Plasminogen Activator Inhibitor-1 and Its Cofactor Vitronectin Stabilize Arterial Thrombi After Vascular Injury in Mice

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Background—The origin and contribution of plasminogen activator inhibitor-1 (PAI-1) and its cofactor vitronectin (VN) to arterial thrombosis/thrombolysis in vivo is controversial.

Methods and Results—Ferric chloride was used to induce carotid artery injury in 97 wild-type (WT), 84 PAI-1−/−, and 84 VN−/− mice. Complete thrombotic occlusion was observed in 70% of PAI-1−/− mice versus 92% of WT (P<0.001) and 87% of VN−/− (P=0.015) mice. In vessels that occluded, mean times to occlusion were significantly longer in PAI-1−/− than in WT or VN−/− mice. The initial thrombotic response of VN−/− mice was similar to that of WT mice, but their thrombi were unstable and frequently embolized. As a result, the patency rate of carotid vessels 30 minutes after injury was as high in VN−/− mice (36%) as in PAI-1−/− mice (which demonstrate progressive thrombolysis) and significantly higher than that of WT mice (12%; P=0.013). Histochemical and reverse transcription–polymerase chain reaction studies revealed an early upregulation of PAI-1 mRNA and protein expression in the thrombus and the vessel wall, which persisted for ≥1 week. VN protein also accumulated after injury, but VN mRNA levels remained low at all times.

Conclusions—PAI-1 and VN participate in the thrombotic response to arterial injury by preventing premature thrombus dissolution and embolization. The accumulation of PAI-1 in the thrombus/vessel wall after injury may result, at least in part, from local synthesis, whereas the VN protein appears to be derived from plasma. (Circulation. 2001;103:576-583.)

Key Words: carotid arteries ■ genes ■ fibrinolysis ■ embolism

Plasminogen activator inhibitor-1 (PAI-1) is the principal physiological inhibitor of both tissue (tPA) and urokinase (uPA) plasminogen activators and is a key regulator of the fibrinolytic system. In humans, up to 90% of circulating PAI-1 is contained within platelet α-granules, and in vitro studies suggest that it may be responsible, at least in part, for the resistance of platelet-rich arterial thrombi to thrombolysis. Clinical studies correlate elevated circulating levels of PAI-1 with an increased risk of both arterial and venous thrombosis, and mice lacking PAI-1 are resistant to venous thrombosis. Despite this, the contribution of PAI-1 to stabilization of arterial thrombi in vivo remains controversial. For example, some studies reported that the thrombotic response to arterial injury was not significantly altered in PAI-1-deficient mice, whereas others demonstrated prolonged times to thrombosis in these mice. PAI-1 is a relatively unstable molecule in solution but is stabilized by binding to plasma-derived vitronectin (VN). Thus, VN may promote thrombosis by localizing active PAI-1 to sites of vascular injury. VN also may contribute to thrombosis by binding to platelet integrins. However, VN-deficient mice demonstrated a significantly enhanced thrombotic reaction to arterial injury, suggesting that it may have an antithrombotic role in vivo. These conflicting results emphasize the need for further studies to define the exact role of PAI-1 and VN in thrombus formation and dissolution.

In addition to their role in thrombosis and thrombolysis, PAI-1 and VN also influence cell migration and the chronic wound-healing response to vascular injury through interactions with uPA, the uPA receptor, and integrins. Although PAI-1 appears to participate in the vascular remodeling process in vivo, its role in injury models that focus on arterial thrombosis and thrombolysis remains to be established. Furthermore, the origin and expression of VN in the vessel wall after arterial injury have not yet been systematically studied.

In this report, we used the FeCl₃, model in gene-inactivated mice to gain further insight into the role of PAI-1 and VN in the response to arterial injury and thrombosis. Our results indicate that PAI-1 and VN both participate in the early stages of the vascular response to injury by stabilizing the initial thrombus and preventing early fibrinolysis and premature embolization. They suggest that the increased PAI-1 in the organizing thrombus and the vessel wall during
the remodeling process reflects local synthesis, whereas the increased VN appears to be derived from plasma.

**Methods**

**Experimental Animals**

C57BL/6J wild-type (WT) mice were from Jackson Laboratories (Bar Harbor, Me), PAI-1–deficient (PAI-1−/−) mice were from Dr P. Carmeliet (Leuven, Belgium), and vitronectin-deficient (VN−/−) mice were from Dr D. Ginsburg (Ann Arbor, Mich). Only animals that were the product of ≥8 backcrosses to the C57BL/6J genetic background were used. Genotyping of PAI-1−/− and VN−/− mice was performed as described previously. All animal care and experimental procedures complied with the Guide for the Care and Use of Laboratory Animals, US Department of Health and Human Services, and were approved by the Animal Research Committee of the Scripps Research Institute.

