Remodeling of the Vessel Wall after Copper-Induced Injury Is Highly Attenuated in Mice with a Total Deficiency of Plasminogen Activator Inhibitor-1

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Clinical studies have indicated that high plasma levels of fibrinogen, or decreased fibrinolytic potential, are conducive to an increased risk of cardiovascular disease. Other investigations have shown that insoluble fibrin promotes atherosclerotic lesion formation by affecting smooth muscle cell proliferation, collagen deposition, and cholesterol accumulation. To directly assess the physiological impact of an imbalanced fibrinolytic system on both early and late stages of this disease, mice deficient for plasminogen activator inhibitor-1 (PAI-1^{-/-}) were used in a model of vascular injury/repair, and the resulting phenotype compared to that of wild-type (WT) mice. A copper-induced arterial injury was found to generate a lesion with characteristics similar to many of the clinical features of atherosclerosis. Fibrin deposition in the injured arterial wall at early (7 days) and late (21 days) times after copper cuff placement was prevalent in WT mice, but was greatly diminished in PAI-1^{-/-} mice. A multilayered neointima with enhanced collagen deposition was evident at day 21 in WT mice. In contrast, only diffuse fibrin was identified in the adventitial compartments of arteries from PAI-1^{-/-} mice, with no evidence of a neointima. Neovascularization was observed in the adventitia and was more extensive in WT arteries, relative to PAI-1^{-/-} arteries. Additionally, enhanced PAI-1 expression and fat deposition were seen only in the arterial walls of WT mice. The results of this study emphasize the involvement of the fibrinolytic system in vascular repair processes after injury and indicate that alterations in the fibrinolytic balance in the vessel wall have a profound effect on the development and progression of vascular lesion formation. (*Am J Pathol* 2001, 158:107-117)

Among the critical proteins that constitute the fibrinolytic system in mammals are the zymogen, plasminogen, and its activated product, plasmin, a serine protease; plas-

minogen activators, eg, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator, which are also serine proteases; receptors for these proteins, eg, the uPA receptor (uPAR); serpin-type inhibitors, eg, plasminogen activator inhibitor-1 (PAI-1); and fibrinogen/fibrin. In addition to the clot-dissolving capacity of this system, a number of *in vitro* and *in vivo* studies have implicated plasmin as playing an important role in proteolytic processes associated with cell migration, which is a pivotal event in the inflammatory response. These effects have been attributed to the ability of components of the fibrinolytic system to assemble on cell surfaces through interaction with specific receptors, such as α -enolase for plasminogen¹⁻³ and uPAR for uPA.⁴ These functions are supported by *in vitro* studies that have identified uPA and uPAR at the leading migratory edges of monocytes and smooth muscle cells.^{5,6} In addition to the involvement of components of the fibrinolytic system in facilitating cell migration through matrix-degrading processes,⁷⁻⁹ these agents have been shown to influence normal and pathological cell migratory events by release and activation of a number of inflammatory mediators,¹⁰⁻¹² as well as by chemotactic processes,¹³⁻¹⁵ integrin-mediated signaling,¹⁶ and other potentially novel mechanisms.¹⁷ Plasmin has also been shown to act directly as a chemoattractant for human peripheral monocytes.¹⁸ Alternatively, PAI-1 has also been implicated in cell migration through its ability to disrupt uPA/uPAR-matrix protein interactions, thus facilitating cell detachment.¹⁹ Indeed, macrophage colony-stimulating factor (or CSF-1) and granulocyte-macrophage colony-stimulating factor have been shown to increase both PAI-1 and PAI-2 expression in human monocytes,²⁰ potentially implicating these proteins in processes involved in inflammation and tissue remodeling.²¹

Atherosclerosis is a chronic inflammatory disease in which the fibrinolytic system plays a major role. For example, several studies have indicated that high plasma

