Plasminogen Activator Inhibitor Type 1 Increases Neointima Formation in Balloon-Injured Rat Carotid Arteries

Mary Beth DeYoung, PhD; Clifford Tom, BS; David A. Dichek, MD

Background—Elevated plasma levels of plasminogen activator inhibitor type 1 (PAI-1) are associated with myocardial infarction, atherosclerosis, and restenosis. PAI-1 is increased in atherosclerotic arteries and failed vein grafts. No experimental data, however, support a causal relationship between elevated PAI-1 expression and vascular lesions. Paradoxically, data generated in PAI-1 knockout mice suggest that PAI-1 might decrease lesion formation after arterial injury and that PAI-1 gene transfer might prevent restenosis.

Methods and Results—Using the rat carotid balloon injury model and a PAI-1-expressing adenoviral vector, we tested whether elevated arterial PAI-1 expression would alter neointima formation. Compared with control-transduced arteries, neointima formation in PAI-1-transduced arteries was initially retarded. By 14 days, however, the intimas of PAI-1-transduced arteries were significantly larger than intimas of control-transduced arteries (1.6±0.1×10^5 versus 1.2±0.1×10^5 μm^2, n=18 to 19, P<0.03). PAI-1 expression in individual arteries correlated with increased cell proliferation at 4 and 8 days after injury (R=0.6, P<0.02 and P<0.006). PAI-1 expression also correlated with fibrin(ogen) accumulation (R=0.77, P<0.001), and fibrin(ogen) accumulation correlated strongly with proliferation (R=0.86, P<0.00001).

Conclusions—Increased expression of PAI-1 in the artery wall promotes neointima growth after balloon injury. Therefore, despite encouraging data generated in other animal models, PAI-1 is not a promising agent for gene therapy to prevent restenosis. Moreover, our data associate elevated PAI-1 expression with fibrin(ogen) accumulation and increased cell proliferation. These data suggest a mechanism to explain the association between elevated PAI-1 expression and the progression of arterial disease. (Circulation. 2001;104:1972-1977.)

Key Words: fibrinogen • gene therapy • restenosis • viruses • plasminogen activators

Increased plasma levels of plasminogen activator inhibitor type 1 (PAI-1) are associated with myocardial infarction, atherosclerosis, and restenosis.1–4 PAI-1 is elevated in atherosclerotic human arteries and failed vein grafts.5–7 In animal models, arterial wall PAI-1 expression is upregulated after injury and is elevated during neointima formation.8,9 Despite the association between PAI-1 expression and poor cardiovascular outcomes, a causal relationship between PAI-1 expression and vascular disease has not been established.

Several groups have used animal models of vascular disease to test, prospectively, whether elevated PAI-1 expression promotes lesion development. In a mouse model of arterial injury, neointima formation was accelerated in the absence of PAI-1 and was retarded by increased systemic levels of PAI-1.10 Similarly, implantation of smooth muscle cells genetically engineered to overexpress PAI-1 transiently limited neointimal growth in rats.11 Experimental manipulation of PAI-1 expression in atherosclerosis-prone mice by gene deletion or systemic overexpression, however, did not affect lesion development.12 Thus, no prospective study has linked elevated PAI-1 expression with the development of vascular lesions. Indeed, the first 2 reports suggested that PAI-1 could retard lesion formation and prompted speculation that PAI-1 might be used as a gene therapy for restenosis.10,11,13

We previously developed an adenoviral vector that increases PAI-1 expression in balloon-injured rat carotid arteries by ~60%.9 This physiological increase in PAI-1 expression is within the range of elevated PAI-1 expression in diseased human arteries.7 Here, we report experiments that test whether elevation of arterial PAI-1 expression with this vector alters neointimal development after balloon injury.