**Carotid Injury and Thrombosis**

Mice (6 to 8 weeks old) were subjected to carotid artery injury with 10% ferric chloride (FeCl₃). Briefly, animals were anesthetized (Metofane, Schering-Plough), the left carotid artery was dissected, and then a 0.5×1.0-mm strip of Whatman No. 1 filter paper soaked in 10% FeCl₃ solution was applied to the surface of the adventitia for 3 minutes. This led to the formation of a 2- to 3-mm-long carotid thrombus (Figure 1). Carotid blood flow was monitored with a miniature ultrasound flow probe (0.5VB, Transonic Systems) interfaced with a flowmeter (model T106, Transonic Systems) and a computerized data acquisition program (WinDaq, DATAQ Instruments). At the end of the 30-minute flow-monitoring period, some of the mice were killed with an overdose of the anesthetic for tissue harvest and processing (see below). For studies at later times, the surgical wound was sutured, the anesthesia was discontinued, and the mice were returned to their cages.

**Tissue Harvest and Processing**

At various times after injury, anesthetized animals were perfusion-fixed with 4% zinc formalin as described. The injured segment of the carotid artery was then excised such that its proximal, thinner edge corresponded to the proximal edge of the thrombus, whereas the distal, thicker edge included the carotid bifurcation. This allowed orientation of the segments for paraffin embedding. The isolated vessel segments were immersed in 4% zinc formalin for 4 hours, fixed with 4% zinc formalin as described, and stored in 70% ethanol, embedded in paraffin blocks, and sectioned at 5-μm thickness for histological studies.

For gene expression studies, the animals were gently perfused with normal saline for 5 to 10 minutes. The thrombosed segment of the vessel was then removed, together with an ~1-mm-long nonthrombosed margin of the carotid vessel on either side. The tissues were then snap-frozen in liquid nitrogen, and pairs of arteries were pooled for preparation of total RNA.

**Histological and Immunohistochemical Studies**

Masson’s trichrome reagent was used to localize fibrin, collagen, smooth muscle cells (SMCs), and platelets in the vessel after injury with FeCl₃, and Perls’ iron stain was used to show the distribution of iron. Endothelial cells were identified with a rabbit anti-human/mouse von Willebrand factor (vWF) primary antibody (Dako; dilution, 1:100); SMCs were studied with a monoclonal anti-mouse α-actin primary antibody (Boehringer Mannheim; 1:20). Migrating and proliferating SMCs were identified by a polyclonal antibody to mouse vimentin (Santa Cruz; 1:50), and tissue-fixed macrophages were detected with a rat monoclonal Cl:A3-1 antibody (BMA; 1:20). Finally, polyclonal rabbit anti-mouse antibodies were used to detect PAI-1 and VN. All incubation, blocking, and antigen unmasking steps were carried out as described. Sections incubated with nonimmune serum instead of the primary antibody served as negative controls.

**Analysis of Gene Expression**

Total RNA was extracted from pooled pairs of mouse carotid arteries with Ultraspec RNA (Bioteck) and chloroform, and PAI-1, VN, and β-actin mRNA levels were determined by reverse transcription–polymerase chain reaction (RT-PCR). With 35 cycles used for VN and β-actin and 30 cycles for PAI-1 and β-actin. Aliquots of the PCR reaction (20 μL) were separated by electrophoresis through a 1.8% agarose gel, and the relative amount of the target (PAI-1 or VN) or the control (β-actin) RNA was determined densitometrically with a computer-based image analyzer (Alpha Imager 2000, Alpha Innotech). Radiolabeled antisense and sense riboprobes for murine PAI-1 and VN were prepared, and in situ hybridization was performed with the pGEM-3Z vector system (Promega) in the presence of 35S-labeled UTP (>1200 Ci/mmol; Amersham). Hybridized slides were exposed in the dark as indicated, developed, and counterstained with hematoxylin and eosin. Parallel sections were hybridized with a sense probe as a control for nonspecific hybridization.

**Statistical Analysis**

Continuous variables are represented by mean values±SD. Differences between genotypes or time points within the same genotype were tested by ANOVA followed by the Bonferroni t test for pairs of means. Qualitative variables (eg, vascular patency rates at specific time points) were tested by χ² and Fisher’s exact test. All statistical tests were 2-sided, with a value of P<0.05 indicating statistical significance.