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levels of fibrinogen and decreased fibrinolytic activity, ie, increased PAI-1, lead to an increased risk for cardiovascular disease.^{22,23} Additionally, components of the fibrinolytic system have been identified in atherosclerotic lesion tissue.^{24,25} Other studies have indicated that insoluble fibrin may promote atherosclerotic lesion formation by affecting smooth muscle cell proliferation and migration, collagen deposition, and cholesterol accumulation.²⁶

The generation of mice deficient for components of the fibrinolytic system has resulted in the development of valuable resources for directly assessing the physiological impact of an imbalanced fibrinolytic system on both early and late stages of a number of inflammation-based diseases. Diminished inflammatory responses have been identified in uPA-deficient (UPA^{-/-}) and plasminogen-deficient (PG^{-/-}) mice challenged with a number of different agents.²⁷⁻²⁹

Murine models for vascular injury/repair are extremely valuable for the study of early and late stage inflammatory disease because the role of genetic factors in inflammation can be investigated effectively using gene-targeted animals. In the current study, a copper-induced model of inflammation has been characterized in WT mice, and applied to mice deficient in the *PAI-1* gene (PAI-1^{-/-}). This model is based on the finding that increased plasma levels of copper have been associated with cardiovascular disease.^{30,31} The results of this study are presented herein.

Materials and Methods

Copper/Silicone Cuff Construction

Prosthetic silicone elastomer, MDX4-4210, (Factor II, Inc., Lakeside, AZ) and copper powder, spherical, -100 + 325 mesh, (Alfa Aesar, Ward Hill, MA) were used to construct the cuffs, which have an inside diameter of 0.028" and an outside diameter of 0.062". The construction was a major modification of a similar cuff used in rats.³² The two-part silicone product was combined with the copper dust and then degassed under vacuum. Stainless steel molds, which mimic the size of the diameter of a mouse carotid artery, were used in the construction. The copper/silicone mixture was spread over both halves of the mold, after which stainless steel rods (0.028" outside diameter/22 gauge) were placed in each of the 15 slots of the mold (0.042" outside diameter of the complete mold slot). The mold halves were combined, pressed closed, and baked at 80°C for ~8 hours. The copper/silicone-coated rods were then inserted in one-half of a larger mold (0.062" outside diameter of the complete mold slot), coated with silicone alone, pressed closed with the other one-half of the similarly coated mold, and then baked as described above.

Placement of Copper/Silicone Cuff around the Artery

Male and female C57 BL/6J wild-type (WT) mice (8 to 12 weeks of age) and mice totally deficient for the PAI-1

gene (PAI-1^{-/-}) were used in this study. All animals were maintained in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and experimental protocols were approved by the Institutional Animal Care and Use Committee.

The left carotid artery of mice anesthetized with an intraperitoneal injection of rodent cocktail (0.015 mg xylazine/0.075 mg ketamine/0.0025 mg acepromazine/g weight of animal) was surgically exposed via a midline incision over an area from the chin to the sternum that had been sterilized with a 1% iodine solution. The salivary gland was separated and the artery dissected proximal to the bifurcation. A copper/silicone cuff, 1 to 1.5 mm in length, was placed around the periphery of the artery proximal to the bifurcation. The surgical site was then closed with a 6-0 nylon suture and the mice allowed to recover. At 7- and 21-days after implantation, the mice were again anesthetized and the left carotid artery re-exposed to remove the cuffed artery. The contralateral right artery served as a negative control.

Histochemistry

Arteries were either fixed in 10% neutral-buffered formalin for processing and paraffin embedding or immersed in 20% sucrose and then frozen in Tissue-Tek OCT (Sakura Fine Tek Co., Torrance, CA) compound for cryosectioning. Paraffin-embedded arteries were sectioned between 3 μ m and 4 μ m. Hematoxylin 2 and eosin Y (H&E; Richard Allen Scientific, Kalamazoo, MI) stains were performed to assess cellular morphology. Masson's trichrome stain³³ was used to identify collagen in the arterial wall and Verhoeff's Van Gieson staining³⁴ was used to identify elastica laminae and to perform morphometric measurements. Cryotomy sections (8 μ m) were used for Oil Red O staining to identify fat deposits in the injured arteries.³⁵