Methods

Adenoviral Vectors

We used 2 E1/E3-deleted adenoviral vectors: AdCMVrPAI-1 (AdPAI-1),9 which expresses a rat PAI-1 cDNA14 from a cytomegalovirus promoter, and AdNull, an essentially identical vector that lacks a transgene expression cassette.15 Three independent preparations of AdPAI-1 and AdNull were used. Titors of viral stocks ranged from 2×10^10 to 2×10^11 plaque-forming units (pfu)/mL, with...
particle-to-plaque ratios of 40 to 300. Both vectors were infused at 2×10^6 to 5×10^6 pfu/mL. The number of pfu infused for the 2 vectors was identical. With this approach, the number of particles infused could be matched only within 2- to 3-fold; however, particles of AdNull either were matched or were in excess of particles of AdPAI-1. The absence of E1A-containing viruses at a concentration exceeding 1 in 10^3 viral genomes was confirmed.\

### In Vivo Transduction and Harvest of Rat Carotid Arteries

Balloon injury, gene transfer, perfusion-fixation, and harvest and embedding of arteries in paraffin were performed as described, except that zinc formalin (Anatech) was used as a fixative.

### Morphometric Analysis

The transduced artery segment most distant from the arteriotomy was analyzed morphometrically. Two sections from this segment, ∼3 mm apart, were stained with Movat’s pentachrome, and intimal and medial areas were measured with computer-assisted planimetry. The mean intimal and medial areas of the 2 sections were calculated for each artery. In 14-day arteries, intimal cell density was measured by counting nuclei in 4 high-power fields in each of 2 sections. The mean density was then calculated for the artery, and the number of intimal cells was calculated by multiplying intimal area by cell density. In 4-day arteries, cells in the intima and first medial layer were counted directly. Measurements were made by an observer blinded to the treatment.

### Measurement of Cell Proliferation

To measure cell proliferation 4 and 8 days after gene transfer, bromodeoxyuridine (BrdU) pellets (50 mg per 350- to 400-g rat) were implanted subcutaneously 24 hours before artery harvest. BrdU incorporation was detected with immunohistochemistry (B20a, 1:50 dilution; Dako). To measure cell proliferation 14 days after gene transfer, sections of harvested arteries were stained with a mouse monoclonal antibody against proliferating cell nuclear antigen (PCNA, 1:40 dilution, Santa Cruz Biotechnology). In both cases, isotype-matched control antibodies did not produce staining. In control experiments performed on 4-day arteries, we analyzed the relationship between BrdU and PCNA staining. The numbers of BrdU- and PCNA-positive cells from serial sections were similar and positively correlated (R = 0.62, P < 0.01). Proliferative indices were calculated from the mean of results obtained by staining 2 sections per artery: BrdU- or PCNA-positive cells per unit area/total cells per unit area × 100. The proliferative index of the intima was calculated from measurements made throughout the intima. Because medial proliferation occurred predominantly in the inner medial layer (≈80% of total medial proliferation), however, we measured medial proliferation only in this layer.

### Immunohistochemistry

We previously reported that infusion of AdPAI-1 at the concentration used in this study increased PAI-1 mRNA and protein expression by ≈60%. In the present study, to permit correlational analysis of PAI-1 expression, fibrin(ogen) accumulation, and cell proliferation in serial sections of individual arteries, we measured PAI-1 expression by staining tissue sections with an antibody against human PAI-1 (No. 3785, 1:25 dilution, American Diagnostica). A thrombosed artery was used as a positive control and an uninjured artery as a negative control. Binding of the PAI-1 antibody was inhibited by preincubation with excess PAI-1 (Molecular Innovations). To detect accumulation of fibrin or fibrinogen, we stained sections with an antisera against human fibrinogen (A0080, 1:1000 dilution; Dako). This antibody reacts with fibrin, fibrin degradation products, and fibrinogen fragments D and E. Sections of a thrombosed artery were used as positive controls, and sections of an aorta from a fibrinogen-knockout mouse (gift of Dr J. Degen, University of Cincinnati) were used as negative controls. Negative control antibodies included an isotype-matched antibody for PAI-1 staining (X0931; Dako) and rabbit IgG (Sigma) for fibrinogen staining. Our confidence in using immunohistochemistry to quantify antigen abundance was based on several factors, including (1) positive experience in our previous studies, (2) positive and negative controls that give anticipated results, (3) presence of a range of positive signals that were reproducible on different days, and (4) presence of biologically plausible staining patterns.