**Results**

**Role of PAI-1 and VN in Thrombosis and Recanalization After Injury**

Carotid artery injury (see Figure 1) was induced in 97 WT, 84 PAI-1−/−, and 84 VN−/− mice, and hemodynamic (Figure 2), histological, and gene expression studies were performed. On induction of injury, rapid and complete thrombotic occlusion occurred in 92% of WT versus 87% of VN−/− mice but in only 70% of PAI-1−/− mice (P<0.001) and PAI-1−/− mice and VN−/− animals, respectively. In vessels that occluded (Figure 2B), the mean time to occlusion was significantly longer in PAI-1−/− versus WT (11.0±3.0 versus 8.7±1.9 minutes; P<0.001) or versus VN−/− (9.2±1.8 minutes; P>0.001) mice. Although no significant differences could be detected between WT and VN−/− animals at early times, the patency rate of injured carotid vessels at 30 minutes after injury (Figure 2C) was as high in VN−/− as in PAI−/− mice (36%) and significantly higher than that of WT animals (12%; P=0.013). Patency was slowly restored in the arteries of all mice, and by 7 to 21 days,
there were no significant differences between the 3 genotypes. Analysis of flow profiles revealed that injury of WT mice (Figure 3A) generally resulted in rapid thrombosis without subsequent recanalization during the first 30 minutes. In contrast, ∼30% of the vessels of PAI-1−/− mice failed to thrombose completely but instead underwent progressive thrombolysis over the 30-minute period (Figure 3B). In the VN−/− mice that occluded, the initial thrombotic response appeared to be similar to that of the WT animals, but the thrombi were unstable and repeatedly embolized (Figure 3C and 3D), leading to early recanalization and restoration of flow.

Morphological Changes in the Carotid Artery After Injury

Figures 4 and 5 display the histochemical changes that occur in the carotid arteries of WT mice after injury (similar results were obtained with the PAI-1−/− and VN−/− mice; not shown). For example, Masson’s trichrome stain (Figure 4, left...
column) revealed the formation of an occlusive, platelet-rich thrombus within 30 minutes after injury, progressive accumulation of fibrin in the thrombus (dark red) at 24 hours, and organization of the thrombus at 7 days with disruption of the internal elastic lamina, migration of cells from the medial layers to the intima, and increased cellularity with marked thickening of the adventitia. At 3 weeks, the adventitial reaction to injury had subsided, and the most prominent finding was the presence of a multilayered neointima.

By 30 minutes after injury, vWF was diffusely present in the platelet-rich thrombus but absent in the intima (Figure 4, middle column). Endothelial denudation persisted at least until the end of the first week, with reendothelialization observed by 3 weeks. Perls' iron stain (Figure 4, right column) revealed that iron accumulated at the interface between the thrombus and the vessel wall at 30 minutes after injury but then was cleared rapidly during the wound-healing process.

FeCl₃ injury resulted in the loss of staining for both α-actin (Figure 5, top row) and the cytoskeletal protein vimentin (Figure 5, middle row) within 30 minutes, and few, if any, intact cells or cell nuclei could be detected in the media or adventitia. These changes persisted for 24 hours after injury, but by 7 days, marked repopulation of the vessel wall was observed, with some α-actin–positive cells in the neointima and abundant vimentin-positive cells throughout the vessel.

**Figure 4.** Histochemical studies of carotid arteries at various times after injury. Left column: Masson's trichrome stain (MTC). Arrow indicates disrupted internal elastic lamina. Middle column: Staining for vWF protein. Arrow indicates endothelial denudation at 7 days. Right column: Perls' iron stain (blue). Arrow indicates migration of cells through disrupted internal elastic lamina 7 days after injury. Magnification: Top 3 panels of left column, ×200; other panels, ×1000.