Immunohistochemistry

A polyclonal goat anti-mouse fibrin(ogen) antibody (Accurate Chemicals, Westbury, NY) was used for immunohistochemical identification of fibrin. The slides were incubated with rabbit serum and then with the anti-fibrin antibody, followed by secondary rabbit anti-goat IgG (DAKO, Carpinteria, CA) and goat peroxidase anti-peroxidase (DAKO). Peroxidase activity was detected with the substrate, 3-amino,9-ethylcarbazole (AEC) (Biomedex, Foster City, CA). A hematoxylin counterstain (Biomedex) was used for all immunohistochemistry. Antigen retrieval was performed under high temperature and pressure with citrate buffer, pH 6.0 (Zymed, South San Francisco, CA), followed by endogenous peroxidase blocking with Peroxoblock (Zymed).

CD45 rat anti-mouse monoclonal antibody (Pharmin-gen, San Diego, CA) was used to identify leukocytes. The secondary antibody consisted of a biotin-conjugated goat anti-rat antibody (DAKO), which was followed by a solution of streptavidin-conjugated horseradish peroxidase (Biogenex). Detection with AEC, antigen retrieval

with citrate buffer, and blocking of endogenous peroxidase were as above. A similar procedure was used for detection of smooth muscle cells with an anti- α -actin monoclonal antibody (Sigma Chemical Co., St. Louis, MO), except that antigen retrieval was accomplished with a limited trypsin treatment.

Immunohistochemical identification of von Willebrand factor (vWF)-positive cell types (endothelial cells, megakaryocytes, and platelets) was accomplished using an EPOS anti-human vWF antibody conjugated to peroxidase (DAKO). Development was accomplished with AEC. Antigen retrieval was performed with limited trypsin digestion. Blocking of endogenous peroxidase was as above.

PAI-1 expression in the vessel wall was evaluated by immunohistochemistry using a rabbit anti-murine PAI-1 polyclonal antibody (Molecular Innovations, Inc., Royal Oak, MI). The second antibody was biotin-conjugated porcine anti-rabbit IgG (DAKO), after which streptavidin-conjugated horseradish peroxidase (Biogenex) was added. Antigen retrieval with citrate buffer, detection with AEC, and blocking of endogenous peroxidase were as above.

Cell Proliferation

Mice were injected intraperitoneally with 50 mg/kg bromodeoxyuridine (BrdU) in physiological saline at times of 24, 16, and 1 hour before sacrificing. A mouse monoclonal anti-human antibody to BrdU was used to identify proliferating cells in the vascular wall. The secondary antibody consisted of a biotin-conjugated rabbit anti-mouse IgG antibody (DAKO), after which was added streptavidin-conjugated horseradish peroxidase, followed by development with AEC. Antigen retrieval was accomplished by incubation in 1 mol/L HCl at 37°C for 10 minutes followed by limited trypsin digestion. Endogenous peroxidase activity was blocked with Peroxoblock. Total cells in the vascular wall compartments were counted and the percentage of BrdU-positive cells was determined for equally spaced sections within the cuffed area of the artery. Five sections per artery, separated by $\sim 50 \mu\text{m}$ /section, of 200 to 300 μm length of injured artery were analyzed. The average values per artery for each genotype were used to determine the mean $\pm$ SEM.

Morphometric Analyses

Morphometric measurements of cross-sectional areas were performed on transverse sections of the artery using a computer-assisted image analysis system (Bioquant True Color Windows software; Biometrics, Nashville, TN) on equally spaced sections within the cuffed area of the artery. The number of sections and length of injured artery analyzed were similar to that described for cell proliferation analyses. The average values per artery for each genotype were used to determine the mean $\pm$ SEM.