### Quantification of Immunohistochemical Staining

Areas of tissue sections that stained positively for PAI-1 or fibrinogen were measured with image-processing tool kit (IPTK) filters (Reindeer Games) and Adobe Photoshop 5.5. Stained sections were photographed with a digital camera, and intensity of immunostaining was quantified. Threshold intensity was based on the intensity range in an individual experiment. Repeated intensity measurements from individual images were reproducible within 10%. Repeated intensity measurements from serial sections stained on different days were highly correlated (R = 0.62; P < 0.006). Concentrations of medial PAI-1 were calculated as the area of media in which staining intensity exceeded threshold/cell number in the inner medial layer.

### Statistical Analysis

Results are reported as mean ± SEM or, for data not normally distributed, as median and range. The significance of intergroup differences was determined with the unpaired t test or, for data not normally distributed, the Mann-Whitney rank-sum test. The strength and significance of correlations between variables were determined by Pearson product-moment correlation. The SigmaStat program (Jandel Scientific) was used for statistical calculations.

### Results

#### PAI-1 Expression and Neointima Formation

We previously reported extensive experiments with 4 PAI-1-expressing adenoviral vectors. These studies confirmed that AdPAI-1 (the highest-expressing vector) achieves a modest increase in PAI-1 expression (≈60%) in balloon-injured rat carotid arteries. After arterial gene transfer, expression of vector-derived PAI-1 mRNA in carotid arteries is elevated at 2 to 3 days, diminished at 4 days, and undetectable at 8 days (Reference 9 and data not shown).

We measured neointima formation 14 days after infusion of AdPAI-1 or AdNull. Neointimas were ≈75% larger in arteries transduced with AdPAI-1 (1.4±0.1×10^5 versus 1.2±0.3×10^5 μm^2; n=8 to 9; Figure 1A); however, this difference did not achieve statistical significance (P = 0.1). We repeated the study with a second group of animals and again found larger neointimas in AdPAI-1–transduced arteries (1.8±0.1 versus 1.4±0.1 μm^2; n=9 to 10, P<0.01; Figure 1B). Combined data from the 2 experiments confirmed a 33% increase in neointima formation in 14-day AdPAI-1 arteries: 1.6±0.1×10^5 versus 1.2±0.1×10^5 μm^2, n=18 to 19, P<0.03; Figure 1C). The medial areas of the 2 groups were not significantly different (1.6±0.1 μm^2 for AdPAI-1 versus 1.4±0.1 μm^2 for AdNull, n=18 to 19, P = 0.1).

To identify whether cellular or extracellular components contributed to the increased neointimal growth at 14 days, we counted cells and measured cell densities in the intimas of the 14-day arteries. Intimal cell number was significantly increased in AdPAI-1 arteries (1500±100 versus 1100±100 for AdNull, n=18 to 19, P<0.05). Intimal cell density tended to be lower in AdPAI-1 arteries (9.7±0.5×10^3 versus 11.0±0.5×10^3 cells/μm^2, n=18 to 19, P = 0.1).

We next examined neointimal growth in arteries harvested 4 and 8 days after balloon injury and gene transfer. At 4 days
after injury, neointimas are just beginning to form and are more accurately measured by cell counting than by planimetry. Surprisingly, the AdPAI-1-transduced arteries had significantly fewer cells than AdNull-transduced arteries after 4 days (12±3 versus 24±4 intimal cells/section; n=9, P<0.04). At 8 days, however, intimal areas of AdPAI-1 and AdNull arteries were not different (8±1×10{sup 5} versus 7±1×10{sup 5} μm{sup 2}, n=8 to 10; P=0.67). Thus, neointima formation in AdPAI-1 arteries is initially retarded but catches up with and surpasses neointima formation in AdNull arteries.

**PAI-1 Expression and Cell Proliferation**

To investigate whether increased cell proliferation contributes to neointima formation in AdPAI-1 arteries, we measured proliferation at 4, 8, and 14 days after balloon injury and gene transfer (Table). At 4 days, medial proliferation was ≈40% higher in AdPAI-1 than AdNull arteries, but this difference fell short of statistical significance (P=0.2). Intima proliferation at 8 and 14 days did not differ in AdNull and AdPAI-1 arteries.

