neointima formation is examined. We propose that the variable extent of thrombus formation and VSMC migration induced by different forms of injury and the wide variation in local PAI−1 concentrations generated in different models account for the reported capacity of PAI−1 to either promote or inhibit neointima formation.

Given the apparent role of mural fibrin deposits in modulating neointima formation, αβ we performed in vitro experiments to further elucidate potential mechanisms by which vitronectin and PAI−1 may regulate fibrin stability and the interactions of VSMCs with fibrin. PAI−1 inhibits APC, an endogenous anticoagulant that may also stimulate fibrinolysis. αβ We found that the capacity of PAI−1 to inhibit APC was markedly attenuated in vitronectin-deficient plasma. Therefore, vitronectin may contribute to neointima formation by attenuating the capacity of APC to reduce intravascular fibrin formation. Our results are consistent with those of Rezaie, αβ who demonstrated in a purified system that vitronectin is a cofactor for the inhibition of APC by PAI−1. The present study extends that of Rezaie by demonstrating that vitronectin exhibits a potent effect on APC inhibition in plasma. We also studied the impact of vitronectin on the inhibition of clot lysis by PAI−1. We found that vitronectin significantly enhanced...
The inhibition of thrombolysis by PAI-1. The effect of vitronectin on PAI-1 in mice with a PAI-1 mutation that does not bind to vitronectin on platelet aggregation and fibrin clot formation was studied. In support of this hypothesis, we found that the capacity of VSMCs to adhere to and spread on fibronectin is enhanced by binding fibronectin in a PAI-1-dependent manner. These results suggest that fibronectin may support neointima formation in mice by enhancing interactions between fibronectin and VSMCs during plasma clot formation in PAI-1-deficient mice. The adhesion of VSMCs to plasma clots was not affected by PAI-1 deficiency. This probably resulted from the low concentrations of PAI-1 in normal mouse plasma (0.02 nmol/L), which would not be expected to bind vitronectin.

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