Neovascularization

Neovascularization was determined by counting the number of vWF-positive vessels in the adventitial compartment of injured arteries and expressed as the number of vessels per mm^2 area. Counts were made over equally spaced sections (4 to 5 fields/vessel) within the cuffed area of the artery and average values per artery for each genotype were used to determine the mean $\pm$ SEM.

Electron Microscopy

Ultrastructural analyses were performed on 3-day injured arteries. The arteries were perfused and fixed with Karnovsky solution³⁶ and postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanol solutions, and embedded in epoxy resins (Polysciences, Warrington, PA). Ultrathin sections (90 nm) were cut and stained in 2% uranyl acetate and Reynolds lead stain.³⁷ Sections were viewed and photographed using a transmission electron microscope (Hitachi H 600; Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.

Statistical Analysis

Where appropriate, values were expressed as mean $\pm$ SEM. Comparisons were made using Student's *t*-test and *P* values < 0.05 were considered significant.

Results

Day 7 Analyses

H&E stains of WT carotid arteries 7 days after injury demonstrated an increase in cellularity and reactivity in medial and adventitial compartments relative to uninjured arteries, with evidence of a small neointima (Figure 1A). Intimal, medial, and adventitial compartments were significantly increased in size in arteries from WT mice relative to arteries from PAI-1^{-/-} mice ($0.011 \pm 0.001 \text{ mm}^2$ versus $0.004 \pm 0.0004 \text{ mm}^2$, $P < 0.001$, respectively, for the intimal compartment; $0.046 \pm 0.004 \text{ mm}^2$ versus $0.020 \pm 0.001 \text{ mm}^2$, $P = 0.003$, respectively, for the medial compartment; and $0.108 \pm 0.009 \text{ mm}^2$ versus $0.075 \pm 0.008 \text{ mm}^2$, $P = 0.021$, respectively, for the adventitial compartment; Table 1). Despite the smaller adventitia in arteries from PAI-1^{-/-} mice relative to WT mice, the proliferative index, as measured by BrdU uptake, was significantly increased in the adventitia in arteries from PAI-1^{-/-} mice compared to arteries from WT mice ($16.7 \pm 3.3\%$ versus $3.5 \pm 1.9\%$, $P = 0.0269$, respectively; Table 2). Medial compartments in arteries from both WT and PAI-1^{-/-} mice exhibited a smooth muscle cell-enriched region (Figure 1, C and D). The neointima in WT-injured arteries consisted primarily of leukocytes (Figure 1, E and F). Additionally, fibrin deposits were significantly enhanced in the medial and adventitial compartments (Figure 1G). This is in sharp contrast to that observed in injured arteries from PAI-1^{-/-} mice, wherein no fibrin was observed in the vessel wall (Figure 1H).

Figure 1. Histological analysis of carotid arteries from WT and PAI-1^{-/-} mice 7 days after implantation of the copper cuff. **A:** H&E stain of WT carotid artery demonstrates a reactive adventitial (asterisk) and medial (arrow) compartments with the presence of a small neointima (arrowhead). Original magnification, ×200. Similar H&E staining of a carotid artery from a PAI-1^{-/-} mouse (**B**) shows a quiescent state of these vascular compartments. Original magnification, ×200. **C:** Anti-α-actin immunostain of a WT carotid artery demonstrates enhanced smooth muscle cells in the medial compartment (arrow) (original magnification, ×200), whereas a PAI-1^{-/-} carotid artery (**D**) indicates no apparent change in the smooth muscle cell population in the medial compartment. Original magnification, ×200. **E:** CD45 immunostain of WT carotid artery demonstrates a leukocyte-enriched adventitia (arrow). Original magnification, ×200. **F:** CD45 immunostain of WT carotid artery demonstrates leukocytes within the neointima (arrow). Original magnification, ×400. **G:** Anti-fibrin(ogen) immunostain of WT carotid artery shows fibrin-rich medial (arrow) and adventitial (asterisk) compartments (original magnification, ×200), whereas similar immunostaining of a PAI-1^{-/-} carotid artery (**H**) demonstrates no apparent fibrin deposition in the vessel wall. Original magnification, ×200.