**Figure 5.** Temporal changes in vascular cells after injury. Immunohistochemical markers were used to study changes in mature SMCs (α-actin; top row), in dedifferentiated (proliferating/migrating) SMCs (vimentin, Vim; middle row), and in tissue-fixed macrophages (Mø; bottom row). Magnification ×1000.
wall. These round/globular cells were tentatively identified as proliferating/migrating SMCs. 19 Three weeks after injury, multiple layers of redifferentiated, flat, \(\alpha\)-actin-positive SMCs could be detected in the neointima. Although isolated macrophages were detected in the adventitia 7 days after injury (Figure 5, bottom row), most had disappeared again by the end of the third week. Proliferation rates (proliferating cell nuclear antigen–stained nuclei; Zymed) in the vessel wall decreased from 13\(\pm\)10% to 7.2\(\pm\)8.1% within 24 hours after injury, then increased dramatically to 55\(\pm\)27% by the end of the first week (\(P<0.001\) versus uninjured vessels), and finally returned to preinjury levels by 3 weeks (5.1\(\pm\)7.7%). The majority of proliferating cells were dedifferentiated migrating SMCs (see Reference 19; not shown).

Changes in PAI-1 and VN Gene Expression in Injured Vessels

RT-PCR revealed relatively low levels of PAI-1 mRNA in the carotid artery before and 30 minutes after injury (Figure 6A and 6B). Increased PAI-1 mRNA was detected 6 hours after injury, however; it remained relatively high from 1 to 7 days after injury, however; it remained relatively high from 1 to 7 days and then returned to baseline levels by 3 weeks. In
contrast, little VN mRNA was detected in the normal vessel, and it did not increase at any time after injury (Figure 6C and 6D). In situ hybridization (Figure 7) revealed that in uninjured vessels and at very early times (30 minutes) after injury, PAI-1 mRNA could be detected only within isolated cells (α-actin-positive; not shown) of the media and adventitia. By 6 hours after injury, some intact endothelial cells (vWF-positive; not shown) and SMCs also began to express PAI-1 mRNA. At 7 days, a strong multifocal signal for PAI-1 mRNA (blue color) was detected in the organizing thrombus and in all layers of the vessel wall (Figures 7 and 8). This pattern was similar to the distribution of PAI-1 antigen (Figure 8). The PAI-1 signal was localized predominantly to proliferating/migrating SMCs in the media and adventitia. Three weeks after injury, the intensity of the PAI-1 signal decreased in accordance with the PCR findings.

Although VN gene expression could not be detected by in situ hybridization in the carotid wall before (not shown) or after (Figure 7) injury, VN antigen was readily detected in the thrombus by 30 minutes after injury (not shown) and in the vessel wall by 6 hours. It remained in the organizing thrombus and in all layers of the vessel wall throughout the wound-healing process (Figure 8). Its diffuse immunosignal, however, was distinctly different from the multifocal signal for PAI-1 mRNA and protein.

**Discussion**

Recent studies6–8,12 yield contradictory results with regard to the thrombotic response of PAI-1−/− and VN-deficient mice to vascular injury and thus raise questions about the true effects of PAI-1 and VN on the balance between thrombosis and fibrinolysis in vivo. The data summarized in this report address these inconsistencies. They demonstrate that PAI-1 and VN are both essential for preventing early fibrinolysis and premature thrombus embolization. For example, arterial injury with FeCl₃ led to thrombotic occlusion in a significantly lower proportion of PAI-1−/− (70%) compared with WT (92%) mice (Figure 2). Moreover, the mean time to thrombosis in vessels that occluded was significantly longer in PAI-1−/− animals, and >30% of the PAI-1−/− vessels were patent within 30 minutes (versus 12% in WT mice). Our results thus confirm and extend the findings of previous studies that reported enhanced endogenous thrombolysis in the absence of PAI-1.7,8,22

VN-deficient mice also exhibited an attenuated thrombotic response to injury (Figures 2 and 3). In this case, the proportion of vessels that initially thrombosed after injury and the mean time to thrombotic occlusion did not differ significantly versus WT mice (Figure 2B). A large proportion of the thrombi were unstable, however, and frequently embolized (Figure 3C and 3D), leading to a high patency rate 30 minutes after injury (Figure 2C). This instability may reflect the absence of the stabilizing effect of VN on PAI-1 at the site of arterial injury3,23 or impaired hemostatic function because of a defect in platelet aggregation caused by VN deficiency.10,11 Further studies are needed to clarify this issue.