Because AdNull arteries express endogenous PAI-1 and because the increase in total PAI-1 expression in AdPAI-1 arteries is modest, we considered whether overlapping PAI-1 expression levels between individual AdNull and AdPAI-1 arteries might obscure a significant relationship between PAI-1 expression and proliferation. We therefore used correlational analysis to investigate whether elevated PAI-1 expression is associated with increased cell proliferation. For all 4-day arteries, the proliferative index in the first layer of the media (the area of maximal medial proliferation and the proximal source of intimal cells) was plotted against the intensity of PAI-1 immunostaining in this layer, measured in adjacent sections of the same artery. There was a significant positive correlation between intensity of PAI-1 immunostaining and the proliferative index of the inner medial layer of the same artery (R=0.60, P<0.02; Figure 2). For the 8-day arteries (in which proliferation is essentially confined to the intima), intimal PAI-1 immunostaining and proliferation remained correlated (R=0.62, P<0.006; data not shown).

**PAI-1 Expression and Fibrinogen Accumulation**

To investigate whether increased PAI-1 expression might augment neointimal mass by inhibiting fibrinolysis, we stained sections from the 4-day arteries for fibrinogen. Medias of the 4-day arteries stained variably for fibrinogen, with staining most prominent in the inner layer (Figure 3). Quantitative analyses revealed a strong positive correlation between fibrinogen and PAI-1 staining in adjacent sections of the same artery (R=0.77, P<0.001; Figure 4A). In contrast, analysis of fibrinogen staining data by comparing the AdPAI-1 and AdNull groups revealed only a trend toward increased fibrinogen staining in AdPAI-1 arteries (1800

### Table: Cell Proliferation in Injured Rat Carotid Arteries Transduced With Adenovirus

<table>
<thead>
<tr>
<th>Time After Balloon Injury and Gene Transfer, d</th>
<th>Location and Vector</th>
<th>4 (n=9 per group)</th>
<th>8 (n=8–10)</th>
<th>14 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdPAI-1</td>
<td>37±5%*</td>
<td>AdNull</td>
<td>27±5%</td>
<td></td>
</tr>
<tr>
<td>Intima</td>
<td></td>
<td>AdPAI-1</td>
<td>15±1%</td>
<td>2.2±1%</td>
</tr>
<tr>
<td>AdNull</td>
<td>14±1%</td>
<td>AdNull</td>
<td>2.5±1%</td>
<td></td>
</tr>
</tbody>
</table>

*P=0.2 vs AdNull.
μm² in AdPAI-1 arteries versus 310 μm² in AdNull arteries, P=0.2; Figure 4B).

Fibrin(ogen) Accumulation and Cell Proliferation
Fibrin(ogen) and fibrin degradation products have been associated with cell proliferation.20,21 Therefore, increased fibrin accumulation due to early inhibition of fibrinolysis by PAI-1 could be a link between a transient increase in PAI-1 expression and increased cell proliferation and neointima formation. We therefore investigated the relationship between fibrin(ogen) staining and cell proliferation in AdNull and AdPAI-1 arteries at 4 days after balloon injury and gene transfer. Fibrin(ogen) staining and cell proliferation in the inner layer of the media were strongly correlated (R=0.86, P<0.00001; Figure 5).

Discussion
We used an adenoviral vector to increase PAI-1 expression in balloon-injured rat carotid arteries. Our major findings were as follows. (1) Increased PAI-1 expression caused a 33% increase in neointima formation 2 weeks after balloon injury.

A. AdNull  
B. AdPAI-1

Figure 3. Fibrin(ogen) staining is localized to inner medial layer. Arteries were harvested 4 days after balloon injury and gene transfer. A, Artery infused with AdNull. B, Artery infused with AdPAI-1. Sections were stained with antibody that detects fibrin, fibrinogen, and fibrin fragments. Arrow indicates medial staining. Hematoxylin counterstain; bar=100 μm.

(2) The neointimas in PAI-1-expressing arteries had significantly more cells. (3) Increased PAI-1 expression transiently decreased neointima formation (4 days after injury), but this effect was no longer present at 8 days. (4) PAI-1 expression in arteries correlated with increased fibrin(ogen) immunoreactivity and cell proliferation. (5) Fibrin(ogen) immunoreactivity in arteries correlated strongly with cell proliferation. Our results do not support the use of PAI-1 for vascular gene therapy but instead suggest a causal relationship between elevated PAI-1 expression and vascular lesion growth.