Table 1. Morphometric Analyses of Carotid Arteries from WT and PAI-1^{-/-} Mice

Time point	Lumen	Intima	Media	Adventitia
DAY 7 (mm ²)*				
WT (n = 6)	0.057 ± 0.006	0.011 ± 0.001	0.046 ± 0.004	0.108 ± 0.009
PAI-1 ^{-/-} (n = 6)	0.059 ± 0.008	0.004 ± 0.0004	0.020 ± 0.001	0.075 ± 0.008
P values	0.846	<0.001	0.003	0.021
DAY 21 (mm ²)*				
WT (n = 4)	0.042 ± 0.013	0.036 ± 0.005	0.063 ± 0.006	0.087 ± 0.009
PAI-1 ^{-/-} (n = 8)	0.023 ± 0.005	0.007 ± 0.002	0.024 ± 0.004	0.182 ± 0.020
P values	0.125	<0.0001	0.0003	0.0093

*P value comparisons between days 7 and 21, within each genotype, indicated that statistically significant differences ($P < 0.05$) were observed in the intimal and medial compartments in WT mice. Luminal and adventitial compartments were not significantly altered. In PAI-1^{-/-} mice significant differences were observed in the lumen, medial, and adventitial but not in the intimal compartment of the vessel.

Day 21 Analyses

H&E stains of carotid arteries from WT mice 21 days after injury revealed an enlarged multilayered neointima (0.036 ± 0.005 mm² for day 21 versus 0.011 ± 0.001 mm² for day 7, $P = 0.0003$; Figure 2A and Table 1), consisting of smooth muscle cells that appeared to be primarily confined to the luminal edges of the neointima (Figure 2C). These arteries also presented enhanced collagen deposition (Figure 2E). WT neointima was also significantly larger than PAI-1^{-/-} neointima at day 21 (0.036 ± 0.005 mm² versus 0.007 ± 0.002 mm², $P < 0.0001$, respectively; Table 1). Cell proliferation in the medial compartment of arteries from WT mice compared to PAI-1^{-/-} arteries were also enhanced ($10.1 \pm 3.9\%$ versus $2.3 \pm 0.3\%$, $P = 0.1168$, respectively; Table 2). This is consistent with a lack of neointima formation in arteries from PAI-1^{-/-} mice, which is derived primarily from proliferating smooth muscle cells from the medial compartment (Figure 2, B, D, and F). Although there did not seem to be any degradation of the elastica laminae in injured arteries from WT mice, as visualized by Verhoeff's Van Gieson staining, stretching and thinning were evident (Figure 3A). In contrast, the elastica laminae appeared unaffected in injured arteries from PAI-1^{-/-} mice (Figure 3B). Immunostaining of vWF in carotid arteries from both WT and PAI-1^{-/-} mice indicated the presence of a normal intact contiguous single-layer intima of endothelial cells adjacent to the lumen of the vessel (Figure 3, C and D). Fibrin deposits in the injured WT arteries remained unresolved, primarily in the adventitial compartment (Figure 4A), most likely because of continued injury to the vessel wall by copper. Injured arteries from PAI-1^{-/-} mice demonstrated little, if any, fibrin in the vessel wall (Figure 4B). Enhanced PAI-1 expression was also

evident in carotid arteries from WT mice (Figure 4C) in the same vascular compartments that fibrin deposits were found, but not in the uninjured contralateral artery (Figure 4D). Fat deposition in the arterial wall, most likely because of copper ion-induced oxidation of low-density lipoprotein (LDL), was evident in arteries from WT mice, primarily confined to the medial layer adjacent to the elastic lamina (Figure 4E), but not in arteries from PAI-1^{-/-} mice (Figure 4F). At day 21, neovascularization in the adventitia was more evident in injured arteries from WT mice than in PAI-1^{-/-} mice (159 ± 6 /mm² versus 105 ± 8 /mm² vessels, $P = 0.0057$; Figure 5). Neovascularization of the adventitial compartment in PAI-1^{-/-} arteries was relatively unchanged in day 21 arteries compared to those analyzed at day 7 (105 ± 8 /mm² versus 106 ± 5 /mm², respectively).