Our results indicating instability of arterial thrombi in VN−/− mice differ from those of recent studies12 showing shortened times to thrombosis in these mice (ie, ≤3.0 minutes). The mouse strains and protocols used to induce arterial injury in the 2 studies were similar, suggesting that the differences might be due to differences in the anesthetics used. To test this hypothesis, we induced carotid artery injury in WT and VN−/− mice after anesthetizing the animals with either 120 mg/kg pentobarbital IP12 or inhaled methoxyflurane according to our protocol. Baseline carotid blood flow was lower in both mouse genotypes under pentobarbital compared with methoxyflurane anesthesia (0.8±0.3 versus 1.5±0.3 mL/min; P=0.002), and the time to thrombotic occlusion after injury was significantly shorter (6.7±0.6 versus 9.5±2.3 minutes; P=0.007). None of the mice, however, whether anesthetized with metofane or pentobarbital, developed occlusive carotid thrombi ≤3.0 minutes after injury. It is possible that the carotid vessels were handled more vigorously in the earlier study, and that this, together with the lower flow rate induced by pentobarbital, contributed to the shortened times to occlusion.
The mechanisms underlying the formation of platelet-rich arterial thrombi after application of FeCl₃ to the adventitia of the mouse vessel are largely unknown. Iron may injure endothelial cells and/or promote platelet activation and thrombosis. Whether changes in plasma components, in local gene expression, or in specific proteins and cells also contribute to the vascular remodeling process in this model is similarly unknown. These issues also were addressed in this study. We found that FeCl₃ accumulates at the interface between the thrombus and the vessel wall (Figure 4), resulting in endothelial denudation and loss of medial SMCs within 30 minutes. The intima, media, and adventitia appeared to be acellular at early times after injury (ie, 30 minutes, 24 hours). By 7 days, however, there was a peak in cellular proliferation (Figures 4 and 5), particularly in the adventitia, and the beginning of cell migration into the media and neointima. The vast majority of the proliferating and migrating cells exhibited characteristics of dedifferentiated SMCs (so-called synthetic phenotype). By 3 weeks, reendothelialization, repopulation of the media, and formation of a multilayered, α-actin–positive neointima had occurred, and the cellular proliferation rate in the vessel wall had returned to preinjury levels. Thus, the temporal response of cells to FeCl₃ injury in the mouse resembles that observed in the electrical and the rose bengal injury models, particularly in the adventitia, and the beginning of cell migration into the media and neointima. The vast majority of the proliferating and migrating cells exhibited characteristics of dedifferentiated SMCs (so-called synthetic phenotype). By 3 weeks, reendothelialization, repopulation of the media, and formation of a multilayered, α-actin–positive neointima had occurred, and the cellular proliferation rate in the vessel wall had returned to preinjury levels. Thus, the temporal response of cells to FeCl₃ injury in the mouse resembles that observed in the electrical and the rose bengal injury models.

Although PAI-1 gene expression was very low in the wall of uninjured mouse carotid vessels, it increased significantly early (6 hours) after injury (Figure 6), consistent with previous reports in the rat and the rabbit. In situ hybridization studies showed that at 6 hours, local PAI-1 synthesis was confined to surviving endothelial cells and SMCs in the injured vascular segment (Figure 7). By 7 days, however, dedifferentiated proliferating/migrating SMCs in the organizing thrombus and throughout the vessel wall appeared to synthesize PAI-1 (Figures 7 and 8). PAI-1 gene expression returned to background levels when arterial remodeling was complete (by 3 weeks). The spatial and temporal changes in PAI-1 mRNA and protein expression observed after injury suggest that the inhibitor may play a role in the regulation of cell migration and neointima formation in vivo. It remains to be determined, however, whether the extent or the rate of restenosis in this model differs significantly between wild-type and PAI-1–deficient mice. Accelerated neointima formation in PAI-1−/− mice was reported after electrical injury, suggesting that PAI-1 may inhibit, or at least retard, excessive thickening of the vessel wall and narrowing of the lumen during the remodeling process. The effects may be different in human atherothrombosis, however, and in models of experimental injury that induce a marked thrombotic response in the vessel (ie, the FeCl₃ model). In such instances, earlier and more complete dissolution of the thrombus in the absence of PAI-1 may remove an important stimulus for ongoing cell proliferation and migration and thus actually reduce neointimal thickening.

Finally, our observations revealed an abundance of VN antigen in the thrombus and the vessel wall over the 3-week period after FeCl₃-induced injury. The diffuse distribution pattern of VN antigen, the presence of very low levels of VN mRNA, and the absence of upregulation of VN gene expression in the thrombus and vessel wall after injury, however, suggest that the VN protein was derived from plasma and not synthesized locally. This hypothesis is consistent with other reports (see, however, a differing view). As in the case of PAI-1, further studies are necessary to confirm the role of VN in vascular remodeling in vivo. In particular, the possibility that the lack of VN may affect the rate and/or extent of neointima formation after arterial injury with FeCl₃ in the mouse needs to be tested.

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References


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