We attempted to uncover the mechanism(s) through which PAI-1 increases neointima formation at 14 days. The increased cell number in 14-day PAI-1 neointimas suggested that PAI-1 increased either cell migration or proliferation. To begin to identify which of these processes contributed to neointima formation, we compared AdPAI-1 and AdNull neointimas 4 days after balloon injury. At this early time point, neointima formation is almost exclusively a consequence of cell migration, with proliferating cells confined to the media.19,22 Surprisingly, neointimas were smaller in the 4-day PAI-1 arteries, indicating that PAI-1 inhibits cell migration early after injury.

Because the PAI-1 neointimas are smaller at 4 days, equal at 8 days, and larger and more cellular at 14 days, PAI-1 could act by promoting cell migration at later time points or by enhancing cell proliferation. The role of PAI-1 in regulating cell migration is complex,23–25 and PAI-1 could have late effects on migration that reverse its early effects. Because there are no techniques to measure late cell migration after arterial injury, however, we cannot confirm or exclude an effect of PAI-1 on late migration. At both 8 and 14 days, intimal proliferation was nearly identical in AdNull and AdPAI-1 arteries (Table). In contrast, 4 days after injury, medial cell proliferation was elevated by 37% in the PAI-1 arteries (P=0.2). We considered the possibility that the lack of statistical significance might represent a type II error, which could be minimized by modestly increasing the sample sizes. Calculations based on the observed variability, how-
earlier reports. Whereas we found that PAI-1 increased fibrin(ogen) with vascular cell proliferation (References 20 and 21, and see Xiao et al.26 for a review). Moreover, this model predicts the primary result of this investigation: that elevated PAI-1 expression promotes neointimal growth.

There are similarities between our results and those of other investigators studying the role of PAI-1 in neointima formation. Carmeliet et al.10 also found an inverse correlation between PAI-1 expression and cell migration early after arterial injury. As in the present study, early differences in lesion formation were transient: PAI-1-knockout mice had larger neointimas at 1 to 2 weeks but not at 3 to 4 weeks. Hasenstab et al.11 also observed increased intimal fibrin in association with PAI-1 overexpression as well as a transient (1- to 2-week) effect of PAI-1 on cell migration and neointima formation.

There are also differences between our results and these earlier reports. Whereas we found that PAI-1 increased neointimal growth, Carmeliet et al.10 reported that increased plasma PAI-1 (generated by adenovirus-mediated overexpression of PAI-1 in the liver 3 days after arterial injury) limited neointima formation. Three aspects of Carmeliet’s study could explain this discrepancy. First, they used electrical injury, in which healing is predominantly due to cell migration. In this regard, the PAI-1–expressing vector did not reduce neointima formation in angioplastied porcine coronary arteries,27 a model in which proliferation contributes significantly to intimal growth.28 Second, they generated supraphysiological plasma levels of PAI-1 (as much as 3000-fold above baseline), which might lead to secondary, systemic effects. Third, they did not infuse the PAI-1 vector until 3 days after arterial injury. Delayed delivery of PAI-1 could miss a window of time during which the injured artery is prothrombotic and elevated PAI-1 may enhance fibrin accumulation.29,30 In the present study, fibrin(ogen) accumulation appears to be a critical pathway through which an early, transient increase in PAI-1 expression enhances neointimal growth at later time points.

In summary, PAI-1 gene transfer to the injured artery wall increases neointima formation. Our data also suggest a mechanism that could account for a causal relationship between elevated PAI-1 expression and arterial disease.1–7 In addition, this study and its predecessors10,11 create an integrated picture of the role of PAI-1 in the formation of arterial lesions. Local PAI-1 expression significantly limits cell migration early after arterial injury, but this affects neointima formation only transiently. Elevated PAI-1 expression also increases fibrin accumulation in injured arteries,11,29,30 which could enhance cell proliferation and compensate for the early, PAI–mediated decrease in cell migration. Taken together, the available data would not support development of PAI-1 gene therapy as a potential treatment for restenosis.

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References


22. DeYoung et al PAI-1 Increases Neointima Formation


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