TEM Analyses

To investigate the effects of a perivascular source of injury on the luminal side of the vessel, electron microscopic analyses were performed 3 days after cuff placement. Although injury was induced from the adventitial side of the vessel, inflammatory cell adhesion and trans-endothelial migration from the lumen were already evident 3 days after injury (Figure 6, A and B). Similar changes were observed in injured arteries from PAI-1^{-/-} mice (Figure 6, C and D). This indicates that the response to injury was also occurring from the luminal side of the vessel as early as a few days after perivascular cuff placement.

Discussion

This study demonstrates that copper-induced injury of murine carotid arteries results in an accelerated progression of pathological events associated with the development of atherosclerotic-like lesions. Clinically, high serum-copper levels have been shown to be associated with increased cardiovascular mortality.³⁰ Other studies have implicated copper-dependent interactions in the atherogenicity of homocysteine,³⁸ an amino acid that exerts its effect through oxidative damage, most probably lipid peroxidation of LDL (OxLDL).³⁹ Lipid peroxidation products are chemotactic for monocytes and T cells, and OxLDL-mediated events lead to the transformation of

Table 2. Percent BrdU(+) Cells in Vessel Wall in WT and PAI-1^{-/-} Arteries

Time point	Intima	Media	Adventitia
DAY 7			
WT (n = 3)	18.5 ± 7.5	6.3 ± 2.1	3.5 ± 1.9
PAI-1 ^{-/-} (n = 3)	10.1 ± 4.1	5.4 ± 2.2	16.7 ± 3.3
P value	N.S.	N.S.	0.0269
DAY 21			
WT (n = 3)	5.9 ± 2.3	10.1 ± 3.9	10.6 ± 3.3
PAI-1 ^{-/-} (n = 3)	3.7 ± 0.9	2.3 ± 0.3	14.4 ± 3.8
P value	N.S.	0.1168	N.S.

Figure 2. Histological analysis of carotid arteries from WT and PAI-1^{-/-} mice 21 days after implantation of the copper cuff. **A:** H&E stain of a WT carotid artery illustrates presence of a multilayered neointima (**arrow**) (original magnification, ×200), whereas similar staining of a PAI-1^{-/-} carotid artery (**B**) demonstrates a lack of neointima formation, with some reactivity in the adventitial compartment (**arrow**) (original magnification, ×200). **C:** Anti-α-actin immunostain of WT carotid artery illustrates a smooth muscle cell-rich luminal side of the neointima (**arrow**) (original magnification, ×200), whereas equivalent immunostaining of a PAI-1^{-/-} carotid artery (**D**) demonstrates the presence of smooth muscle cells primarily confined to the medial compartment (original magnification, ×200). **E:** Masson's trichrome stain for collagen (blue) in a WT carotid artery illustrates a collagen-rich neointima (**arrow**) (original magnification, ×200), whereas a PAI-1^{-/-} carotid artery (**F**) indicates that collagen is primarily confined to the medial (**arrow**) and adventitial compartments (original magnification, ×200).

Figure 3. Histological analysis of carotid arteries from WT and PAI-1^{-/-} mice 21 days after implantation of the copper cuff. Verhoeff's Van Gieson stain (black) of WT carotid artery (**A**) shows a thinning and stretching of the elastica laminae (**arrow**) (original magnification, $\times 200$), whereas a PAI-1^{-/-} carotid artery (**B**) presents a normal thick-layered elastica laminae (**arrow**) (original magnification, $\times 200$). **C:** Anti-vWF immunostain of endothelial cells in a WT carotid artery demonstrates a single endothelial layer of the luminal side of the neointima (**arrowhead**) with evidence of neovascularization in the adventitial compartment (**arrow**) (original magnification, $\times 200$). **D:** Anti-vWF immunostain of endothelial cells in PAI-1^{-/-} carotid artery demonstrates a single layer endothelial intima (**arrow**) (original magnification, $\times 200$).

monocytes into macrophages with subsequent uptake of OxLDL via scavenger receptors, resulting in the formation of fatty streaks.⁴⁰ Other atherogenic effects of OxLDL contribute to later stages of atherosclerosis.^{41,42} OxLDL has been shown to increase the expression of PAI-1 in vascular smooth muscle cells and endothelial cells.^{43,44} Indeed, expression of PAI-1 is increased in the atherosclerotic vessel wall.⁴⁵ Additionally, lipid-enriched macrophages demonstrate increased expression of PAI-1 and the procoagulant protein, tissue factor.⁴⁶ Clinically, it has been shown that atheromatous plaque macrophages from patients produce PAI-1 and stimulate its production by endothelial cells and vascular smooth muscle cells.⁴⁷ These multiple effects indicate that OxLDL, originating through copper-dependent mechanisms, may indirectly contribute to the thrombotic phenotype associated with atherosclerosis.

A number of animal models have evolved that attempt to differentiate among specific mechanisms involved in atherosclerotic lesion development.⁴⁸ Transgenic mice have been developed in which genes that regulate the atherosclerotic process have been altered, ie, apo E and LDL receptor (LDLR).^{49,50} However, because of the complexity of human atherosclerotic development, the relevance of the mouse model has been questioned. Despite this, the mouse has become increasingly useful for studying atherosclerosis and its risk factors. For example, this animal presents a classic model for genetic studies because inbred strains and complete linkage maps are available. Additionally, a number of strains exhibiting genetic variations relevant to atherosclerosis have been identified and have provided an opportunity to examine the involvement of candidate genes. Finally, techniques

Figure 4. Histological analysis of carotid arteries from WT and PAI-1^{-/-} mice 21 days after implantation of the copper cuff. **A:** Fibrin immunostain of a WT carotid artery illustrates fibrin deposits that remain unresolved in the medial (**asterisk**) and adventitial (**arrow**) compartments (original magnification, $\times 200$). **B:** Fibrin immunostain of a PAI-1^{-/-} carotid artery (original magnification, $\times 200$) demonstrates only small amounts of fibrin in the adventitial compartment (**arrow**). **C:** PAI-1 immunostain of a WT carotid artery shows enhanced PAI-1 expression in the medial (**asterisk**) and adventitial (**arrows**) compartment compared to the (**D**) contralateral uninjured artery (original magnification, $\times 200$). **E:** Oil Red O stain illustrating the presence of lipid (**arrows**) in a WT carotid artery (original magnification, $\times 400$), whereas similar staining of a PAI-1^{-/-} carotid artery (**F**) shows a lack of lipid accumulation (original magnification, $\times 400$).

Figure 5. Vessel counts per mm² area of the adventitial compartment of arteries from WT and PAI-1^{-/-} mice 7 and 21 days after perivascular placement of the cuff. WT *versus* PAI-1^{-/-} at day 21, $P = 0.0057$.

for genetic manipulation *in vivo* are more advanced in the mouse than in other animals.

In the current study, copper-induced oxidative damage to the vascular wall resulted in accelerated neointima

formation, matrix protein and lipid deposition, and neovascularization; all clinical features of atherosclerosis. It has been indicated that neovascularization of the vessel wall may be an important feature of early atherosclerosis development and could potentially serve as an alternative means for transport of leukocytes and lipid into the vessel wall.⁵¹ Interestingly, in this study fibrin deposits persisted throughout the progression of lesion formation and were major components of the diseased artery. Indeed, fibrin and fibrin degradation products have been shown to stimulate the migration of smooth muscle cells and, therefore, may contribute directly to neointima formation.^{26,52} Advanced human atherosclerotic lesions also contain significant amounts of fibrin and, therefore, this model is ideal for studying the thrombotic response associated with the progression of this disease.⁵³

Because clinical studies have shown that increased plasma PAI-1 levels are conducive to the development of cardiovascular disease,^{22,23} a number of *in vivo* investigations have been undertaken to assess the effects of PAI-1 on vascular repair processes. A murine model of pulmonary embolism showed an accelerated clot lysis

Figure 6. Transmission electron microscopy of carotid arteries of WT and PAI-1^{-/-} mice 3 days after cuff implantation. **A:** A neutrophil with vesicular transport activity (arrow) is seen attached to the luminal endothelium in WT carotid artery close to a cellular junction (asterisk) (original magnification, $\times 11,500$). **B:** A polymorphonuclear leukocyte (arrow) is present in an open subendothelial space in WT carotid artery (original magnification, $\times 6,200$). **A** and **B** suggest that a diapedesis process is occurring. **C:** Altered cellular morphology of an endothelial cell (E) is evident in PAI-1^{-/-} carotid artery, the result of copper-induced injury (original magnification, $\times 6,800$). **D:** Activated macrophages with visible phagolysosomes (P) are evident in the medial compartment of a PAI-1^{-/-} carotid artery (original magnification, $\times 2,700$).

response to a preformed thrombus in PAI-1^{-/-} mice.⁵⁴ Additionally, these mice demonstrated delayed effects on the development of occlusive arterial and venous thrombosis after photochemical injury.⁵⁵ Together, these studies indicate that an imbalance in the expression of fibrinolytic components dictate the extent and severity of vascular thrombosis.

Although alterations in plasma PAI-1 levels could have a profound effect on the extent of luminal thrombosis, localized arterial wall responses to thrombosis could influence PAI-1 expression in endothelial and vascular smooth muscle cells⁵⁶ and lead to an antifibrinolytic environment with resultant arterial wall fibrin deposition and eventual neointima formation. Indeed, PAI-1 levels were enhanced in the vessel wall of WT animals. However, the effect of PAI-1 on neointima thickening remains controversial. A study using transfected smooth muscle cells that overexpress PAI-1 seeded on denuded rat carotid arteries led to enhanced neointimal thrombosis, but reduced neointima thickening.⁵⁷ Additionally, in an electrical injury model, PAI-1 inhibited arterial neointima formation.⁵⁸ Another investigation using mice either deficient in, or overexpressing, PAI-1, with a combined deficiency of apoE or LDLR, and challenged with a high-fat diet, concluded that PAI-1 did not influence the development or characteristics of atherosclerotic lesions in the dietary-challenged apoE^{-/-} or LDLR^{-/-} mice.⁵⁹ This latter study, although differing from the results reported herein, relies on a simple pathophysiological effect, namely, lipid deposition, to drive this disease. On the other hand, the copper cuff model detailed herein relies on oxidative injury events that result in fatty streak formation as well as deposition of fibrin in the vascular wall. These features are apparent in advanced complex lesions observed in humans. Because of the thrombotic response to injury featured in this model, the effects of alterations in endogenous fibrinolysis on the progress of oxidative-induced lesion formation can be assessed.

In conclusion, the data obtained in this investigation show that enhanced fibrinolytic potential, because of the absence of PAI-1, results in attenuated thrombotic lesion development, with resultant lack of neointima formation. We suggest that a lack of vascular wall fatty streak formation in PAI-1^{-/-} mice is a consequence of limited leukocyte accumulation in the vessel wall possibly due in part to diminished neovascularization and that an imbalance in the spatial expressions of components of the fibrinolytic system, within the vascular wall compartments, could have a profound effect on the progression and persistence of vascular lesions. Thus, the expression of fibrin-containing lesions within the vessel wall of challenged WT animals, which result from a prothrombotic environment driven by increased expression of plasminogen activator inhibitors and procoagulant factors, culminates in the development of occlusive vascular lesions. This study clearly demonstrates that deficiencies in PAI-1 expression alter the development and progression of vascular lesions by affecting the local hemostatic environment of the vessel wall.

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